Early Steps in Inducing Type III Secretion in *Shigella*

The *Shigella* type III secretion apparatus tip complex matures in stepwise fashion before effector proteins are injected into target cells

Wendy L. Picking and William D. Picking

Gram-negative bacteria have several means for secreting proteins from the cytoplasm to the surrounding milieu. Among them, the type III secretion system (TTSS) proves to be a highly coordinated means for exporting proteins across the inner and outer membranes of bacterial pathogens and the cytoplasmic membrane of the eukaryotic cells that they target. Such TTSSs are found in a wide range of gram-negative bacterial pathogens, including *Shigella*, *Yersinia*, *Salmonella*, *Pseudomonas, Burkholderia*, and enteropathogenic *Escherichia coli* (EPEC). In each case, the TTSS provides a means by which the bacteria communicate directly with targeted eukaryotic cells, subverting normal processes and benefiting the pathogen. Although some parts of the type III secretion apparatus (TTSA) are highly conserved among these organisms, the secreted effector proteins vary greatly and tend to be pathogen specific.

Induction of type III secretion in *Shigella* can be dissected into three distinct steps—formation of the TTSA needle tip complex from IpaD, recruitment of IpaB to the tip complex, and recruitment of IpaC to the complex concomitant to secretion induction. This model, which provides a starting point for comparisons with other gram-negative pathogens, is the only one of its kind. Although this stepwise model simplifies the overall dynamic processes leading to host-cell subversion, these stepwise snapshots provide a platform for advancing our understanding of specific mechanisms shared among TTSA needle tip complexes immediately before injection of effector proteins into target cells.

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Architecture Helps To Determine TTSA Functions

Much of what is known of the ultrastructure of the TTSA stems from transmission electron microscopy analysis of these systems from *Shigella flexneri*, *Salmonella typhimurium*, and *Yersinia* spp. In these microorganisms, the TTSA resembles a syringe and needle, leading many researchers to refer to it as the injectisome. The syringe portion is composed of 25 or more different proteins, which form a basal body that spans the inner and outer membranes of the

Summary

- Many gram-negative bacterial pathogens produce type III secretion systems (TTSSs) as important virulence factors.
- The type III secretion apparatus (TTSA) consists of a complex basal structure, which spans the inner and outer bacterial membranes, and an external needle with a tip complex.
- Environmental ligands such as bile salts can elicit the first discrete step in *Shigella* type III secretion, recruiting the translocator protein IpaB into the maturing tip complex.
- Contact with membranes containing sphingolipids and cholesterol triggers the final step of assembling the *Shigella* TTSA tip complex and leads to induction of type III secretion.
- The *Shigella* type III secretion system can be activated and induced in a stepwise fashion.

Wendy and William Picking: in Life and the Lab Together, Plus He Makes Pickles

When the former Wendy Tiemann met William Picking in graduate school, she could not get to first base—at first. "We met on the softball field," she says. "Bill's roommate said their team needed more women. Bill was the coach—and a great pitcher—and he put me in right field. I hate right field. So I got to know the coach a little better, and [later] got to play first base—which I love."

"By the time two seasons had passed, we were an item," Bill Picking adds. And more than two decades later they remain partners in life and the lab. When they met on the softball fields of the University of Kansas, they were both doctoral students. They went on to postdoctoral fellowships at the University of Texas at Austin, then to St. Louis University.

Today, Bill, 49, is professor and head of the department of microbiology and molecular genetics at Oklahoma State University, while Wendy, 46, is associate professor in the same department. Together, they study how certain pathogenic bacteria inject proteins into human cells through the Type III secretion system. The goal in better understanding that mechanism is to learn how to prevent diseases that these bacteria cause.

With such closely aligned careers, the Pickings often deal with questions about how a married couple can work together and preserve both relationships. "If there is any particular misconception about our roles as a research team, it has to be that I am the one running the show," Bill says. "In reality, Wendy is the driven one, and the person from whom the best ideas arise."

