Genetic Reporter System for Positioning of Proteins at the Bacterial Pole

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<td>Published Version</td>
<td>doi:10.1128/mBio.00251-11</td>
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The proper localization of proteins in bacterial cells is critical to many cellular processes, including virulence, DNA replication, chromosome segregation, cell division, chemotaxis, gene transfer, and motility. Detailed molecular understanding of how proteins localize in bacteria is incomplete. A major challenge in the investigation of the mechanism of protein localization has been the paucity of methods that enable screening of large numbers of mutants for defects in protein localization. While previously published large-scale screens have yielded important insights, they have depended on microscopic visualization of individual mutants (e.g., see references 1, 2), which is tedious and time-consuming.

In rod-shaped bacteria, a subset of proteins localize to the bacterial pole. Among these are autotransporter proteins, the largest group of secreted proteins in Gram-negative bacteria. Autotransporters are outer membrane proteins that are secreted at the bacterial pole, contain a large domain exposed on the bacterial surface (see Fig. S1a in the supplemental material), and commonly play a role in virulence (3–5). The model autotransporter IcsA from Shigella mediates assembly of an actin tail at the bacterial pole that propels the intracellular bacterium through the intestinal epithelium during infection in humans (6). Like other autotransporters, IcsA is secreted across the cytoplasmic membrane via the Sec secretion apparatus (7), is inserted into and translocated across the outer membrane in a process that requires the outer membrane insertase BamA (YaeT, Omp85) (8), and is localized to the bacterial pole (4).

Localization of IcsA to the bacterial pole occurs in the cytoplasm prior to secretion across the cytoplasmic membrane (7, 9). Although IcsA is present only in Shigella spp., the molecular mechanism that localizes IcsA to the pole is conserved among a wide variety of Gram-negative bacteria (9, 10).

Currently, to our knowledge, there are no methods that enable large-scale genetic screening of spatial positioning of proteins in bacteria. Using IcsA, we developed a reporter assay for protein localization within the bacterial cytoplasm that allows saturation screening for mutants in which protein localization is altered. Utilizing this system, we identify the conserved cell division protein FtsQ as being required for positioning of IcsA to the bacterial pole. We demonstrate further that the requirement for FtsQ for polar positioning applies to other autotransporters that display polar positioning within bacterial cells.
screening for mutants in which protein localization is altered. Utilizing this assay, we identify proteins required for proper positioning of IcsA and other autotransporters at the bacterial pole. We demonstrate further that this system can be successfully applied to the study of proteins other than autotransporters that localize to the bacterial pole.

RESULTS

Reporter system for IcsA localization in the bacterial cytoplasm. To identify proteins involved in polar localization of IcsA, we developed a reporter system for IcsA localization in the bacterial cytoplasm based on the premise that fusion of a transcription regulator to IcsA in the cytoplasm might sequester the transcription regulator at the pole and disrupt its ability to regulate its cognate operons. CRP (cyclic AMP receptor protein), a transcription factor required for uptake and utilization of maltose (11), was translationally fused to the carboxy terminus of a derivative of IcsA that localizes to the pole and yet remains in the cytoplasm because it lacks a signal peptide (the entire domain, residues 53 to 757 [see Fig. S1b in the supplemental material]) (9). On maltose utilization indicator (maltose MacConkey) medium, wild-type (WT) Escherichia coli (strain MG1655), which can utilize maltose as a carbon source, formed red colonies and a crp mutant, which cannot utilize maltose, formed white colonies; similarly, on maltose minimal medium, the wild-type strain grew well, but the crp mutant did not grow (Fig. 1a). A crp mutant directing the synthesis of CRP alone or an IcsA-CRP fusion protein lacking the IcsA polar localization signal (IcsA53-757-CRP) was able to use maltose on both media (Fig. 1a). Because an IcsA53-757-CRP fusion protein localizes to the bacterial pole and an IcsA507-729-CRP fusion protein is diffuse (see Fig. S1c) (9), the inability of cells containing IcsA53-757-CRP to utilize maltose is likely due to sequestration of the fusion protein at the pole, thereby disrupting CRP activity, and the ability of IcsA507-729-CRP to utilize maltose is likely due to delocalization of the fusion protein. To further test the hypothesis that sequestration of the fusion protein at the pole is responsible for the maltose utilization-minus phenotype of cells containing IcsA53-757-CRP, we provided excess full-length untagged IcsA from a multicopy vector, which is capable of displacing localized (nonaggregated) IcsA-green fluorescent protein (GFP) fusion proteins from the pole (9, 12, 13). Cells containing the IcsA53-757-CRP fusion protein together with excess full-length IcsA were once again able to utilize maltose (Fig. 1a), providing strong support for the inference that the IcsA53-757 moiety can sequester CRP at the cell pole.

