Supporting Information

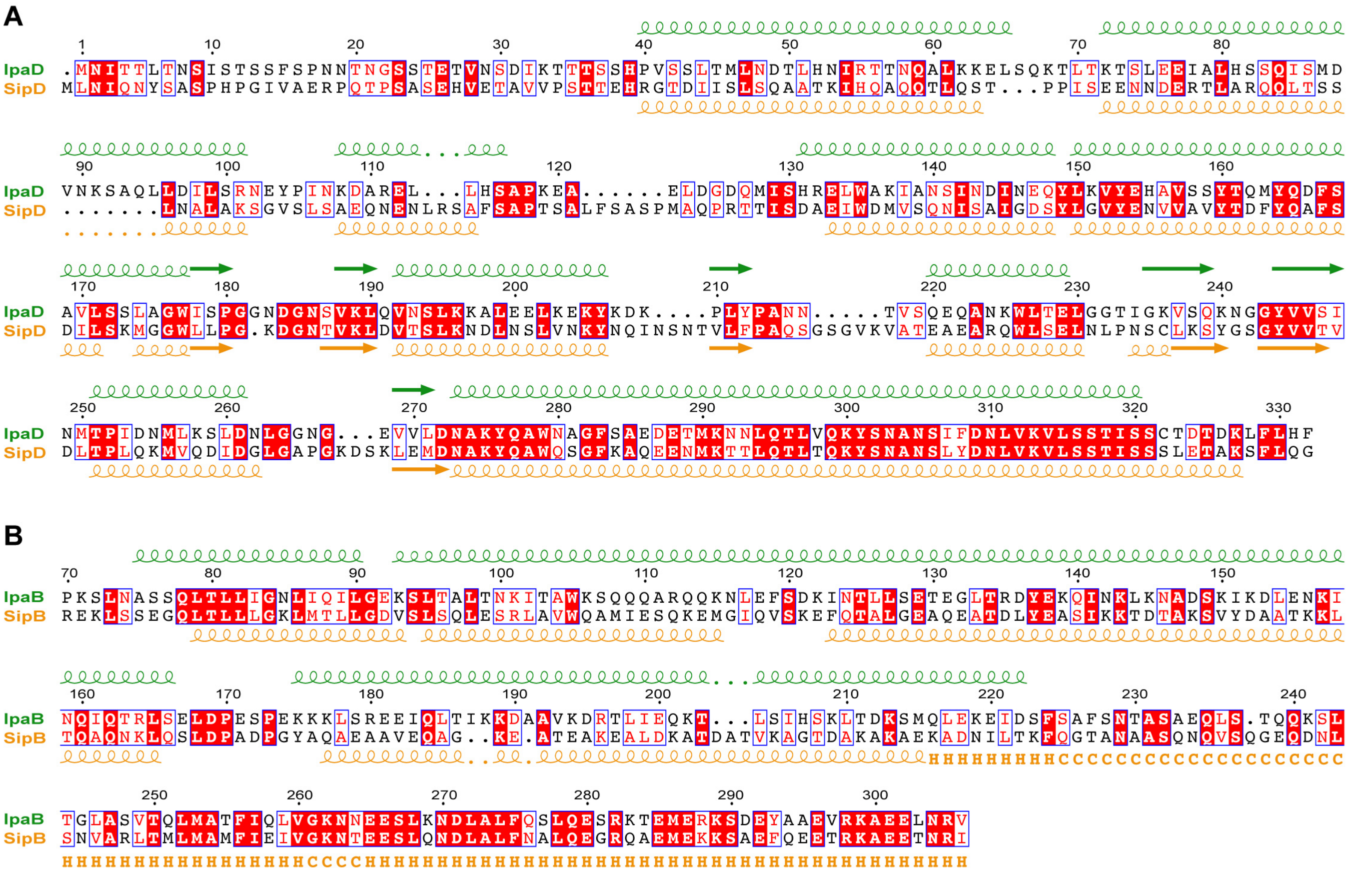

Figure S1. Sequence alignment of tip and translocon proteins from *Shigella* and *Salmonella* T3SS. Sequence alignment of **(A)** tip proteins IpaD and SipD and **(B)** N-terminal ectodomain of major translocon proteins IpaB and SipB from *Shigella* and *Salmonella* T3SS, respectively. Numbers above the sequences correspond to *Shigella flexneri* proteins. Secondary structure elements are shown above and below the alignment and color-coded with its corresponding protein. (Legend: β-strands, arrows; predicted helices, H; predicted coils, C). Alignment was generated using Clustal Omega and rendered with ESPript (Robert and P. Gouet, *Nucl. Acids Res* **2014**, 42 (W1): W320-W324).

