Regulation of Surface Presentation of IcsA, a Shigella Protein Essential to Intracellular Movement and Spread, Is Growth Phase Dependent

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After lysing the phagocytic vacuole, Shigella spp. accumulate filaments of polymerized actin on their surface at one pole, leading to the formation of actin tails that enable them to move through the cytoplasm. We have recently demonstrated that the Shigella protein IcsA is located at the pole that is adjacent to the growing end of the actin tail (M. B. Goldberg, O. Barzu, C. Parsot, and P. J. Sansonetti. J. Bacteriol. 175:2189–2196, 1993). Not every bacterium that is observed within the cytoplasm has an actin tail. The factors that determine when a bacterium will form a tail are unknown. Here we demonstrate that at the moment of initiation of movement, Shigella spp. are frequently in the process of division. Furthermore, the expression of IcsA on the surface of the bacteria occurs in a growth phase-dependent fashion, suggesting that the surface expression of IcsA per se determines the observed association of bacterial division with movement.

Shigella flexneri induces diarrhea in humans by invading and killing colonic epithelial cells. Colonic mucosal lesions are a result of the spread of the cytoplastically located bacterium into adjacent epithelial cells and the concurrent inflammatory response. Shortly after being phagocytosed by the host cell, S. flexneri lyases the phagocytic vacuolar membrane (19) and is thereby released into the cytoplasm. Once within the host cell cytoplasm, the bacterium may begin to accumulate short filaments of polymerized cellular actin on its surface at one pole (1, 14). Then, in conjunction with bacterial movement, these actin filaments form a tight bundle, known as an actin tail, that extends behind the bacterium from that pole as it moves through the cytoplasm. The moving bacterium spreads from the cytoplasm of one cell into an adjacent cell by way of finger-like protrusions from the cell surface (1, 10, 14, 15, 18).

We have recently characterized a secreted Shigella surface protein, IcsA, that interacts with the actin tail (6). IcsA is a 120-kDa protein that is located at a single pole on the surface of the bacterium. The pole that contains IcsA is that which is derived from the old pole at the previous division. During the process of division, small amounts of IcsA appear at the pole that is derived from the septum at the previous division. This unipolar localization of IcsA necessarily places it adjacent to the growing end of the actin tail. Thus, IcsA is ideally situated to participate in actin tail formation. Furthermore, previous studies have demonstrated that a deletion in the gene encoding IcsA produces a mutant that does not polymerize actin on its surface, does not form actin tails, does not move within the host cell cytoplasm, does not form protrusions from the cell surface, and does not spread from cell to cell (1, 11, 21, 22).

Previous analyses using fixed specimens have shown that not every wild-type organism that is observed within the cytoplasm of an infected cell will have an actin tail attached to it. Many are without actin tails, suggesting that they are stationary at the time of fixation of the sample. The factors that determine when a bacterium will move are unknown. Prevost et al. (15) have observed previously that 85% of bacteria at the tips of protrusions are in the process of division; all organisms at the tips of protrusions have actin tails. We were therefore interested in examining whether the initiation of directional movement within the cytoplasm is associated with bacterial division.

The association of the initiation of bacterial movement with bacterial division was analyzed by using a video recording of PtK2 (potoroo kidney epithelial) cells infected with S. flexneri wild-type serotype 5 strain M90T (17). PtK2 cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum on 25-mm-diameter round glass coverslips. After infection, as described previously (19), infected coverslips were transferred to an aluminum chamber maintained at 37°C by a circulating water bath. Infected cells were observed on a Nikon Diaphot 300 inverted microscope equipped with phase-contrast optics and a Hamamatsu Newvicon camera. Time-lapse sequences were recorded by using Metamorph (Universal Imaging) and stored on a Panasonic optical disc. Typically, one frame was recorded every 10 s for a total of approximately 10 min.

Two approaches were used to analyze whether bacterial division is associated with the initiation of directional movement. First, for each bacterium observed to divide on video recordings, it was determined whether the organism (i) initiated movement within 1.5 min (real time) following division, (ii) was already moving at the time of division, or (iii) remained stationary for the 1.5 min following division. Second, for each bacterium observed on video to initiate directional movement, it was determined whether or not the organism divided within 1.5 min (real time) following the initiation of movement. For each infected cell, all bacteria were analyzed. A total of 1,250 bacteria in 38 distinct cells were observed over a total of 500 min (real time).

In any 1-min window of observation, 44% ± 9% (mean ± standard deviation) of the organisms were moving. Of the 136 organisms observed to divide, 68 (50%) also initiated directional movement as they divided and 47 (35%) were already moving at the time of division. Thus, 85% of those observed to...
divide either began moving or were already moving at the time; division was significantly associated with movement (chi-square test, $P < 0.001$). Of the 109 organisms observed to initiate movement, 73 (67%) also divided as they initiated movement; the initiation of directional bacterial movement was significantly associated with bacterial division (chi-square test, $P < 0.001$).

