Genetic Reporter System for Positioning of Proteins at the Bacterial Pole

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ABSTRACT Spatial organization within bacteria is fundamental to many cellular processes, although the basic mechanisms underlying localization of proteins to specific sites within bacteria are poorly understood. The study of protein positioning has been limited by a paucity of methods that allow rapid large-scale screening for mutants in which protein positioning is altered. We developed a genetic reporter system for protein localization to the pole within the bacterial cytoplasm that allows saturation screening for mutants in Escherichia coli in which protein localization is altered. Utilizing this system, we identify proteins required for proper positioning of the Shigella autotransporter IcsA. Autotransporters, widely distributed bacterial virulence proteins, are secreted at the bacterial pole. We show that the conserved cell division protein FtsQ is required for localization of IcsA and other autotransporters to the pole. We demonstrate further that this system can be applied to the study of proteins other than autotransporters that display polar positioning within bacterial cells.

IMPORTANCE Many proteins localize to specific sites within bacterial cells, and localization to these sites is frequently critical to proper protein function. The mechanisms that underlie protein localization are incompletely understood, in part because of the paucity of methods that allow saturation screening for mutants in which protein localization is altered. We developed a genetic reporter assay that enables screening of bacterial populations for changes in localization of proteins to the bacterial pole, and we demonstrate the utility of the system in identifying factors required for proper localization of the polar Shigella autotransporter protein IcsA. Using this method, 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 and that the method can be applied to polar proteins other than autotransporters.
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 IcsA53-757-GFP fusion protein displayed a polar focus (Fig. 1b, top row, arrows), whereas IcsA53-757-CRP-GFP fusion protein was diffuse (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 concentration of CRP in the cytoplasm. To further test the hypothesis that sequestration of IcsA53-757-CRP 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 IcsA-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 terminus of IcsA, IcsA53-757-CRP, and IcsA507-729-CRP-GFP and IcsA507-729-CRP-GFP in MG1655 crp. Arrows, polar foci of IcsA53-757-CRP-GFP. (b) Maltose utilization phenotype of MG1655 crp or crp cheA, carrying a plasmid carrying cheY::crp or cheZ::crp. Predicted CRP position, putative positioning of fusion based on observed maltose utilization phenotype. (c) Colony phenotype on indicator medium containing various sugars. Note the correlation of the specificity of the phenotype with multiple CRP-dependent operons being required for sugar utilization. N/A, not applicable. Bars, 5 mm.
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). Thus, the two GFP fusion proteins were produced at similar levels (see Table S2). Moreover, inactivation of the ClpXP protease complex 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 rbsB 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 rbsB 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 rbsB also formed red colonies, and maltose nonutilization was rescued by expression of rbsB in trans (Fig. 2a; Table 1), indicating that the maltose utilization phenotype of the rbsB mutant was due to the transposon insertion in rbsB. 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}–GFP [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 rbsB mutation caused IcsA delocalization in the cytoplasm, since IcsA\textsubscript{507-620}–GFP was diffuse in crp rbsB 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 rbsB, 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 rbsB mutant were 10-fold higher than those in crp cells (see Fig. S2c), as seen previously (20, 21). IcsA delocalization was due to the high levels of RpoS, since disruption of rpoS in the crp rbsB p–IcsA\textsubscript{53-757}–CRP strain caused loss of maltose utilization (Fig. 2a; Table 1) and in the crp rbsB p–IcsA\textsubscript{507-620}–GFP strain restored polar GFP foci (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.

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>
</tr>
</thead>
<tbody>
<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>-</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>-</td>
<td>White</td>
<td>BD</td>
</tr>
<tr>
<td>crp rssB rpoS p-icsA53-757::crp strain</td>
<td>-</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>

a  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.
b ND, not determined.
c BD, below the level of detection.

