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Stochastic expression of lactate dehydrogenase A induces *Escherichia coli* persister formation

Naoki Yamamoto,[1,‡] Rino Isshiki,[1,‡] Yuto Kawai,[1,‡] Daiki Tanaka,[2] Tetsushi Sekiguchi,[2] Shinya Matsumoto,[3] and Satoshi Tsuneda[1,2,*]

Department of Life Science and Medical Bioscience, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan,[1] Research Organization for Nano and Life Innovation, Waseda University, 513 Waseda-tsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan,[2] and Graduate School of Medicine, Nagoya University, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan[3]

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Bacterial persisters are phenotypic variants that survive the treatment of lethal doses of growth-targeting antibiotics without mutations. Although the mechanism underlying persister formation has been studied for decades, how the persister phenotype is switched on and protects itself from antibiotics has been elusive. In this study, we focused on the lactate dehydrogenase gene (*ldhA*) that was upregulated in an *Escherichia coli* persister-enriched population. A survival rate assay using an *ldhA*-overexpressing strain showed that *ldhA* expression induced persister formation. To identify *ldhA*-mediated persister formation at the single-cell level, time-lapse microscopy with a microfluidic device was used. Stochastic *ldhA* expression was found to induce dormancy and tolerance against high-dose ampicillin treatment (500 μg/ml). To better understand the underlying mechanism, we investigated the relationship between *ldhA*-mediated persister formation and previously reported persister formation through aerobic metabolism repression. As a result, *ldhA* expression enhanced the proton motive force (PMF) and ATP synthesis. These findings suggest that *ldhA*-mediated persister formation pathway is different from previously reported persister formation via repression of aerobic metabolism.

[**Key words:** Persister; *ldhA*; *Escherichia coli*; Single cell; Microfluidic device; Stochastic expression; Energy metabolism; Proton motive force]

Over the years, it has been believed that a clonal bacterial population derived from a single colony is homogeneous. However, single-cell image analysis reveals that the gene expression level or phenotype of each cell is different even within the clonal population (1). This phenotypic heterogeneity is believed to engage in a bet-hedging strategy (1). As an example of diverse phenotypic heterogeneity, persisters are defined as subpopulations that are tolerant and survive against lethal doses of antibiotics without genetic mutation and can regrow after antibiotic removal (2–10). Therefore, persister populations pose a serious threat of recurrence in chronic infectious diseases (7,11,12).

In many previous studies, it is suggested that persister formation is caused by dormancy (2–9). For example, toxin/antitoxin (TA) modules and the stringent response regulated by a global regulator (p)ppGpp were reported to inhibit the growth and translation activity of cells and then induce persister formation (3,8,13–17). In addition, metabolic change in a small fraction of the bacterial population has been revealed to be associated with persister formation (10,16,18–21). However, in these studies, the persister formation pathway was inferred from phenotypic differences between the wild type and gene overexpression/knockout strain. Thus, the detailed mechanism, i.e., how gene expression regulates the metabolic pathway in the natural state and induces persister formation, remains poorly understood.

Our group has successfully sorted persisters, using a recombinant *Escherichia coli* strain in which non-dividing and dividing cells are distinguishable based on Z-ring visualization during cell division (22). Microarray analysis with sorted persisters showed that the lactate dehydrogenase gene (*ldhA*) together with previously reported persister genes such as *relA* and *cyaA* (22) was significantly upregulated. In this study, we hypothesized that *ldhA* expression was involverd in *E. coli* persister formation. Therefore, we examined the influence of *ldhA* expression level on the persister fraction by assaying the survival rate in the *ldhA*-overexpressing *E. coli* strain. In addition to the above-mentioned "bulk-population based" assay, we performed an "individual-cell level" assay using a microfluidic device in which both native *ldhA* expression and antibiotic tolerance levels of individual *E. coli* cells could be visualized with time in the natural cell state. A combination of these two assays could illuminate the relationship between *ldhA* expression and persister formation.

In previous studies, it has been reported that the repression of aerobic metabolism such as proton motive force (PMF) and ATP synthesis induces persister formation (6,18,20). To better understand the underlying mechanism, we investigate the relationship between pathways of *ldhA*-mediated persister formation and

* Corresponding author at: Department of Life Science and Medical Bioscience, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan. Tel./fax: +81 3 5369 7325.
E-mail address: stsuneda@waseda.jp (S. Tsuneda).
‡ The first three authors contributed equally to this work.

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TABLE 1. Bacterial strains and plasmids used in this work.