To examine the position of the CRP reporter fusions in these cells, we placed a GFP tag at the C termini of IcsA53-757-CRP and IcsA507-729-CRP. Cells containing IcsA53-757-CRP-GFP displayed foci that were polarly localized (Fig. 1b, top row, arrows), whereas cells containing IcsA507-729-CRP-GFP displayed a diffuse signal (Fig. 1b, third row). The GFP foci were visualized only upon induction of expression by addition of low concentrations of the inducer anhydrotetracycline (50 ng/ml), which reflects the low minimal medium, whereas the same mutant directing the synthesis of CRP alone or an IcsA-CRP fusion protein lacking the IcsA polar localization signal (IcsA507-729-CRP) was able to use maltose on both media (Fig. 1a).
level of synthesis of IcsA\textsubscript{53-757}−CRP and IcsA\textsubscript{507-729}−CRP from the same vector in the experiments described above, which were performed in the absence of inducer (see comparison to level of synthesis of native IcsA [see Fig. S2a in the supplemental material]). The two GFP fusion proteins were produced at similar levels (see Fig. S2b). These data provide additional evidence for the inference that IcsA\textsubscript{53-757}−CRP sequesters CRP at the pole and IcsA\textsubscript{507-729}−CRP does not.

Further validation of this reporter system was provided by the observation that fusion of CRP to each of two other polar proteins, the soluble chemotaxis proteins CheY and CheZ, also disrupted its activity (Fig. 1c). The localization of CheY or CheZ to the pole is dependent on the polar membrane-associated histidine kinase CheA (14), and expression of either the cheY::crp or cheZ:crp fusion from a multicopy plasmid in a cheA crp strain rescued maltose utilization (Fig. 1c), consistent with sequestration of CRP at the pole by the fusion proteins being dependent on CheA. These results demonstrate that IcsA is not unique in its ability to interfere with the function of a fused transcription regulator by sequestering it at the pole and suggest that this capability may be a general feature of polar proteins. Thus, the inability of a CRP−minus strain containing a polar protein fusion to CRP to utilize maltose as a carbon source correlates with polar localization of the protein−CRP fusion.

As CRP is required for the transcriptional activation of operons that encode proteins for the utilization of any of several sugars as carbon sources by the bacterium, we tested whether utilization of sugars other than maltose could serve as a readout for this reporter system. On indicator medium that contained rhamnose, growth of the E. coli crp strain carrying the polar IcsA\textsubscript{53-757}−CRP reporter construct or the nonpolar IcsA\textsubscript{507-729}−CRP control construct reproduced the phenotypes observed on maltose indicator medium, with cells carrying IcsA\textsubscript{53-757}−CRP growing as white colonies and cells carrying IcsA\textsubscript{507-729}−CRP growing as red colonies (Fig. 1d). However, on indicator medium that contained lactose, ribose, or xylose, both the cells carrying IcsA\textsubscript{507-729}−CRP and the cells carrying IcsA\textsubscript{53-757}−CRP grew as red colonies (Fig. 1d). The parent E. coli crp strain was unable to metabolize any of these sugars, since in all cases it grew as white colonies (Fig. 1d), indicating that the red colony color of cells carrying IcsA\textsubscript{53-757}−CRP upon growth on lactose, ribose, or xylose was due to binding of the CRP moiety of the fusion protein to cognate CRP promoters in these cells. We speculate that these differences reflect the ability of IcsA-CRP molecules that are sequestered at the pole to access the transcriptional units required for utilization of each of these sugars. For the utilization of maltose or rhamnose, CRP regulates multiple operons, and multiple CRP binding sites at a distance from one another must be occupied to activate transcription of these operons (11, 15). In contrast, for utilization of lactose, ribose, or xylose, either sugar utilization itself depends on transcription of only a single operon, or if transcription of multiple operons is required, CRP activates only one of them (16−18). The position of the CRP binding sites relative to the E. coli origin was not sufficient to explain the sugar utilization phenotype on the basis of the origin being generally positioned near the bacterial pole (see Table S1 in the supplemental material). Instead, when CRP−dependent activation of only a single operon is required, we speculate that, within a subpopulation of cells in each colony, the chromosome will at times be positioned such that the cognate CRP binding site will be sufficiently close to the bacterial pole to permit binding and activation, leading to sugar utilization and red colony color (see Fig. S3). Since only 3 kb separates the two sites required for rhamnose utilization, relatively short distances between CRP binding sites may be sufficient to prevent coordinated binding of sequestered CRP; alternatively, the two rhamnose CRP binding sites may not be simultaneously accessible due to other aspects of chromosome positioning or structure. Alternatively, these findings could potentially be explained by the level of production of IcsA\textsubscript{53-757}−CRP being sufficient to occupy one binding site but insufficient to occupy two or three binding sites or by differences in the affinities of IcsA\textsubscript{53-757}−CRP for the various binding sites. However, the findings described below, in which elevated levels of RpoS resulting from disruption of rssB lead both to rescue of maltose utilization (Fig. 2a) with no change in the level of production of IcsA\textsubscript{53-757}−CRP (see Fig. S2a) and to delocalization of IcsA\textsubscript{507-620}−GFP (Fig. 2b), indicate that this level of production of IcsA\textsubscript{53-757}−CRP is sufficient to occupy all three CRP binding sites within the maltose operons and that differences in affinities for these sites do not determine maltose utilization. These findings suggest that IcsA-CRP sequestered at the pole retains its ability to activate CRP−dependent promoters and that the limitation on its ability to activate cognate promoters may result from the positioning of the chromosome in the cells.

