Bile Salts Stimulate Recruitment of IpaB to the *Shigella flexneri* Surface, Where It Colocalizes with IpaD at the Tip of the Type III Secretion Needle[▽]

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Shigella flexneri uses its type III secretion apparatus (TTSA) to deliver invasins into human cells. This TTSA possesses an external needle with IpaD at its tip. We now show that deoxycholate promotes the stable recruitment of IpaB to the needle tip without inducing a rapid burst of type III secretion. The maintenance of IpaB at the needle tip requires a stable association of IpaD with the Shigella surface. This is the first demonstration of a translocator protein being stably associated with the TTSA needle.

Shigella flexneri uses its type III secretion system (TTSS) to invade colonic epithelial cells and trigger the onset of shigellosis (2, 4). A prominent feature of the TTS apparatus (TTSA) is an external needle composed of MxiH monomers (3). We recently demonstrated that IpaD localizes to the tip of the *Shigella* TTSA needle, where it controls the secretion of the translocators IpaB and IpaC (6). Neither IpaB nor IpaC was detected on the *Shigella* surface in this previous study (6). Because there have been reports of IpaB association with the *Shigella* surface (11, 14, 15), we explored different conditions that might favor controlled mobilization of IpaB to the surface without inducing the burst of type III secretion that is seen upon host cell contact. In particular, bile salts were examined based on observations that, when added to tryptic soy broth (TSB), they lead to increased *Shigella* adherence to and invasion of HeLa cells (12). The molecular basis for this effect is not known and has not been explored in detail.

IpaB and IpaD colocalize on the *S. flexneri* surface in the presence of DOC. IpaD and IpaB were detected on the *Shigella* surface by using immunofluorescence microscopy as previously described (6). *S. flexneri* was grown to early log phase in TSB supplemented with 2.5 mM (0.1%, wt/vol) deoxycholate (DOC), a concentration well within the physiological range seen in the human intestine (Fig. 1) (5). As expected, IpaD continued to be detected on the bacterial surface by using rabbit anti-IpaD antiserum and Alexa Fluor 568 goat anti-rabbit immunoglobulin G (IgG) under these conditions (Fig. 1A). In the presence of DOC, however, IpaB was also detected on the *Shigella* surface by using rabbit anti-IpaB antiserum (Fig. 1B). Both proteins gave rise to similar punctate staining

patterns. In contrast, IpaB was not surface exposed in either an *mxiH*-null (Fig. 1C) or an *ipaD*-null (Fig. 1D) mutant strain, indicating that IpaB surface localization relies upon a functional TTSS needle and IpaD. The maintenance of IpaD at the *Shigella* surface appears to be required for IpaB recruitment in the presence of DOC because a small C-terminal deletion that allows IpaD and IpaB secretion without concomitant retention of IpaD at the bacterial surface fails to permit IpaB labeling on the bacterial surface (Table 1).

We previously reported that IpaB is not detected on the surface of early-log-phase *Shigella* in the absence of DOC (6); however, immunoblot analysis suggested that IpaB is associated with the TTSS needle (6). When bacteria were grown to early log phase in TSB and then DOC was added, IpaB mobilized to the bacterial surface within 15 min of DOC exposure. IpaB and IpaD were also surface localized when *S. flexneri* was exposed to chenodeoxycholate and taurodeoxycholate (data not shown). IpaC was never seen at the *Shigella* surface during these experiments, and the levels of IpaB in the bacterial cytoplasm did not appear to change (data not shown), presumably due to the minimal effect that DOC addition had on the short-term induction of type III secretion (see below).

To determine whether IpaD and IpaB actually colocalize on the *Shigella* surface, bacteria were double labeled with anti-IpaD monoclonal antibody and rabbit anti-IpaB antiserum. Primary antibodies were detected using Alexa Fluor 488 goat anti-mouse IgG (Fig. 1J) and Alexa Fluor 568 goat anti-rabbit IgG (Fig. 1K). When the images were merged (Fig. 1L), IpaD and IpaB appeared to colocalize. While IpaD could be readily observed without IpaB costaining, IpaB was not typically observed on the surface without colocalizing with at least some population of IpaD (Fig. 1, panels J to L). The fact that a short C-terminal truncation of IpaD eliminated its association with the *Shigella* surface (Table 1) but allowed uncontrolled secretion of IpaD and IpaB indicated that none of the observed results are due to the readsorption of secreted IpaD, IpaB, or IpaD/IpaB complexes to the bacterial surface. These findings prompted a closer look at the localization of IpaB and IpaD with respect to the *Shigella* TTSA.

