Recruitment of ZipA to the Septal Ring of Escherichia coli Is Dependent on FtsZ and Independent of FtsA

CYNTHIA A. HALE AND PIET A. J. DE BOER*

Department of Molecular Biology and Microbiology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4960

Received 13 July 1998/Accepted 26 October 1998

Cell division in prokaryotes is mediated by the septal ring. In Escherichia coli, this organelle consists of several essential division proteins, including FtsZ, FtsA, and ZipA. To gain more insight into how the structure is assembled, we studied the interdependence of FtsZ, FtsA, and ZipA localization using both immunofluorescence and Gfp tagging techniques. To this end, we constructed a set of strains allowing us to determine the cellular location of each of these three proteins in cells from which one of the other two had been specifically depleted. Our results show that ZipA fails to accumulate in a ring shape in the absence of FtsZ. Conversely, depletion of ZipA does not abolish formation of FtsZ rings but leads to a significant reduction in the number of rings per unit of cell mass. In addition, ZipA does not appear to require FtsA for assembly into the septal ring and vice versa. It is suggested that septal ring formation starts by assembly of the FtsZ ring, after which ZipA and FtsA join this structure in a mutually independent fashion through direct interactions with the FtsZ protein.

Cell division in bacteria occurs by the coordinated invagination of the cell envelope layers that make up the cell wall. Work in the past few years has revealed that this process is mediated by a membrane-associated, cytoskeleton-like organelle which assembles at the prospective division site before the onset of septal invagination and which remains associated with the ingrowing cell wall until septal closure (24, 32). In Escherichia coli, septum formation requires at least nine gene products, FtsA, -I, -K, -L, -N, -P, -Q, -W, -Z, and ZipA, which are specifically dedicated to this process. All these essential division proteins have recently been shown to be part of the septal ring structure in E. coli (2–4, 17, 25, 35, 39, 40) with the exception of FtsL and -Q. A distant homolog of FtsQ (DivIB), however, was recently localized to the septal ring of Bacillus subtilis (19), and it may be expected that FtsQ, as well as FtsL, is associated with the organelle during some stage of the division cycle in E. coli.

The phylogenetically highly conserved FtsZ protein is a major component of the septal ring and plays a key role in the division process. This protein is a tubulin-like GTPase which can form large protofilament-like polymers in vitro (9, 15, 23, 26–28, 31, 38, 41). In vivo, FtsZ moves from the cytoplasm to accumulate in a ring-like structure at the prospective division site early in the division cycle (4). Based on the in vitro properties of FtsZ, this ring is thought to be formed by a self-assembly reaction and to consist of a homopolymeric form of the protein. How formation of the FtsZ ring is initiated and by what mechanism it is normally restricted to certain sites on the cell envelope remains unclear. It is conceivable, however, that it involves the interaction of FtsZ with a hypothetical membrane factor (factor X) which marks potential division sites and stimulates polymerization of the protein at these sites.

We recently used an in vitro assay to search for factors in E. coli which interact directly with the FtsZ protein. This approach led to the discovery of a previously unknown division protein, which we called ZipA (17). ZipA is essential for cell division, and our results indicate not only that ZipA and FtsZ associate with each other in vitro but that this interaction also occurs in vivo and is required for cell constriction. Evidence suggests that ZipA is a bitopic integral membrane protein of type Ib of which the N terminus traverses the inner membrane once and of which the rest of the protein resides in the cytoplasm. Furthermore, localization studies indicate that ZipA and FtsZ interact primarily within the septal ring structure. Thus, like FtsZ, a ZipA-Gfp fusion protein was found to accumulate very early in the division cycle in a ring structure at the prospective division site and to remain associated with the invaginating septum during the cell constriction process (17).

In addition to its interaction with ZipA, recent two-hybrid experiments (14, 37) as well as localization studies (3, 25) have indicated that FtsZ also directly interacts with the FtsA protein, supporting previous genetic experiments suggesting such an interaction (8, 13). FtsA is a phosphoprotein which is peripherally associated with the membrane. It appears to belong to a family of ATPases which also includes actin, sugar kinases, and Hsp70 proteins (5, 33), but its role in the division process is presently not understood.