High levels of RpoS (sigma S) disrupt localization of IcsA to the pole. To identify nonessential genes involved in targeting IcsA to the pole, we performed a large-scale transposon mutagenesis of crp cells containing IcsA\textsubscript{53-757}−CRP, first selecting for mutants that displayed rescue of growth on maltose minimal medium and subsequently screening these mutants for red colony color on maltose indicator medium. Utilization of maltose was rescued only by transposon insertions in rssB or in the plasmid carrying icsa\textsubscript{53-757}−crp. Insertions in the plasmid most likely led to production of nonsequestered CRP and were thus not studied further. A strain with a nonpolar deletion of rssB also formed red colonies, and maltose nonutilization was rescued by expression of rssB in trans (Fig. 2a; Table 1), indicating that the maltose utilization phenotype of the rssB mutant was due to the transposon insertion in rssB. To visualize localization of IcsA in the cytoplasm, we tagged IcsA with GFP, using for these studies IcsA residues 507 to 620 (IcsA\textsubscript{507-620}) [see Fig. S1d in the supplemental material], the minimal internal fragment of IcsA\textsubscript{53-757} that localizes to the pole, because this fragment is slightly more efficient at localizing the fused GFP moiety to the pole than are residues 53 to 757 (9). The rssB mutation caused IcsA delocalization in the cytoplasm, since IcsA\textsubscript{507-620}−GFP was diffuse in crp rssB cells and yet polar in crp cells (Fig. 2b and 2c; see also Table S2) while being produced at similar levels under the two conditions.

The protein encoded by rssB, RssB (SprE), targets the sigma factor RpoS (sigma S) to the ClpXP protease complex during the exponential phase of growth, leading to RpoS degradation (19) (see Fig. S2c in the supplemental material). During late exponential phase, RpoS levels in the crp rssB mutant were 10-fold higher than those in crp cells (see Fig. S2e), as seen previously (20, 21). IcsA delocalization was due to the high levels of RpoS, since disruption of rpoS in the crp rssB p-icsa\textsubscript{53-757}−CRP strain caused loss of maltose utilization (Fig. 2a; Table 1) and in the crp rssB p-icsa\textsubscript{507-620}−GFP strain restored polar GFP focus (Fig. 2b; see also Table S2). Moreover, inactivation of the ClpXP protease complex by disruption of clpP in the crp p-icsa\textsubscript{53-757}−CRP strain restored maltose utilization in a manner dependent on rpoS (Fig. 2a; Ta-
The fainter red color of the crp clpP colonies was not due to lower levels of RpoS (see Fig. S2f) but may be due to either the slower growth of Clp strains (22) or the accumulation of another ClpP substrate that also influences maltose utilization. Of note, insertions in clpP and clpX were not isolated in our transposon mutagenesis selection, likely because the slower growth of these strains may cause the mutants to be missed. We did not attempt to overexpress rpoS from an inducible promoter, since it is difficult to increase levels of RpoS by this approach due to its tight post-translational regulation. Altogether, these data demonstrate that high levels of RpoS disrupt polar localization of IcsA and validate the ability of the CRP reporter system to identify cells in which IcsA is delocalized.

