NMR Model of Prgl–SipD Interaction and Its Implications in the Needle-Tip Assembly of the Salmonella Type III Secretion System

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Abstract

Salmonella and other pathogenic bacteria use the type III secretion system (T3SS) to inject virulence proteins into human cells to initiate infections. The structural component of the T3SS contains a needle and a needle tip. The needle is assembled from Prgl needle protomers and the needle tip is capped with several copies of the SipD tip protein. How a tip protein docks on the needle is unclear. A crystal structure of a Prgl–SipD fusion protein docked on the Prgl needle results in steric clash of SipD at the needle tip when modeled on the recent atomic structure of the needle. Thus, there is currently no good model of how SipD is docked on the Prgl needle tip. Previously, we showed by NMR paramagnetic relaxation enhancement (PRE) methods that a specific region in the SipD coiled coil is the binding site for Prgl. Others have hypothesized that a domain of the tip protein—the N-terminal α-helical hairpin—has to swing away during the assembly of the needle apparatus. Here, we show by PRE methods that a truncated form of SipD lacking the α-helical hairpin domain binds more tightly to Prgl. Further, PRE-based structure calculations revealed multiple Prgl binding sites on the SipD coiled coil. Our PRE results together with the recent NMR-derived atomic structure of the Salmonella needle suggest a possible model of how SipD might dock at the Prgl needle tip. SipD and Prgl are conserved in other bacterial T3SSs; thus, our results have wider implication in understanding other needle-tip complexes.

Introduction

Many Gram-negative bacterial pathogens utilize the type III secretion system (T3SS) to inject virulence proteins into eukaryotic cells to cause disease [1,2]. The main structural component of the T3SS is the needle complex, which is a macromolecular structure assembled in the bacterial envelope from over 20 different proteins and consists of a membrane-bound base, an external needle, a tip, and a translocon (Fig. 1a). In Salmonella, the needle is assembled from the polymerization of approximately 120 copies of the needle protein Prgl [3–5]. Prgl (80 residues) [6,7] and other T3SS needle protomers [8,9] have α-helical hairpin structures flanked by flexible tails. At the distal end of the T3SS needle docks a tip complex [10,11], which in Salmonella is formed by the tip protein SipD (343 residues) [12]. The crystal structure of SipD [13] and homologs [14,15] show three common structural features: an N-terminal α-helical hairpin domain, a long central coiled coil, and a distal domain (Fig. 1b). Prior to contact with the host cell membrane, the T3SS needle apparatus is pre-assembled where the SipD tip complex is docked at the needle tip but the translocon comprising the membrane proteins SipB and SipC is still missing [12]. When Salmonella detect eukaryotic cells, a conformational change occurs at the needle tip in a process that is not yet well understood, allowing the translocon proteins SipB and SipC to transit through the needle and dock at the SipD tip complex. SipB and SipC form a
translocon pore on the host cell membrane to complete the assembly of the needle apparatus and allow the passage of effector proteins directly into the host cell [12]. The effectors then modulate host cell biology for the pathogen’s benefit.