The observations that ZipA is an early component of the septal ring and that it binds to both FtsZ and the cytoplasmic membrane raise the possibility that the protein is directly involved in the assembly of FtsZ and/or other components, such as FtsA, into the septal ring organelle. In assessing the possible roles of ZipA in the division process, we have used both direct immunofluorescence and Gfp tagging techniques to study the interdependence of ZipA, FtsZ, and FtsA localization. To this end, we constructed a set of strains allowing us to determine the cellular location of each of these three proteins in cells from which one of the other two had been specifically depleted.

Our results show that ZipA fails to accumulate in a ring shape in the absence of FtsZ. Conversely, depletion of ZipA does not abolish formation of FtsZ rings but leads to a significant reduction in the number of rings per unit of cell mass. In...
addition, ZipA does not appear to require FtsA for assembly into the septal ring and vice versa. It is suggested that septal ring formation starts by assembly of the FtsZ ring, after which ZipA does not appear to require FtsA for assembly into the septal ring and vice versa. It is suggested that septal

### MATERIALS AND METHODS

#### Strains

Unless stated otherwise, cells were grown at 37°C in Luria-Bertani (LB) medium supplemented, where appropriate, with antibiotics at concentrations of 50 μg/ml (ampicillin, kanamycin, and spectinomycin), 25 μg/ml (chloramphenicol), and 12.5 μg/ml (tetracycline).

Strains GC13109 (his rpsL sulA366 leu), GC13301 (his rpsL sulA366 leu recA0), DR120 (imm21 bla), and DR120 as described previously (17). To obtain CH50, CH5 CH50 as described previously (17). Strain PB103 (ftsA (Ts), ftsZ (Ts)) ftsA (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (Ts) ftsZ (T
RESULTS

Localization of ZipA, FtsZ, and FtsA. Assembly of ZipA, FtsZ, and FtsA into the septal ring was monitored by the application of both indirect immunofluorescence and Gfp tagging techniques.

Immunofluorescence microscopy was performed essentially as described previously (1). To detect FtsZ, we used a mixture of monoclonal antibodies kindly provided by Jan Voskuil (34). For detection of ZipA and FtsA, we used affinity-purified polyclonal antibodies. Using this technique, all three division proteins were found to localize to the septal ring in normally dividing cells (Fig. 2a to c). The specificity of each antibody preparation was confirmed by strongly diminished signals after depletion of the corresponding antigen from cells, as judged by both Western analyses and immunofluorescence microscopy (results not shown) (see below).

Gfp tagged derivatives of ZipA, FtsZ, or FtsA were expressed from lysogenic λ phages containing the appropriate gene fusion downstream of the lac promoter. Phage λACH50 [lac::zipA-gfp] was described before (17) and encodes a ZipA-Gfp fusion in which Gfp65ST (20) is fused to the C terminus of ZipA. In addition, we constructed λADR120 [lac::gfp::ftsZ] and λCH75 [lac::gfp::ftsA] encoding Gfpmut2 (7) fused to the N terminus of FtsZ and FtsA, respectively. At sufficiently high levels of expression, all three Gfp tagged proteins prevented cell division, leading to the formation of filamentous cells (not shown). At the low levels of expression used in this study, in contrast, none of the fusion proteins interfered substantially with the division process, and all three localized to the septal ring in normally dividing cells (Fig. 2d to f).

Both when using each of the antibodies as well as when using each of the Gfp tagged proteins, we detected a single ring structure in the majority (71 to 88%) of exponentially grown cells with a normal division phenotype (Tables 2 and 3). In all cases, the subpopulation that lacked a clearly detectable ring structure consisted primarily of the smallest cells in the population, and the percentage of cells with more than one ring structure was less than 1%. We found that, particularly when using immunofluorescence detection, the exact percentage of cells with a clearly visible fluorescent ring could vary by as much as 20% between some repeated experiments (not shown). Differences in the 70 to 90 percentile range are, therefore, unlikely to be of biological significance, but rather due to inherent experimental variables.

We conclude that all three Gfp tagged division proteins used in this study are suitable markers for the septal ring structure.

Strains for HID or CID of division proteins. Throughout this study, we used two types of strains which allowed for the specific, temperature-dependent depletion of ZipA, FtsZ, or FtsA (Table 1). For convenience, we use the acronyms HID (for heat-induced depletion) to indicate one type and CID (for cold-induced depletion) to indicate the other type.