RpoS accumulates in stationary phase. In wild-type cells, IcsA507-620-GFP localized to the pole during exponential phase but was delocalized at high cell density in a manner dependent on rpoS (Fig. 2c; see also Table S2 in the supplemental material), while IcsA507-620-GFP levels were similar. In Shigella flexneri, the disruption of rssB led to undetectable IcsA on the bacterial surface (IcsA as polar on 99% ± 2% of wild-type [WT] cells versus <1% ± 0% of rssB cells, \( P = 6 \times 10^{-8} \) [see Fig. S4a and Table S3]) and in cell lysates (see Fig. S4b). The absence of IcsA in this strain could be consistent with the presence of high levels of RpoS interfering with proper targeting of IcsA that in turn causes it to be unstable. We were unable to directly test whether the block in IcsA secretion and/or stability in these cells was due to elevated RpoS levels, since introduction of a mutation in rpoS into the S. flexneri rssB (but not the E. coli rssB) background led to a high rate of cell lysis (see Table S3). We have previously observed a significant decrease in IcsA signal and localization to the pole in stationary-phase S. flexneri serotype 5 strain M90T (23); however, this effect or the levels of RpoS in stationary phase appear to be somewhat strain depen-

**TABLE 1** Maltose utilization and RpoS levels in *E. coli* MG1655 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Maltose utilization</th>
<th>Colony color on indicator medium</th>
<th>RpoS levela</th>
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<tr>
<td>WT, stationary phase</td>
<td>+</td>
<td>Red</td>
<td>1.0</td>
</tr>
<tr>
<td>WT, exponential phase</td>
<td>NDb</td>
<td>ND</td>
<td>BDc</td>
</tr>
<tr>
<td>crp p-icsA53-757::crp strain</td>
<td>ND</td>
<td>White</td>
<td>BD</td>
</tr>
<tr>
<td>crp rssB p-icsA53-757::crp strain</td>
<td>+</td>
<td>Red</td>
<td>1.4</td>
</tr>
<tr>
<td>crp rssB p-icsA53-757::crp p-rssB strain</td>
<td>ND</td>
<td>White</td>
<td>ND</td>
</tr>
<tr>
<td>crp rpoS p-icsA53-757::crp strain</td>
<td>ND</td>
<td>White</td>
<td>BD</td>
</tr>
<tr>
<td>crp rssB rpoS p-icsA53-757::crp strain</td>
<td>ND</td>
<td>White</td>
<td>BD</td>
</tr>
<tr>
<td>crp clpP p-icsA53-757::crp strain</td>
<td>ND</td>
<td>Red</td>
<td>1.3</td>
</tr>
<tr>
<td>crp clpP rpoS p-icsA53-757::crp strain</td>
<td>ND</td>
<td>White</td>
<td>BD</td>
</tr>
</tbody>
</table>

* RpoS levels were determined for exponential-phase growth in rich media, except where otherwise indicated, and were normalized to the level in WT stationary-phase cells.
* ND, not determined.
* BD, below the level of detection.
The conserved cell division protein FtsQ is required for IcsA localization to the bacterial pole. RpoS activates the transcription of a large number of genes (19). We postulated that a component of the IcsA polar localization machinery might be inhibited by a factor synthesized under the control of RpoS, in which case overproduction of this component of the polar localization machinery might titrate out the inhibitory factor, thereby rescuing polar localization of IcsA. From among plasmids of a multicopy chromosomal library introduced into the WT (parental) strain, the essential cell division gene ftsQ, which are in the KS272 background.

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FIG 3 Conserved cell division protein FtsQ is required for localization of IcsA to the pole. (a) Hierarchical recruitment of cell division proteins (33). (b) Position of IcsA-GFP at 37°C or an ftsQ1(Ts) derivative visualized by immunofluorescence (IF). Growth was at 37°C, the restrictive temperature for ftsQ1(Ts) cells, for 1 h. Aztreonam was used to filament WT (WT + aztr) and complemented ftsQ1(Ts) cells during the 1-h period; addition of aztreonam per se had no effect on distribution of IcsA. (d) Rescue of cell division upon shift of S. flexneri 2457T ftsQ1(Ts) to 30°C following 2 h of growth at 37°C. Bars, 10 μm.

TABLE 2 Distribution of GFP- or mCherry-tagged cytoplasmic derivative of IcsA (IcsA507-620-GFP or IcsA507-620-mCherry) in E. coli