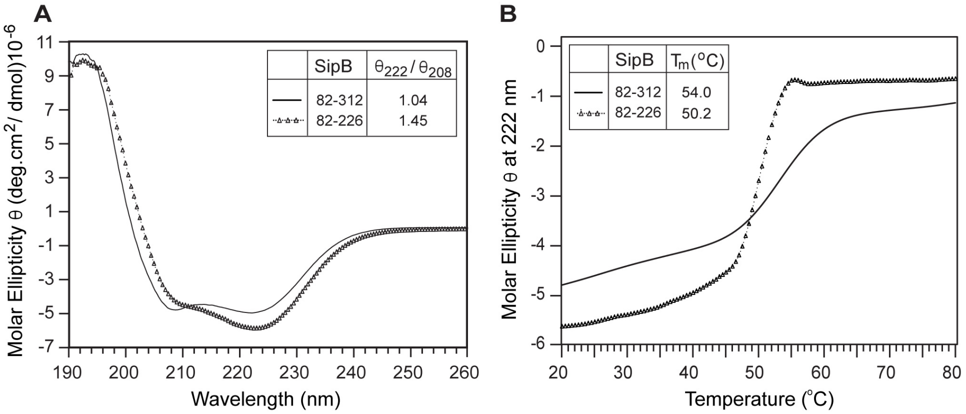

Figure S2. Circular Dichroism and thermal denaturation spectra of SipB constructs. (A) CD spectra for SipB$^{82-312}$ and SipB$^{82-226}$ shows predominantly α-helical protein. **(B)** Thermal denaturation curves at 222 nm show a T_m of 54 °C and 50.2 °C, for SipB$^{82-312}$ and SipB$^{82-226}$, respectively.

