N-terminal residues of SipB are required for its surface localization on *Salmonella enterica* serovar Typhimurium

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SipB, one of the invasion proteins encoded in *Salmonella* pathogenicity island 1 (SPI-1), is known to be secreted outside the cell, where it functions as a translocon by assembling into a host-cell plasma membrane-integral structure. Here, we confirmed that wild-type SipB could be localized to the bacterial outer membrane, and further showed that its localization was dependent on extracellular secretion, and was independent of the presence of the SipD protein. Proteinase K susceptibility and immunofluorescence assays indicated that SipB was not incorporated into the outer membrane, but rather was displayed on the bacterial surface. Finally, mutation studies revealed that the N-terminal 100–140 aa (especially amino acids 135–138) of SipB were required for its localization on the bacterial outer membrane.

INTRODUCTION

Salmonella spp., and other Gram-negative pathogenic bacteria, have evolved sophisticated mechanisms to infect and colonize their hosts. One such mechanism is termed the virulence-associated type III secretion system (T3SS), which is composed of specialized organelles that deliver bacterial proteins (effectors) directly into the host-cell cytoplasm (Cornelis & Van Gijsegem, 2000; Galán & Collmer, 1999; Hueck, 1998; Kimbrough & Miller, 2002; Ramamurthi & Schneewind, 2005). The T3SS is composed of a basal body that crosses both bacterial membranes, and an external needle through which effector proteins are secreted (Blocker *et al.*, 2001; Hueck, 1998; Kimbrough & Miller, 2002).

Various studies have shown that the *Salmonella* invasion proteins (Sips) encoded by the T3SS genes of *Salmonella* pathogenicity island 1 (SPI-1) are central to initiation of the entry process. For example, individual non-polar *sipB*/

sipC/*sipD* deletion mutants have been found to be entry-deficient, due to their inability to deliver other effectors into cultured mammalian cells (Collazo & Galán, 1997). The effector proteins SipB and SipC form an extracellular complex following their secretion through the SPI-1 T3SS, and they are thought to assemble into a plasma membrane-integral structure (translocon) that mediates effector delivery (Hayward & Koronakis, 1999; Scherer *et al.*, 2000). The IpaB and IpaC proteins in *Shigella flexneri*, BipB and BipD in *Burkholderia pseudomallei*, YopB and YopD in *Yersinia enterocolitica*, EspB and EspD in enteropathogenic *Escherichia coli* (EPEC), BopB and BopD in *Bordetella bronchiseptica*, and PopB and PopD in *Pseudomonas aeruginosa* are thought to be homologous components of the translocation apparatus formed by SipB and SipC in *Salmonella*. All of these secreted proteins have been shown to be delivered to the host-cell membrane, where they form pore complexes (Daniell *et al.*, 2001; Davis *et al.*, 1998; Hayward *et al.*, 2000; Ide *et al.*, 2001; Kuwae *et al.*, 2003; Neyt & Cornelis, 1999; Nogawa *et al.*, 2004; Scherer *et al.*, 2000; Suparak *et al.*, 2005; Faudry *et al.*, 2006; Johnson *et al.*, 2006). In addition to its role as a translocon, SipB can also reportedly be phagocytized by macrophages and subsequently translocated via SPI-1 T3SS into the cytoplasm of the macrophage, where it induces apoptosis of the host cell

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Abbreviations: DOC, sodium deoxycholate; IFA, immunofluorescence assay; SPI-1, *Salmonella* pathogenicity island 1; T3SS, type III secretion system.

by associating with the proapoptotic protease caspase-1 (Hersh *et al.*, 1999).