<table>
<thead>
<tr>
<th>Relevant genotype (growth phase)</th>
<th>Distribution of IcsA-GFP or IcsA-mCherry (% of cells)</th>
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<tbody>
<tr>
<td></td>
<td>Foci at poles Diffuse</td>
</tr>
<tr>
<td><strong>crp</strong> B p-vector (exponential)</td>
<td>15 ± 5 \textsuperscript{a} 86 ± 5</td>
</tr>
<tr>
<td><strong>crp</strong> B p-ddlB-ftsQ (exponential)</td>
<td>85 ± 1 \textsuperscript{b} 15 ± 1</td>
</tr>
<tr>
<td><strong>crp</strong> B p-ftsQ (exponential)</td>
<td>75 ± 8 \textsuperscript{c} 26 ± 8</td>
</tr>
<tr>
<td><strong>crp</strong> B p-ddlB (exponential)</td>
<td>14 ± 3 \textsuperscript{d} 86 ± 3</td>
</tr>
<tr>
<td>WT p-vector (stationary)</td>
<td>19 ± 8 \textsuperscript{e} 81 ± 8</td>
</tr>
<tr>
<td>WT p-ddlB-ftsQ (stationary)</td>
<td>97 ± 1 \textsuperscript{f} 3 ± 1</td>
</tr>
<tr>
<td>WT p-ftsQ (stationary)</td>
<td>91 ± 4 \textsuperscript{g} 9 ± 4</td>
</tr>
<tr>
<td>WT p-ddlB (stationary)</td>
<td>7 ± 1 \textsuperscript{h} 93 ± 1</td>
</tr>
<tr>
<td>WT attt-gfp (stationary)</td>
<td>16 ± 6 \textsuperscript{i} 84 ± 6</td>
</tr>
<tr>
<td>WT attt-gfp::qqq (stationary)</td>
<td>82 ± 6 \textsuperscript{f} 18 ± 6</td>
</tr>
<tr>
<td>WT attt-gfp::ffq (stationary)</td>
<td>85 ± 4 \textsuperscript{f} 15 ± 4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All strains are MG1655 and its derivatives, except for chromosomal integrants of gfp, gfp::qqq, and gfp::ffq, which are in the KS272 background.

\textsuperscript{b} P < 0.03.

\textsuperscript{c} P < 0.01.

\textsuperscript{d} P < 0.03.

\textsuperscript{e} P < 0.003.

\textsuperscript{f} P < 0.007.

\textsuperscript{g} P < 0.005.
which FtsQ encoded by the *ftsQ1* (Ts) allele had been inactivated by growth at 37°C, IcsA was distributed circumferentially around the filamneted cells (Fig. 3c), whereas in wild-type cells grown at 37°C, similar to previous results (4), IcsA was localized to the poles on 43% ± 6% of the cells, reflective of its localization under these growth conditions to the older of the two bacterial poles of most cells. To test whether filamentation *per se* influenced IcsA localization, wild-type cells were filamented with azetronam, which inhibits the cell division protein FtsI; IcsA localized to the poles of these cells (Fig. 3c) [filament poles with IcsA at 37°C, 2% ± 2% of *ftsQ1* (Ts) cells versus 98% ± 2% of WT cells, *P* = 5.5 × 10⁻⁷]. The presence of IcsA at both poles of most filamented wild-type cells, but only the older pole of dividing wild-type cells, has been noted previously (12) and suggests that the newer pole matures during filamentation in such a way as to enable localization to these sites. Polar localization of IcsA in the *ftsQ*1 strain grown at 37°C was rescued by the presence of *ftsQ* in trans (Fig. 3c). Altered IcsA localization was not due to loss of cell viability, since shifting cells to 30°C after growth at 37°C rescued both cell division (Fig. 3d) and polar localization of IcsA (IcsA was polar on 17% ± 7% and 27% ± 1% of cells after shifting to 30°C for 1 and 2 h, respectively). These findings indicate that FtsQ function is required for localization of native IcsA to the poles of *S. flexneri*.

**Cell division proteins other than FtsQ are not required for establishing IcsA polarity.** The cell division proteins FtsB and FtsL form a complex with FtsQ (Fig. 3a) (25). We examined whether these and cell division proteins that lie downstream of FtsQ in the cell division recruitment cascade might also be required for proper IcsA localization. Chromosomal overproduction of FtsB was able to rescue polarity of cytoplasmic IcsA in stationary-phase *E. coli* (see Fig. S5a in the supplemental material), as did multicopy or chromosomal overproduction of FtsQ (Fig. 3b; see also Fig. S5a), while chromosomal overproduction of all other downstream cell division proteins did not (see Fig. S5a), raising the possibility that FtsB might be required for establishing IcsA polarity. However, on *S. flexneri* cells that had been depleted of FtsB, IcsA localized to the pole similarly to its localization on wild-type *S. flexneri* (see Fig. S5b), suggesting that FtsB is not required for establishing IcsA polarity and that overproduction of FtsB under our experimental conditions may increase FtsQ stability or alter FtsQ function.