"When we started at St. Louis University, we subconsciously had an imaginary line of duties," Wendy Picking says. "I did my duties, and he did his. He did not cross the line, and when I did, I was quickly reminded why we had those lines. We still have those lines today, even though they are a little less rigid. I cannot do certain things, and BP cannot do certain things. We know this, and don't even try to cross the line anymore."

Bill Picking says each brings different qualities to their collaboration, and these differences strengthen it. "Being around each other as much as we are can result in tensions at times. However, we've always managed to work through any difficulties," he says. "I think the useful thing is that we have very different but complementary personalities. Wendy can be quite intense and single-mindedly focused in what she's doing, while I tend to be more laid back in my overall attitude. She is go-go-go and ready to take on the world. I tend to weigh things a bit more carefully. She drags me along, while I sometimes have to temper her zeal."

"The best and worst part is that we are together 24/7/365," she adds. "We can talk science at any time, day or night. We can shut science off, or turn it on anytime. We can be watching [television], and I'll have some idea and we'll talk about it. I don't have to talk to my collaborator during normal business hours, or by e-mail. Of course, when things aren't going well in the lab, or it is 'grant season'—as our kids call it—it is more stressful because there is no real escape, but we just get through it." He agrees. "Any time a new idea pops up, the other one is there to hear it out in real time," he says. "We've now been married long enough to know that the differences in opinion we have are small in the grand scheme of things."

They come from similar backgrounds. Both were born and raised on Kansas farms—Wendy in Lincoln, Bill in Abilene. Wendy's father was a farmer and electrician; Bill's father was a farmer and mechanic. His mother was a bookkeeper, while her mother held a variety of jobs, including teacher's aide and bookkeeper. Their agricultural origins inspired both to appreciate and enjoy science.

"I grew up in small-town America, with a graduating class of 36," she says. "My love of science came from growing up on a farm and experiencing science every day. The wheat/soybeans/milo [sorghum] was planted and grew, and then had to be harvested. The leftovers in the fridge grew moldy if we didn't eat them, the bugs came and went, the river runs or floods, and people grow up, have families, and die. It's a circle of life."

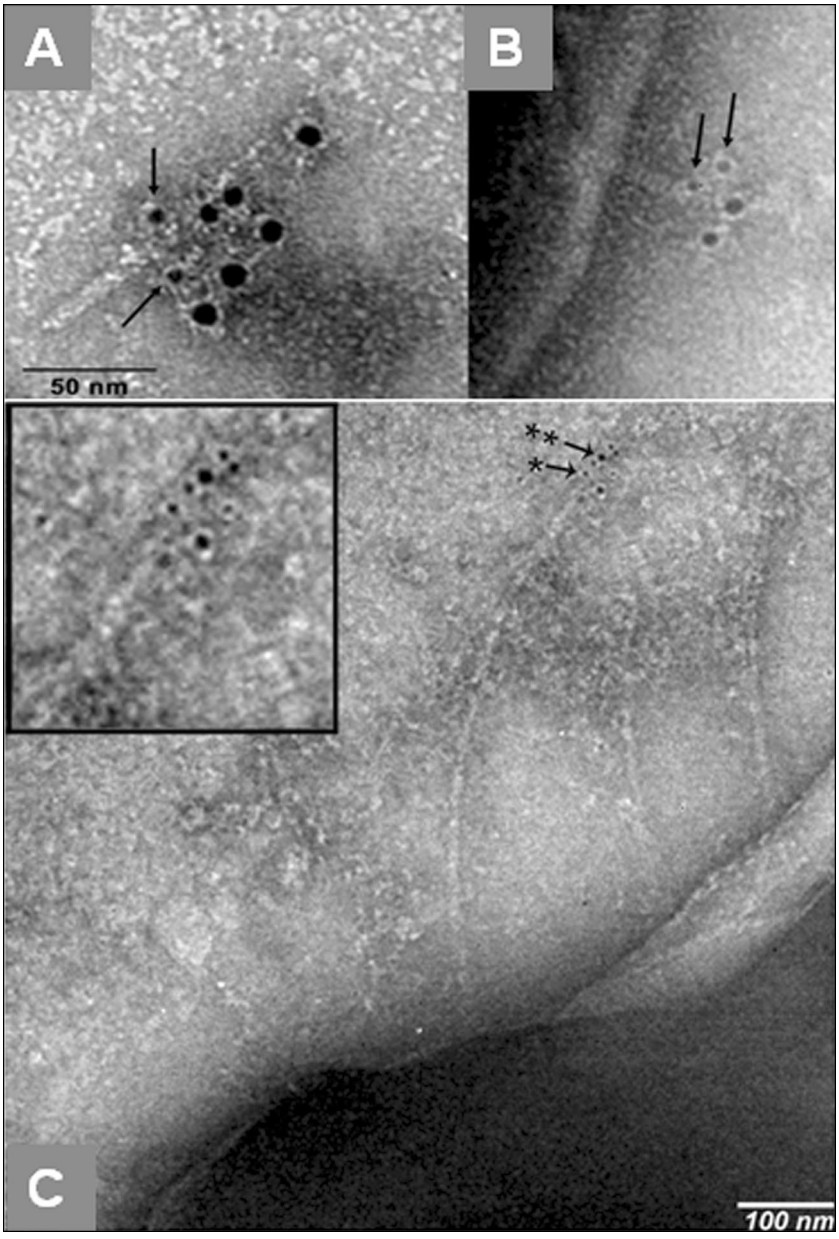
"Like most kids who have a love a nature and biology, I believed that I would go to medical school and become a physician," he says. "But interactions with my professors as an undergraduate convinced me that research was the direction meant for me." He earned a B.S. degree in 1984 at Kansas State University. She earned a B.A. degree at the University of Kansas in 1985. He was a semester ahead in graduate school; he earned his doctorate in 1989, she earned hers in 1990.

