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Polar positional information in *Escherichia coli* spherical cells

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Abstract

Shigella surface protein IcsA and its cytoplasmic derivatives are localized to the old pole of rod-shaped cells when expressed in *Escherichia coli*. In spherical *mreB* cells, IcsA is targeted to ectopic sites and close to one extremity of actin-like MamK filament. To gain insight into the properties of the sites containing polar material, we studied the IcsA localization in spherical cells. GFP was exported into the periplasm via the Tat pathway and used as a periplasmic space marker. GFP displayed zonal fluorescence in both *mreB* and *rodA-pbpA* spherical *E. coli* cells, indicating an uneven periplasmic space. Deconvolution images revealed that the cytoplasmic IcsA fused to mCherry was localized outside or at the edge of the GFP zones. These observations strongly suggest that polar material is restricted to the positions where the periplasm possesses particular structural or biochemical properties.

Keywords: Periplasm; Polar localization information; Spherical cells; MreB; GFP; mCherry

Polarity is an intrinsic property of cells. Many different bacterial species utilize polar localization for diverse cellular processes including chromosome segregation, protein secretion, macromolecule delivery, chemotaxis, adherence, motility, cell division, cell shape and virulence [1]. The *Shigella* outer membrane protein IcsA (VirG) is one of the most extensively studied polar proteins. It localizes to the pole of *Shigella*, where it mediates assembly of the actin tail and movement of this intracellular pathogen within the cytoplasm of a host cell [2]. IcsA is targeted and exported at the bacterial pole [3]. Each of two segments, residues 1–104 ($IcsA_{1–104}$) or 507–620 ($IcsA_{507–620}$), is sufficient for targeting IcsA to the pole on the cytoplasmic side of the inner membrane of rod-shaped cells [3,4]. The bacterial cytoskeletal protein MreB forms helical structures along the long axis of the cell, just beneath the cytoplasmic membrane (for review see [5]). Interestingly, the *mreB* mutation leads to ectopic localization of IcsA and its truncated $IcsA_{1–104}$ and $IcsA_{507–620}$ derivatives in the spherical cells [6,7]. Janakiraman and Goldberg have shown that polar positional information required for IcsA localization is independent of both known components of the cell division machinery and nucleoid occlusion [8]. Recently, two laboratories have independently identified a landmark protein, TipN, which acts as a spatial and temporal cue for setting up the correct polarity in the bacterium *Caulobacter crescentus* [9,10]. In contrast to IcsA, TipN does not localize to the division site in cells depleted of the division initiation protein FtsZ [9,10] or in cells in which the division site peptidoglycan synthesis protein FtsI has been inactivated [9].

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These observations would suggest that different mechanisms might be used for polar localization of IcsA and TipN. However, the polar positional information that mediates proper localization of the polar proteins and the mechanisms by which polar proteins recognize this positional information are poorly understood [1].

MamK actin proteins are found only in magnetotactic bacteria and involved in positioning bacterial organelles, magnetosomes [11,12]. When expressed in *Escherichia coli*, MamK appears as a linear filament which is structurally and functionally distinct from the MreB filament [13]. Interestingly, one extremity of the MamK filament is located close to the co-expressed IcsA in spherical *mreB* mutant cells [13], suggesting that the filament may not be randomly positioned within a geometrically symmetric spherical cell. In *E. coli*, like the *mreB* mutation, *rodA-pbpA* mutations affect periplasmic sidewall peptidoglycan synthesis and lead to spherical cells [5]. The cellular localization of penicillin-binding protein 2 (encoded by *pbpA*) depends upon the localization of both MreB and MreC in *C. crescentus* [14–16]. To gain more information regarding the polar material localization information in spherical cells, we compared the localization of IcsA in *mreB* and *rodA-pbpA* spherical cells. Green fluorescence protein (GFP) was exported into the periplasm via the twin-arginine translocation pathway and used as a periplasmic space marker under physiological conditions. Periplasmic GFP exhibited zonal localization in both mutants. Interestingly, $IcsA_{507–620}$-mCherry was located outside or at the edge of the GFP zones, but not overlaid on the GFP zones. These results would suggest that polar positional information recognized by IcsA resides at positions where the periplasm possesses particular structural or biochemical properties.

Materials and methods

Bacterial strains, plasmids, and media. Escherichia coli strains used in this study are: MC1000 (*araD139* (*ara*, *leu*)7697 *lacX74 galU galK rpsL*) and its derivative YLS3 (*mreB*) [6], GC3904 (*argS* Δ(*pbpA-rodA*)::Kan, *zbf*::Tn*10* [17] and TG1 (Δ(*lac-pro*) *supE thi hsdD5*/F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15).