**The periplasmic domain of FtsQ is sufficient for establishing IcsA polarity.** FtsQ is a bitopic membrane protein with a short cytoplasmic tail (residues 1 to 24), a transmembrane segment (residues 25 to 49), and a 227-residue periplasmic domain (residues 50 to 276). During septation, FtsQ localizes to the cytokinetic ring at midcell (Fig. 4a) (26). In stationary-phase cells, GFP-tagged FtsQ localized to the membrane circumferentially around the entire cell, whereas cytoplasmic IcsA<sub>507-620-mCherry</sub> localized to the pole (Fig. 4b). An FtsQ chimeric protein in which the cytoplasmic and transmembrane segments of MalF have replaced these domains of FtsQ (FFQ) is mildly defective in localization to midcell but recruits downstream cell division proteins (26). The FFQ construct rescued IcsA<sub>507-620</sub>-localization (Fig. 4c and Table 2), indicating that the periplasmic domain of FtsQ is sufficient to establish IcsA polarity. The ability of the periplasmic domain of FtsQ to rescue polar localization of cytoplasmic IcsA indicates that the role of FtsQ in IcsA polarity is due to an indirect relationship between the two proteins.

**FtsQ is required for establishing polarity of other autotransporter proteins.** Like IcsA, other bacterial autotransporters are secreted at the cell pole (3). We tested whether the *Shigella* autotransporter SepA required FtsQ for localization to the pole in the bacterial cytoplasm by determining the localization of a GFP-tagged derivative of SepA that remains in the cytoplasm by virtue of lacking the Sec recognition sequence within its signal peptide (SepA<sub>1-24/57-1042-GFP</sub>) (3). Like IcsA, during exponential phase of growth (3). Also like IcsA, during stationary-phase growth, this GFP-tagged SepA was diffuse in wild-type *E. coli* but, in the presence of multicopy FtsQ, localized to the pole (see Fig. S6a in the supplemental material), indicating that FtsQ is required for the establishment of the polar positional information recognized by both IcsA and SepA in the bacterial cytoplasm.

We tested whether the localization of autotransporters other than IcsA to the pole on the bacterial surface is dependent on FtsQ. SepA is not well suited to this analysis because surface-exposed SepA is efficiently cleaved and is thus difficult to detect (3). BrkA, an autotransporter from the distantly related Gram-negative cocccobacillary pathogen *Bordetella pertussis*, is secreted at the pole of *B. pertussis* and, upon heterologous expression, at the pole of *E. coli* as well (3). We introduced a plasmid carrying *brkA* into an *S. flexneri* derivative that lacks *icsA* and *sepA* by virtue of having been cured of the *Shigella* large plasmid that carries these genes (strain BS103). On the surface of BS103, BrkA was distributed asymmetrically (see Fig. S6b in the supplemental material). Its distribution was less polarized than that of IcsA, likely because IcsA is maintained in tight pole caps on *Shigella* in part as a result of its regulated cleavage from the surface by specific proteases (3, 29).
27, 28). In BS103 ftsQ1(Ts) cells in which FtsQ had been depleted by growth at 37°C, BrkA was absent from the poles and localized along the length of the cell (see Fig. S6c). In contrast, in the presence of FtsQ, either by virtue of growing the ftsQ1(Ts) strain at 25°C or by complementing cells grown at the restrictive temperature with a wild-type allele of ftsQ, BrkA localized asymmetrically at and near the poles of the cell (see Fig. S6c). On the other hand, FtsQ had no effect on localization to the pole of the nonautotransporter polar proteins CheY and CheZ. These findings indicate that FtsQ is required for localization to the pole of multiple autotransporters from distantly related organisms, suggesting that FtsQ may be generally required for the localization of autotransporters to the pole.

**DISCUSSION**

Spatial positioning of proteins is critical to multiple bacterial cellular processes. Bacterial proteins critical to virulence, DNA replication, chromosome segregation, cell division, chemotaxis, and other processes are positioned at one or both poles, at midcell, on the chromosome, to an endospore, or to another specific site within the cell. The proper positioning of many of these proteins is a prerequisite for normal function. Enzymatic assays have existed that indicate whether a specific protein is located within the cytoplasm or in an extracytoplasmic compartment; these assays typically use beta-galactosidase or alkaline phosphatase as a reporter and permit the determination of the subcellular localization of a protein on a large scale within the cells of bacterial colonies (29, 30). However, because their ability to distinguish bacterial compartments is based on the redox potential of the compartment, which is constant throughout the compartment, these enzymatic assays provide no information on protein positioning within the compartments.

Despite the importance of spatial positioning within the cytoplasm, to our knowledge, no methods have existed previously for detecting on a large scale the position of specific proteins within the cytoplasm of bacterial cells. The reporter assay that we describe here provides this functionality. We demonstrate the ability not only to discriminate in individual bacterial strains whether the polar protein IcsA is at the pole or is delocalized from the pole but also to perform large-scale selection for second-site mutations that lead to its delocalization and large-scale screening for loci that rescue its polar positioning in a strain background in which it is delocalized. This reporter system is adaptable to proteins other than IcsA that localize to the pole in the cytoplasm, since the polar chemotaxis proteins CheY and CheZ each functioned similarly to IcsA in the assay (Fig. 1c).