It has been reported that *Shigella* IpaB, a homologue of SipB, is associated with the bacterial envelope (Menard *et al.*, 1994; Mills *et al.*, 1988; Watarai *et al.*, 1995) and can be localized to the *Shigella* cell surface in the presence of T3SS (Olive *et al.*, 2007). Recently, Johnson *et al.* (2006) have shown that surface localization of IpaB is dependent on the presence of IpaD. Veenendaal *et al.* (2007) have also suggested that the interaction between IpaB and IpaD at the needle tip is a key to host-cell sensing, and allows insertion of the translocation pore into the host-cell membrane. Interestingly, Hayward *et al.* (2000) have shown that *Salmonella* SipB can also be localized to the cell surface of bacteria during invasion of mammalian target cells, and furthermore that the protein fractionated with outer-membrane proteins. However, other evidence on SipB localization is conflicting. SipB can be detected (1) in the culture medium, (2) associated with the bacterial outer membrane, and (3) inserted into the target-cell plasma membrane (Hayward & Koronakis, 1999; Hayward *et al.*, 2000; Kubori & Galan, 2002; Scherer *et al.*, 2000).

Here, we confirmed that wild-type SipB protein could be localized on the surface of *Salmonella*, and showed the essential domain of SipB for mediating outer-membrane localization.

METHODS

Bacterial strains and growth conditions. Bacterial strains and their genotypes are listed in Table 1. Strains were grown on L-agar or in L-broth supplemented with 0.3 M NaCl to allow optimal expression of the components of the invasion-associated T3SS. When required, the following antibiotics were added at the indicated concentrations: kanamycin, 50 µg ml⁻¹; ampicillin, 100 µg ml⁻¹; streptomycin, 100 µg ml⁻¹; tetracycline, 10 µg ml⁻¹.

Genetic techniques. DNA cloning was carried out using the method described elsewhere (Sambrook *et al.*, 1989). Restriction enzymes, T4 ligase and *Taq* polymerase were purchased from Boehringer Mannheim or Takara. General transduction was performed with P22 HT105/int, and non-lysogenic segregants were identified by sensitivity to P22 H5 (Davis *et al.*, 1980). Bacterial mutant strains (Δ sipB and Δ sipD) were constructed by the λ Red recombinase method (Datsenko & Wanner, 2000).

Plasmid construction. All plasmids used in this study are listed in Table 1. For expression of the 140 aa SipB and its derivatives, we used plasmid pSSV160 (Kim *et al.*, 2007) as a template. To construct pSSV140, the region encoding the N-terminal 140 aa of SipB was amplified using appropriate primers (forward primer PlacL, 5'-ccgactagtaatacgaacacgcctct-3'; reverse primer Sip140R, 5'-ccgggtaccgagatccgtcgctcct-3'). The underlined sequences indicate the restriction enzyme sites for *SpeI* and *KpnI*, respectively. The amplified fragments (Plac and SipB 140 aa) were digested with *SpeI* and *KpnI*, and then ligated with T4 ligase (Takara) to pSSV160, which had been pre-digested with *SpeI* and *KpnI* and eluted. The same strategy was used to construct plasmids pSSV120 (SipB₁₋₁₂₀-LLO-M45), pSSV100 (SipB₁₋₁₀₀-LLO-M45), pSSV72 (SipB₁₋₇₂-LLO-M45), pSSV30

(SipB₁₋₃₀-LLO-M45), pSSV15 (SipB₁₋₁₅-LLO-M45), pSSV8 (SipB₁₋₈-LLO-M45) and pSSV1 (SipB₁-LLO-M45) (see Table 2). To construct pSSV Δ 2-30, the region encoding the N-terminal 31–160 aa of SipB was amplified as a template pSSV160 using appropriate primers (forward primer SipB31F, 5'-ccggatcatcatgacggactttttaaagcgcg-3'; reverse primer Sip160R, 5'-ccgggtaccttacgcagctcataaacact-3'). The underlined sequences indicate the restriction enzyme sites for *EcoRV* and *KpnI*, respectively. The amplified fragments (SipB 31–160 aa) were digested with *EcoRV* and *KpnI*, and then ligated with T4 ligase (Takara) to pSSV160, which had been pre-digested with *EcoRV* and *KpnI* and eluted. The DNA sequences of the ligated clones were confirmed by DNA sequencing (Macrogen).

