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Unique transcriptional profile of native persisters in *Escherichia coli*

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Non-dividing persisters, bacteria that can survive in the presence of antibiotics by pausing their metabolic activity, are among the many causes of the refractory nature of bacterial infections. Here we constructed a recombinant *Escherichia coli* strain that enables to distinguish non-dividing from dividing cell based on Z-ring during cell division. Then, non-dividing cells and dividing cells were successfully separated using a fluorescence activated cell sorter. The sorted non-dividing cells showed significantly higher tolerance toward ofloxacin than dividing cells, which indicates that persisters were concentrated with the methodology. Transcriptional analysis revealed that genes involved in guanosine tetraphosphate synthesis are upregulated in persisters, which represses transcription and DNA replication and leads to ofloxacin tolerance. Lactate dehydrogenase and several ATP-binding cassette transporters were upregulated in persisters to adapt to anaerobic metabolism. In addition, nitrite and dimethyl sulfoxide (DMSO) may be used as reducible substrates for alternative energy generation pathways. Our methodology revealed a unique transcriptional profile of *E. coli* persisters.

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Bacterial persisters are a small subpopulation of cells tolerant toward antibiotics (1). In contrast to antibiotic-resistant bacteria, persisters do not grow in the presence of antibiotics and the tolerance arises from phenotypic variations rather than genetic mutations (2,3). Balaban et al. (4) demonstrated that persisters are abundant in slow growing or growth-arrest population using a microfluidic device to monitor cell growth. From the clinical point of view, persisters may cause recurrence of infection once the treatment is ended. Indeed, *in vivo* clinical studies have indicated that persistence in uropathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* presents a therapeutic challenge during the treatment of these infective agents (5–7).

Though the isolation of a gain-of-function high-persister allele, *hipA7*, showed enhanced persistence up to 1000-fold (8,9), a complete understanding of persister formation remains elusive. Candidate genes responsible for persister formation have been found using an *E. coli* gene knockout library (10) and subsequent analysis revealed the pathways and mechanisms of persister formation (11). However, no knockout strain completely lacked persisters (11), which indicates that these cells apparently arise through highly redundant pathways.

Another obstacle to the analysis of persister genes has been a lack of methods for the segregation of persister cells (12). The fact that persisters form a small and temporary population makes their segregation challenging. The simplest approach was to lyse the *E. coli hipA7* mutant with a β-lactam antibiotic, which regards unlysed cells as persisters (13). Their subsequent transcriptional analysis indicated overexpression of chromosomal toxin-antitoxin (TA) modules in *hipA7* mutants. A more advanced method (14) used *E. coli* expressing a degradable GFP under the control of a ribosomal promoter. Persisters showed a weak GFP signal because of low levels of translation, allowing to sort native persister cells based on GFP expression without exposing the cells to antibiotics. Persisters segregated using this technique were shown to be tolerant to ofloxacin, confirming that they are persisters. Transcriptional analysis of the segregated cells indicated the downregulation of biosynthesis genes and increased expression of several TA modules. Nevertheless, to proficiently segregate bacterial persisters more methodology is needed in order to explore with deepest detail the mechanisms involved in the induction, maintenance and exit from the persister state (15).

Most prokaryotes employ a tubulin homolog known as FtsZ for cell division (16,17). Bacterial cell division is initiated by the formation of the Z-ring, which is a ring-like structure formed at midcell that integrates at least 10 additional essential proteins that will ultimately separate the two daughter cells (18,19). During Z-ring formation, FtsZ polymerizes into protofilaments by the head-to-tail association of individual subunits (20,21).

We reasoned that Z-ring formation can be a target to distinguish non-dividing cells from other cells. Using a fluorescently tagged version of the FtsZ protein we developed a method of detecting

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E. coli native non-dividing cells based on Z-ring formation with fluorescence resonance energy transfer (FRET) (Fig. 1).

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions Bacteria were grown in Luria–Bertani (LB) medium at 37°C. The $P_{T5\text{-}lac}$ and P_{BAD} (22) promoters were induced by addition of 10 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) (23) and 0.01% arabinose, respectively. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; ofloxacin, 5 μg/ml; kanamycin, 20 μg/ml; and chloramphenicol, 25 μg/ml.