In HID strains, a chromosomal null allele of one of the division genes is complemented by a wild-type allele which is present on \(repA(Ts)\) derivative of plasmid \(pSC101\) (18). HID strains used for depletion of ZipA, FtsZ, and FtsA were CH5/pCH32 [zipA::aph::repA(Ts) zipA ftsZ], PB143/pCX41 [ftsZ\(^{repA(Ts)}\) repA(Ts) ftsZ\(^{repA} \)], and CH2/pDB280 [ftsA\(^{repA(Ts)}\) repA(Ts) ftsA\(^{repA} \)]. All strains were grown at 30°C but formed long filaments at 42°C due to a failure of the complementing plasmids to repli-
appropriate antibodies. Cells in panels d to f were grown at 30°C in the presence of 25 μM IPTG and 50 μM (d and e) or 50 μM (f) IPTG, and were observed immediately after chemical fixation. Results with a number of other normally dividing lysogens [e.g., PB103 (ACH50), CH3 (ACH75), and PB103 (ADR120)] were similar to those shown in panels d to f. Bar in panel a represents 2.0 μm.

To study whether FtsA is required for the localization of ZipA-Gfp in cells from which FtsZ had been specifically depleted. For this experiment, the FtsZCID strain PB143/pDB346 (ftsZΔ/cI857 P_{AR}:ftsZ), containing zipA-gfp downstream of the IPTG-inducible lac promoter (17). Cells were grown at 30°C in the presence of 25 μM IPTG and observed by fluorescence microscopy. As shown in Fig. 4a, cells formed long nonseptate filaments due to depletion of the FtsZ protein. Moreover, ZipA-Gfp failed to accumulate in ring structures but appeared to be evenly distributed along the periphery of the filaments. Of 133 filaments, representing a total cell length of 2,564 μm, only a single ring structure was seen in each of six relatively short filaments (Table 3). These results suggested that ZipA-Gfp was still associated with the cytoplasmic membrane, but required FtsZ in order to localize to the septal ring.

To confirm these observations, we used indirect immunofluorescence to determine the location of native ZipA protein in cells of strain PB143/pCX41 [ftsZΔ repA(Ts) ftsZ−] which had been depleted of FtsZ by growth at 42°C. Similar to the distribution of ZipA-Gfp in the experiment described above, native ZipA failed to accumulate into ring structures but was located along the periphery of the FtsZ filaments (Fig. 4b). Also in this experiment, only a small minority (4%) of the filaments contained a single fluorescent ring (Table 2). We conclude that FtsZ is required for localization of ZipA to the septal ring structure.

FIG. 2. Localization of ZipA, FtsA, and FtsZ in wild-type cells. Fluorescence (a through f) and corresponding differential interference contrast (a' through f') micrographs showing the location in normally dividing cells of both native (a to c) and Gfp tagged (d to f) ZipA, FtsA, and FtsZ proteins, respectively. Cells shown are from strains PB103 (wild type [wt]) (a to c), CH3 (ACH50) pDB355 [wt(P_{lac}:zfpr-gfp)/cI857 P_{AR}:ftc4A] (d), PB103 (CH75) [wt(P_{lac}:zfpr-gfp)/cI857 P_{AR}:ftc4A] (e), and CH3 (ADR120) pDB361 [wt(P_{lac}:zfpr-gfp)/cI857 P_{AR}:ftc4A] (f). Cells in panels a to c were grown at 37°C prior to immunofluorescence staining with the correspondent ZIPA-GFP failed to accumulate into ring structures but was located along the periphery of the FtsZ filaments. Cells in panels d to f were grown at 37°C prior to immunofluorescence staining.
ZipA and ZipA filaments showed multiple fluorescent ring structures. As shown in Fig. 4c and d, the majority of both FtsZ filaments in an FtsA-independent fashion. Whereas over 90% of these filaments contained ring structures, the number of rings per unit of cell length was approximately half that found in wild-type cells. Thus, as indicated in Tables 2 and 3, one ZipA ring was present approximately every 3 μm on average in wild-type cells, whereas we detected on average only one ZipA ring per every 6 μm in FtsA-depleted filaments (CID or HID). This increase in the ratio of unit length per ZipA ring in these filaments parallels a similar increase in the length/FtsZ ring ratio after depletion of FtsA (see below and Tables 2 and 3) and presumably reflects the loss of a stabilizing effect of FtsA on the septal ring structure (1). These results indicate that ZipA joins the FtsZ ring in an FtsA-independent fashion.