Strain/Plasmid	Genotype	Sources
Strain		
MG1655	wild type *E. coli*	NBRP, 23
MG1655_pCA24N	MG1655 pCA24N	This work
ldhA OE	MG1655 pCA24N-*ldhA*	This work
ΔfliC	BW25113 *ΔfliC*	NBRP, 24
ldhA reporter	BW25113 rfliC pSC101_PldhA_*venus*	This work
Plasmid		
pCA24N	*cat*, *lacI*, pT5lac promoter	NBRP, 25
pCA24N-*ldhA*	*cat*, pCA24N pT5lac::*ldhA*	This work
pSC101	*tetR*, ptet promoter	NBRP, 27
pSC101_PldhA	*tetR*, pSC101 p*ldhA*	This work
pSC101_PldhA_*venus*	*tetR*, pSC101 p*ldhA*::*venus*	This work

previously reported persister formation through the repression of aerobic metabolism such as PMF, ATP synthesis, and $NADH/NAD^+$.

MATERIALS AND METHODS

Reagents and bacterial strains The *E. coli* K-12 MG1655 (23) strain was used for assaying persister formation and *E. coli* BW25113 Δ*fliC* strain purchased from the Keio collection (24) was used for single cell image analysis. All strains were cultured in LB medium (Difco, Wako, Tokyo, Japan) at 37°C. Antibiotics were used as follows: 500 μg/ml (high dose) or 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 10 μg/ml tetracycline, or 5 μg/ml ofloxacin. Isopropyl-ß-D-thiogalactopyranoside (IPTG) at 0.1 mM was used as an overexpression inducer. All the strains and plasmids used in this study are listed in Table 1.

Construction of ldhA-overexpressing strain To assess the relationship between *ldhA* expression level and persister formation frequency, we constructed an *ldhA*-overexpressing strain and a vacant vector strain as negative control. These strains harbor the high-copy plasmid pCA24N capable of replicating 300−500 copies per cell. This plasmid was derived from ASKA clone, which is *E. coli* overexpression library (25). Also, this plasmid contains IPTG-inducible pT5lac. For constructing the *ldhA*-overexpressing strain, pCA24N and *ldhA* sequence fragments were digested with BseRI and HindIII, and then ligated using T4 ligase. The constructed plasmid was introduced into *E. coli* MG1655 and insertion of the plasmid into each strain was checked by colony PCR, using the YFP_f2 primer. All primers for constructing *ldhA*-overexpressing strain are listed in Table 2.

Construction of the ldhA reporter strain To visualize native *ldhA* expression in *E. coli*, we constructed an *ldhA* reporter plasmid containing the *E. coli ldhA* promoter region at the upstream of *venus* encoding yellow fluorescent protein. We selected the *ldhA* promoter region, which contains a promoter and transcriptional regulator, between approximately 250 bp upstream and 80 bp downstream of the *ldhA* start codon (26). Venus protein is expressed earlier for preventing a time lag between *ldhA* expression and Venus fluorescence. For constructing the *ldhA* reporter strain, pSC101 (27) and the Venus sequence fragment were digested with XhoI and EcoRI, and ligated. Then, the pSC101 containing *venus*, and the *ldhA* promoter region fragment were digested with XhoI and PvuII, and ligated. An insert check was performed by colony PCR, using the primers reporter_f and reporter_Venus_r. All primers for constructing *ldhA* reporter strain are listed in Table 2.

Persister assay For assessing persister formation, we determined the bacterial survival rate against antibiotic challenge. First, 50 μl of overnight culture was diluted in 5 ml LB medium in the presence of IPTG as an inducer, and cultured at 37°C for 3 h to the exponential phase. Then, 2 ml of each sample was transferred to 5 ml tube and incubated for 1, 2, 3, 5, and 20 h after the antibiotics treatment. One hundred microliters of sample before and after the treatment was resuspended and serially diluted in phosphate-buffered saline (PBS). From each sample, a 20-μl aliquot was spotted on an LB agar plate and incubated overnight. Colony forming units (CFUs) were counted and the survival rate was calculated by the ratio of CFUs before and 3 h after antibiotics treatment. In this assay, *ldhA* expression was continuously induced during IPTG induction followed by CFU measurement. In the persister assay inhibiting PMF, the uncoupler agent, carbonylcyanide *m*-chlorophenyl hydrazone (CCCP), was added to the medium diluting overnight culture at a final concentration of 20 μM and then cultured at 37°C for 3 h to exponential phase.