FIG 2  High levels of RpoS disrupt localization of IcsA to the bacterial pole. (a) Maltose utilization phenotypes on maltose MacConkey indicator agar of the indicated E. coli MG1655 strains, each carrying the icsA53-757::crp reporter. (b and d) Positions of IcsA507-620-GFP in each of the indicated derivatives of E. coli MG1655. GFP fluorescence and phase micrographs. (c) Levels of IcsA507-620-GFP in indicated derivatives of MG1655, corresponding to panel b. For each construct, breakdown of fusion protein yields some free GFP, which is likely responsible for the diffuse signal in those strains with polar foci and for part of the diffuse signal in the crp rssB strain. Numbers at left are molecular masses in kilodaltons. Bars, 5 mm (a) and 10 μm (b and d).
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 over-production 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 E. coli crp rssB mutant, carrying vector, p-ddlB-ftsQ, p-ddlB, or p-ftsQ, grown to stationary or exponential phase.

In its native context, IcsA localizes to the poles in the outer membrane of Shigella sp. (4). The ftsQ(Ts) allele of ftsQ contains an E125K mutation, which leads to dysfunction of FtsQ at elevated temperatures, inhibition of septation, and filamentation (24). Since IcsA expression is optimal at 37°C, this was used as the restrictive temperature in these experiments. In S. flexneri cells in

![FIG 3](https://example.com/fig3.jpg) 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 IcsA507-620-GFP in E. coli MG1655 or an MG1655 crp rssB mutant, carrying vector, p-ddlB-ftsQ, p-ddlB, or p-ftsQ, grown to stationary or exponential phase. (c) Distribution of native IcsA on the surface of intact cells of WT S. flexneri 2457T or an ftsQ(Ts) derivative visualized by immunofluorescence (IF). Growth was at 37°C, the restrictive temperature for ftsQ(Ts) cells, for 1 h. Aztreonam was used to filament WT (WT + aztr) and complemented ftsQ(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 ftsQ(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>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Foci at poles</td>
</tr>
<tr>
<td><strong>cmp B</strong> p-vector (exponential)</td>
<td>15 ± 5c</td>
</tr>
<tr>
<td><strong>cmp B</strong> p-ddlB-ftsQ (exponential)</td>
<td>85 ± 1b</td>
</tr>
<tr>
<td><strong>cmp B</strong> p-ddlB (exponential)</td>
<td>75 ± 8g</td>
</tr>
<tr>
<td><strong>cmp B</strong> p-ddlB (exponential)</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>WT p-vector (stationary)</td>
<td>19 ± 8lx</td>
</tr>
<tr>
<td>WT p-ddlB (stationary)</td>
<td>97 ± 1d</td>
</tr>
<tr>
<td>WT p-ftsQ (stationary)</td>
<td>91 ± 4r</td>
</tr>
<tr>
<td>WT p-ddlB (stationary)</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>WT p-ddlB (stationary)</td>
<td>16 ± 6f'</td>
</tr>
<tr>
<td>WT p-ftsQ (stationary)</td>
<td>82 ± 6f</td>
</tr>
<tr>
<td>WT p-ddlB (stationary)</td>
<td>85 ± 4f</td>
</tr>
</tbody>
</table>

* All strains are MG1655 and its derivatives, except for chromosomal integrants of *gfp*, *gfp::cppq*, and *gfp::ffq*, which are in the KS272 background.

b P = 0.03.

c P = 0.01.

d P = 0.03.

e P = 0.003.

f P = 0.007.

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 filamented 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 aztreonam, 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 ftsQ1 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.

The 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 duplocytosis 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 bipartite 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>24-57;1042-GFP</sub>) (3). Like IcsA, the GFP-tagged cytoplasmic derivative of SepA localizes GFP to the pole of E. coli during the 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 cococobacillary 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 icaA 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 polar caps on Shigella in part as a result of its regulated cleavage from the surface by specific proteases (3).
27, 28). In BS103 ftsQ(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 ftsQ(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 function 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 (tryptone casein soy broth) at 37°C. ftsQ1(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-icsA507-729-GFP, and pBAD-icsA507-729-mCherry, 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-icsA53,757-525:crp, a single-step PCR cloning strategy was used. The icsA open reading frame (ORF) was amplified by PCR with primers icsA53,757-525F and icsA53,757-525R; the resulting product was digested with HindIII and SphI and ligated into the HindIII and SphI sites of pAYC184.

