Bacteria under the physical constraints of periodic micro-nanofluidic junctions reveal morphological plasticity and dynamic shifting of Min patterns

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Morphological plasticity is an important survival strategy for bacteria adapting to stressful environments in response to new physical constraints. Here, we demonstrate *Escherichia coli* morphological plasticity can be induced by switching stress levels through the physical constraints of periodic micro-nanofluidic junctions. Moreover, the generation of diverse morphological aberrancies requires the intact functions of the divisome- and elongasome-directed pathways. It is also intriguing that the altered morphologies are developed in bacteria undergoing morphological reversion as stresses are removed. Cell filamentation underlies the most dominant morphological phenotypes, in which transitions between the novel pattern formations by the spatial regulators of the divisome, i.e., the Min system, are observed, suggesting their potential linkage during morphological reversion. © 2014 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4892860>]

Most bacteria have evolved sophisticated systems to manage their characteristic morphology by orchestrating the spatiotemporal synthesis of the murein sacculus (peptidoglycan exoskeleton), which is known to be the stress-bearing component of cell wall and presides over *de novo* generation of cell shape.¹ Morphological plasticity is attributed to a bacterial survival strategy as responding to stressful environments such as innate immune effectors, antimicrobial therapy, quorum sensing, and protistan predation.² It comes of no surprise that stress-induced diversified morphology and mechanisms, ascribed to shape control and determination, have drawn great attention in both fundamental and clinical studies.^{3–6} The molecular mechanism to form filamentous bacteria has been revealed that both β -lactam antibiotics³ and oxidative radicals produced by phagocytic cells⁵ trigger the SOS response, promoting cell elongation by inactivating cell division via the blockade of tubulin-like FtsZ, known as the divisome initiator. While apart from the scenario of length control by the divisome-directed filamentation, the elongasome assembled by proteins associated with actin-like MreB complex^{1,7,8} helps the insertion of peptidoglycans into lateral cell wall, suggesting the role in the determination of cell diameter during cell elongation.

Recently, additional mechanisms other than the divisome/elongasome-directed pathways of shape maintenance are discovered to regenerate normal morphology *de novo* from wall-less lysozyme-induced (LI) spheroplasts of *E. coli* via a plethora types of aberrant division intermediates.⁹ Similar morphological reversion from different aberrant bacterial shapes has been observed as squashed wild-type bacteria generated through sub-micron constrictions are released into connected microchambers.¹⁰ Previous work using the microfluidic approach focuses on the septation accuracy and robustness of constricted bacteria,¹¹ but the reversion process of stress-released

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bacteria is not well studied and analyzed. In particular, the aberrant bacterial shape is mainly branched-type with bent and curved variants in the reverting bacteria, analogous to the aberrant intermediate found in the morphological reversion of LI spheroplasts with PBP5-defective mutant.⁹ Since bacteria suffering from starvation¹² or confronting mechanical stresses exerted by phagocytosis and protistan grazing⁶ can induce morphological alterations, one could manipulate the stress levels of physical constraints by adopting repeated structures of sub-micron constricted channels (nanoslits) and microchambers,^{10,11} to select and enrich bacteria converting to specified aberrant intermediates. The stress incurred by the nanoslit on bacteria is about the mechanical intervention over *de novo* synthesis of the cell wall, which is the major factor causing morphological aberrancy, while the second environmental stress comes from bacterial growth in the restricted space of microchamber as bacteria proliferate to full confluency, resulting in growth pressure of high population density, nutrient deficiency, and the size reduction of bacteria.

Here, we report the selection of distinctive bacterial morphologies by size shrinkage in the outlet cross-section ($W \times H = 1.5 \times 1.5 \mu\text{m}$) of the terminal microchamber in the periodic structures of nanoslit-microchamber (Figs. 1(a) and 1(b)). The fluidic structures were micropatterned on fused silica wafers by photolithography, fabricated through reactive ion etching (RIE) and inductively coupled plasma (ICP) etching, and encapsulated by cover glasses coated with polydimethylsiloxane (PDMS) or polysilsesquioxane (PSQ) layer as described earlier.^{13,14} Two days after the outgrowth of *Escherichia coli* (imp4213 [MC4100 $\Delta\text{lamB106 imp4213}$]) loaded to the microfluidic device at 25 °C, bacteria started to penetrate into the nanoslit as they proliferated to full confluency in the first microchamber (Fig. 1(c)). It takes about 10 days for bacteria traversing 500 μm long (5 repeated nanoslit-microchamber units) via proliferations and being released from the outlet of the terminal microchamber. The narrowed outlet allows only bacteria with smaller diameters to be squeezed into the spacious and nutrient-rich region, thus it acts as a spatial filter to avoid the passage of branching bacteria with cross-sectional size larger than that of the outlet. The rationale of this design is to select aberrant bacteria prone to promote *de novo* shape regeneration other than the branched-type, which is the dominant morphology of