To construct the template plasmid for the alanine substitution mutant (pSM), we amplified the region encoding the native promoter *sicA* along with the *sipB* gene (forward primer CoSPaS, 5'-ggcgtcga-catttctgaccatgaagatatg-3'; reverse primer SipB593R, 5'-ggcagctcgc-gactctggcgcagaataa-3') using the *Salmonella* chromosome as template. The underlined sequences indicate restriction enzyme sites for *Sall* and *SacI*, respectively. The amplified fragments (Pnative, *SicA* and SipB 593 aa) were digested with *Sall* and *SacI*, and ligated into pMW118 plasmids pre-cut with *Sall* and *SacI*. To construct the alanine substitution mutants, site-directed mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene). Briefly, complementary oligonucleotides were designed to include the desired codon change to alanine (see Table 2). Plasmid pSM was used as template, and the resulting mutagenized PCR products were digested with *DpnI* and transformed into *E. coli* DH5 α cells. Plasmids obtained from these transformed colonies were sequenced to confirm the desired mutation (Macrogen).

Preparation of culture supernatant proteins and Western blotting. For preparation of culture supernatant proteins, bacterial supernatants (10 ml) were passed through a 0.45 µm pore-size syringe filter in order to remove the bacteria. The proteins present in the bacteria-free medium were precipitated to 10% (v/v) by the addition of cold TCA followed by incubation on ice for 2 h. The proteins were collected by centrifugation at 10 000 g for 20 min at 4 °C. The resulting pellets were washed in 0.8 ml cold acetone, dried, and resuspended in PBS buffered with 80 mM Tris/HCl, pH 8.0. Samples corresponding to 100 µl whole bacterial culture or 200 µl culture supernatant were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes. Wild-type SipB, truncated SipB or reporter-fused SipB proteins were detected by immunoblot analysis. Western blots were treated with a mAb against the M45 tag (a kind gift from P. Hearing, State University of New York, Stony Brook, NY) or a polyclonal anti-SipB antibody (Kim *et al.*, 2007) separately, followed by incubation with horseradish peroxidase-labelled anti-mouse or anti-rabbit antibodies, respectively. The blots were developed using a chemiluminescence detection kit (Amersham ECL Western Blotting Detection Reagent).

***Salmonella* subcellular fractionation.** The outer-membrane fraction was prepared by a modification of the lysozyme–osmotic shock method (Osborn & Munson, 1974). Cultures were grown in LB broth to OD₆₀₀ 0.9 and then centrifuged at 7000 g for 10 min, and supernatants were saved for analysis of secreted proteins. The cell pellets were resuspended in 800 µl 100 mM Tris/HCl buffer (pH 8.6) containing 500 mM sucrose and 0.5 mM EDTA. Hen egg white lysozyme (40 µl of a 4 mg ml⁻¹ stock solution) was added, followed immediately by the addition of 3.2 ml 50 mM Tris/HCl buffer (pH 8.6) containing 250 mM sucrose, 0.25 mM EDTA and 2.5 mM MgCl₂. After gentle agitation, the suspension was incubated for 15 min on an ice bath. The resulting pellet was resuspended in 4 ml 20 mM Tris/HCl (pH 8.6), disrupted by two passages through a French pressure cell (82 800 kPa; American Instrument Company) and centrifuged at 7000 g for 6 min at 4 °C in order to remove