Bacterial strains and plasmids All DNA manipulations, analyses and bacterial transformations were performed essentially according to standard protocols (24). Bacterial strains used and plasmid constructions are described in Table 1; PCR primers are listed in Table 2. When part of the cloning strategy was PCR amplification, the constructs were confirmed by sequencing at Fasmac (Kanagawa, Japan). The VIP205/pSMV1 strain was constructed as follows. Genes mCyPet and mYPet were amplified from pET15bmCyPet and pET15bmYPet (gifts from Dr. Harold P. Erickson, Duke University, NC, USA), respectively, by PCR using PrimeSTAR MAX DNA polymerase (Takara, Tokyo, Japan) with the primers CFP_f and CFP_r, and YFP_f and YFP_r, respectively. The GFP fragment of pCA24N_FtsZ-GFP (ASKA clone (25) JW0093) was removed by NotI digestion and the vector was linearized. The PCR product of mCyPet was cloned into linearized pCA24N_FtsZ using the In-Fusion PCR cloning kit (Clonetech, Mountain View, CA, USA) to generate pCA24N_FtsZ-mCyPet. pCA24N_FtsZ-mCyPet was linearized by BseRI digestion and the PCR product of mYPet was cloned into the linearized pCA24N_FtsZ-mCyPet using the In-Fusion PCR cloning kit to generate pCA24N_mYPet-FtsZ-mCyPet. The mYPet-FtsZ-mCyPet gene was isolated by BseRI and HindIII digestion. pBAD24 was amplified by PCR using PrimeSTAR MAX DNA polymerase with the primers YFP-FtsZ-CFP_f and YFP-FtsZ-CFP_r to provide the backbone for pBAD24_mYPet-FtsZ-mCyPet. The isolated mYPet-FtsZ-mCyPet was cloned into the PCR product of pBAD24 to generate pSMV1. All plasmid DNAs were amplified in *E. coli* strain JM109 (Takara), and then isolated and purified using the PureYield Plasmid Miniprep System (Promega, Madison, WI, USA).

Imaging Images of the cells were acquired with a FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with 440 nm, 515 nm, 568 nm and 633 nm lasers. FRET was measured with the 440 nm laser and FRET emission was obtained with a 535/26 nm filter.

Cell sorting VIP205/pSMV1 was cultured in a 15 ml culture tube containing 10 mL of LB broth, and incubated at 37°C for 24 h prior to sorting. Cells were collected by centrifugation (8000 ×*g*, 10 min) and resuspended in phosphate buffered saline (pH 7.2) containing 0.01% arabinose for CFP-YFP FRET analysis using a FACSAria instrument (BD Biosciences, San Jose, CA, USA) equipped with 405 nm, 488 nm and 633 nm lasers. FRET was measured by exciting cells with the 405 nm laser and FRET emission was obtained with a 530/30 nm filter. Cells were sorted directly into 5 mL sampling tubes that contained 670 μL PBS. A total of 100,000 events were sorted for each fraction, which resulted in fractions containing 1000 μL (330 μL sorted cells and 670 μL PBS).

Persister assays Ofloxacin was added to 100 μL aliquots of sorted cells at a final concentration of 5 μg/ml and incubated for 3 h at 37°C. The cells were diluted 1:100 in PBS. The number of colony forming units (CFUs) were determined using LB agar plates containing 0.01% arabinose.

