Title

Role of toxin-antitoxin-regulated persister population and indole in bacterial heat tolerance

Running title

Heat tolerance of bacterial persister cells

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YafQ is an endoribonuclease toxin that degrades target gene transcripts such as that of tnaA, a gene encoding tryptophanase to synthesize indole from tryptophan. DinJ is the cognate antitoxin of YafQ and YafQ-DinJ system was reported to regulate persister formation by controlling indole production in Escherichia coli. In this study, we investigated the role of YafQ-DinJ, indole production, and persister population in bacterial heat tolerance. Single-gene knockout mutants of yafQ (ΔyafQ), dinJ (ΔdinJ), and tnaA (ΔtnaA) showed approximately tenfold higher heat tolerance than wild type E. coli BW25113 (WT). Persister fractions of all mutants were slightly larger than that of WT. Interestingly, these persister cells showed approximately hundredfold higher heat tolerance than their normal cells, but there was no difference among the persister cells of all mutants and WT in terms of heat tolerance. Indole and its derivatives promoted drastic reduction of bacterial heat tolerance by just 10 min of pretreatment, which is not sufficient to affect persister formation before heat treatment. Surprisingly, indole and its derivatives also reduced the heat tolerance of persister cells. Among the tested derivatives, 5-iodoindole exhibited the strongest effect on both normal and persister cells.

**IMPORTANCE** Our study demonstrated that a small persister population exhibits significantly higher heat tolerance and this small fraction contributes to the heat tolerance of the total bacterial population. This study also demonstrated that indole, known to inhibit persister formation, and its
derivatives are very promising candidates to reduce the heat tolerance of not only normal bacterial
cells but also persister cells.

INTRODUCTION

Antimicrobials are generally fed to livestock to prevent infections by pathogens, but unfortunately
consumptions of such livestock pose a severe threat to human health due to development of
drug-resistant bacteria. Even vegetables can be causative when they grow with manure containing
fecal contaminated by drug-resistant bacteria (1). Drug-resistance in bacteria normally occurs due to
gene mutations, which could inherit from one generation to the next (2). Strictly controlled usage of
antimicrobials in agriculture and livestock production can prevent emergence of drug-resistance,
because drug-resistance generally arises by unnecessary and uncontrolled overdoses. On the other
hand, it is not “easy” to combat another uninherited sneaky bacterial population of so-called persister
cells since they evade antimicrobial attack. Persister cells are genetically identical to parental cells
but are somehow in metabolically inactive state, therefore they exhibit remarkable tolerance against
antibiotics targeting cellular activities such as cell division or protein synthesis (3, 4).

Toxin-antitoxin (TA) system is a key regulation factor of stress tolerance and persister formation (5–
7). TA system consists of toxin and the corresponding antitoxin encoded in a single operon. Toxin
inhibits bacterial metabolic systems such as DNA replication, transcription, and translation, or destabilizes bacterial membrane homeostasis (7, 8). Antitoxin encoded in front of toxin gene is a dedicated immune protein or mRNA for its toxin. In the case of type II TA system, antitoxin functions as a protein that inhibits toxin by forming a protein complex during non-stress conditions. While, environmental stresses such as heat shock, oxidative stress, antibiotic attacks, or starvation trigger proteolytic degradation of antitoxin, resulting in free cytosolic toxin that induces a non-growing state to survive stress conditions (9). Several type II TA systems have been demonstrated to be involved in persister formation, and HipAB is the first one to be discovered (10–12). Toxin HipA inhibits translation by phosphorylation of the elongation factor EF-Tu, preventing its interaction with tRNA. Toxin RelE is the best characterized ribosome-dependent RNA interferase, which cleaves mRNA at the A site of ribosome with site preferences manner (13). YafQ is also characterized as a ribosome-dependent mRNA interferase that targets the mRNAs of several genes such as those of elongation factors or tryptophanase (14, 15). These toxins eventually interfere with translation, resulting in drug-tolerant persister cell formation. Indole production in E. coli is directly regulated by YafQ-DinJ system where YafQ degrades the mRNA of tmaA, which encodes tryptophanase that metabolizes tryptophan to indole (16). Furthermore, it was demonstrated that indole production controlled persister formation of E. coli and pre-treatment with a high
concentration of indole (~2.0 mM) before antibiotic treatments, significantly reduced the persister population.