**Localization of FtsZ in ZipA** and FtsA** filaments.** The finding that ZipA failed to localize to the septal ring after depletion of FtsZ suggests either that the incorporation of both proteins into the structure depended on the protein partner or that formation of the FtsZ ring can occur independently of ZipA. To assess which of these possibilities is most likely correct, we studied the localization of FtsZ in cells from which ZipA had been depleted. To this end, the ZipA** CID** strain CH5/pDB361 (zipA::aph/c857 Paph::zipA) was lysogenized with phage ADR120 (Pg::gfp-ftsZ), and cells of a resulting lysogen were shifted from 42 to 30°C in the presence of 50 μM IPTG. As illustrated in Fig. 5a, many of the resulting ZipA-depleted filaments showed multiple fluorescent ring structures, suggesting that FtsZ rings were still being formed. Not all filaments showed rings, however, and of 64 filaments examined, rings were absent in 12 (19%) cells. In addition, the majority of filaments contained fewer ring structures than one might expect. Thus, whereas in the ring-containing portion of a wild-type population the ratio of cell length to FtsZ ring was approximately 2.7 μm, this ratio was significantly higher (8.6 μm) in the portion of ZipA CID filaments that contained ring structures. In addition, there was a large variation in the value of this ratio among individual filaments, ranging from 4.9 to 40.2 μm (Table 3).

Quite similar results were obtained after staining native FtsZ in heat-induced filaments of the ZipA** HID** strain CH5/pCH32 (zipA::aph/c857 Paph::zipA) (Fig. 5b; Table 2). In this case, 28% of the ZipA-depleted filaments showed no rings at all, and the length-to-ring ratio in the portion of filaments that did contain one or more rings was 17.2 μm.

Multiple FtsZ rings were previously also observed in filaments of a strain carrying a ts allele of ftsA, indicating that the formation of the FtsZ ring does not require fully functional FtsA (1). Consistent with those results, we found that FtsZ rings were also still present in filaments from which FtsA had

### Table 2. Quantitation of cell length and fluorescent rings after HId of FtsZ, FtsA, or ZipA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Depleted protein</th>
<th>Antibody</th>
<th>% (n)</th>
<th>Avg length (μm) (range)</th>
<th>% (n)</th>
<th>Avg length (μm) (range)</th>
<th>L/R (μm) (range)</th>
<th>Rings/cell (range)</th>
<th>L/R (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB103</td>
<td>NA</td>
<td>Anti-FtsZ</td>
<td>29 (21)</td>
<td>1.5 (1.3–1.8)</td>
<td>71 (52)</td>
<td>2.1 (1.4–2.8)</td>
<td>2.1 (1.4–2.8)</td>
<td>0.71 (0–2.7)</td>
<td>2.7</td>
</tr>
<tr>
<td>CH2/pDB280</td>
<td>FtsA</td>
<td>Anti-FtsZ</td>
<td>0 (0)</td>
<td>NA</td>
<td>100 (27)</td>
<td>29.7 (10.5–55.6)</td>
<td>6.2 (4.6–10.1)</td>
<td>4.78 (10–16.2)</td>
<td>6.2</td>
</tr>
<tr>
<td>CH5/pCH32</td>
<td>ZipA</td>
<td>Anti-FtsZ</td>
<td>28 (41)</td>
<td>45.4 (10.3–75.9)</td>
<td>72 (103)</td>
<td>37.7 (10.1–83.2)</td>
<td>17.2 (3.3–89.5)</td>
<td>1.56 (0–5)</td>
<td>25.5</td>
</tr>
<tr>
<td>PB103</td>
<td>NA</td>
<td>Anti-FtsA</td>
<td>18 (14)</td>
<td>1.6 (1.3–1.8)</td>
<td>82 (63)</td>
<td>2.3 (1.3–3.7)</td>
<td>2.3 (1.3–3.7)</td>
<td>0.82 (0–1)</td>
<td>2.6</td>
</tr>
<tr>
<td>CH2/pCX41</td>
<td>FtsZ</td>
<td>Anti-FtsA</td>
<td>96 (90)</td>
<td>14.5 (2.4–39.2)</td>
<td>4 (4)</td>
<td>2.9 (2.5–3.7)</td>
<td>2.9 (2.5–3.7)</td>
<td>0.04 (0–1)</td>
<td>329.8</td>
</tr>
<tr>
<td>CH5/pCH32</td>
<td>ZipA</td>
<td>Anti-FtsA</td>
<td>21 (9)</td>
<td>40.8 (9.1–65.4)</td>
<td>79 (36)</td>
<td>27.6 (8.6–90.8)</td>
<td>12.0 (5.8–40.8)</td>
<td>1.81 (0–5)</td>
<td>16.7</td>
</tr>
<tr>
<td>PB103</td>
<td>NA</td>
<td>Anti-ZipA</td>
<td>25 (14)</td>
<td>1.7 (1.3–1.90)</td>
<td>75 (41)</td>
<td>2.3 (1.3–3.6)</td>
<td>2.3 (1.3–3.6)</td>
<td>0.75 (0–1)</td>
<td>2.9</td>
</tr>
<tr>
<td>PB143/pCX41</td>
<td>FtsZ</td>
<td>Anti-ZipA</td>
<td>96 (78)</td>
<td>17.3 (2.3–53.4)</td>
<td>4 (3)</td>
<td>8.1 (2.2–18.8)</td>
<td>8.1 (2.2–18.8)</td>
<td>0.04 (0–1)</td>
<td>457.6</td>
</tr>
<tr>
<td>CH2/pDB280</td>
<td>FtsA</td>
<td>Anti-ZipA</td>
<td>21 (1)</td>
<td>16.1 (NA)</td>
<td>98 (40)</td>
<td>24.6 (7.1–50.0)</td>
<td>5.3 (3.9–21.5)</td>
<td>4.34 (0–10)</td>
<td>5.6</td>
</tr>
</tbody>
</table>