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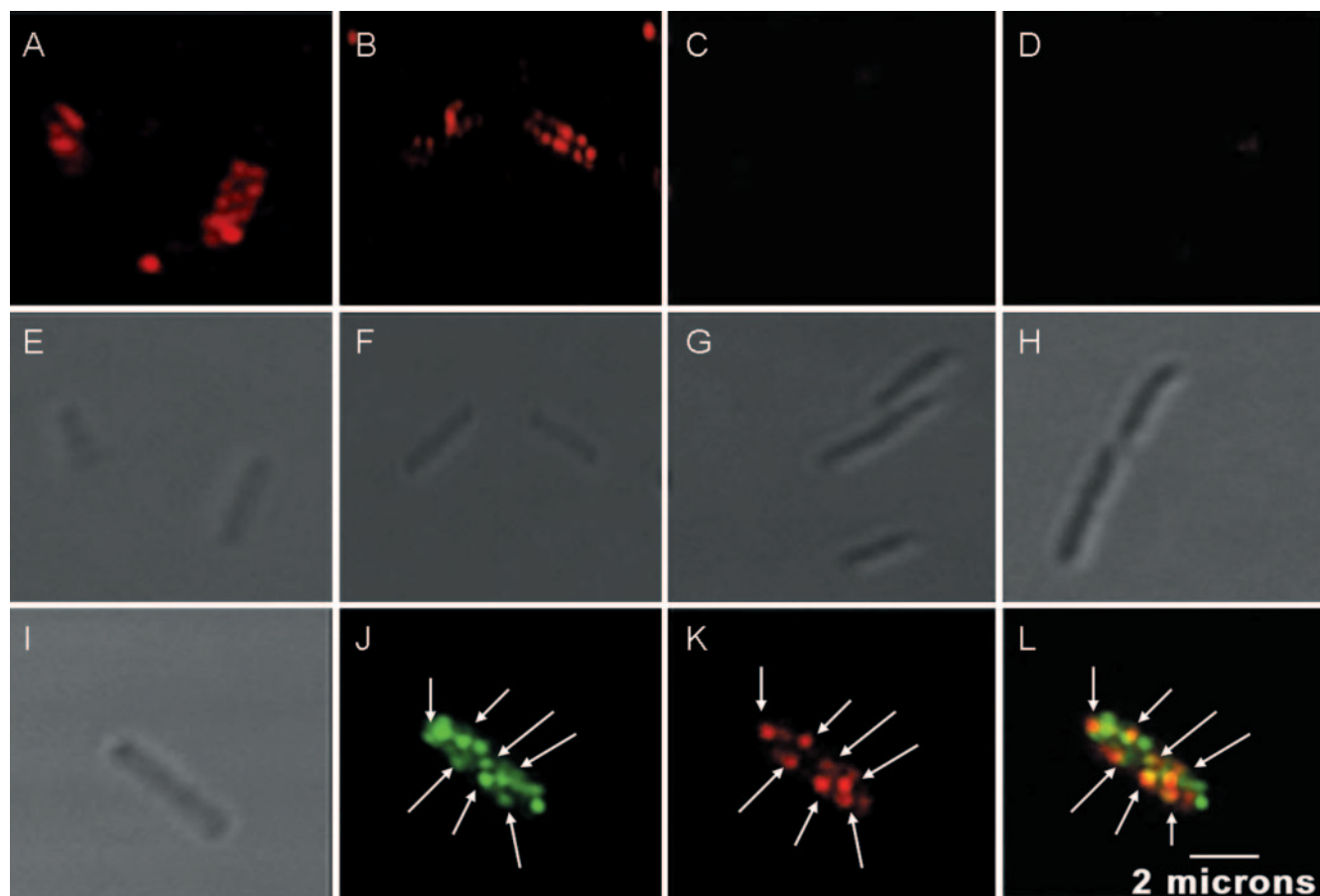▽ Published ahead of print on 12 February 2007.