How the tip protein binds to the needle protein is not well understood. Using an intact form of SipD, we recently showed by paramagnetic relaxation enhancement (PRE) that PrgI interacts with a specific region of the SipD coiled coil [16]. Others have hypothesized that the α-helical hairpin domain in the tip proteins functions as a self-chaperone [15] that prevents the tip protein from self-oligomerizing within the bacterial cytosol. Moreover, upon assembly at the needle tip, the α-helical hairpin domain is not required for interaction with the needle and thereby swings away [17]. Thus, a truncated form of SipD (here referred to as SipD1; Fig. 1c) lacking the α-helical hairpin domain should interact with PrgI. To determine how a truncated SipD interacts with PrgI and dock at the needle tip, Lunelli et al. determined the crystal structure of a PrgI–SipD1 fusion protein comprising PrgI at the N-terminus, a short 5-residue (GGSGG) linker, and a truncated SipD (residues 127–343) lacking the α-helical hairpin domain (Fig. 2a) [18]. The crystal structure of the PrgI–SipD1 fusion protein could be docked at the needle tip of the model of the needle derived by electron microscopy (EM) [18]. Since then, however, a seminal contribution from solid-state NMR (ssNMR) by Loquet et al. showed a completely different orientation of PrgI [3] compared to the EM-derived atomic models of T3SS needles [4,9,19]. The ssNMR atomic model of the needle places the N-terminus of needle protomers outside the needle and the C-terminus inside the needle channel (Fig. 2b and c) [3,20], which is completely opposite to the orientation of needle protomers suggested by EM [4,9,19]. When the crystal structure of the PrgI–SipD1 fusion protein [18] is docked on the current model of the Prgl needle, a massive steric clash of SipD occurs at the needle tip (Fig. 2d). The crystal structure of the PrgI–SipD1 fusion protein is thus incompatible with the current atomic model of the needle and could not be used in modeling SipD at the needle tip.

Here, we extend our previous PRE studies characterizing the PrgI–SipD interaction by using a truncated form of SipD lacking the α-helical hairpin. In contrast to the crystallography results of PrgI–SipD1 fusion protein wherein PrgI packs on one contiguous surface of the SipD coiled coil [18], our PRE results suggest that there are multiple PrgI binding surfaces on the SipD coiled coil. Based on our PRE results, we propose a model of SipD docked on the needle tip without resulting in steric clash of SipD.

**Results**

**PRE of truncated SipD–Prgl interaction**

To identify how SipD1 (a truncated form of SipD lacking the α-helical hairpin) binds to PrgI, we used our previous PRE approach [16]. Single-time-point PREs were determined for 10 SipD1 cysteine mutants spread across the length of the coiled coil in residues D136, A144, D147, N155, K173, T303, L318, K324, S331, and E335 (Fig. 4). The structure of SipD1 shown in Fig. 3 is oriented with an arbitrary “top/bottom” or “upper/lower” perspective used here to describe the various parts of the coiled coil. Spin labels attached to residues on the lower half of the coiled coil on helix α4 (D136, A144, and D147; Fig. 3b) and helix α8 (L318, K324, S331, and E335; Fig. 3) induced strong PRE effect upon titration into PrgI and peak intensity ratios ($I_{\text{par}}/I_{\text{dia}}$) were in the range 0.30 and 0.67 (Fig. 4). D136 at the extreme bottom of helix α4 exhibited the strongest PRE effect with ($I_{\text{par}}/I_{\text{dia}}$) ratio of 0.30 (Fig. 4e). On the other hand, when the spin label was attached to K173 (Fig. 4a) or T303 (Fig. 4f), which were located on the upper half of the coiled coil (Fig. 3), SipD1 showed relatively weaker PRE effect on PrgI with $I_{\text{par}}/I_{\text{dia}}$...
ratios at 0.91 and 0.83, respectively. In line with these, N155 (Fig. 4b) in the middle of helix α4 showed relatively moderate PRE effect \( \left( \frac{I_{\text{par}}}{I_{\text{dia}}} = 0.73 \right) \). Thus, single-time-point PREs showed that Prgl mostly interacted with the lower half of the SipD1 coiled coil spanned by residues D147 to D136 on helix α4 (Fig. 4c–e) and residues L318 to S331 on helix α8 (Fig. 4g–j).

**Fig. 2.** Docking of Prgl–SipD1 fusion protein at the needle tip. (a) Structure of Prgl–SipD1 fusion protein (PDB ID 3ZQB [18]) colored as green (SipD1) and red (Prgl). (b and c) Atomic model of the Salmonella needle derived by ssNMR (PDB ID 2LPZ [3]) shown as orthogonal views. (d) Docking two Prgl–SipD1 molecules at the needle tip by superposition of Prgl in the fusion protein (red) with two adjacent Prgl protomers at the needle tip results in steric clash of SipD1 (green and cyan).