Our findings are consistent with a model in which, in any given cell, when CRP is sequestered at the bacterial pole, it cannot access all of the binding sites necessary to enable sugar utilization (see Fig. S3 in the supplemental material). Our results suggest that the high specificity of the reporter system for maltose (or rhamnose) utilization is due to the requirement for CRP to bind to multiple promoter binding sites for utilization of that particular sugar (Fig. 1d). Consequently, we anticipate that the system will be adaptable to proteins that are not polar and have specific nonpolar positions, since distant binding sites will be equally inaccessible in aggregate to an activator that is sequestered anywhere in the cytoplasm. Similarly, it seems likely that transcriptional reporters other than CRP that must bind multiple chromosomal sites to produce a particular phenotype will also work as reporters. Thus, this reporter system is likely to be applicable to the study of a wide range of bacterial proteins. The ability to discern the distribution of a protein within the bacterial cytoplasm in a high-throughput fashion will likely have broad-reaching applications.

One potential limitation of this system is the requirement that the readout (e.g., maltose utilization) depend on the reporter binding to multiple sites on the chromosome; the requirement for CRP in either maltose utilization or rhamnose utilization meets these requirements. A second limitation of the system is that the reporter must retain its activity upon fusion to the targeting protein. Given that multiple transcription activators are functional when fused to bait proteins in two-hybrid systems, this limitation is unlikely to be a major restriction. Finally, a potential limitation is that because the number of copies of the fusion protein in our cells is very low compared to native IcsA (see Fig. S2a in the supplemental material), it can be difficult to visualize the position of the IcsA-CRP reporter fusion protein in individual cells. We were not able to detect it by immunofluorescence using antibodies to IcsA or CRP; we were able to detect it only after fusion to a GFP tag and induction of expression with low concentrations of anhydro-tetracycline (Fig. 1b). However, with different reporters or different antibodies, detection of an untagged reporter by immunofluorescence might be possible.

Our initial selection, designed to identify nonessential genes required for polarity of an IcsA-CRP reporter, resulted in the demonstration that elevated levels of RpoS block IcsA polarity. RpoS could theoretically block IcsA polarity by negatively regulating expression of ftsQ; instead, however, RpoS is known to be an inducer of expression from one of two ftsQ promoters in E. coli (31, 32), indicating that RpoS regulation of ftsQ is not the cause of the FtsQ-dependent phenotype that we observed. RpoS regulates entry into stationary phase, yet levels of IcsA in S. flexneri are diminished during stationary phase (23). Moreover, during infection of mammalian cells, Shigella spp. are continuously dividing and using IcsA to spread into adjacent cells, such that bacterial cell density likely never reaches that of stationary phase.

We demonstrate that the cell division protein FtsQ is required for localization of IcsA and other autotransporters to the bacterial pole. In most bacteria, FtsQ is required for cell division, a process that involves assembly of a cytokinetic ring of FtsZ at midcell, followed by constriction of that ring, leading to invagination of the cell envelope and formation of a septum at midcell. FtsQ functions in the middle of the cell division cascade (Fig. 3a), is dependent on FtsK for its recruitment to midcell, and in turn is required for recruitment of FtsB and FtsL (33). The fact that the periplasmic domain of FtsQ is sufficient for its function in IcsA localization suggests that an extracytoplasmic activity of FtsQ contributes to the establishment of bacterial poles. Other than its role in recruitment of FtsB and FtsL, the molecular function of FtsQ in the cell division process is unknown. We speculate, as others have done previously (34, 35), that the periplasmic domain of FtsQ is critical to the remodeling of peptidoglycan that normally occurs in conjunction with cell division and that may contribute to the establishment of autotransporter polarity. The methodology for assaying protein position described here, which enabled the identification of a role for FtsQ in polar localization of autotransporters, has the potential to be a powerful tool for the study of positional information relevant to a wide range of proteins that localize to the bacterial pole.
MATERIALS AND METHODS

Bacterial strains, plasmids, and libraries. Strains and plasmids used in this study are listed in Table S4 in the supplemental material. Unless otherwise indicated, E. coli strains were grown in LB at 37°C, and S. flexneri strains were grown in TCS (tryptose casein soy broth) at 37°C. fisQ1(Ts) strains were maintained at 30°C. Maltose MacConkey agar base (Difco) supplemented with 0.2% maltose. Maltose minimal medium was M63 medium supplemented with 0.2% maltose. Where appropriate, antibiotics were added at the indicated concentrations: kanamycin, 50 μg/ml; ampicillin 100 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 12.5 μg/ml; spectinomycin, 100 μg/ml.