FIG. 1. IpaD and IpaB localize to the *S. flexneri* surface. IpaD and IpaB were detected on the surface of *S. flexneri* grown in TSB containing 2.5 mM DOC by confocal immunofluorescence microscopy using rabbit anti-IpaD antiserum (A) or rabbit anti-IpaB antiserum (B). IpaB was not detected on the surface of *mxiH* (C)- or *ipaD* (D)-null strains. Panels E to H are differential interference contrast (DIC) micrographs of panels A to D, respectively. IpaD and IpaB appeared to colocalize on the *S. flexneri* surface by double labeling. Panel I shows the DIC micrograph of panels J to L. Panel J is a green pseudocolored image indicating IpaD staining by using anti-IpaD monoclonal antibody. Panel K is a red pseudocolored image indicating IpaB staining by using rabbit anti-IpaB antiserum. Panel L is a merged image of panels J and K. The arrows point to regions where both IpaD and IpaB appear to be labeled.

IpaB and IpaD colocalize at the *S. flexneri* TTSA needle tip. Bacteria and sheared MxiH needles were examined by transmission electron microscopy with negative staining and immunogold labeling. *Shigella* was grown to early log phase in TSB containing 2.5 mM DOC. The bacteria or needles sheared from the bacteria were applied to carbon-Formvar grids and

TABLE 1. IpaB recruitment to the TTSS needle tip of mutant *S. flexneri* strains[a]

S. flexneri strain	IpaD localization		IpaB localization
	Before DOC	After DOC	
ipaD-null mutant (SF622)	NA	NA	−
ipaB-null mutant (SF621)	+	−	NA
IpaD$^{\Delta328-332}$ mutant	−	−	−

[a] Surface localization of IpaD and IpaB was determined by immunofluorescence microscopy. At least 100 bacteria in each of three randomly selected fields were counted. Strains with >80% of bacteria demonstrating surface staining for the given protein were considered positive. In most cases, surface localization was seen on either all bacteria or none. NA, not applicable.

treated with monoclonal anti-IpaD IgG, followed by 5-nm gold particle-labeled goat anti-mouse F(ab′)$_2$ and rabbit anti-IpaB antiserum and 20-nm gold particle-labeled goat anti-rabbit IgG (Fig. 2). The grids were then stained with 2% uranyl acetate and imaged using a Jeol 1200 EX II transmission electron microscope at an electron acceleration voltage of 120 keV as described previously (6). In Fig. 2A and B, needles sheared from the bacterial surface show IpaD labeling (5-nm gold particles) near the needle tip with IpaB labeling (20-nm gold particles) associated with the same needle. Similar micrographs showing bacterium-associated needles indicated that the observed staining was at the needle tip and not at the base of sheared needles (Fig. 2C).