**Fig. 3.** Location of PRE spin labels on SipD1. (a and b) The PRE spin labels on the SipD1 coiled coil are depicted as spheres and colored based on weak (yellow), medium (orange), and strong PRE effect with Prgl. SipD1 as depicted here is oriented with an arbitrary “top/bottom” or “upper/lower” used herein to describe the various parts of the coiled coil.
To quantify intermolecular PREs and obtain restraints for PRE-based structure calculations of the PrgI–SipDt complex, we determined the PRE 1H relaxation rates ($\Gamma_2$) using the two-time-point method [21]. As the total acquisition time for the two-time-point PRE method was much longer compared to that of the single-time-point method, the two-time-point PREs were only acquired for the four cysteine mutants in positions A144 and D147 on helix $\alpha_4$ and in positions K324 and E335 on helix $\alpha_8$ that showed the strongest single-time-point PREs. As a negative control, $\Gamma_2$ were also determined for K173, which showed the weakest single-time-point PRE. Results of two-time-point PREs showed that the four spin labels at A144, D147, K324, and E335 showed strong PRE effect, with average $\Gamma_2$ values of 27.51 s$^{-1}$, 32.9 s$^{-1}$, and 22.5 s$^{-1}$, respectively (Fig. 5), whereas K173 showed the lowest $\Gamma_2$ average value of 7.0 s$^{-1}$.

Results of PRE structure calculations using $\Gamma_2$ values from the spin labels at A144, D147, K324, and E335 as distance restraints revealed that two populations of PrgI were localized around the lower half of the SipDt coiled-coil region (Fig. 6). One PrgI population was partly localized at the position previously occupied by the $\alpha$-helical hairpin facing the lower half of the SipD coiled coil (labeled site X in Fig. 6a and b). PRE results suggested that PrgI binds to SipD at the position previously occupied by the $\alpha$-helical hairpin. The binding site X on SipD was not identified as a PrgI binding surface by the crystal structure of the PrgI–SipDt fusion protein and is a unique contribution from PRE. Our PRE results are the first direct experimental demonstration that a truncated T3SS tip protein interacts with its cognate needle protomer at the site previously occupied by the $\alpha$-helical hairpin, and this information has a direct bearing on determining how a tip protein docks at the needle tip. A second PrgI population was localized on another face of the coiled coil opposite from binding site X (labeled site Y in Fig. 6a and b). Results of PRE structure calculations therefore suggested that a truncated SipD could bind simultaneously to two PrgI molecules at two sites and that both PrgI binding sites X and Y were located at the lower half of the SipD coiled coil and on opposite faces of the coiled coil (Fig. 6a and b). Another unique contribution revealed by PRE is that there are two PrgI binding sites on SipD, and this information is also important in modeling SipD at the needle tip. Results from crystallography [18], isothermal titration calorimetry [18], and fluorescence polarization (FP) (this work) thus far have suggested only one binding site. Binding site Y (Fig. 6a) overlaps with the PrgI binding site on SipD in the crystal structure of the PrgI–SipDt fusion protein (Fig. 2a) [18].

Fig. 4. Single-time-point PREs of spin labeled SipDt titrated into PrgI. The 10 SipDt spin labels are at position (a) K173, (b) N155, (c) D147, (d) A144, (e) D136, (f) T303, (g) L318, (h) K324, (i) S331, and (j) E335. The locations of the spin labels on helices $\alpha_4$ and $\alpha_8$ are indicated by black spheres and the average $I_{\text{par}}/I_{\text{dia}}$ values are indicated on each plot.
PRE-based model of SipD docked at the needle tip