*Cells were treated and analyzed as described in the text. Some parameters were calculated separately for cells without fluorescent ring structures (R– cells), cells with one or more rings (R+ cells), and all cells combined (R+ + R– cells). NA, not applicable.*

### Table 3. Quantitation of cell length and fluorescent rings after CID of FtsZ, FtsA, and ZipA

<table>
<thead>
<tr>
<th>Strain</th>
<th>Depleted protein</th>
<th>Marker</th>
<th>% (n)</th>
<th>Avg length (μm) (range)</th>
<th>% (n)</th>
<th>Avg length (μm) (range)</th>
<th>L/R (μm) (range)</th>
<th>Rings/cell (range)</th>
<th>L/R (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB103(ADR120)</td>
<td>NA</td>
<td>Gfp-FtsZ</td>
<td>12 (14)</td>
<td>2.1 (1.7–3.1)</td>
<td>88 (104)</td>
<td>2.7 (1.6–4.4)</td>
<td>2.7 (1.6–4.4)</td>
<td>0.88 (0–1)</td>
<td>3.0</td>
</tr>
<tr>
<td>CH2(ADR120)/pDB55</td>
<td>FtsA</td>
<td>Gfp-FtsZ</td>
<td>7 (4)</td>
<td>56.1 (18.7–98.9)</td>
<td>93 (50)</td>
<td>34.4 (11.9–58.1)</td>
<td>6.3 (4.7–35.7)</td>
<td>5.04 (10–11)</td>
<td>7.2</td>
</tr>
<tr>
<td>CH5(ADR120)/pDB361</td>
<td>ZipA</td>
<td>Gfp-FtsZ</td>
<td>19 (12)</td>
<td>36.1 (16.8–63.4)</td>
<td>81 (52)</td>
<td>35.5 (5.7–85.5)</td>
<td>8.6 (4.9–40.2)</td>
<td>3.36 (0–9)</td>
<td>10.6</td>
</tr>
<tr>
<td>PB103(ACH75)</td>
<td>NA</td>
<td>Gfp-FtsA</td>
<td>15 (20)</td>
<td>2.3 (1.6–4.0)</td>
<td>85 (114)</td>
<td>2.9 (1.8–6.3)</td>
<td>2.9 (1.8–6.3)</td>
<td>0.85 (0–1)</td>
<td>3.3</td>
</tr>
<tr>
<td>CH2(ACH75)/pDB346</td>
<td>FtsZ</td>
<td>Gfp-FtsA</td>
<td>100 (50)</td>
<td>36.7 (5.3–66.3)</td>
<td>0 (0)</td>
<td>NA</td>
<td>NA</td>
<td>0 (NA) &gt;2054.7</td>
<td></td>
</tr>
<tr>
<td>CH5(ACH75)/pDB361</td>
<td>ZipA</td>
<td>Gfp-FtsA</td>
<td>8 (7)</td>
<td>35.4 (15.0–56.3)</td>
<td>92 (79)</td>
<td>43.0 (16.8–84.0)</td>
<td>9.4 (6.1–64.9)</td>
<td>4.20 (0–10)</td>
<td>10.1</td>
</tr>
<tr>
<td>PB103(ACH50)</td>
<td>NA</td>
<td>ZipA-Gfp</td>
<td>14 (16)</td>
<td>2.0 (1.5–3.9)</td>
<td>86 (100)</td>
<td>2.7 (1.5–4.2)</td>
<td>2.7 (1.5–4.2)</td>
<td>0.86 (0–1)</td>
<td>3.0</td>
</tr>
<tr>
<td>CH2(ACH50)/pDB355</td>
<td>FtsZ</td>
<td>ZipA-Gfp</td>
<td>95 (127)</td>
<td>19.7 (1.9–62.5)</td>
<td>5 (6)</td>
<td>10.5 (2.6–26.9)</td>
<td>10.5 (2.6–26.9)</td>
<td>0.05 (0–1)</td>
<td>427.3</td>
</tr>
</tbody>
</table>