Genetic manipulations. pBAD-icsA507-620-GFP, pBAD-icsA507-104-GFP, and pBAD-icsA507-729-GFP, which encode translational fusions of IcsA polypeptides to GFP or mCherry under the control of the arabinose promoter, as well as pBR322-icsA, which carries icsA under the control of the native promoter, have been described elsewhere (9, 36). pBAD-icsA507-620-mCherry is pBAD33 carrying a translational fusion of the coding sequence for icsA507-620 to mCherry. To generate p-icsA53,757-::crp, sequence encoding IcsA amino acids 53 to 757 with an initiating methionine, fused in frame via a 3-alanine linker (GGG GGC GCA) to amino acid 2 of CRP, was cloned into a derivative of pZ2E1-MCS-1 (37). The C-terminal CRP fusion to icsA507-729 (endothelial protein) was recombined into the chromosome by PCR. The resulting product was digested with BamHI and NotI and ligated into the XbaI and SphI sites of pACYC184. To create p-icsA507-729-GFP, in which case cells were harvested following induction with 0.2% L-arabinose to overnight stationary phase at 37°C. For determination of icsA507-620-GFP or -mCherry localization in mid-exponential-phase CRP+ cells, t-arabinose was added to 0.2% and growth was continued for an additional 30 min at 30°C. Because CRP is transcriptionally active at arabinose promoters, cells carrying a deletion of crp displayed reduced expression from arabinose-inducible promoters; therefore, induction of arabinose-inducible icsA fusions from these promoters was for 90 to 120 min at 37°C, which resulted in levels of icsA507-620-GFP comparable to those in CRP+ cells. To determine icsA localization in stationary phase, expression of icsA507-620-GFP or -mCherry was induced by the addition of 0.2% l-arabinose to overnight cultures for 0 to 90 min at 30°C. For stationary-phase colocalization of GFP-FtsQ or expression of FFQ or QQQ, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was included in overnight cultures. SepA1-2457-1042-GFP expression was induced by the addition of l-arabinose to 0.2% for 40 min at 30°C in stationary-phase MG1655. Depletion of functional FtsQ in fisQ1 strains was accomplished by shifting to growth at 37°C or 42°C for 60 to 150 min. Depletion of FtsQ or FtsL in DJS86 and DJS87 was accomplished by growth in the absence of arabinose for 60 to 180 min. Filamentation with azetromin was performed by adding it to 1 μg/ml for 50 to 60 min. Fixation and detection of icsA on the surface of bacteria were performed as described previously (4). Alternatively, cells were labeled with primary and secondary antibodies prior to fixation.

Whole-cell blot analysis. Whole-cell proteins were prepared from bacterial cultures immediately before microscopy. Protein loading was normalized to cell density, and all sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels consisted of 5% stacking and 10% separating layers. Western blot analysis was carried out using standard protocols, using antibodies to icsA (4) at a 1:10,000 dilution, isocitrate dehydrogenase (ICDH; gift from A. L. Sonenschein) at a 1:10,000 dilution, RpoS (Neclone) at a 1:5,000 dilution, or GFP (Molecular Probes or Roche) at 1:1,000,000 dilution. RpoS levels were determined by band densitometry using IP Laboratory (Scanalytics) software.

Microscopy. For microscopic observation, cells were microtomed onto 1% agarose pads or glass coverslips. Microscopy was performed by using a 100× oil-immersion objective lens on a Nikon TE300 microscope with Nikon or Chroma Technology filters. Images were captured digitally using a black-and-white CoolSnap HQ charge-coupled device camera and IP Laboratory (Scanalytics) software.

Statistical analysis. Tabulation of protein localization was conducted in a blinded fashion. For each set of experiments, 30 or more cells were analyzed in each of three independent experiments. For GFP fusions, if a focus was present at a cell pole, the cell was scored as polar, and if there were no foci, it was scored as diffuse. We never observed cells with foci that were only at sites other than a pole. The statistical significance of differences between experimental results was determined using a Student t test.
ACKNOWLEDGMENTS

This work was supported by Public Health Service grant R01 AI035817 from the National Institutes of Health (to M.B.G.) and American Heart Association fellowship 042585ST (to A.J.).

We thank C.-R. Yi and L. Stamm for careful reading of the manuscript; J. Kahane, D. Raj, and W. Lane for technical assistance; and A. L. Sonenchein, J. Beckwith, and R. Fernandez for reagents.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.orglookup/suppl doi:10.1128/mBio.00251-11/DCSupplemental.

Figure S1, TIF file, 1.3 MB.
Figure S2, TIF file, 1.6 MB.
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Figure S6, TIF file, 1 MB.
Table S1, DOC file, 0.1 MB.
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Table S4, DOC file, 0.1 MB.

REFERENCES