Despite their togetherness at work, each has separate outside interests. Wendy makes quilts, preferring traditional designs. Bill collects postage stamps, a hobby he started in elementary school. He also likes to hunt, fish, garden, cook, and make dill pickles. "I believe anyone who can do biochemistry can cook," he says, although "my cooking is much more ad lib and experimental than my research."

They have two children, Lydia, 17, and Daniel, 15. "We even have managed to include our children in the lab science," he says. "Our son is pretty good at purifying recombinant proteins, and our daughter has done great plasmid preps. This has given us all a bit of common thread to build upon as a family. I don't for a second suspect that our kids will be microbiologists, but, no matter what road they take, they will understand and appreciate microbiology."

Marlene Cimons
Marlene Cimons lives and writes in Bethesda, Md.

FIGURE 1



Immunogold labeling with transmission electron microscopy was used to show that TTSA needles sheared from (A) or retained on (B) the *Shigella* surface possess both IpaD (5-nm particles shown by arrows) and IpaB (10-nm particles without arrows) following incubation of the bacteria with deoxycholate (DOC). In panel C, induction of type III secretion using liposomes possessing phosphatidylcholine, sphingomyelin and cholesterol is accompanied by the recruitment of IpaC (shown by labeling with 5-nm gold particles [*]) and the continued presence of IpaB (10-nm gold particles [**]) on needles still attached to the *Shigella* surface. The presence of the different sized particles is more clearly evident in the inset. (Adapted from Epler et al., Infect. Immun. **77**:2754–2761, 2009.)

bacterium. The cytoplasmic bulb of the basal structure contributes to controlling the order of assembly of this apparatus and the subsequent release from it of effector proteins. The cytoplasmic bulb includes a protein with ATPase activity that is similar to the F_1 ATPases and provides at least a portion of the catalytic power of the TTSS. ATP hydrolysis may also be involved in separating secretion substrates from their cognate chaperones and in loading the TTSA with cargo.

Additional regulatory proteins that remain to be fully characterized act as substrate switches to coordinate assembly of the needle and nascent tip complex and to set the hierarchy of translocator and effector protein secretion. The remaining structural components of the TTSA basal body include the inner membrane rings embedded in the cytoplasmic membrane, the periplasmic or inner rod that spans the periplasm, and the outer membrane ring that connects the base with the extracellular portions of the TTSA. Extending outward from the base is the external needle of the TTSA. The monomers making up the needle pack in a helical fashion, forming a 50- to 60-nm cylinder, with a 7-nm outer diameter and having a 2- to 3-nm inner channel. At the distal end, the needles of the *Yersinia, Shigella*, and likely other gram-negative TTSAs possess a needle-tip protein complex.