*Cells were treated and analyzed as described in the text. Columns list the same parameters as those described in the footnotes to Table 2 except that the third column (Marker) indicates the Gfp fusion that was used to mark septal rings. NA, not applicable.*
been depleted. Our results are illustrated in Fig. 5c and d, showing filaments of, respectively, the FtsA CID lysogen CH2 (ADR120)/pDB355 [ftsA0(Plac::gfp-ftsZ)/cI857 PllR::ftsA], which was grown at 30°C in the presence of 50 μM IPTG, and of the FtsA HID strain CH2/pDB280 [ftsA0/repA(Ts)ftsA1], which was grown at 42°C and immunostained with FtsZ-specific antibodies. One or more FtsZ rings were present in over 90% of both the FtsA CID and FtsA HID filaments, and the average length-to-ring ratio in both types was approximately 6.3 μm (Tables 2 and 3).

We conclude that FtsZ can still assemble into a ring structure under conditions in which the cellular concentration of either ZipA or FtsA is very low. It seems clear, however, that depletion of either protein leads to a significant reduction in the number of FtsZ ring structures per unit of cell mass. Localization of FtsA in FtsZ and ZipA filaments. In a previous study, it was shown that formation of the FtsZ ring is a prerequisite for the localization of FtsA to the septal ring structure (3). Consistent with the results of this study, we found that FtsA failed to localize in filaments from which FtsZ had been depleted. Thus, no fluorescent rings were observed in filaments of the FtsZ CID strain PB143/pDB346 [ftsZ0(Plac::gfp-ftsZ)/cI857 PllR::ftsZ], lysogetic for λCH75 (Pλ::gfp-ftsA) and grown at 30°C in the presence of 25 μM IPTG (Fig. 6a; Table 3). Similarly, FtsA-specific antibodies failed to stain septal ring structures in the vast majority (96%) of FtsZ HID filaments of strain PB143/pCX41 [ftsZ0(Plac::repA(Ts)ftsZ)] (Table 2).

Experiments described above indicated that the localization of ZipA to the septal ring requires FtsZ but occurs independently of the FtsA protein. To test whether FtsA localization is dependent on ZipA, we studied how depletion of ZipA affected the localization of FtsA. For this purpose, phage λCH75 (Pλ::gfp-ftsA) was introduced into the ZipA CID strain CH5/pDB361 (zipA::aph/cI857 PllR::zipA), and a resulting lysogen was incubated at 30°C in the presence of 25 μM IPTG and inspected by fluorescence microscopy. Many of the resulting filaments (92%) showed one or multiple fluorescent ring structures (Fig. 6b; Table 3), suggesting that depletion of ZipA does...
not prevent FtsA from associating with the septal ring. Again, similar results were obtained when heat-induced filaments of CH5/pCH32 (zipA::aph/repA$^{ts}$ zipA ftsZ) were subjected to immunostaining with FtsA-specific antibodies (Fig. 6c; Table 2). As was observed for FtsZ rings above, the number and distribution of FtsA rings were quite heterogeneous among individual filaments of both the ZipA$^{CID}$ and ZipA$^{HID}$ populations. These results indicate that FtsA does not require ZipA in order to associate with the septal ring.