Microfluidic device description and fabrication A microfluidic device was used to observe the growth and antibiotic susceptibility of the *ldhA* reporter strain at the single-cell level (Fig. 1). We referred to the microfluidics system described by Aldridge et al. (28). This device allows cell growth in shallow growth channels (1 μm height; and 20, 50, and 100 μm width) that are connected to medium channels (25 μm height; and 100 μm width). The microfluidic device was prepared by standard soft lithography techniques. We used a SU-8 3025 photoresist (MicroChem, Westborough, MA, USA) for the first layer of the medium channels and AZ5214E (Clariant, Muttenz, Switzerland) for the second layer of the growth channels to prepare the master mold on silicon wafers. On the mold, silicon tubes (1 × 2 mm; TGK, Tokyo, Japan) were attached for the inlet and outlet ports. Then, the polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, MI, USA) prepolymer was mixed with a crosslinker at a 10:1 weight ratio and poured on the master mold. PDMS was then degassed under negative pressure, and cured by incubation at 80°C for 16 h. The cured PDMS replicas were then cut out around the structures on the wafers and removed from the molds. Finally, both the glass slides (50×70×0.12−0.17 mm; Matsunami Glass, Kishiwada, Osaka, Japan) and the PDMS replicas were subjected to a brief oxygen plasma treatment, and immediately bonded to obtain the final devices.

Single-cell observation, image acquisition, and analysis Cells were grown in LB medium to the mid-exponential phase ($OD_{600} = 0.3−0.5$) at 37°C, and then concentrated 5-fold by centrifugation for adjusting cell density in the device. This culture was loaded into the device by diffusion. The device was assembled in a heating chamber attached to the microscope with temperature maintained at 37°C. Medium was pumped through the device using a syringe pump SPE-1 (As One, Osaka, Japan) at a flow rate of 4 μl/h. LB medium was supplied for observation of *ldhA* expression and the growth of individual cells. To observe the response of individual cells to antibiotics exposure, LB medium with 500 μg/ml ampicillin was supplied for 90 or 180 min, or 5 μg/ml ofloxacin was supplied for 180 min. At the endpoint of the antibiotics exposure phase, we measured the proportion of cells without morphological changes, regarded as the antibiotic-tolerant cells. After antibiotic exposure, LB medium without antibiotics was resupplied and the regrowth behavior of the tolerant cells was observed for 16 h. Time-lapse images were acquired with a fluorescent microscope IX81 (Olympus, Tokyo, Japan) equipped with a CCD camera on the phase-contrast and YFP channels at 2-min intervals. Venus fluorescence exhibited an emission maximum at 528 nm that could be detected using a standard YFP filter set. Statistic analysis was performed using an odds ratio (OR) for 95% confidence intervals (CI) to judge significant difference in the survival rate between *ldhA*-expressing and *ldhA*-non-expressing cells.

PMF measurement Proton motive force measurement was performed using the BacLight Bacterial Membrane Potential Kit (Thermo Fisher Scientific, Yokohama, Kanagawa, Japan). In this assay, 3,3′-diethyloxa- carbocyanine iodide ($DiOC_2$) is used as a dye that exhibits green fluorescence, but shifts into emitting red fluorescence by self-association as the intracellular concentration of $DiOC_2$ increases in a cell with higher membrane potential (29). In this study, 50 μl of overnight culture from each strain was diluted in 5 ml LB medium and cultured for 3 h to the exponential phase. Then, 100 μl of this culture was centrifuged at 13,000 rpm for 2 min, resuspended in 1x PBS, and diluted to approximately 1×10^6 cells/ml in PBS. Samples stained with $DiOC_2$ at 30 μM, samples depolarized with $DiOC_2$ and CCCP at 30 μM and 5 μM, respectively, and unstained samples without $DiOC_2$ were analyzed using FACS Aria IIu (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Proton motive force was determined as the ratio of red to green fluorescence intensities.

TABLE 2. PCR primers used in this work.

PCR primer	Sequence (5′-3′)	Reference
pCA24N_ldhA_f2	TTAAAGAGGAGAAATTAACATGAAACTCGCCGTTTATAGCACAAAAC	This work
pCA24N_ldhA_r	TAATTAAGCTTTTAAACCAGTTCGTTCGGGCAGGTTTC	This work
Inv_pCA24N_f	GCAGCCAAGCTTAATTAGCTGAGC	This work
Inv_pCA24N_r	GCATGAAGCTTGGTGAGATCCTCTCATAGTTAATTTCTCCTC	This work
YFP_f_2	TATCACGAGGCCCTTTCGTC	This work
ldhA_qRT_f	GCGTACCCGTGATGCTAACTTCTC	This work
ldhA_qRT_r	GCGCCGCTGCACTTGGATAC	This work
ldhA_reporter_f	CCGTACAGCTGCAGTAATAACAGCGCGAGAACGG	This work
ldhA_reporter_r	CCGTACTCGAGCCAGCTCAAAGCCAAAGGACTCG	This work
reporter_f	CAGATTACAACCGTCGCTCGATAGC	This work
reporter_Venus_r	CTGTGATAAACTACCGCATTAAAGCTTATC	This work

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