The PRE results regarding the PrgI–SipD\(^1\) interaction together with the ssNMR-derived atomic model of the Salmonella needle [3] constrain the possible orientations of SipD\(^1\) at the needle tip and thus suggest a possible model of how SipD might dock at the needle tip (Fig. 6c–e). In the atomic structure of the Salmonella needle [3], PrgI molecules are arranged in a spiral symmetry with the PrgI α-helical hairpin slightly tilted away from the wall of the needle. Further, at the needle tip, the PrgI α-helical hairpins are separated from each other by 25 Å [3], which is the distance between the center of mass of two adjacent PrgI α-helical hairpins. Because our PRE data suggested that SipD\(^1\) could interact with two PrgI molecules, we propose that the SipD central coiled coil could fit into the gaps between the PrgI α-helical hairpins at the needle tip. The average distance between the C\(^{\alpha}\) atoms that spans the coiled-coil region at the bottom of SipD is \(\sim 17\) Å, which could be easily accommodated between the two PrgI α-helical hairpins at the needle tip. In this model, the region corresponding to the SipD N-terminal α-helical hairpin will be splayed outside the needle (Fig. 6f and g). Consistent with the pentameric state proposed by others [15,17,18], a pentameric SipD complex could be accommodated at the needle tip (Fig. 6f and g). Further, our PRE model of how SipD docks at the needle tip reveals the regions of SipD that are exposed at the needle tip and therefore could be potential sites for interaction with the translocon. This could be experimentally tested using recombinant SipD and translocon proteins. Finally, we expect SipD to follow the spiral symmetry of the needle protomers; however, how this symmetry translates into the translocon remains unknown.

Biological tests of the model

To test the biological relevance of our PRE-derived PrgI–SipD model, we assayed the effect of mutations on the PrgI binding site at the SipD coiled-coil region on the ability of Salmonella to invade cultured human epithelial cells. The ability of Salmonella to invade eukaryotic cells has been shown to be strictly dependent on SipD function [22]. Point mutations were introduced in five residues (S148, V157, S313, D320, and V323) located at the lower half of the SipD coiled coil at the binding interface with PrgI. The mutations were designed to alter the bulkiness of the side chain (S148E, V157K, S313E, and V323K) or the surface electrostatics (S148E, V157K, S313E, D320R, and V323K). Results showed that the S148E mutation did not alter invasiveness (Fig. 7a), suggesting that S148 may not be crucial for PrgI–SipD interaction. Other mutations, such as point mutations on the lower half of helix α4 (V157K) and helix α8 (D320R and V323K), significantly decreased invasiveness. Among these, D320R and V323K located at the bottom of helix α8 showed only 25% invasiveness, while V157K (on helix α4) and S313E (on helix α8) showed invasiveness between 50% and 60% of wild

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Fig. 5. Two-time-point PREs. Two-time-point \(\Gamma_2\) values of backbone \(^1\)H\(_N\) were acquired for the SipD\(^1\) spin labels at the following positions: (a) A144, (b) D147, (c) K324, (d) E335, and (e) K173.
type. We reasoned that point mutations may not cause large alterations in protein–protein interaction surfaces to drastically affect invasiveness; hence, double and triple mutations that alter contiguous surfaces were designed. The double and triple mutations on SipD rendered Salmonella completely non-invasive (Fig. 7a). Thus, altering the PrgI binding surface on SipD as predicted by the PRE can drastically affect SipD function and Salmonella invasiveness.
To correlate the observed loss of function of SipD to its binding interaction with PrgI, we used FP to quantify the binding affinities of PrgI with the various SipD mutants. PrgI bound to SipDt with an apparent $K_D$ of $108 \pm 10 \mu M$ (Fig. 7c); however, the dissociation constant of PrgI with SipD could not be determined by FP because it was too weak as indicated by the FP plot failing to reach saturation (Fig. 7b). Because the FP assay could be only carried out with the SipD<sup>T</sup> construct, mutations were introduced in SipD<sup>T</sup> and tested for in vitro binding affinity with PrgI. The S148E point mutant, which showed a wild-type phenotype in the invasion assay, displayed a binding affinity (apparent $K_D$ of $189 \pm 20 \mu M$) to PrgI similar to SipD<sup>T</sup>. The other SipD point mutants that showed decreased invasiveness (S313E, V157K, D320R, and V323K) also showed decreased binding affinities to PrgI compared to SipD<sup>T</sup> (Fig. 7b). In contrast, the double and triple mutations that rendered Salmonella totally non-invasive resulted in $K_D$ values that were too weak to be determined by FP (Fig. 7c). Thus, the decreased in vitro binding affinities between PrgI and SipD correlated with decreased Salmonella invasion assay.