DISCUSSION

The septal ring in E. coli is a complex structure consisting of at least seven different protein products. Important challenges concerning this organelle are to understand its precise molecular architecture, its mode of assembly, and the mechanism whereby it mediates the coordinated invagination of the cell envelope layers during septum formation. The order in which the different components assemble to form a mature septal
ring has been studied in several laboratories by determining the localization of FtsA, -I, -N, -K, and -Z in filaments in which one of the essential division proteins has either lost function due to a conditional mutation or drug treatment or is largely lacking, due to a specific block in gene expression (2, 3, 6, 22, 29, 35, 39, 40). The picture emerging from these studies is that, first, FtsZ assembles at the prospective division site to form the FtsZ ring. FtsA then joins the FtsZ ring, followed by FtsI, FtsK and FtsN. The best evidence supporting early assembly of the FtsZ ring has come from the observations that FtsA (reference 3 and this study), FtsI (35), FtsK (40) and FtsN (2) completely failed to localize properly in FtsZ² filaments whereas, on the other hand, FtsZ rings were still present in FtsA² (reference 1 and this study), FtsI² (1, 29, 35, 39), FtsN² (2), FtsK² (40), FtsQ² (1), and FtsW² (6, 22) filaments.

We recently identified ZipA as a novel division factor and showed that it is an integral inner membrane protein which interacts directly with FtsZ. A ZipA-Gfp fusion protein, furthermore, localized to the septal ring at an early stage of the division cycle (17). Here, we confirmed this localization pattern for native ZipA by immunofluorescence microscopy.

The combined properties of ZipA raised the possibility that the protein plays a role in assembly of the FtsZ ring by attracting FtsZ to the prospective division site. The principal conclusion of this study is that this possibility is most likely incorrect. In FtsZ-depleted filaments, the bulk of both native ZipA or ZipA-Gfp clearly failed to accumulate at potential division sites but, rather, appeared to be evenly distributed over the cell membrane. Conversely, both native FtsZ and a Gfp-FtsZ fusion protein still assembled into ring structures in the majority of ZipA-depleted filaments. It is most likely, therefore, that the localization of ZipA to the septal ring is secondary to that of FtsZ.

In support of the conclusion that FtsA localization is also
dependent on FtsZ (3), we found that FtsA and Gfp-FtsA failed to localize after depletion of FtsZ, but that FtsZ rings could still be formed in FtsA-depleted filaments. Interestingly, however, ZipA rings were still present in FtsA-depleted filaments, and FtsA rings were still present in ZipA-depleted filaments, indicating that the incorporation of ZipA into the septal ring does not require the prior localization of FtsA and vice versa. Combined with the knowledge that both ZipA and FtsA localize early in the division cycle and that both can bind FtsZ directly, we propose that both proteins become associated with the FtsZ ring either during or very soon after formation of the latter. In this regard, it is interesting that whereas the vast majority of FtsZ- or ZipA-depleted filaments appeared completely smooth, more than half of the FtsA-depleted filaments (HID or CID) showed one or more marked indentations of the cell wall (Fig. 4 to 6). This suggests that despite the early localization of FtsA, the early stages of separation are less sensitive to depletion of the protein than are later stages.

Although the scenario proposed above represents the most straightforward interpretation of our results, it is difficult to completely rule out an essential role for ZipA in the assembly of the FtsZ ring. Although the ZipA-depleted filaments used in this study clearly contained an insufficient level of ZipA to support cell division, it cannot be excluded that this level (~10% of normal [Fig. 3]) may have been sufficient to actively stimulate formation of FtsZ rings. This same argument also applies to FtsA, -I, -N, -K, -Q, and -W in the studies in which stimulus formation of FtsZ rings. This same argument also applies to FtsA, -I, -N, -K, -Q, and -W in the studies in which.