While the sensing and triggering mechanisms for induction of type III secretion are only now beginning to be studied, it is becoming clear that contact between the TTSA tip complex and environmental signals is critical for this process. The first step in type III secretion induction is generally thought to be recruitment of translocator proteins to the needle tip complex, where they form a translocon pore in the target cell membrane. It is through the translocon that effector proteins gain access to the host interior. The size of the translocon pore has been determined for several systems, and it is generally considered to have an inner diameter between 1.5 and 3.5 nm. The translocon remains part of the needle

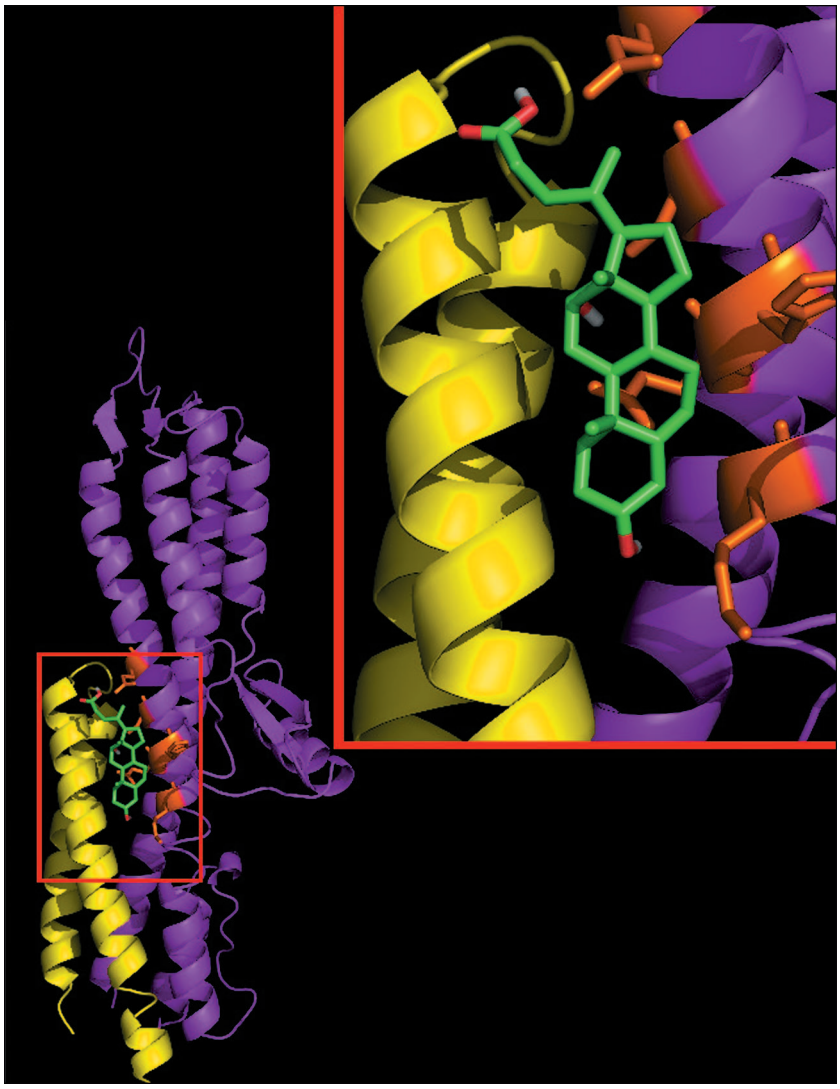
tip protein complex following pore formation to complete a delivery conduit composed of the TTSA basal body, needle, tip protein, and translocon, and through which subsequent effector proteins are injected to enter the host cell cytoplasm.