To further explore the relationship between bacterial growth and movement, we examined the effects of two types of bacteriostatic antibiotics on movement. Tetracycline inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit (3). Ampicillin interferes with normal cell wall synthesis and at low concentrations inhibits septation and leads to the formation of long filamentous bacteria (20).

PtK2 cells were infected as described above, and tetracycline was added to the culture medium at concentrations between 1 and 10 $\mu$g/ml. At these concentrations, bacterial growth was completely halted within 15 min. Moving bacteria continued to move at unidirectionally at approximately normal rates for at least 45 min after the cessation of protein synthesis. However, no stationary bacteria were observed to initiate movement during this time period. After wash-out of the antibiotic, apparently normal bacterial movement and division resumed within 20 to 25 min. Therefore, continuous bacterial protein synthesis is not required for the continuation of actin-based motility, but it is required for the initiation of movement. While under normal conditions, bacteria start and stop movement frequently; bacteria moving in the presence of tetracycline were not observed to stop. This preliminary observation suggests that IcsA may be periodically degraded or inactivated by some mechanism requiring protein synthesis.

Infected PtK2 cells were also treated with 20 to 40 $\mu$g of ampicillin per ml. Under these conditions, intracellular bacteria continue to grow without septating for at least 3 h, resulting in bacterial filaments up to 10 times the average length of single bacteria. Surprisingly, the bacterial filaments continued to exhibit some actin-based motility throughout this time period. Some filaments moved unidirectionally, while others occasionally switched their direction of movement. This change in direction is never observed under normal conditions. Many filaments underwent an undulating or back-and-forth movement, and a few curved into a horseshoe shape and moved in the direction of the curved edge. Movement was observed to start and stop periodically in the presence of ampicillin, in contrast to what was observed in the presence of tetracycline. These observations suggested that the filaments might be expressing IcsA and forming actin tails at both poles; surface labelling of bacteria treated with ampicillin revealed localization of IcsA at both ends of the filaments (data not shown), supporting this hypothesis.

The expression of IcsA on the surface of strain M90T was assessed at multiple phases of bacterial growth by examining the percentage of bacteria that was labelled as a function of the optical density at 600 nm (OD<sub>600</sub>) and time from 1/100 back-dilution of the culture (Fig. 1 and 2). Bacteria were grown in Trypticase soy broth (Diagnostics Pasteur, Marnes la Coquetter, France) at 37°C. Surface labelling was performed as described previously (6). To determine the percentage of bacteria labelled at a given time point, labelled and total numbers of bacteria were counted in the same microscopic field under immunofluorescence and phase-contrast visualization, respectively. At each time point, at least 200 bacteria were counted. Counting was performed blinded to the time point and OD of the particular preparation. The data presented are from a single experiment. Independent experiments were performed on three separate occasions, and the results of all three were similar.

The percentage of bacteria that were labelled increased steadily throughout the early and late exponential phases of growth, from a minimum of 13% just after back-dilution (at an OD<sub>600</sub> of 0.023) to a maximum of 87% after 4 h 45 min (at an OD<sub>600</sub> of 2.2), and subsequently decreased during stationary phase to 28% at 24 h (at an OD<sub>600</sub> of 2.6) (Fig. 1 and 2). Thus, the expression of IcsA on the bacterial surface is maximal during periods of rapid bacterial division and is markedly diminished on nondividing bacteria.

A slight reduction in the percentage of bacteria that was labelled was observed at 3 h 15 min of growth (at an OD<sub>600</sub> of 0.90) in repeated experiments. An explanation for this observation is based on the fact that the bacteria undergo multiple cell divisions during the 24-h period of study (approximately one division every 40 min) and that the cell cycle of the individual bacteria in the culture population is relatively more synchronized towards the beginning of the period of study. Given this, the 3 h 15 min time point may correspond to a moment at which relatively more individual bacteria are early in the cycle of division. In support of this theory is our observation that at the 3 h 15 min time point, 38% of the bacteria were short (less than 3 $\mu$m in length), whereas at the 2 h 30 min, 4 h, and 4 h 45 min time points, considerably fewer bacteria were short (18, 6, and 26%, respectively). Short bacteria are likely to be those that have divided recently. These data suggest that surface expression of IcsA may be linked to the cell cycle.

To determine whether the growth phase-dependent surface expression of IcsA was due to a growth phase dependence in the amount of IcsA present in the cell, whole-cell proteins were prepared at the same time points of bacterial growth as those used to assess surface labelling described above. To normalize the amount of protein in each preparation, at each time point, proteins were prepared from a volume of bacteriologic culture that

![Figure 1](http://iai.asm.org/)

**FIG. 1.** Surface expression of IcsA on S. flexneri through the growth cycle. (a) Growth curve indicating time points at which bacteria were harvested for analysis; (b) percentage of bacteria labelled at each time point. Labelling was performed with affinity-purified IcsA antibody.
was calculated from the OD_{600} to represent a fixed number of bacteria [assuming that the number of bacteria = OD_{600} × (5 × 10^8)]. Western blotting (immunoblotting) was performed as described previously, using rabbit antiserum to IcsA (6), except that visualization was performed with ECL Western blotting detection reagents (Amersham). Scanning densitometry was performed with a Molecular Dynamics laser scanning densitometer using ImageQuant software. These analyses show that the presence of IcsA in whole-cell protein occurs in a pattern similar to its expression on the bacterial surface, with greater amounts being produced in the mid and late exponential phases than in the stationary phase (Fig. 3). However, during the stationary phase, a significant amount of IcsA is present but not expressed on the bacterial surface. These data indicate that the growth phase dependence of surface expression of IcsA appears to be associated with a growth phase-related variation in the presence of IcsA as well as an alteration of the presentation of IcsA on the surface during the stationary phase.