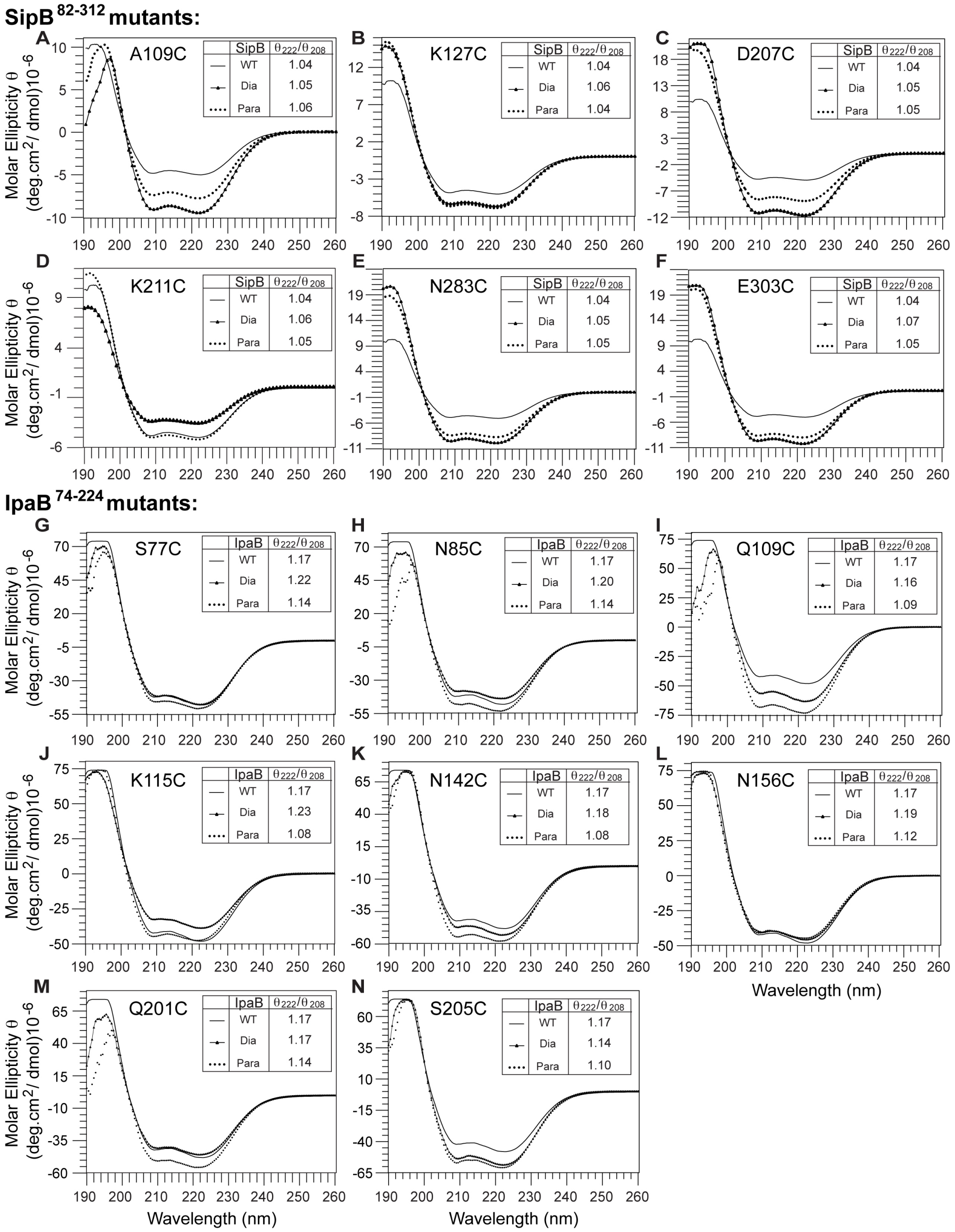

Figure S3. CD spectroscopy of SipB$^{82\text{-}312}$ and IpaB$^{74\text{-}224}$ cysteine mutants used in the PRE studies. **(A-F)** The CD spectrum of wild type SipB (WT) compared to cysteine point mutants before and after the attachment of the MTSL spin label. **(G-N)** The CD spectrum of wild type IpaB (WT) compared with cysteine point mutants before and after attachment of the MTSL spin label. (Legend: dia, diamagnetic; para, paramagnetic).