When IpaB labeling alone was examined for *Shigella* grown in the presence of DOC, labeling at the needle tip was readily apparent for both bacterium-associated (Fig. 2D) and sheared (Fig. 2E) needles by using secondary F(ab′)$_2$ labeled with 5-nm gold particles. Likewise, IpaD staining alone (using rabbit antiserum in this case) continued to be seen for needles prepared from *Shigella* grown in the presence of DOC (Fig. 2F), as was
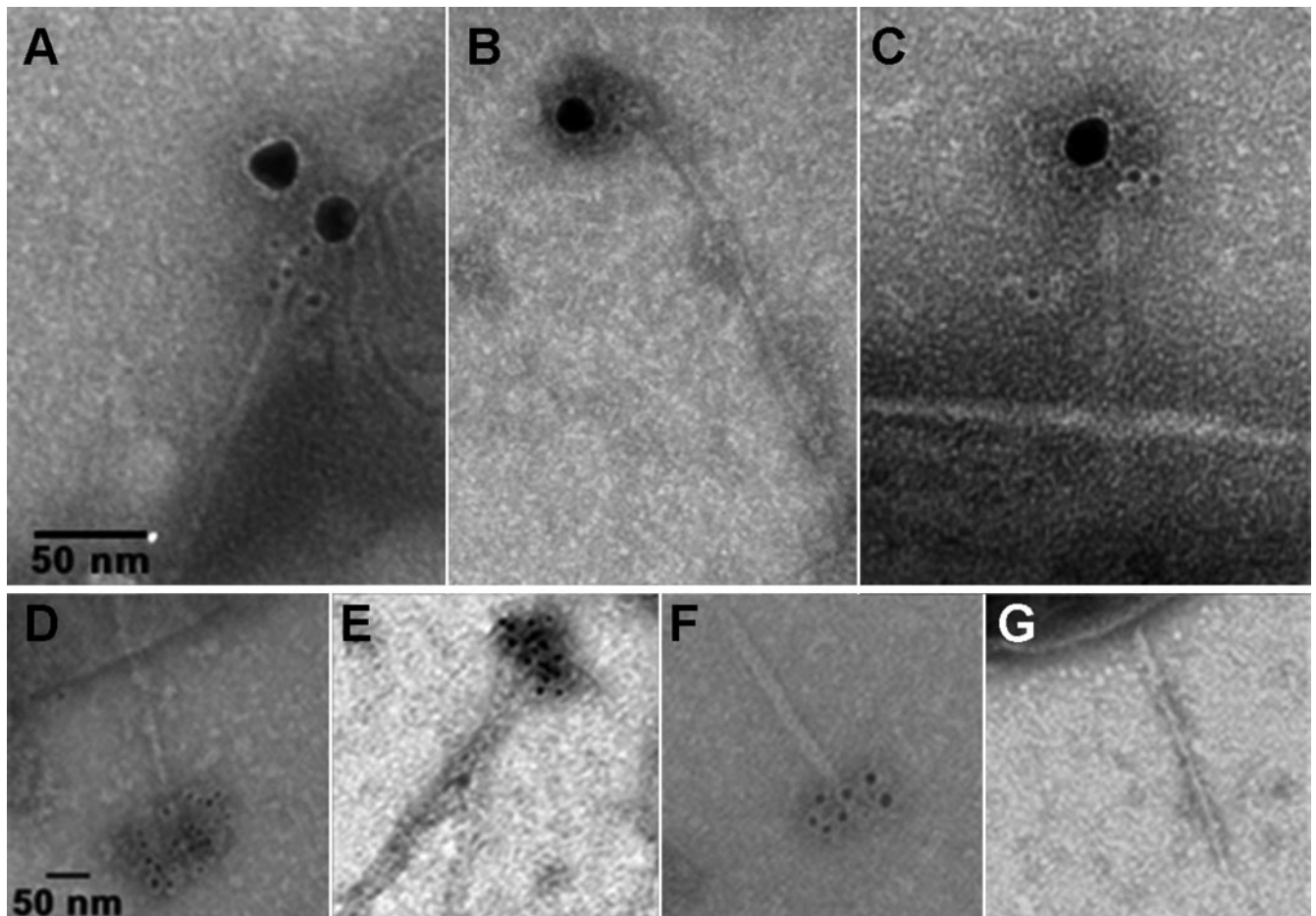
FIG. 2. Transmission electron micrographs of immunogold-labeled IpaD and IpaB at the *S. flexneri* TTSA needle tip. Free TTSA needles (A, B, E, and F) or *S. flexneri*-associated needles (C, D, and G) were immunolabeled with secondary gold-labeled IgG or F(ab′)$_2$ fragments and negatively stained as described in the text. Typical dissociated needles are shown in panels A and B after being double labeled for IpaD (5-nm gold particles) and IpaB (20-nm gold particles), respectively. In panel C, a bacterium-associated needle is labeled for IpaD with 5-nm gold particles and for IpaB with 20-nm gold particles. A *Shigella*-associated needle (D) and a needle dissociated from *Shigella* (E) (another needle with neither end exposed crosses the field) are shown after being stained with anti-IpaB IgG only following exposure to DOC. A needle dissociated from *Shigella* (F) is shown after being stained with anti-IpaD only following exposure to DOC. In panel G, a *Shigella*-associated needle stained for IpaB after the bacteria were grown without any exposure to DOC is shown. Although needle breakage could account for the lack of IpaB detection, no IpaB staining was found to be associated with needles from multiple samples.