The 120-kDa full-length form of IcsA is cleaved at the bacterial surface, releasing a 95-kDa form into the culture...
supernatant (6). Because secreted IcsA is found associated with the actin tail, we have previously hypothesized that cleavage might occur in conjunction with actin tail formation and therefore also in conjunction with bacterial movement (6). To assess whether IcsA cleavage and secretion are growth phase dependent in a fashion that parallels the presence of IcsA in whole cells, we examined the relative amounts of IcsA in the culture supernatant as a function of phase of growth. As can be seen in Fig. 3a and c, the presence of the cleaved 95-kDa form in the supernatant occurs simultaneously with IcsA expression on the bacterial surface (Fig. 1 and 2), suggesting that cleavage and secretion of IcsA occur simultaneously with the formation of the actin tail.

Data presented here indicate that surface presentation of IcsA is growth phase dependent. Bacteria in the exponential phase of growth are actively dividing, whereas bacteria in the stationary phase are not undergoing division. Therefore, one explanation for the data presented here is that IcsA expression and surface presentation are associated with the cell cycle, occurring maximally during cell division. In this case, the mechanism of the association of bacterial division to bacterial movement may be a cell cycle-dependent surface presentation of IcsA.

The presence of IcsA in the supernatant throughout the log phase and then its absence at 24 h of growth indicate that supernatant IcsA is degraded. One possibility is that variations in supernatant IcsA might be due to the secretion of a growth phase-dependent protease. The recent demonstration by Nakata et al. (12) that the *Escherichia coli* surface protease OmpT cleaves IcsA from the surface of *E. coli* strains into which *icsA* has been transformed might support this hypothesis. We anticipated that this would not be the case, however, since we had previously demonstrated that *E. coli* protease activity does not fully degrade IcsA but rather leads to the release of two major protein bands (at 95 and 80 kDa) into the supernatant (6).

To further clarify whether the growth phase variations in IcsA were due to growth phase alterations in *icsA* transcription or in IcsA production or degradation, the relative amount of *icsA* transcript was examined at multiple phases of bacterial growth (7). An equivalent quantity of RNA, as determined from ethidium bromide staining of 23S rRNA (Fig. 4b), was loaded into each slot. As a probe, the 2.8-kbp *XbaI*-*CiaI* fragment, which is internal to *icsA*, was isolated from plasmid pHS3199 (4) and labelled with a 32PQuickPrime kit (Pharmacia). On a Northern (RNA) blot (Fig. 4a), a band at 3.3 kb, which corresponds to the calculated molecular weight of the *icsA* transcript, is weakly present in RNA isolated from the early exponential phase (lane 1), increases in intensity in RNA isolated from the mid-exponential phase (lanes 2 and 3), and is undetectable in RNA isolated from the late exponential phase (lane 4). Two additional extremely weak bands were seen at equivalent intensities in all four lanes at the same apparent molecular weights as the heavy bands of 23S and 16S rRNA; these bands are likely the result of nonspecific binding of the probe. Thus, *icsA* transcription closely parallels IcsA surface expression, indicating that the expression of large amounts of IcsA on the surface during exponential growth is regulated at the transcriptional level.

Other bacterial proteins that are expressed as a function of the cell cycle have been characterized. For example, transcription of the *E. coli* cell division gene *fzZ* has been shown to vary during the cell cycle, reaching a maximum around the time of initiation of DNA replication (5) or at about 20 min after cell division in the 60-min division cycle (16). In *Caulobacter crescentus*, assembly and disassembly of the flagellum and stalk as well as expression of the methyl-accepting chemotaxis proteins and the heat shock protein DnaK occur in a cell cycle-dependent fashion (2, 8, 9, 13). Cell cycle-dependent expression of IcsA in *Shigella* spp. would directly link bacterial division to bacterial movement and spread.

In sum, the initiation of actin-based *Shigella* movement is significantly associated with bacterial division. In addition, the expression of IcsA on the surface is growth phase dependent and regulated at the transcriptional level. Furthermore, de novo protein synthesis is required for the initiation of movement. While surface expression is growth phase dependent, it is not dependent on the formation of the septum per se during division. Distribution of IcsA to both poles, which occurs in the presence of ampicillin, leads to inefficient unidirectional movement. Together, these data indicate that de novo surface expression of IcsA per se determines the association of bacterial division with movement. Furthermore, the function of this association appears to be to ensure the unidirectionality and efficiency of actin-based movement by keeping the surface-expressed IcsA concentrated at a single pole.

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