Plasmids pBAD-$IcsA_{1–104}$-GFP, pBAD-$IcsA_{507–620}$-GFP (=pMPR402), pBAD-$IcsA_{\Delta 507–730}$-GFP and P*tac*-$IcsA_{507–620}$-mCherry (pAWY3) express hybrid proteins consisting of various segments of IcsA fused in frame to green fluorescent protein or mCherry under the control of the arabinose promoter of pBAD24 or the *tac* promoter [7]. To improve signal/noise ratio of periplasmic GFP fluorescence, the gfpmut2 in our previously described pRR-GFP plasmid [17] was changed with the gene encoding pH sensor derivative of GFP, the ratiometric pHluorin [18]. Compared to the wild type GFP, pHluorin displays a reversible excitation ratio change between pH 7.5 and 5.5; the major excitation peak is at 475 nm under acidic conditions [18]. Since the periplasm is more acidic than the cytoplasm due to the proton motive force, ratiometric pHluorin increases the signal/background ratio comparing to other green fluorescent protein derivatives when the same portion of the marker proteins are exported into the periplasm. The DNA fragment encoding for ratiometric pHluorin was amplified by PCR using PHR5NHE (5′-cgg tgc tag caa agg aga aga act ttt cac-3′) and PHR3H3 (5′-gaa tta agc tta ttt gta tag ttc atc cat gcc-3′) as primers, and the reaction was performed by using the Expand High Fidelity PCR System according to the manufacturer's instructions (Roche). The amplified fragment was purified, double digested by *Nhe*I and *Hin*dIII and cloned into the corresponding sites of the plasmid pRR-GFP, resulting to plasmid p8799.

The bacteria were routinely grown in Luria–Bertani (LB) medium or on LB plates [19]. As required, ampicillin (Amp) (100 μg/ml), chloramphenicol (30 μg/ml), glucose (0.2% w/v), arabinose (0.2% w/v) or IPTG (0.5 mM) were added.

Microscopy and fluorescence spectrometry. Overnight cultures were diluted 1:100 for wild type strain and 1:50 for the mutants in LB+Amp+glucose medium and incubated at 37 °C with shaking for 3 h. Cells were centrifuged, washed once with LB+Amp medium, and resuspended in LB+Amp with 0.2% arabinose or/and IPTG (0.5 mM), and grown at 20 °C for 80 min with shaking. The cells were examined directly, or after the addition of 0.15 M NaCl or other treatment as described in the text. Protein synthesis was blocked by the addition of rifampicin (0.15 μg/ml) to the culture. Samples were taken 1 h later and examined under fluorescence microscope. Cells were fixed in 0.25% agarose on slides. Images and *Z*-stack of 25–33 images were captured with step distance ranging from 0.15 or 0.25 μm with Zeiss Axiovert 200M connected with a Hamamatsu ORCA ER camera. Image restoration was obtained by deconvolution using Huygens Essential software (SVI). Three-dimensional visualization was performed with Imaris software package (Bitplane). Immuno-gold staining of ultra-thin frozen sections were performed by using 7 nm gold-conjugated protein A as described by Anba et al. [20]. Antisera used are polyclonal rabbit anti GFP [17] or RFP (Chemicon International, Temecula, California, USA) at dilution of 1/500 and 1/100, respectively.

Results and discussion

Localization of polar $IcsA_{507–620}$-GFP fusion in spherical rodA-pbpA cells

In *E. coli*, mutation of the *mreB* gene results in spherical cells [21,22]. Interestingly, residues 507–620 of IcsA, which are sufficient to localize a GFP fusion to the pole of rod-shaped cells, localizes GFP to ectopic sites in spherical *mreB* cells [6,7]. In addition, the *Vibrio cholerae* type II secretion protein EpsM [7] and the *E. coli* chemotaxis protein Tar [6] are also localized at ectopic sites in the *mreB* mutant. Recent studies showed that MreB and its homologue Mbl govern spatial localization of proteins involved in peptidoglycan biosynthesis and influence the cellular morphology [14,15,23]. Penicillin-binding protein 2, encoded by *pbpA,* and RodA play important roles in peptidoglycan synthesis [24], and mutation of either the *rodA* or the *pbpA* gene results in round cells [25,26]. MreB localization is disrupted in RodA-depleted cells [27]. We sought to assess whether the distribution of the polar protein IcsA in *rodA-pbpA* spherical cells would be similar to its distribution in *mreB* cells.

As has been reported for the *mreB* cells [7], $IcsA_{507–620}$-GFP appeared as fluorescent foci in *rodA-pbpA* cells. The number of foci per cell ranged from 1 to 4, increasing proportionally to the cell volume (Fig. 1A). The similar localization of the fluorescent foci to multiple sites in *mreB* and *rodA-pbpA* cells is consistent with the observation that *mreB* depletion leads to mislocalization of Pbp2, hence spherical cellular morphology [16]. Whereas cells of stationary phase cultures could have more foci, most cells of exponential phase cultures had only one spot per cell, probably because they have recently divided and have not yet grown. To avoid formation of inclusion bodies due to over-