Inducing Type III Secretion in *Shigella*

Shigella species cause shigellosis, a potentially life-threatening bacillary dysentery that is restricted to humans and other higher primates. While shigellosis is perceived as a disease of the developing world, it is an underreported problem in industrialized nations. After being ingested, *Shigella* travels to the colon, where it infects the intestinal epithelium. The bacteria cross M cells, invading and killing underlying macrophages to gain access to the basal side of the colonic epithelium. *Shigella* then enters epithelial cells by inducing macropinocytosis. This ability to kill macrophages and invade epithelial cells depends entirely on the TTSS, which is encoded within the 31-kB entry region of the *Shigella* virulence plasmid. The *mxi/spa/ipa* loci encode the TTSA proteins, the translocators, and the major effector proteins.

While IpaB and IpaC are first and foremost translocator proteins, they also are effectors. IpaB activates caspase 1 in macrophages, leading to cell death, while IpaC activates the Cdc42 pathway and nucleates actin to enable epithelial cell entry. Because so much is known about the ultrastructure of the *Shigella* TTSA, the molecular structures of the *Shigella* needle and tip proteins, the roles of IpaD and IpaB in secretion control, and the *Shigella* translocon, this system provides an excellent model for further dissecting type III secretion.

FIGURE 2

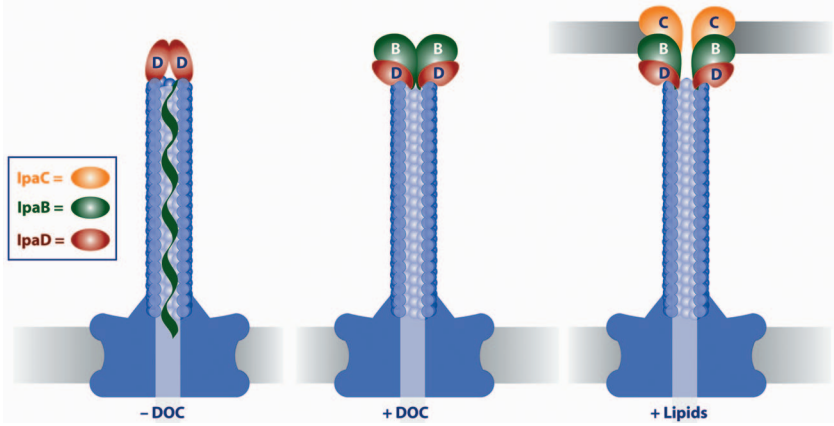


Simulated docking of DOC onto the crystal structure of IpaD. The yellow region indicates the "N-terminal domain" which folds separately from the main body of the protein whose structure is dominated by a central, stabilizing coiled-coil (composed of α3 and α7). The N-terminal domain may be replaced by a terminal MxiH unit during IpaD docking at the *Shigella* TTSA needle tip. DOC docks into a pocket on the coiled-coil (positions of interacting proteins shown in orange) and this is shown more clearly in the inset. The C terminus of IpaD would be at the bottom of the shown structure and it is this region that is implicated in stable association with the MxiH needle. (Generated by G. Lushington, Molecular Modeling Laboratory, University of Kansas.)

Components of the *Shigella* Needle Tip Complex

IpaD stably resides at the tip of the MxiH needle. Similarly, LcrV from *Y. enterocolitica*, PcrV from *P. aeruginosa*, and AcrV from *Aeromonas hydrophila* are examples of proteins that stably associate with the needle tips of comparable gram-negative TTSAs. No translocator proteins have yet been observed as part of the *Yersinia* tip complex. However, the *Shigella* translocator protein IpaB can be detected by immunoblot

FIGURE 3



Working model for the stepwise induction of type III secretion in *Shigella*. The addition of deoxycholate (DOC) promotes the first step of secretion induction. The subsequent addition of liposomes containing sphingomyelin and cholesterol triggers the final step of secretion induction concomitant with translocon insertion into the host cell membrane. (Generated by G. Ortiz, University of Kansas.)

analysis in needles sheared from the bacterial surface.

IpaD has an overall dumbbell shape with an intramolecular coiled-coil providing the handle and governing the overall stability of the protein. The intramolecular coiled-coil is a common feature among the tip proteins, while the sizes of the globular ends tend to be more diverse, probably due to functions that are specific for each pathogen and tailored to different environments and target cells. As IpaD docks at the *Shigella* TTSA needle tip, its N-terminal globular domain and C-terminal tail appear to lie near the outermost end of the needle, with the second globular "distal" domain positioned away from the needle.