Table 1. Strains, phages and plasmids used in this study

Name	Properties	Reference or source
<i>S. typhimurium</i>		
SF530 (χ 3761)	Wild-type	Curtis <i>et al.</i> (1991)
χ 4370	SL1344, <i>invA</i> ::Km, Km ^r	R. Curtiss, Washington University
SF586 (JR501)	<i>hsdSA29 hsdSB121 hsdL6 metE551 trpC2 ilv452 rpl120 gale719 H1-6 H2-c, n, x nml fla-66</i>	Bullas & Ryu (1983)
YKJ013	SF530, Δ <i>sipB</i>	This study
YKJ019	SF530, Δ <i>sipD</i>	This study
YKJ028	SF530, <i>invA</i> ::Km, Km ^r , SF530 $\times$ HT(χ 4370)	This study
<i>E. coli</i>		
DH5 α	F [−] <i>endA1 recA1 hsdR17</i> (r _K [−] m _K ⁺) <i>supE44 thi-1 gvrA96 relA1 ϕ80dlacZ ΔM15 Δ(lacZYA-argF)U169</i>	Gibco-BRL
Phages		
P22HT int 105	Used for generalized transduction	Davis <i>et al.</i> (1980)
H5	P22 <i>c2</i> mutant	Davis <i>et al.</i> (1980)
Plasmids		
pGEM-T Easy	High-copy PCR cloning vector; Ap ^r	Promega
pMW118	Low-copy plasmid, Ap ^r	N. Okada, Kitasato University
pKD46	Red recombinase plasmid	Datsenko & Wanner (2000)
pCP20	FLP ⁺ , λ cI857 ⁺ , λ P _R Rep ^{ts} , Ap ^r , Cm ^r	Datsenko & Wanner (2000)
pSSV160	pGEM-T Easy inserted with Lac promoter, nucleotide region encoding N-terminal 160 aa of SipB (SipB160), LLO, and M45 epitope tag, Ap ^r	Kim <i>et al.</i> (2007)
pSSV160-1	Derivatives of pSSV160, in which LLO was deleted. Ap ^r	Kim <i>et al.</i> (2007)
pSSV140	Derivative of pSSV160 encoding only 140 aa of the SipB N terminus, Ap ^r	This study
pSSV120	Derivative of pSSV160 encoding only 120 aa of the SipB N terminus, Ap ^r	This study
pSSV100	Derivative of pSSV160 encoding only 100 aa of the SipB N terminus, Ap ^r	This study
pSSV72	Derivative of pSSV160 encoding only 72 aa of the SipB N terminus, Ap ^r	This study
pSSV30	Derivative of pSSV160 encoding only 30 aa of the SipB N terminus, Ap ^r	This study
pSSV15	Derivative of pSSV160 encoding only 15 aa of the SipB N terminus, Ap ^r	This study
pSSV8	Derivative of pSSV160 encoding only 8 aa of the SipB N terminus, Ap ^r	This study
pSSV1	Derivative of pSSV160 encoding only 1 aa of the SipB N terminus, Ap ^r	This study
pSSV Δ 2-30	Derivative of pSSV160 encoding only 31–160 aa of the SipB N terminus, Ap ^r	This study
pSM	pMW118 inserted with SipB promoter, SicA and SipB amino acids 1–593, Ap ^r . Expression for wild-type SipB and template for alanine substitution.	This study
pSA1	Derivative of pSM, 111–114 aa of SipB replaced with alanine	This study
pSA2	Derivative of pSM, 115–118 aa of SipB replaced with alanine	This study
pSA3	Derivative of pSM, 119–122 aa of SipB replaced with alanine	This study
pSA4	Derivative of pSM, 123–126 aa of SipB replaced with alanine	This study
pSA5	Derivative of pSM, 127–130 aa of SipB replaced with alanine	This study
pSA6	Derivative of pSM, 131–134 aa of SipB replaced with alanine	This study
pSA7	Derivative of pSM, 135–138 aa of SipB replaced with alanine	This study
pSA8	Derivative of pSM, 139–142 aa of SipB replaced with alanine	This study

unbroken cells. The supernatant was then centrifuged at 132 000 *g* for 1 h at 4 °C to separate the soluble fraction and insoluble cell envelopes. The soluble fraction was taken as containing the cytoplasmic proteins. For isolation of the outer-membrane fraction, total envelope pellets were suspended in 4 ml 20 mM Tris/HCl (pH 8.6) containing 1 % Sarkosyl and incubated for 30 min on ice. The outer-membrane fraction was obtained as a pellet after centrifugation at 132 000 *g* for 1 h at 4 °C. The pellet was resuspended in 20 mM Tris/HCl buffer (pH 8.6). The original culture supernatant was filtered (0.22 μ m pore-size filter), and precipitated with 10 % TCA (2 h, 4 °C). Each fraction was separated by SDS-PAGE for Western blot analysis.