To further explore the binding of SipD to PrgI in vivo, we used a previously described surface display immunofluorescence-based assay that specifically detects SipD only when anchored at the needle tip [12]. In contrast to wild type, the SipD triple mutant (D320R/D323K/S327R) was not detected at the bacterial surface (Fig. 8a–c) despite the fact that this mutant was expressed and secreted by the T3SS at wild-type levels (Fig. 8d). It has been previously shown that loss-of-function mutations in the SipD family of proteins lead to enhanced type III secretion. It is thought that the properly positioned SipD complex at the tip acts as a "lid" that prevents the premature secretion of effector proteins before contact with cells. We found that a Salmonella strain expressing the SipD triple mutant (D320R/D323K/S327R) exhibits enhanced type III secretion equivalent to the absence of SipD.

EM examination of needle complexes obtained from a Salmonella strain expressing the SipD triple mutant showed that they were properly assembled and exhibited a normal appearance (Fig. 9). These results confirmed that the phenotypes associated with mutations in SipD were not the result of gross morphological changes in the needle complex. Taken together, these results indicate that the SipD triple mutant, which showed a marked decrease in PrgI binding affinity, was unable to assemble at the tip of the needle complex in vivo. In other words, the lack of PrgI–SipD interaction observed in the SipD triple mutant is not due to overt structural defects of the needle complex itself but rather due to the specific disruption of the PrgI binding surface.

**Discussion**

An essential step in the assembly of the T3SS needle complex is the anchoring of several copies of SipD at the tip of the needle. Recently, we showed by using PRE that the Salmonella PrgI protein binds at the lower half of the SipD coiled coil [16]. Here, we extended our results by showing that PrgI binds...
more tightly to a truncated form of SipD (termed SipDt here) lacking the N-terminal α-helical hairpin (Fig. 1). This work was motivated by observations that the N-terminal α-helical hairpin functions mainly as self-chaperone to the tip protein [15] and that it is partially dispensable for bacterial invasiveness [23]. Based on structural analysis, others have proposed that the N-terminal α-helical hairpin may be splayed out to allow the anchoring of SipD at the tip [15,17]. Furthermore, using isothermal calorimetry, Lunelli et al. showed that the PrgI–SipD interaction could only be detected when the N-terminal α-helical hairpin was removed from SipD [18]. Understanding how SipDt interacts with PrgI is important because this information is needed to model how SipD is docked onto the assembled PrgI needle [18]. When the PrgI–SipD fusion protein was docked onto the current model of the PrgI needle [3], however, there was a massive steric clash between SipD molecules at the needle tip (Fig. 2). Thus the PrgI–SipD crystal structure could not be used to dock SipD at the needle tip. We reasoned that the 5-residue linker (with the sequence GGSGG) between PrgI and SipD was perhaps too short and artificially constrained PrgI to SipD in an orientation that could not be used to dock SipD at the needle tip without resulting in steric clash of SipD molecules. Here we used PRE methods (Figs. 3–6) to identify specific regions in the SipD coiled coil as the binding sites for PrgI (Fig. 6) and propose an alternate model (Fig. 6) that did not result in the steric clash of SipD at the needle tip.