Although many studies have described the practical issues and fundamental drug-resistance mechanisms of bacterial persister cells, no study so far has discussed their tolerance against physical stresses such as heat, acidic, and oxidative stress, except the study of bet hedging in yeast by Levy et al., in which persister cells of yeast showed more heat tolerance than normal cells (17). Therefore, this study aimed to investigate the role of persister population, YafQ-DinJ toxin-antitoxin system, and its regulating indole production in bacterial heat tolerance.

RESULTS

Heat tolerance, injury, and recovery of ΔyafQ and ΔdinJ

To investigate the role of YafQ-DinJ system in E. coli heat tolerance, first, single gene knockout mutants of yafQ (ΔyafQ) and dinJ (ΔdinJ) and wild type E. coli BW25113 (WT) were exposed to heat stress (55°C) in 5 mL of TSB in test tube followed by recovery culture for 4 h. The cell viabilities were determined before and after heat treatment, and once every hour during recovery culture by plating on Tryptic soy agar (TSA), where both intact and heat-injured cells could form colonies, and deoxycholate-hydrogen sulfide-lactose (DHL) agar plates, where only intact cells could form colonies.
colonies as a biological surfactant, sodium deoxycholate inhibited the growth of heat-injured cells. Therefore, the difference between the colony forming unit (CFU) of TSA and DHL was evaluated as the number of injured cells in this study. According to the viable counts on TSA, cell amounts of all three tested strains were approximately $10^9$ CFU/ml before heat treatments. The viable count of WT on DHL agar did not show difference compared to the count on TSA, whereas the counts of ΔyafQ and ΔdinJ on DHL showed lower tendencies than those of TSA even without heat stress (Fig. 1 (A; $t = -1$)). The differences between TSA and DHL counts of ΔyafQ and ΔdinJ were 0.28 ± 0.19 and 0.25 ± 0.14 Log CFU/ml, respectively, which indicates that more than 50% of their cells were somehow already injured without heat stress. After heat treatment as shown in Fig. 1 (A; $t = 0$), intact cell count of WT (on DHL) decreased to 3.65 ± 0.32 Log CFU/ml, while the total cell count including intact and injured cells (on TSA) decreased to 5.76 ± 0.54 Log CFU/ml, indicating that more than 99% of the survivors were injured but culturable by this heat treatment. The TSA count of ΔyafQ also decreased (6.07 ± 1.00 Log CFU/ml) similarly, however, the DHL count (4.78 ± 0.35 Log CFU/mL) after heat treatment was significantly higher than that of WT (3.65 ± 0.32 Log CFU/ml). In other words, approximately 1% of survived WT cells were intact cells, while approximately 12% of survived ΔyafQ were intact, indicating that the survived ΔyafQ cells were more heat tolerant compared to those of WT. On the other hand, compared to WT, ΔdinJ showed higher viable counts...
on both TSA and DHL (7.24 ± 0.50 and 5.16 ± 0.61 Log CFU/ml, respectively), and the ratio of intact cells in survived cells was approximately 1 %, indicating the higher heat tolerance of total population of \( \Delta \text{din}J \). Heat tolerance was also evaluated as cell survival rate (%) on both TSA and DHL plates after heat treatment (Fig. 1 (B)). Both \( \Delta \text{yaf}Q \) and \( \Delta \text{din}J \) exhibited higher survival rates after heat treatment as compared to WT, particularly, the survival rates of their intact cells were more than tenfold compared with WT. As shown in Fig. 1 (A), during recovery culture, only DHL counts of the three strains had increased, while TSA counts were the same for the first 1 h, which means that only injured cells had recovered but no cell divisions had occurred during this period. DHL and TSA counts were same level at 2 h, and thereafter both counts simultaneously increased, indicating that the complete recovery from heat injury was achieved at 2 h and cell division restarted spontaneously. Recovery behaviors of \( \Delta \text{yaf}Q \) and \( \Delta \text{din}J \) were similar to that of WT.