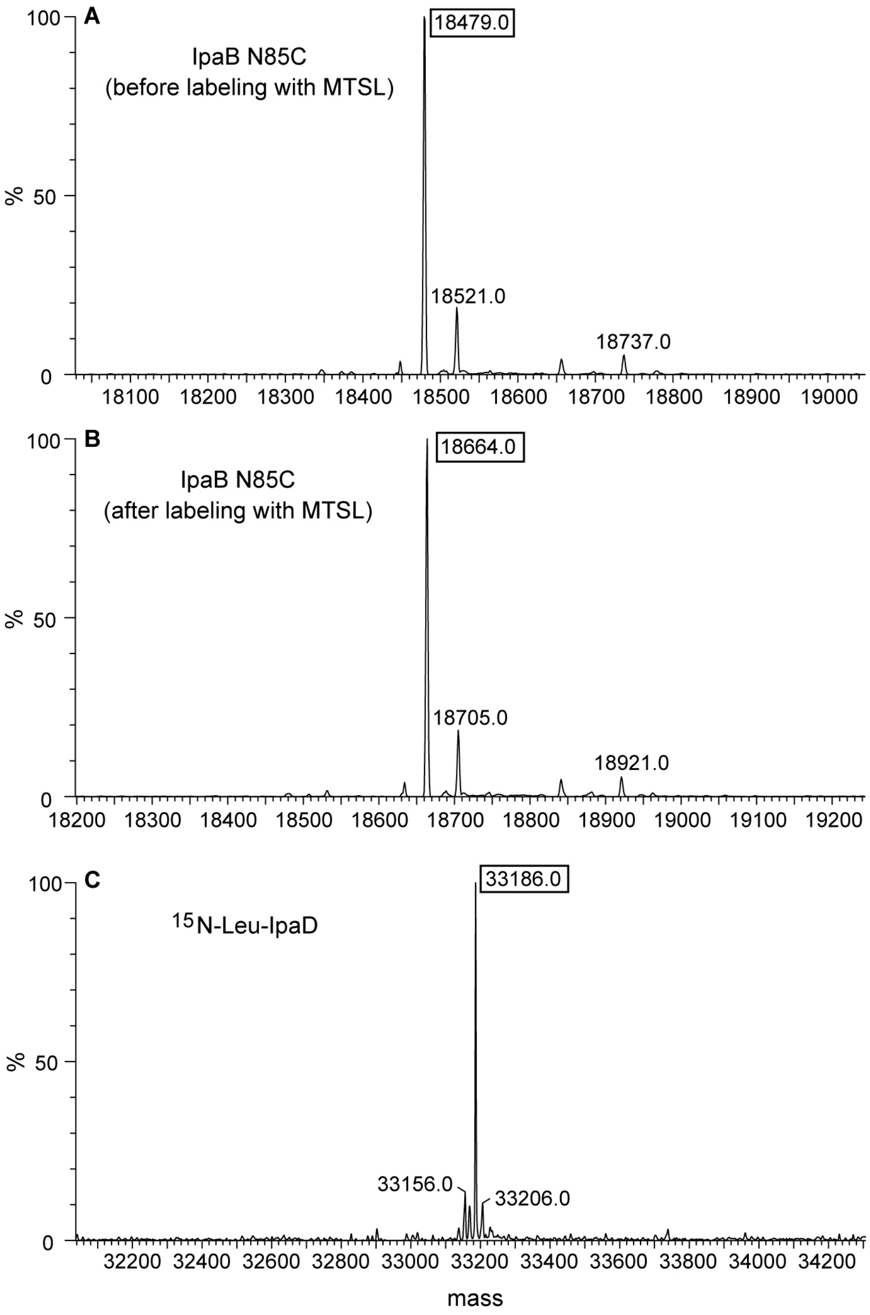

Figure S4. Representative electrospray ionization mass spectrometry (ESI-MS) on IpaB and IpaD to confirm extent of labeling. ESI-MS on IpaB N85C mutant **(A)** before MTSL-labeling (theoretical mass, 18479 Da), and **(B)** after MTSL labeling (theoretical mass, 18664). MTSL conjugation resulted in an expected increase in mass of 185 Da. **(C)** ESI-MS of ^{15}N-Leu labeled IpaD (theoretical mass, 33186).

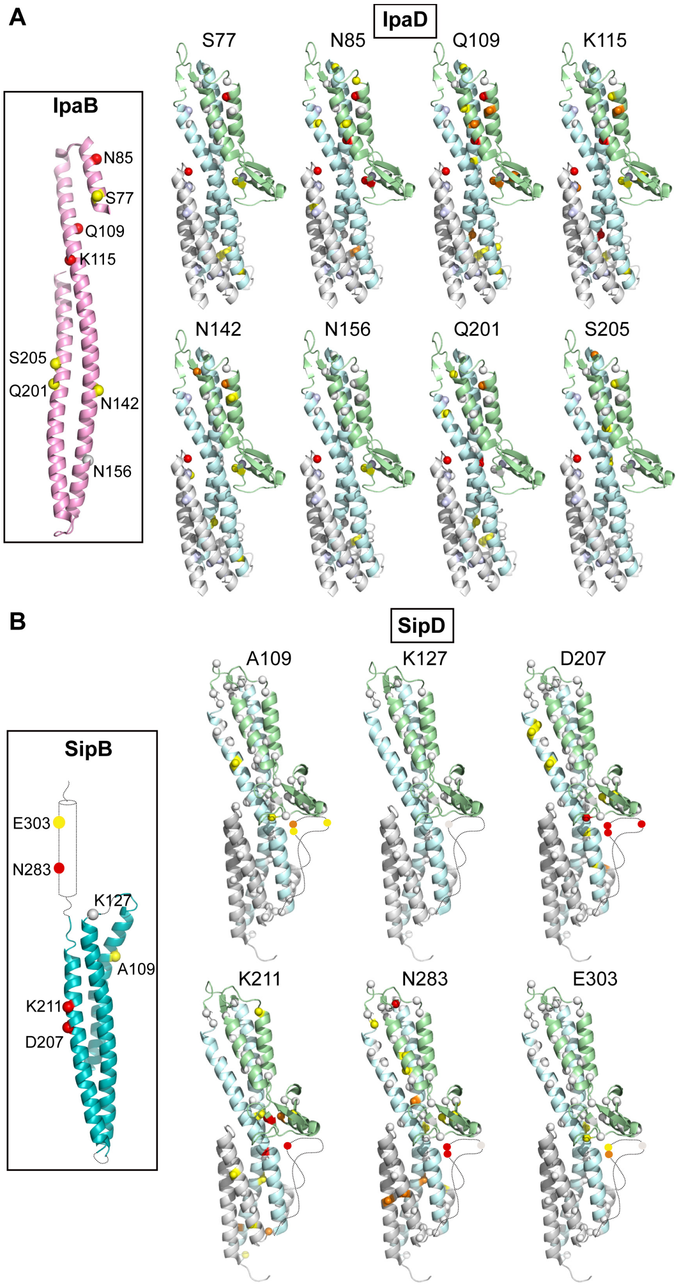

Figure S5. PRE effect produced by different spin labels in *Shigella* IpaD-IpaB and *Salmonella* SipD-SipB interaction. (A) Results of *Shigella* PRE experiments with spin-labeled IpaB mapped on the structure of IpaD. **(B)** Results of *Salmonella* PRE experiments with spin-labeled SipB mapped onto the structure of SipD. Color scheme is followed from Figure 4 and Figure 5.