Our PRE results identified two binding sites for PrgI on the SipD coiled coil and suggested that the SipD coiled-coil region could insert between PrgI protomers at the needle tip (Fig. 6). Our model predicts that, upon assembly at the needle tip, the N-terminal α-helical hairpin of SipD will be splayed outside the needle [15,17]. This scenario is consistent with the observation that the major epitopes of the Shigella tip protein IpaD that are exposed on the bacterial surface are in residues 13–121, which correspond to the region encompassing the N-terminal α-helical hairpin [24]. It should be noted that, during the assembly of the needle apparatus, the tip protein must pass through a narrow, 25-Å-diameter needle channel [4,9], which at best could only fit two tightly packed α-helices. Hence, the tip protein likely passes through the channel in a partially unfolded state. Others have also suggested that the tip protein N-terminal region could swing away to allow the passage of the rest of the tip protein through the needle channel [14]. Subsequently, upon reaching the needle tip, the central coiled coil could be exposed for interaction with the needle protomer.

Fig. 8. A non-invasive SipD triple mutant (D320R/V323K/S327R) failed to be displayed on the bacterial surface. (a) Salmonella sipD knockout strain probed with antibodies to lipopolysaccharide (red) and Flag-tag (green) transformed with plasmid that supplied no SipD (left panel); Flag-tagged SipD (middle panel), Flag-tagged mutant SipD (right panel). (b) Protein expression and secretion of SipD and other T3SS proteins were not affected as probed by immunoblotting.

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Thus, we have shown using PRE methods to characterize the binding interaction of SipD with PrgI, based on which site-directed mutagenesis could render Salmonella non-invasive (Fig. 7). The Salmonella invasion assay by itself, however, could not directly link the non-invasiveness of a particular SipD mutation to defective SipD–PrgI interaction because of the multifunctional nature of SipD because it binds to other proteins and bile salts and defects in those interactions could render SipD nonfunctional. Additionally, there are many things that could go wrong during the assembly of the needle apparatus, which after all is assembled from over 20 different proteins in a hierarchical and multi-step manner. Thus, we used a series of experimental approaches to link the non-invasiveness of SipD mutations specifically to the disruption of the PrgI–SipD binding interface that was identified by PRE methods.

We used FP to correlate the relative binding affinities of the PrgI–SipD interaction with respect to SipD loss of function mutants (Fig. 7). Determination of the binding affinities between PrgI and SipD is challenging because of the poor solution behavior of the native forms and weak binding affinities. Currently, there are only two methods that have been successfully used to measure the binding affinities of PrgI with SipD— isothermal calorimetry [18] and, shown herein, FP. In both isothermal titration calorimetry and FP methods, the truncated version of SipD lacking the N-terminal α-helical hairpin was used because it yielded a measurable binding affinity with PrgI, whereas the intact SipD construct was beyond the detection limit of FP. The results of FP (Fig. 7) suggested that the non-invasiveness of the SipD triple mutants, which were designed to disrupt the PrgI–SipD interaction on the basis of the PRE results, was due to the decreased binding affinities to PrgI at a molecular level. Further, a cell-based assay using Flag-tagged SipD demonstrated that the non-invasive triple mutation (D320R V323K S327R) prevented SipD from localizing on the bacterial surface (Fig. 8). EM showed that Salmonella was able to properly assemble the base and the PrgI needle itself (Fig. 9); therefore, the non-invasive phenotype of the SipD mutants was due solely to the inability of SipD to bind to PrgI and anchor on the needle tip. Without SipD anchored at the needle tip, the translocon cannot be assembled rendering Salmonella incapable of injecting effectors directly into eukaryotic cells and thereby incapable of invading eukaryotic cells.

The model of SipD at the needle tip is also useful in developing a working model of the interface between SipD and the translocon. PrgI and SipD are conserved in other T3SSs; thus, our results will advance...
understanding of the needle-tip interaction in other bacterial pathogens.