Separation of inner- and outer-membrane fractions by sucrose gradient. *Salmonella enterica* serovar Typhimurium (*S. typhimurium*)

stationary-phase cultures were diluted 1:20 into LB medium, and incubated to OD₆₀₀ 0.9 under type III secretion induction conditions (0.3 M NaCl) with mild aeration. Cells were harvested by centrifugation (8000 *g*, 10 min), resuspended in 0.03 culture volumes of 20 mM Tris/HCl (pH 7.4) containing 1 mM PMSF, and disrupted in a French press (82 800 kPa, American Instrument Company). The lysate was clarified (8000 *g*, 10 min, 4 °C) and the resulting supernatant was centrifuged again (132 000 *g*, 1 h, 4 °C) to separate the soluble cytosolic proteins (supernatant) from the whole membranes (pellet). Isolated whole membranes were resuspended in 20 mM Tris/HCl (pH 8.6) containing 5 mM EDTA and layered onto a six-step sucrose gradient (1.0–2.0 M sucrose, 5 mM EDTA). The gradient was centrifuged (100 000 *g*, 36 h, 4 °C) and fractionated into 14 fractions, and the proteins from each fraction were precipitated with 10 % (v/v) TCA and analysed by immunoblotting.

Table 2. Primers used in this study

Primer	Oligonucleotide sequence 5'–3'
General plasmid construction*	
SipB120R	ccgggtaccctcttttgtgactcaatcatc
SipB100R	ccgggtaccgaaacatcgccagtagg
SipB72R	ccgggtaccgtaggcggttttagacc
SipB30R	ccgggtaccgttcttacgaacgcttcaa
SipB15R	ccgggtacctgggtatatccgctacg
SipB8R	ccgggtaccaatgctactgtcttctttgccat
SipB1R	ccgggtaccatcagctgttctctgtgaaat
Alanine substitution mutagenesis†	
pSA1F	cgtctggcgtagccgcgccggttagtcacaaaag
pSA1R	cttttgtgactcaatggcgccggtacgagcagc
pSA2F	ggcaggcgatggcgccgaggaagatgg
pSA2R	ccatctctttcgcgccggtatcgctgcc
pSA3F	gattgagtcacaagccgcgccggttagtcg
pSA3R	cgatactgaatcgcgccggttagtcactcaatc
pSA4F	caaaaagatggggcgccggttagtcacgagc
pSA4R	cgtctgaattctttggcgccggttagtcactcttttg
pSA5F	ggattcaggatcgcgccggttagtcacggtctggg
pSA5R	cccagagcgtggcgccggttagtcacggttagtc
pSA6F	gaaagaattccagcgccggttagtcacggttagtc
pSA6R	cctgagcctcgcgccggttagtcacggttagtc
pSA7F	ggctctgggagcgccggttagtcacggttagtc
pSA7R	gatccgtcgcgccggttagtcacggttagtc
pSA8F	ggctcaggagcgccggttagtcacggttagtc
pSA8R	ctggctcatagcgccggttagtcacggttagtc
Mutant construction‡	
musipB-F	gggaattgacattactgcttggcaagttaatgacctacgttaggctggagctgcttc
musipB-R	ggcggtatccgtcttttgatactggcttagatagatcccatatgaatatcctccttag
musipD-F	cagccgagaacaaccatttctgatgctgagatttgggatagttaggctggagctgcttg
musipD-R	actgtttaaatcatttttgatgaggttaacatctagcttagatgaatatcctccttag

*Underlined sequences represent restriction enzyme *KpnI*.

†Alanine-substituted sequences are underlined.

‡Underlined sequence are FLP recombination target (FRT) sites which allow the kanamycin cassettes to be removed with the FLP recombinase.