**Persister isolation and heat tolerance of persister cells**

As shown in Fig. 1 (B and C), \( \Delta \text{yaf}Q, \Delta \text{din}J \), and the single gene knockout mutant of \( \text{tnaA} \ (\Delta \text{tnaA}) \) exhibited tendencies of higher heat tolerance than WT. Therefore, to confirm whether persister frequency in total population affects the difference of heat tolerance, persister cells of all strains were isolated and their heat tolerances were examined. In order to determine the viable counts of both
intact and injured but recoverable cells, only TSA was used for viable counts in the following experiments. All strains were treated with ciprofloxacin (CPFX, 1.0 μg/ml) for 4 h and viable counts were determined once every hour (Fig. 2 (A)). After 4 h treatment, killing curves reached plateau phases where only persister cells survived, so these survived cells were collected, and the cell densities were readjusted for the subsequent heat tolerance assay. All deletion mutants showed tendencies of higher persister frequency than WT (1.15 ± 0.22 × 10^2 %) (Fig. 2 (B)). The viable counts of CPFX-untreated cells (normal cells) of all strains after heat treatments with thermal cycler (57°C, 5 min) were below the detection limit (10^2 CFU/ml). Therefore, the viable counts of all untreated strains after heat treatments were considered as less than 10^2 CFU/ml for the calculation of cell survival rates. While, all CPFX-treated persister cells exhibited at least more than hundredfold survival rates as compared to their parental normal cells (Fig. 3). Interestingly, there was no difference in the survival rates among the persister cells of WT and all deletion mutants.

**Effect of tryptophan on persister formation and heat tolerance**

Additional tryptophan (Trp) in culture medium increases indole production and reduces persister formation of *E. coli* (16). To confirm the contribution of persister cells to heat tolerance of *E. coli*, persister frequency and heat tolerance with additional Trp were examined in WT and ΔtnaA. Indole
production of WT increased from $108 \pm 4.69$ to $293 \pm 14.0 \mu M/OD_{600}$, and the persister frequency decreased from $1.15 \pm 0.22 \times 10^{-2}$ to $0.29 \pm 0.01 \times 10^{-2}$% in the medium with additional Trp as compared to that in a medium without additional Trp (Fig. 4 (A)). In addition, the survival rate of the cells cultured with additional Trp (0.88 ± 0.22 %) was significantly lower than that of cells cultured without additional Trp (3.37 ± 0.44 %) (Fig. 4 (B)). On the contrary, there was no difference in both persister frequency and heat tolerance between ΔtnaA cells cultured with and without additional Trp.

Indole production of ΔtnaA was lower than the detection limit (0.01 mM) both with and without additional Trp. These results confirm that the highly heat-tolerant persister fraction contributes to the heat tolerance of the total bacterial population, and indole controls the heat tolerance via persister frequency.

Direct effect of indole on heat tolerance

Hu et al. (2015) demonstrated that an addition of a certain amount of indole strictly reduced persister formation (16). Similarly, in our study, highly heat tolerant persister cells were controlled by the synthesized indole, and these cells could survive under the heat stress conditions where normal cells could not. Therefore, the direct effect of indole on the heat tolerance of bacterial cells was investigated. To avoid persister-mediated effects on heat tolerance, indole was added 10 min before
heat treatments, as this time period was not sufficient to change the persister frequency. As shown in Fig. 5 (A), 10 min indole (2.0 mM) pre-treatment reduced the survival rates of both WT and ΔtnaA to less than one-tenth of the survival rates of untreated cells. However, there was no significant effect of indole at a concentration less than 1.0 mM. Interestingly, 10 min indole pre-treatment also significantly reduced the survival rates of persister cells of both WT (from 8.17 ± 1.54 to 3.92 ± 0.83 %) and ΔtnaA (from 7.92 ± 1.68 to 1.34 ± 0.78 %) (Fig. 5 (B)). Furthermore, indole also greatly reduced the survival rate of indole-non-producing Salmonella enterica Typhimurium NBRC12529 (1.39 ± 0.41 × 10^{-2} to 0.26 ± 0.06 × 10^{-3} %). (Fig.5(C))