Materials and Methods

Protein expression and purification

The expression and purification of a monomeric form of PrgI lacking the C-terminal five residues and replaced with a his-tag has been described previously [6]. SipD^{1} (residues 101−343) was subcloned in the NdeI/SalI sites of the expression plasmid pDZ1 [13] and expressed and purified following similar protocol used for the intact SipD [13]. To eliminate complications arising from multiple cysteines during the spin labeling, all the SipD^{1} constructs used in the PRE experiments contained the background C244S mutation. A total of 10 cysteine point mutations were introduced by PCR in the following residues of SipD^{1}: D136, A144, D147, N155, K173, T303, L318, K324, S331, and E335 (Fig. 3). The PRE experiments contained the background C244S mutation. For PRE data acquisition, two 2D (dimensional) 1H-15N heteronuclear correlation spectra were acquired for two samples, one for 15N-labeled PrgI complexed with diamagnetic SipD^{1} and another for 15N-labeled PrgI complexed with paramagnetic SipD^{1}. The protein concentrations, buffer conditions, NMR data acquisition, and processing parameters were kept identical for both samples. Initially, single-time-point PREs were determined for the 10 SipD^{1} cysteine mutants as described previously [16]. All the 10 SipD^{1} cysteine mutants were 100% spin labeled as confirmed by electrospray ionization mass spectrometry.

Bioassays

Site-directed mutations in SipD within the plasmid pRK2 [13] were introduced by PCR and used to determine the effect of SipD mutations on the ability of Salmonella to invade a cultured human epithelial cell line Henle 407 (American Type Culture Collection #CCL6) following protocol described previously [13]. The Salmonella typhimurium strains (wild type and sipD^{−} knockout) used in the Salmonella invasion assay have been described previously [13].

For EM, needle complexes were purified as described elsewhere [5,25]. Purified needle complexes were applied to glow-discharged, carbon-coated Cu grids and stained with 2% phosphotungstic acid, pH 7.0. Images were acquired using a Tecnai Biotwin TEM (FEI Company, Hillsboro) at 80 kV using the Morada Soft Imaging system and a 6 M pixel CCD camera (Olympus, Munster, Germany).

Immunoblotting was used to determine the level of protein expression and secretion of T3SS proteins and performed as previously described [12,26] using antibodies specific to the SptP\textsuperscript{a} effector protein and the structural proteins SipB, SipC, InvJ, PrgH, PrgK, InvG, PrgJ, and PrgL.

Fluorescence polarization

For FP, a PrgI double mutant, V65A/V67A, which rendered full-length PrgI to be soluble and functional [7], was used. A site-directed mutant L31C was used to attach the fluorophore as follows: PrgI was dialyzed into 1× PBS (phosphate-buffered saline), pH 7.4, with a 10-fold excess of tris(2-carboxyethyl)phosphine to reduce disulfide bonds for 30 min at 25 °C. A 20-fold excess of Alexa Fluor 488-CH\textsubscript{2}-Maleimide (Invitrogen) was resuspended in 1× PBS, pH 7.4, and added dropwise to the protein solution with mixing and allowed to react for 3 h at 25 °C in the dark. Excess dye was removed by dialysis in 1× PBS, pH 7.4, followed by passage through a centrifugal filter unit with molecular mass cutoff of 3 kDa (Millipore). Increasing concentrations of SipD were titrated into 25 nM PrgI-fluorophore and FP data were collected at 25 °C for 30 min at 25 °C. A 20-fold excess of Alexa Fluor 488-CH\textsubscript{2}-Maleimide (Invitrogen) was resuspended in 1× PBS, pH 7.4, and added dropwise to the protein solution with mixing and allowed to react for 3 h at 25 °C in the dark. Excess dye was removed by dialysis in 1× PBS, pH 7.4, followed by passage through a centrifugal filter unit with molecular mass cutoff of 3 kDa (Millipore). Increasing concentrations of SipD were titrated into 25 nM PrgI-fluorophore and FP data were collected at 25 °C using a Varian Cary Eclipse Fluorescence Spectrophotometer. The average of 5 data points was subtracted from a base polarization value of 25 mM PrgI-fluorophore in 1× PBS, pH 7.4, alone. The difference in millipolarization (ΔmP) was plotted against increasing concentrations of SipD. The FP binding curve was fitted using the Hill equation and Origin (OriginLab, Northampton, MA).