Role of *Shigella* Needle Tip Complex during the Second Type III Secretion Step

Based on its position as the outermost point of the TTSA, the needle tip complex is poised to sense environmental signals and to play a key role in controlling type III secretion status. Indeed, IpaD is a regulatory component of the *Shigella* TTSS. For instance, IpaD and IpaB both play roles in controlling effector protein secretion in *Shigella*, and these proteins have been suggested to form a plug to prevent secretion prior to host cell contact. The absence of either protein results in uncontrolled secretion of the remaining Ipa proteins and other type III secretion substrates.

As a regulator of type III secretion, IpaD is able to serve as an environmental sensor. For example, it can specifically bind bile salts, leading to recruitment of IpaB to the *Shigella* TTSA needle tip complex (Fig. 1). While IpaB is not required for IpaD localization to the TTSA needle tip, IpaB cannot localize to the *Shigella* TTSA needle tip in the absence of IpaD. Further, when bile salts are added to an *ipaB* null mutant, the IpaD that is docked at the needle tip is released from the bacterial surface. This finding suggests that the mature form of the tip complex requires both secretion-controlling proteins—IpaD and IpaB— on the surface.

Nevertheless, bile salt-induced recruitment of IpaB to the maturing needle tip complex does not result in detection of IpaC as part of the needle tip complex, nor does it induce active type III secretion. This finding suggests that sequential signals are required for full induction of type III secretion in *Shigella*. The first signal would be detected by IpaD and the second by IpaB. How IpaD actions relate to those of other TTSA needle tip complex proteins is not yet known.

IpaD apparently senses bile salts through its central coiled coil, namely helices α3 and α7 (Fig. 2). While we cannot be absolutely certain that deoxycholate (DOC) is the trigger for IpaB recruitment to the *Shigella* surface during natural infection, *Shigella* does pass through a bile salt-rich environment as it travels to the colon. Furthermore, once IpaB is recruited to the TTSA needle tip complex, it remains there for at least 1 to 2 hours. Thus, the initially generated IpaB-IpaD-MxiH ternary complex could be involved in events during and following transcytosis across M cells and those following passage between cells as spaces are opened by migrating PMNs.

Bile salts might not be the only small molecules that induce the first step of type III secretion. For example, small molecules in serum induce full type III secretion in *Shigella*, and serum-free tissue culture medium promotes recruitment of IpaB to the *Shigella* surface in the absence of full secretion induction, much as bile salts do. Nevertheless, bile salts may very well be

the ligands that prime the TTSA needle tip complex before *Shigella* invades enterocytes.

Completing the Conduit during Type III Secretion in *Shigella*

The MxiH, IpaD, IpaB ternary complex forms in the absence of IpaC recruitment to the needle tip complex and is not associated with an increase in type III secretion. Thus, a third step is required for secretion induction. Further, because liposomes rich in sphingolipids and cholesterol enhance type III secretion by *Shigella*, that next signal may depend on host-cell contact.

Incubation with liposomes mimics what is expected to occur as *Shigella* encounters a target cell. Liposomes derived from erythrocytes or epithelial cells, or prepared from a defined mixture of lipids that includes sphingomyelin and cholesterol, efficiently induce *Shigella* type III secretion; this coincides with recruitment of IpaC, the second and final translocator protein, to the bacterial surface (Fig. 1 and 3).

The stepwise formation of the final TTSA needle tip complex (with IpaD, IpaB and IpaC) results in decreased *Shigella* invasion of cultured cells, presumably because "premature" assembly of the IpaB/IpaC translocon as part of the needle tip complexes prevents efficient delivery of effector proteins into the host cell cytoplasm. In other words, the translocon forms prior to actual insertion into the targeted cells cytoplasmic membrane. Because IpaB also controls secretion and is a cholesterol binding protein, it appears at this point that it serves as the sensor for host-cell contact and the trigger for the final step in type III secretion induction.

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