previously seen for needles prepared from bacteria grown without DOC (6). In the absence of DOC, no IpaB was seen at the TTSA needle tip (Fig. 2G). Thus, the postulated IpaB-IpaD secretion plug appears to be located at the *Shigella* TTSA needle tip. From the presented data, one might also speculate that IpaB assumes a position that is external to IpaD relative to the needle tip and bacterial surface, but this will require further work to confirm. It is therefore possible that IpaD localizes initially to the needle tip, where it detects environmental signals that trigger the recruitment of IpaB to a distal position at the needle tip to form an MxiH-IpaD-IpaB ternary complex.

DOC promotes maturation to a secretion-competent state. The small amphipathic dye Congo red (CR) is known to induce a burst of *Shigella* type III secretion which mimics that seen upon host cell contact (1). DOC causes a minor, low-level increase in type III secretion during sustained growth (12), but whether it induces a rapid burst of secretion has not been explored. Thus, early-log-phase bacteria were resuspended in

phosphate-buffered saline containing 35 μg/ml CR or 2.5 mM DOC for 30 min. The supernatants were then collected, and the levels of IpaB, IpaC, and IpaD were determined by immunoblot analysis using monoclonal anti-IpaB, anti-IpaC, and anti-IpaD antibodies followed by IRDye 800 goat anti-mouse secondary antibodies. Immunoblots were visualized using an Odyssey infrared imager (LI-COR, Lincoln, NE). Unlike CR, DOC does not induce the burst of secretion associated with TTSS activation (Fig. 3). CR and DOC therefore seem to influence the TTSS differently. Because DOC does not actually induce a rapid burst of type III secretion, it is unlikely that IpaB localization at the needle tip is an artifact of the secretion process. The molecular basis for DOC-mediated recruitment of IpaB to the *Shigella* TTSA needle tip remains to be determined. We speculate that DOC association with IpaD results in a conformational change that allows IpaB to exit the TTSA and remain bound to IpaD at the needle tip in the absence of further secretion stimuli.
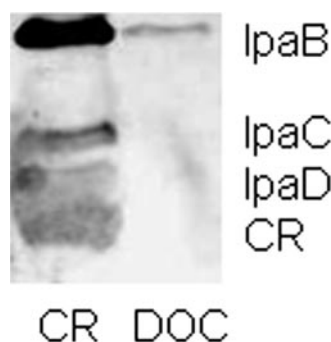
FIG. 3. Analysis of protein secretion by *S. flexneri*. IpaB, IpaC, and IpaD from the supernatants of equal numbers of *S. flexneri* following 30 min of incubation in phosphate-buffered saline containing 35 μg/ml CR or 2.5 mM DOC were analyzed by immunoblotting using monoclonal anti-IpaB, anti-IpaC, and anti-IpaD antibodies and IRDye 800 goat anti-mouse IgG.

This is the first report of a TTSS translocator protein being specifically recruited to the tip of the TTSA needle in a controlled fashion. IpaB localization to the needle tip requires the presence of IpaD and MxiH. This event can be triggered by bile salts, leading to speculation that IpaD could serve as an environmental sensor that gives rise to a secretion-competent or secretion-primed TTSA. These findings fit well with observations that both IpaB and IpaD are needed to control type III secretion (10). In the same respect, this important observation suggests that a needle tip complex composed of IpaB and IpaD (with IpaB perhaps taking up a more distal position relative to the bacterial surface) may represent the primary sensor of host cell contact. This is consistent with the fact that IpaB is a cholesterol binding protein (7, 8) and that invasion occurs at lipid rafts on host cells (9, 13). It will now be important to determine the fate of this needle complex following host cell contact.

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