Effect of indole derivatives on heat tolerance

Among the great variety of indole derivatives, halogenated indoles are capable of eradicating persister cells and biofilm (18). Therefore, we investigated the potential of three indole derivatives in reducing heat tolerance of both normal and persister cells. Owing to the antibacterial activity of 5-iodoindole against E. coli, firstly, normal cells of E. coli were treated by 0.5, 1.0, and 2.0 mM of 5-iodoindole to determine the optimal concentration for heat tolerance assay, where it could not exhibit antibacterial activity. A significant reduction of viable count was obtained with 1.0 and 2.0 mM of 5-iodoindole treatment, but no reduction was obtained in the case of 0.5 mM treatment (data...
not shown). Therefore, subsequent heat tolerance assays were performed with 0.5 mM of 5-iodoindole and 2.0 mM of indole, 5-hydroxyindole, and indole-3-acetic acid. As shown in Fig. 6 (A), 5-iodoindole exhibited a stronger effect on heat tolerance of normal cells with even at a concentration less than that of indole. The survival rate of cells treated with 5-iodoindole (1.75 ± 0.20 × 10^{-4} \%) was approximately 0.005 \% of the survival rate of untreated cells (3.54 ± 0.95 \%) and approximately 0.09 \% of the survival rate of indole-treated cells (0.19 ± 0.04 \%). Whereas, 5-hydroxyindole and indole-3-acetic acid did not reduce the heat tolerance of normal cells. In addition, 5-iodoindole also exhibited a higher potential in reducing the heat tolerance of persister cells as compared to indole (Fig. 6 (B)), although the difference was not as clear as that observed in normal cells (Fig. 6 (A)). The survival rate of persister cells treated with 5-iodoindole (0.10 ± 0.01 \%) was approximately 1.26 \% of untreated cells (7.92 ± 1.68 \%) and approximately 7.5 \% of indole-treated cells (1.34 ± 0.78 \%). However, the other two derivatives did not show any difference compared to untreated cells.

DISCUSSION

Indole plays many important roles in bacterial physiologies such as biofilm formation, virulence, spore formation, acid resistance, and persister formation (19–23). The conversion of tryptophan to
indole by tryptophanase (TnaA) is the only one indole synthesis pathway in bacteria, and over 85 bacterial species, including both Gram-negative and Gram-positive bacteria, utilize this pathway (24–26). The concentration of excreted indole into a liquid medium can normally reach 0.5 – 1.0 mM and the presence of sufficient amounts of Trp can increase its production (25). The secreted indole acts as an intercellular signaling molecule, such as quorum sensing (QS) molecules, in microbial communities (23, 26, 27). Various bacteria coexist in nature, therefore, indole-non-producing bacteria can encounter a significant amount of indole produced by indole-producing bacteria, and indole in a broad range of concentrations can have harmful or beneficial effects on individual bacteria (23, 26, 28, 29). In this study, we demonstrated that the persister fraction in total bacterial population considerably contributed to bacterial heat tolerance, and indole played a key role in regulating bacterial heat tolerance with or without influencing persister frequency.

YafQ-DinJ is a type II toxin-antitoxin system and known to regulate persister formation by controlling indole production. Overexpressed YafQ, an endoribonuclease-type toxin, strictly reduced tnaA expression, and consequently indole production and persister formation (16). As shown in Fig. 1, ΔyafQ, ΔdinJ and the indole-non-producing mutant ΔtnaA exhibited higher heat tolerance than WT strain. The higher heat tolerances of these three mutants could be explained by their higher persister frequencies (Fig. 2) and the higher heat tolerance of those persister cells (Fig. 3). The higher persister
frequency and heat tolerance of ΔtnaA may be due to the absence of indole production, as the
enhanced indole production in WT by additional Trp significantly reduced persister frequency and
heat tolerance (Fig. 4).

As antitoxin strongly inhibits the effects of toxin, the phenotype of antitoxin knock-out mutant can
be influenced by free toxin, while the phenotype of toxin knock-out mutant can show contrary or no
influence. However, both ΔyafQ and ΔdinJ somehow exhibited the higher persister frequencies and
heat tolerances than WT (Fig. 1 and 2). Moreover, the indole productions by ΔyafQ (181 ± 4.83 μM/
OD₆₀₀) and ΔdinJ (148 ± 15.47 μM/ OD₆₀₀) were significantly higher than that by WT (108 ± 4.69
μM/OD₆₀₀). Higher indole production of ΔyafQ might be attributed to the lack of YafQ, which results
in non-ribosomal degradation of tnaA transcripts. Contradictorily, however, this increased indole
production did not reduce the persister frequency and heat tolerance of ΔyafQ compared to WT.