FIG. 1. (a) Schematics of the microfluidic device used in this study with an H-shaped geometry (left upper panel), where repeated nanoslit ($L \times W \times H = 50 \times 10 \times 0.4 \mu\text{m}$)–microchamber ($L \times W \times H = 50 \times 50 \times 1.5 \mu\text{m}$) structures are bridged between two arms of the H-shaped microchannels (left lower panel and enlarged view in right panel). (b) Top-view layout of an individual channel in (a) with close view of the outlet in the terminal microchamber (orange: nanoslits; blue: micro-chambers). (c) Fluorescence micrograph of *E. coli* imp4213 penetrating a nanoslit (scale bar: 5 μm). (d) Bright-field micrographs for various cell morphology of the selected imp4213 released from the outlet (magenta arrows: cells with vesicles; scale bar: 5 μm). (e) Sequential bright-field micrographs of morphological reversion. T1–T3 indicate the time after bacteria escaping from the outlet. T1: 3 h; T2: 6 h; T3: 24 h. Scale bar: 10 μm .

reverting bacteria in the prior microfluidic constriction study.¹⁰ As anticipated, the stress-released bacteria through the narrowed outlet are therefore mostly filamentous (see statistical analysis for cell morphology in the supplementary material).¹⁵ However, it is noted that the aberrant morphology of lemon-like shape with tubular poles (Figs. 1(d-1), 1(d-3), and 1(d-11)) is developed about 3 h after the stress-released bacteria escaped through the outlet. Though the generation of the lemon-like aberrancy in bacteria has been reported in PBP5/7-defective *E. coli* mutant subjected to a high-level inhibition of both MreB and FtsZ, while the same mutant treated with low-level MreB inhibitor, together with antagonized-FtsZ, displays filamentous shape with varying diameters,¹⁶ these morphological aberrances can be observed in our system (Figs. 1(d-2) and 1(d-12)). Besides, a high-level inhibition of MreB in *E. coli* with an intact divisome function is known to cause round bacteria, resembling to the cell morphology of the bacteria shown in Fig. 1(d-4). Interestingly, parallel experiments using bacteria mutants carrying impaired regulatory functions in either the divisome (Min^-) or the elongasome (MreB^-) do not develop morphological plasticity (supplementary Fig. S1).¹⁵ Taken together, the filamentous and lemon-like variants selected from our microfluidic platform, while elaborating the morphological plasticity and reverting progression, require both the functional divisome/elongasome. Alternatively, the selection by the spatial filter does not fully exclude cells with aberrant shapes such as the branched-type with initial budding (Fig. 1(d-7)), cells with asymmetric cross-section perpendicular to the longitudinal axis (Figs. 1(d-2), 1(d-8), 1(d-9), 1(d-9'), and 1(d-10)), and those resembling to the morphological phenotypes of the division intermediates reported in the LI-spheroplasts carrying genetic defects on some non-cytoskeletal proteins (Figs. 1(d-5) and 1(d-6)). In particular, intracellular vesicles and cell autolysis are observed in some reverting bacteria (Figs. 1(d-5) and 1(d-6)), which are reminiscent to the phenomena reported in the division intermediates of the LI-spheroplasts lacking stress response system (Rcs) or some accessory proteins (PBP1B and LpoB). Unlike the bacteria grow with odd shapes under the stress of nano-fluidic confinement only¹⁰ (Fig. 1(c)), all the morphological aberrancy reported here are developed in the reverting bacteria, which grow in the spacious and nutrition-rich environment and are free from physical constraints. Further investigations over the expression levels of the divisome/elongasome networks and the stress-response system in bacterial cells subjected to micro-nano fluidic junctions could be insightful in understanding their role in bacterial shape control.⁹

During the morphological reversion, the stress-released bacteria rapidly increase their size in the first 3 h after escaping from the terminal microchamber (T1 in Fig. 1(e)). Some filamentous bacteria even grow over 50 μm long, though such a morphological phenotype implicates the cessation of functional divisome. With active growth and proliferation, the progeny of stress-released bacteria increase their population but gradually reduce their size about 6 h after being released from the constriction stress (T2 in Fig. 1(e)). Fig. 2 displays the marginal histograms for different shape factors, where Fig. 2(a) is the plot of the *minimal Feret diameter* (cell diameter) versus *Feret diameter* (cell length), i.e., the shortest versus the longest distance between any two points with parallel tangents along the cell peripheral, respectively, indicating that cell diameters are larger for reverting bacteria at T1 (mean $\pm$ S.E.M. = $1.89 \pm 0.08 \mu\text{m}$) with respect to T2 ($1.51 \pm 0.06 \mu\text{m}$). Moreover, the histogram of Feret diameter depicts two major populations of the cell length for reverting bacteria at T1, which mostly resume to typical cell length at T2 (the median of Feret diameter = $3.33 \mu\text{m}$; see statistical analysis for Fig. 2 in the supplementary material).¹⁵ The shape factors of circularity ($4\pi \times [\text{area}]/[\text{perimeter}]^2$) and aspect ratio ($[\text{major axis}]/[\text{minor axis}]$ for the cell geometry fitted to an ellipse) confirm the existence of dual populations for bacteria at T1 as well (Fig. 2(b)). About 24 h after escaping (T3 in Fig. 1(e)), almost all the progeny of stress-released bacteria regained the rod shape.