In these same studies, the ratio of cell length to the number of FtsZ rings in most types of FtsZ filaments was much higher than expected, and a large variability in this ratio between individual filaments was observed. Similarly, we observed that the average length-to-FtsZ ring ratios in the ring-containing populations of both ZipA-depleted (17.2 μm in ZipA-HID and 8.9 μm in ZipA-TD) and FtsA-depleted (6.2 μm in FtsA-HID and 6.3 μm in FtsA-TD) filaments was significantly higher than that in wild-type cell (~2 to 3 μm). In addition, this ratio varied widely between individual filaments. For instance, after heat-induced depletion of ZipA, 72% of the filaments (ranging in size from 10.1 to 83.1 μm) showed from one to five FtsZ rings per cell with a length-to-ring ratio ranging from 3.3 to 89.0 μm, and the remaining 28% (ranging in size from 10.3 to 76.9 μm) showed no ring at all (Table 2). After CID of ZipA, 81% of the population (ranging in size from 5.7 to 85.5 μm) contained from one to nine Gfp-FtsZ rings per cell with a ratio ranging from 4.9 to 40.2 μm, and the remaining 19% (ranging in size from 16.8 to 63.4 μm) were devoid of rings (Table 3). The length-to-ZipA ring ratio in FtsA- filaments and length-to-FtsA ring ratio in ZipA- filaments were also relatively high and variable which, given the high and variable length to FtsZ ring ratio in FtsA- and ZipA- filaments, is consistent with the interpretation that the incorporation of FtsA and ZipA into the septal ring is dependent on formation of the FtsZ ring component.

Why the numbers of FtsZ rings are so low and variable in ZipA-depleted cells (and other types of filaments) relative to wild-type cells is an intriguing question. The possibility that depletion of ZipA simply leads to a reduction in the cellular concentration of FtsZ was ruled out by quantitative immunoblot analyses showing that depletion of 90% of ZipA affected the FtsZ concentration by less than 5% (not shown). Inefficient fixation and/or staining of cells could lead to an underestimation of the number of ring structures by immunofluorescence, but we consider it unlikely that this was a determining factor in our experiments, especially since we obtained largely comparable results using Gfp tagged proteins as septal ring markers. One reasonable hypothesis is that binding of ZipA to the FtsZ ring stabilizes the structure, for instance, by providing an anchor to the cell membrane. If correct, and when combined with the results of previous studies (1, 2, 6, 22, 29, 39, 40), this would mean that most of the septal ring components contribute substantially to the stability of the structure. As pointed out by Pogliano et al. (29), an interesting alternative possibility is that the state of maturation or activity of a septal ring at one potential division site somehow affects the formation or stability of rings at additional sites in the cell. Clearly, more work is needed to test these possibilities.

The results of this study indicate that ZipA is not required for the initiation of FtsZ ring formation or for the recruitment of FtsA to the ring but suggest that it contributes significantly to the stability of the organelle. Many additional roles for the protein are possible. Because ZipA is very likely among the first factors to become associated with the FtsZ ring, it is conceivable that the protein helps recruit other components to the structure. It will, therefore, be interesting to determine the localization pattern of other Fts proteins in ZipA-depleted cells. Filaments lacking ZipA are smooth, which is consistent with a role for the protein throughout the cell wall invagination process. It is possible that ZipA simply ensures that the inner membrane remains anchored to the shrinking FtsZ ring. In addition, the protein may well play a more active role(s), such as stimulating constriction of the FtsZ ring or coordinating the activity of the FtsZ ring in the cytoplasm with the peptidoglycan synthesizing machinery in the periplasm. Additional experimentation is needed to define the role(s) of ZipA more precisely.

To date, assembly of the FtsZ ring at the prospective division site is the first recognizable step in the formation of the septal ring organelle. Although we cannot be absolutely certain, this and other studies have rendered it unlikely that any of the other known division proteins, including ZipA, perform an essential function prior to this step. To determine what defines a potential division site and how FtsZ assembly is initiated at this site remain important unanswered questions.

ACKNOWLEDGMENTS

Parts of this study were initiated in the laboratory of Larry Rothfield at the University of Connecticut Health Center, and we thank him for exceptional support, the gift of materials, and critical comments on the manuscript. We, furthermore, thank David Raskin, Robin Crossley, and Gregory Matera for technical assistance and advice and Jan Voßkuil for the gift of monoclonal antibodies. This work was supported by NIH grants GM-57059 (to P.D.B.) and GM-53276 (to L.R.), as well as by an NSF young investigator award (MCB94-58197) and by generous donations from Wyeth-Ayerst Research, The Elizabeth M. and William C. Treuhaft Fund, The Frank K. Griesinger Trust, Arline H. Garvin, James S. Blank, Charles E. Spahr, Alfred M. Taylor, and Theodore J. Castele (to P.D.B.).

REFERENCES