Higher indole production by ΔdinJ seems to be inconsistent with its higher persister frequency and
heat tolerance as compared to WT. In 2012, Vega et al. demonstrated that addition of 0.5 mM of
indole to culture medium increased persister frequency of ΔtnaA (23). The extracellular indole
concentrations of overnight culture supernatants of WT, ΔyafQ and ΔdinJ were 0.41 ± 0.04 mM, 0.54
± 0.38 mM, and 0.57 ± 0.05 mM, respectively. These results indicate that in the range of these low
concentration, enhanced indole productions of ΔyafQ and ΔdinJ could increase their persister
frequencies and heat tolerances. On the other hand, additional Trp enhanced indole production to approximately 1.0 mM, which was enough to reduce the persister frequency according to the study of Hu et al. (16); therefore, the heat tolerance of total bacterial population was also reduced subsequently (Fig. 4).

The direct effect of indole on bacterial heat tolerance was also evaluated in this study, and the results indicated that indole somehow directly acts on bacterial cells to reduce the heat tolerance without influencing the number of persister cells (Fig. 5). As described above, different concentrations of indole can have different effects on such as enhancing biofilm formation and reducing or increasing persister frequency. The concentration of extracellular indole required to reduce heat tolerances of WT and ΔtnaA was 2.0 mM; this concentration of indole could also reduce the heat tolerances of their persister cells and S. Typhimurium cells. These results suggest that the decrease in heat tolerance by additional Trp (Fig. 4)) was caused by reduction in persister formation by indole production and not by the direct effect of indole on the heat tolerance, as the indole concentration of the culture supernatant with additional Trp was only 1.01 ± 0.04 mM, which is less than the effective indole concentration (2.0 mM) required to directly reduce the heat tolerance. In 2013, Kim et al. demonstrated that 1.0 mM indole inhibited the refolding of denatured protein in vitro (30), which could explain the direct effect of indole on bacterial heat tolerance. In addition, it has been
demonstrated that high concentration of indole (2.0–5.0 mM) prevented the cell division of *E. coli* by modulating membrane potential (31), which could be the mechanism involved in reduction of heat tolerance demonstrated in this study. On the other hand, the halogenated indole 5-iodoindole exhibited remarkable reduction in the heat tolerance of both normal and persister cells at a concentration at which it did not show antimicrobial activity (i.e., 0.5 mM) (Fig. 6). It has already been demonstrated that 1.0–2.0 mM of 5-iodoindol inhibited the growth of *E. coli* and *S. aureus*, and also eradicated the persister cells when used in combination with antibiotics (18). However, the pretreatment time before heat treatment in our study was only 10 min, while the persister eradication effect of 5-iodoindole was detected after 3 h of incubation, indicating that, like indole, 5-iodoindole also directly reduced heat tolerance. Interestingly, 5-hydroxyindole did not reduce heat tolerance, which may suggest the importance of hydrophobicity of indole in interacting with denatured proteins and preventing their refolding.

In conclusion, a small fraction of persister cells regulated by TA systems and indole production possessed remarkably high heat tolerance and it contributed to the heat tolerance of total bacterial population. Therefore, this small persister population cannot be neglected in bacterial heat tolerance and food safety. Furthermore, as indole can reduce bacterial heat tolerance with or without
influencing persister cells, indole and its derivatives can enhance the antimicrobial effect of heat treatments or other treatments for food production or hygienic purposes.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Wild type strain, *Escherichia coli* BW25113 (WT) and the knockout mutants, Δ*yafQ*, Δ*dinJ*, and Δ*ttnA* (Keio collection), were obtained from National BioResource Project (NBRP). *Salmonella enterica* serovar *Typhimurium* NBRC 12529 was purchased from Institute of Fermentation, Osaka (IFO). Tryptic soy broth (TSB) (BD) and Luria-Bertani (LB) broth (BD) were used for heat treatment and persister isolation, respectively. Tryptic soy agar (TSA) (BD), LB agar (nakarai tesque, Japan), and deoxycholate-hydrogen sulfide-lactose (DHL) agar (NISSUI PHARMACEUTICAL, Japan) were used for determination of cell viability. Phosphate buffered saline (PBS, pH 7.4) was used for cell dilution and washing steps.