A multicopy library of the E. coli MC4100 chromosome was constructed by partially digesting MC4100 chromosomal DNA with Sau3AI, isolating DNA fragments between 3 and 10 kb by agarose gel electrophoresis, and ligating the size-selected DNA into the medium-copy-number vector pACYC184. Within this library, 80% of clones contained inserts larger than 3 kb. The library was transformed into E. coli crp rssB (p-icsA53,757:crp) cells, and transformants were plated on maltose MacConkey agar. A total of 7,500 colonies were screened, 8% of which were white. Plasmid libraries were isolated from 73 randomly selected white colonies, and the 5′ and 3′ ends of the inserts were sequenced to identify the genetic context of the plasmid insert.

To create p-ddIB, the coding sequence of ddIB along with 762 bp upstream of its translation start codon was amplified from p-ddIB-fisQ as an XbaI-SphI fragment by PCR. The resulting product was digested with XbaI and SphI and ligated into the XbaI and SphI sites of pACYC184. To create p-fisQ, the coding sequence of fisQ along with 575 bp upstream of its start codon was amplified from p-ddIB-fisQ as a HindIII and SphI fragment by PCR. The resulting product was digested with HindIII and SphI and ligated into the HindIII and SphI sites of pACYC184.

Sequence analysis was performed to verify that each construct was correct. Transduction was performed with P114 phage by standard procedures. The sequences of primers used in PCR and sequencing are available from the authors upon request.

The fisQ1(Ts) allele was introduced into S. flexneri wild-type strain 2457T and virulence plasmid-cured strain BS103 by P1 transduction of the allele, which is linked to leu::Tn10, from E. coli strain MDG149. The presence of the fisQ1(Ts) allele was verified by testing transductants for temperature-sensitive growth and filamentation at elevated temperatures.

Transposon mutagenesis. A transposon library was generated in E. coli crp (p-icsA53,757:crp) cells using a mini-Tn10 (Cmr) delivered by lambda NK1324, as described elsewhere (38). The selection of transposon insertions that result in utilization of maltose was performed by growth on maltose minimal medium. Approximately 13,000 individual transposon insertion mutants were screened, as estimated by plating in parallel on maltose MacConkey agar. The ability of colonies that grew on maltose minimal medium to utilize maltose was verified by red colony color upon replica plating on maltose MacConkey agar.

Localization of icsA, FtsQ, and CRP fusions. The localization of IcsA507-620-GFP or -mCherry was determined by induction of protein synthesis from an arabinose-inducible promoter following growth either to mid-exponential phase or overnight to 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% t-arabinose to overnight cultures for 60 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. SepA1-24/57-1042-GFP expression was induced by the addition of t-arabinose to 0.2% for 40 min at 30°C in stationary-phase MG1655. Depletion of functional FtsQ in ftsQ1 strains was accomplished by shifting to growth at 37°C or 42°C for 60 to 150 min. Depletion of FtsB or FtsL in DJS86 and DJS87 was accomplished by growth in the absence of arabinose for 60 to 180 min. Filamentation with aztreonam was performed by adding it to 1 μg/ml for 50 to 60 min. Fixation and detection of IcsA on the surface of bacteria was performed as described previously (4). Alternatively, cells were labeled with primary and secondary antibodies prior to fixation.

Western 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. Sonenshein) at a 1:10,000 dilution, RpoS (Neoclonie) at a 1:5,000 dilution, or GFP (Molecular Probes or Roche) at a 1:1,000 dilution. GFP levels were determined by band densitometry using IP Laboratory (Scanalytics) software.

Microscopy. For microscopic observation, cells were mounted 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.
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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org.

Figure S1, TIF file, 1.3 MB.
Figure S2, TIF file, 1.6 MB.
Figure S3, TIF file, 0.3 MB.
Figure S4, TIF file, 2.6 MB.
Figure S5, TIF file, 2.5 MB.
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