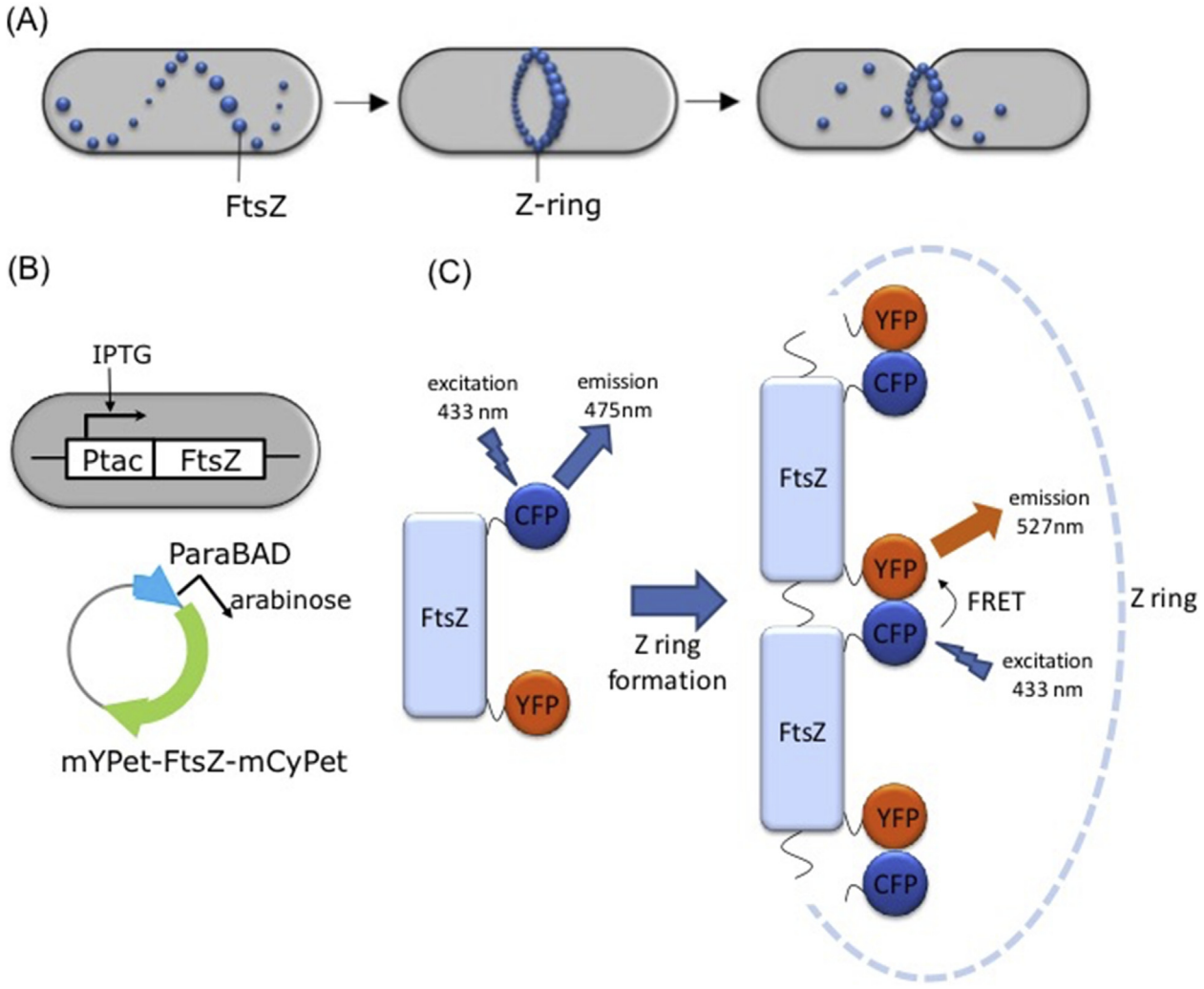

FIG. 1. Proposed mechanism of bacterial cell division marker. (A) FtsZ (blue spheres) polymerizes at the midcell through *E. coli* cell division. (B) The native *ftsZ* gene on chromosome is under control of the *tac* promoter, by which the expression is repressed without IPTG. The mYPet-FtsZ-mCyPet fusion protein is induced by arabinose (araBAD promoter). (C) In non-dividing cells, the mYPet-FtsZ-mCyPet monomer excited by 433 nm laser emits only CFP fluorescence. In dividing cell, the mYPet-FtsZ-mCyPet polymerization brings CFP and YFP in close proximity, and emit FRET fluorescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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