All strains were refreshed from glycerol stocks at -80 °C by overnight culture at 37 °C with aeration, and subsequently streaked on TSA plates and incubated overnight at 37 °C. For refreshing cultures of knockout mutants from -80°C stocks, 5.0 μg/ml of kanamycin (nacalai tesque, Japan) was added to TSA or TSB. A single colony from each plate was transferred to fresh TSB or LB broth and
precultured overnight at 37 °C with aeration, and subsequently used for the experiments. To investigate the influence of tryptophan (Trp), single colony from each plate was transferred to TSB containing 4.0 mM of Trp (TSBW), cultured at 37 °C with aeration for 24 h, and subsequently used for the experiments.

Cell viability assay

Cell viability was determined by counting the number of colonies (colony forming unit, CFU) on selective (DHL) or non-selective (TSA) agar plates. In this study, the number of colonies on DHL plates represented the number of intact cells and that on TSA plates represented the number of intact and injured but recoverable cells. Therefore, the number of heat injured cells was determined as ‘CFU_{TSA} – CFU_{DHL}’ and evaluated as \( \log \left( \frac{\text{CFU}_{\text{TSA}}}{\text{CFU}_{\text{DHL}}} \right) \). LB agar plate was used to determine the cell viability for persister cells. Colony counts were performed using the spotting method as described previously with certain modifications (32, 33). Briefly, the samples were serially diluted with PBS and subsequently spot-plated (10 μl) or spread-plated (100 μl) on plates. The detection limit for spot-plating and spread-plating was 100 and 10 CFU/ml, respectively. The plates were incubated at 37 °C for 24 h; then, the number of colonies was counted.
Isolation of persister cells

LB-precultures of all strains were diluted in fresh LB and OD$_{600}$ was adjusted to 0.4 (~$10^8$–$9$ CFU/ml).

For TSB- and TSBW-precultures, cells were collected by centrifugation (10,000 × g, 1 min, 25 °C) and washed with PBS, and subsequently resuspended in fresh LB to be OD$_{600}$ = 0.4 (~$10^8$–$9$ CFU/ml).

Ciprofloxacin (CPFX) (nacalai tesque, Japan) was added to adjusted cells at a final concentration of 1.0 μg/ml, and cells were incubated at 37 °C with aeration (120 rpm) for 4 h. After 0, 1, 2, and 4 h of incubation, cells were collected from 500 μl of cultures by centrifugation and resuspended in 500 μl of PBS. The cells were washed, again centrifuged, and resuspended in 500 μl of PBS, and cell viability was determined by plating LB agar (CFU/ml). Persister frequency was calculated as the ratio of cells that survived 4 h CPFX treatment to the parental population before antibiotic treatment.

The cells that survived 4 h CPFX treatment were collected by centrifugation, washed with PBS, and subsequently used for heat tolerance assays.

Heat tolerance assay

Heat treatments were performed using two methods, the test tube method and the thermal cycler method. The test tube method was used only for heat injury and recovery assays. Briefly, TSB-preculture was diluted in fresh TSB and to obtain OD$_{600}$ = 0.1 (~$10^8$ CFU / ml). Five milliliters
of this cultures were placed in a test tube and incubated in a water bath (120 rpm) at 25 °C for 10
min, followed by 60 °C for 10 min, and finally 25 °C for 10 min; next, the test tube was incubated at
37 °C for 4 h (120 rpm) to allow recovery of heat-injured cells. Cell viability was determined at each
time-point (before heat treatment, and 0, 1, 2, 3, and 4 h after heat treatment).