The bacterial size reduction of filamentous and lemon-like shape variants, though involving negative control of the divisome positioning by the spatial regulators of MinCDE system,¹⁷ is not completely understood as to how they coordinate in aberrant geometries. Besides, the filamentation of stress-released bacteria during the period of T1 to T2 implicates the inhibition of functional divisome. With minimal perturbation of the divisome by leaky expression of GFP-MinD and MinE (imp4213/*P_{lac}-gfpmut2::minD minE*), the patterning dynamics of GFP-MinD in different bacterial morphology were time-lapse imaged during morphological reversion.

FIG. 2. Marginal histograms for shape factors measured from the reverting imp4213 at T1 and T2. (a) Minimal Feret diameter (cell diameter) versus Feret diameter (cell length). (b) Circularity versus aspect ratio. $N = 366$ for T1 and $N = 494$ for T2.

Intriguingly, more than the standing-wave-like pattern of MinD denoted in filamentous *E. coli*,¹⁸ we discovered bidirectional drifting of two standing-wave-like patterns of MinD occur in most reverting bacteria filaments (supplementary Figs. S2(a) and S2(b)).¹⁵ The bidirectional drifting in the longitudinal direction of the cells may be emanating from the cell poles (the blue upper panel of Fig. 3(a) and supplementary Fig. S2(c)¹⁵) and the cylinder region (the blue lower panel of Fig. 3(a) and supplementary Fig. S2(d)¹⁵). Furthermore, the MinD pattern transitions from the standing to traveling waves are occasionally observed (the lower panel of Figs. 3(a) and supplementary Fig. S2(e)¹⁵). Notably, the standing-wave-like MinD patterns exhibit bidirectional drifting along the cell longitudinal direction and intermittently change directions, implying the competition between coexisting MinD patterns can be supported under filamentous geometry. Despite there have been observations of multiple wave-packet of traveling waves in filamentous cells,¹⁹ the mixture of distinct wave-like MinD patterns have never been experimentally reported. While most intriguingly, multiple drifting movements of wave-like MinD patterns potentiate the mitigation of periodic minima in time-averaged Min gradient in the

FIG. 3. Kymographs for GFP-MinD dynamics in selected imp4213 cells during morphological reversion: (a) Mixture modes of standing wave packets and traveling wave. The left panel is the stacked fluorescence micrograph displaying cell shape (scale bar = $5 \mu\text{m}$). The kymograph is derived from the filamentous cell indicated by the green arrow (scale bar: 120 s horizontal; $5 \mu\text{m}$ vertical), where the lower panel follows the upper panel in time. The yellow windows indicate bidirectional-drifting standing wave packets, while the green indicates traveling waves (see also supplementary Fig. S2).¹⁵ (b) Sequential fluorescence micrographs of GFP-MinD in lemon-shape imp4213 show amoeba-like motion, with the first left a bright-field image (scale bar: $10 \mu\text{m}$). (c) Mixed modes of amoeba-like motion and waves in selected filamentous imp4213 cell indicated by the green arrow in the left panel (scale bar = $5 \mu\text{m}$). The filamentous cells depicted in (a) and (c) locate at the top region while the lemon-shape cell in (b) at the central region of the movie (time stamp in min:s). (Multimedia view) [URL: <http://dx.doi.org/10.1063/1.4892860.1>]

reverting filamentous bacteria, suggesting the disability of proper divisive positioning for recovering the typical rod shape. Apart from the wave-like movements, amoeba-like motion of Min proteins has been shown *in vitro* upon synthetic minimal system, but never been verified *in vivo*.²⁰ Strikingly, here amoeba-like motion of MinD is the dominant mode in lemon-like bacteria and the transitions between wave-like patterns and amoeba-like motion are supported even under filamentous geometry (Figs. 3(b) and 3(c), Multimedia view).

In summary, we have demonstrated that the development of bacterial morphological plasticity can be stress-induced by periodic physical constraints with intact functions of the divisive and elongasome-directed pathways. Through size exclusion, the constricted outlet structure designed in our microfluidic device is useful in selecting bacteria with plethora morphological aberrancies other than the branched type. Interestingly, disparate morphological changes, rather than those being directly induced under a stressful environment, can be generated in the stress-released bacteria experiencing morphological reversion. Further, the discovery of novel transitions between the Min patterns in most reverting bacteria implicates its regulatory effect of cell filamentation. However, by exploiting the micro-nanofluidic approach, further investigations of the mechanism underlying the development of morphological plasticity in bacteria adapting to physical constraints are expected in future studies to gain more insights into the molecular basis of shape generation.

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