The outer-membrane protein OmpW (a kind gift from H. Y. Kang, Pusan National University, Pusan, Korea) was detected as an outer-membrane marker by immunoblotting.

Immunofluorescence assay (IFA). Cultures were grown in LB broth containing 0.3 M NaCl to OD₆₀₀ 0.9. In order to stain the cells without leakage of the cell envelope, Hoechst 33342 (Sigma) was added to the bacterial culture at a final concentration of 1.5 g ml⁻¹, followed by incubation for 15 min as described by Walberg *et al.* (1999). Bacterial cells were harvested by centrifugation (3000 g, 10 min). The cell pellets were gently washed three times with PBS, and then the cells were fixed for 1 h with freshly prepared 4 % paraformaldehyde, washed twice with PBS, and blocked with PBS containing 3 % BSA for 1 h at room temperature. The blocked cells were incubated for 1 h at room temperature with anti-M45 mAb, gently washed seven times with PBS, and then incubated with FITC-conjugated goat anti-mouse IgG secondary antibodies (Sigma). The cells were washed five times with PBS and briefly rinsed with water before being mounted onto slides for observation under fluorescence microscopy. For confocal microscopy, bacterial cells were grown in LB broth containing 0.3 M NaCl or 2.5 mM sodium deoxycholate (DOC; Sigma) to OD₆₀₀ 0.9, fixed with

4 % paraformaldehyde, overlaid on gelatin-coated slides (0.1 % gelatin, 0.01 %, v/v) and then stained with polyclonal anti-SipB/AlexaFluor 488-conjugated anti-rabbit IgG.

In situ protease treatment. *Salmonella* cells harbouring pSSV160 were grown to the late-exponential phase and pelleted by centrifugation for 5 min at 5000 r.p.m. The pellet was washed once with 1 × PBS and resuspended in the same buffer to a final concentration of 2 × 10¹⁰ bacteria ml⁻¹. The bacteria were then incubated at 37 °C with proteinase K (20 or 200 µg ml⁻¹) for 10, 20, 40 or 60 min, whereupon digestion was terminated by addition of PMSF to a final concentration of 1.6 mg ml⁻¹.

RESULTS

SipB could be localized on the surface of *S. typhimurium*

Previous studies have suggested that SipB can be detected on the surface of wild-type *Salmonella* strains (Collazo &

Fig. 1. SipB localization to the bacterial outer membrane requires its secretion and is independent of the presence of SipD. (a) Expression and secretion of SipB protein was detected from whole-cell lysates (equivalent to 100 μ l bacterial culture) or culture supernatants (600 μ l) from wild-type (lane 1), $\Delta sipB$ (lane 2), *invA::Km* (lane 3), and $\Delta sipD$ (lane 4) cells. Blots were probed with polyclonal anti-SipB160 antibodies, and then reprobed with anti-DnaK mAbs as a control for cytosolic protein contamination (lower panel). (b) Expression and outer-membrane localization of SipB in wild-type (lane 1), $\Delta sipB$ (lane 2), *invA::Km* (lane 3) and $\Delta sipD$ (lane 4) cells. Outer-membrane fractions were prepared by sucrose density-gradient fractionation as described in Methods. Since we showed previously the non-specific signal in fraction numbers 7–13, the bottom fractions (nos 1–6) were pooled and blotted, and the blots were separately probed with polyclonal anti-SipB160, polyclonal anti-OmpW and monoclonal anti-DnaK antibodies. OmpW was used as an outer-membrane marker (middle panel) and DnaK was used as a control for cytosolic protein contamination (lower panel). Asterisks indicate non-specific signals. (c) SipB localizes to the *S. typhimurium* surface. *S. typhimurium* wild-type and $\Delta sipB$ mutants were grown in LB broth containing 0.3 M NaCl or 2.5 mM DOC to OD₆₀₀ ~0.9, fixed, overlaid on gelatin-coated slides (0.1 % gelatin, w/v), and then stained with polyclonal anti-SipB/AlexaFluor 488-conjugated anti-rabbit IgG. Confocal immunofluorescence microscopy revealed SipB expression on the bacterial surface of wild-type cells (upper panel, right) but not $\Delta sipB$ mutant cells (lower panel, right). The left-hand panels show differential interference contrast (DIC) micrographs of the above two strains.