Other heat treatments were carried out using the Thermal Cycler Dice Touch (TaKaRa, Japan) with
0.2 ml PCR tube (NIPPON Genetics, Japan). Briefly, the preculture was diluted in fresh TSB to
obtain OD$_{600}$ = 0.1 (~10$^8$ CFU/ml). Aliquots of 75 μl were placed in PCR tubes and subjected to heat
treatment in the thermal cycler (25 °C for 10 min, 55 °C for 5 min, 25 °C for 10 min). For heat
tolerance assay of persister cells, isolated persister cells were resuspended in fresh TSB to obtain a
cell density of approximately 10$^5$ CFU/ml and heat treatments were performed using a temperature
of 57 °C instead of 55 °C. CPFX-untreated normal cells could spontaneously involve persister
fractions less than 0.1 % of the total population. The influence of persister cells in normal cell
population was insignificant in this study as the cell density of normal cells was adjusted to be
approximately 10$^5$ CFU/ml where the number of persister cells was below the detection limit (10$^3$
CFU/ml). Cell viability was evaluated before and after heat treatments.

Evaluating the effects of indole and its derivatives on heat tolerance
To evaluate the effects of indole and its derivatives on heat tolerance, precultures of WT and ΔtnaA in TSB were diluted in fresh TSB with 0.5–2.0 mM of indole (Tokyo Chemical Industry, Japan), 5-iodoindole (Sigma-Aldrich), 5-hydroxyindole (Tokyo Chemical Industry, Japan), or indole-3-acetic acid (Tokyo Chemical Industry, Japan), and applied to following heat tolerance assays. Persister cells of WT and ΔtnaA were also resuspended in fresh TSB with indole or its derivatives and applied to following heat tolerance assays. The final concentration of indole was 2.0 mM for the heat tolerance assay of indole-non-producing Salmonella enterica serovar Typhimurium NBRC 12529.

**Indole measurement**

Indole measurements were carried out according to previous reports (20) with certain modifications. Briefly, standard solutions of indole in the range of 0 to 800 μM concentration were prepared in butanol (nacalai tesque, Japan). Cell-free supernatants were prepared by centrifuging 1 ml of cultured samples (OD$_{600} = 0.1$). In order to measure the indole productions, 50 μl of supernatant was mixed with 400 μl of butanol and 50 μl of Kovac’s reagent (Merck), while 50 μl of standard solution was mixed with 350 μl of butanol, 50 μl of fresh TSB, and 50 μl of Kovac’s reagent. After incubation at 25 °C for 5 min, the absorbance of standard solutions and samples at 590 nm was measured, and
indole concentrations were calculated from the established standard curve. When the concentration of indole was higher than 800 μM, the supernatants were optimally diluted with fresh TSB to obtain a concentration within the range of the standard curve.

**Statistical analysis**

Data points of all experiments represent means of the results from at least three independent experiments and error bars indicate standard deviations. Significance of the differences was determined using the T-test or Tukey’s test in Excel.
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Fig. 1 Heat tolerance, injury, and recovery of *E. coli* BW25113 and its single gene knockout mutants. (A) *E. coli* BW25113 (WT), *ΔyafQ* and *ΔdinJ* were cultured in TSB at 37 °C for overnight and subsequently subjected to heat treatments in test tube (60 °C, 10 min). The viable counts of WT and single-gene knockout mutants during heat treatments and recovery cultures were determined. Data points at t = -1 and 0 h mean viable counts before and just after heat treatments. (B) Heat tolerance of each strain was determined as a ratio of the number of the cells that survived heat treatment (t = 0) to the number of untreated (before heat treatment, t = -1) cells. (C) Heat tolerances of intact cells were determined by viable counts on DHL plates before and after heat treatments with the thermal cycler method. Data points represent means of results from at least three independent experiments, and error bars indicate standard deviations. Significance of difference between the data of WT and each knockout mutant strain on TSA or DHL plates was analyzed by the T-test. (*p <0.1, **p <0.05)

Fig. 2 Persister cells were isolated by 4 h treatments with CPFX. (A) Precultures of WT, *ΔyafQ*, *ΔdinJ*, *ΔtnaA* were diluted in fresh LB to obtain 10^8–9 CFU and subjected to CPFX treatments (1.0 µg/ml, 37°C, 120 rpm). Viable count was monitored during CPFX treatments. Data points at 0 h mean numbers of viable counts before treatment. (B) Persister frequency was determined by
calculating the ratio of the cells survived 4 h CPFX treatment to the number of cells before treatment.