NMR spectroscopy

NMR data were acquired at 25 °C on a Bruker Avance 800-MHz spectrometer equipped with a cryoprobe, processed with NMRPipe (version 2.1)[28], and analyzed using NMRView (version 2.1) [29]. The spin label MTSL-S-(2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methane-sulfonothioate (Toronto Research Chemicals) was attached to the SipD^{1} cysteine mutants as described previously [16]. All the 10 SipD^{1} cysteine mutants were 100% spin labeled as confirmed by electrospray ionization mass spectrometry. For PRE data acquisition, two 2D (2-dimensional) 1H-15N heteronuclear correlation spectra were acquired for two samples, one for 15N-labeled PrgI complexed with diamagnetic SipD^{1} and another for 15N-labeled PrgI complexed with paramagnetic SipD^{1}. The protein concentrations, buffer conditions, NMR data acquisition, and processing parameters were kept identical for both samples. Initially, single-time-point PREs were determined for the 10 SipD^{1} cysteine mutants (D136C, A144C, K324C, and E335C) in complex with 15N-labeled PrgI following the PRE method described previously [16]. In the single-time-point PRE method, two identical 2D 1H-15N heteronuclear single quantum coherence (HSQC) [30] spectra were acquired for 0.6 mM 15N-labeled PrgI complexed with 0.3 mM diamagnetic or paramagnetic SipD^{1}. Typical acquisition parameters were 8 scans, 18 ppm 15N sweep width centered at 118 ppm, 216 15N complex points, and a recycle delay of 5 s to ensure that the magnetization returned to equilibrium before the next scan. Each 2D 1H-15N HSQC spectrum took about 2.30 h to complete. The single-time-point PRE for each non-overlapped PrgI backbone amide peaks was calculated from the peak intensity ratio (I_{PrgI}/I_{PrgI}).

For the SipD^{1} mutants (D136C, A144C, K324C, and E335C) that showed strong single-time-point PREs with
Prgl, two-time-point PREs [21] were acquired following the method described previously [16]. As a control, two-time-point PREs were also acquired for K173C, which showed weak single-time-point PRE. For acquiring two-time-point PREs, the optimal delay between the two time points [21] was found to be 10 ms. Typically, for a Prgl–Spd D complex at 1:0.5 molar ratio, the two-time-point PRE data were acquired with 108 scans and 18 ppm 15N sweep width centered at 118 ppm. Each 2D 1H–15N HSQC spectra had 2048 1H complex points and 300 15N complex points. The total acquisition time to complete the two HSQC spectra for each cysteine mutant was ~23 h. For each non-overlapped peak, the PRE 1H transverse relaxation rate ($r_2$) was determined as previously described [16] and used as restraints in structure calculations of the Prgl–Spd D complex using Xplor-NIH [31]. The protocol for PRE structure calculation has been described earlier [16]. Briefly, each paramagnetic probe was represented by three conformers with the structure of Spd D fixed. An ensemble of three conformers was used to represent Prgl and these were allowed to freely rotate and translate. Jointly refining against intermolecular PREs of A144C, D147C, K173C, K324C, and E335C, 128 structures were calculated and 60 structures were selected for their lowest PRE Q-factors below 0.35 [21]. All structure figures were rendered using PyMOL [32].

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**Abbreviations used:**
PRE, paramagnetic relaxation enhancement; T3SS, type III secretion system; EM, electron microscopy; ssNMR, solid-state NMR; FP, fluorescence polarization; HSQC, heteronuclear single quantum coherence.

**References**