Data points represent means of results from at least three independent experiments, and error bars indicate standard deviations. Significance of difference between the data of WT and each knockout mutant strain was analyzed by the T-test. P values for ΔdinJ, ΔyafQ, and ΔtnaA were 0.19, 0.52, and 0.24, respectively.

Fig. 3 Heat tolerance of persister cells were greatly higher than intact cells. (A) Scheme illustrating the set up for persister isolation and heat tolerance assay. (B) Isolated persister cells and normal (untreated) cells of WT, ΔyafQ, ΔdinJ, and ΔtnaA were adjusted to be approximately $10^5$ CFU/ml in fresh TSB and subjected to heat tolerance assay with the thermal cycler method (57°C for 5 min). The viable counts of normal cells after heat treatments were below the detection limit ($10^2$ CFU/ml), therefore, their viable counts after heat treatments were considered as $10^2$ CFU/ml for the calculation of the cell survival rates here. Data points represent means of results from at least three independent experiments, and error bars indicate standard deviations. Significance of difference between the data of normal and persister cells was analyzed by the T-test (*p < 0.05).

Fig. 4 Additional Trp reduced persister frequency and heat tolerance of E. coli. WT and ΔtnaA
were cultured at 37 °C with aeration for 24 h in TSB with or without additional Trp (4.0 mM), and

(A) subsequently resuspended in fresh LB to be $10^{8-9}$ CFU/ml and subjected to CPFX treatments to
determine persister frequencies or (B) diluted in fresh TSB to be approximately $10^8$ CFU/ml and
subjected to heat tolerance analysis using the thermal cycler method (57°C, 5 min). Data points
represent means of results from at least three independent experiments, and error bars indicate
standard deviations. Significance of difference between the data of Trp- and Trp+ was analyzed by
the T-test (*$p <0.05$).

**Fig. 5** Indole can directly reduce bacterial heat tolerance. (A) Precultures of WT and ΔtnaA were
diluted in fresh TSB to obtain approximately $10^8$ CFU/ml and subjected to heat tolerance analysis
(55°C, 5 min) with several final concentrations of indole (0 ~ 2.0 mM). (B) Isolated persister cells of
WT and ΔtnaA were resuspended in fresh TSB with or without 2.0 mM of indole to obtain
approximately $10^5$ CFU/ml and subjected to heat tolerance assay (57°C, 5 min). (C) The preculture
of *S. Typhimurium* was diluted in fresh TSB with or without 2.0 mM of indole to obtain
approximately $10^8$ CFU/ml and subjected to heat tolerance analysis (55°C, 5 min). Data points
represent means of results from at least three independent experiments, and error bars indicate
standard deviations. Significance of difference between the data of Indole- and Indole+ was analyzed
by the T-test (*p <0.05).

Fig. 6 Effects of indole derivatives on heat tolerance of ΔtnaA. (A) the preculture of ΔtnaA was diluted in fresh TSB with indole (2.0 mM), 5-iodoindole (0.5 mM), 5-hydroxyindole (2.0 mM), or indole-3-acetic acid (2.0 mM) to obtain approximately 10^8 CFU/ml and subjected to heat tolerance assays (55°C, 5 min). (B) Isolated persister cells of ΔtnaA were resuspended in fresh TSB with indole (2.0 mM), 5-iodoindole (0.5 mM), 5-hydroxyindole (2.0 mM), or indole-3-acetic acid (2.0 mM) to obtain approximately 10^5 CFU/ml and subjected to heat tolerance assays (57°C, 5 min). Data points represent means of results from at least three independent experiments, and error bars indicate standard deviations. Significance of difference among the all data was analyzed by the Tukey’s test, and bars with different letters are statistically different (*p <0.05).
(A) Antibiotic treatment

Untreated Persister cells

CPFX (1 µg/ml) for 4 h

Normal cells

Centrifugation

10,000 xg
1 min

Washing

With PBS

Resuspension

Heat treatment

Adjusting to 10⁵ CFU/ml

57°C
5 min

Viability assay

On TSA plate
37°C, 24 h

(B) Cell survival (%)

10²

10

1

10⁻¹

10⁻²

Normal Persister

Normal Persister

Normal Persister

Normal Persister

Normal Persister

WT

ΔyafQ

ΔdinJ

ΔnaA

*