membranes were isolated and fractionated by sucrose density-gradient centrifugation. Consistent with earlier observations (Collazo & Galan 1996; Hayward *et al.*, 2000), wild-type SipB co-migrated with the bacterial outer-membrane marker OmpW (data not shown). To further confirm that wild-type SipB could be localized to the *Salmonella* cell surface, bacterial cells were labelled with a polyclonal anti-SipB antibody, as described in Methods. As shown in Fig. 1(c), SipB was successfully localized to the bacterial cell surface.

The SipB protein crosses both the inner and the outer membrane through the T3SS needle during the translocation process. Therefore, it was possible that the outer-membrane localization of SipB resulted from random association with cell membranes during the protein-preparation process. To address this possibility, we examined whether the SipB protein was present in outer-membrane fractions from an *invA* mutant *Salmonella*, which is known to be defective for T3SS (Galan & Curtiss, 1991). Culture supernatants, cell lysates and outer-membrane fractions were prepared from the wild-type, *sipB* and *invA* strains, and Western blotting of the combined bottom fractions (fractions 1–6; see Fig. 1 legend) was used to observe the localization of the wild-type SipB proteins. As shown in Fig. 1(a), SipB was not secreted to the culture supernatant or detected in outer-membrane fractions from the *invA* strain. This supports

Galan, 1996), particularly during invasion of mammalian target cells, when it may be fractionated with outer-membrane proteins (Hayward *et al.*, 2000). It has been also reported that *Shigella* IpaB, a homologue of *Salmonella* SipB, may be associated with the bacterial surface (Menard *et al.*, 1994; Mills *et al.*, 1988; Watarai *et al.*, 1995; Veenendaal *et al.*, 2007). Therefore, we first sought to confirm that SipB protein was present on the bacterial outer membrane.

Wild-type and *sipB* deletion mutants were cultured under SPI-1-inducing conditions (0.3 M NaCl), and total cell

our contention that the presence of SipB in the outer-membrane fractions of wild-type preparations (Fig. 1b) was not due to random association with the cell membrane during the preparation process.

Recent reports have shown that localization of *Shigella* IpaB to the bacterial cell surface is dependent on the presence of IpaD, a homologue of *Salmonella* SipD, and have further shown that interaction of IpaB and IpaD at the needle tip is necessary for their insertion into the host-cell membrane (Johnson *et al.*, 2006; Veenendaal *et al.*, 2007). Since no earlier study has examined whether outer-membrane localization of SipB is dependent on SipD, we prepared culture supernatants, cell lysates and outer-membrane fractions from a *sipD* deletion strain, and examined SipB localization by Western blotting. As shown in Fig. 1(b), SipB was detected in the outer-membrane fractions from the *sipD* deletion strain. Collectively, these results indicate that SipB is localized on the *Salmonella* surface in a SipD-independent manner.

The N-terminal 160 residues of SipB could direct localization to the bacterial cell surface

In a recent study, we were able to show that a recombinant protein containing the N-terminal 160 aa of SipB (SipB160) could be successfully secreted into the culture supernatant (Kim *et al.*, 2007). We next examined whether the same fragment of SipB (SipB160) could direct localization to the outer membrane, by using a recombinant plasmid containing SipB160 protein fused to a C-terminal M45 epitope tag (pSSV160).

To investigate whether recombinant SipB160 fusion proteins were displayed on the bacterial surface in a manner similar to that seen for wild-type SipB, immunofluorescence microscopic analysis was carried out with a mouse anti-M45 mAb. Hoechst 33342 dye was added to the bacterial culture to facilitate cell staining without leakage of the cell envelope, as described in Methods, and stained cells expressing the recombinant protein were probed with an anti-M45 primary mAb followed by an FITC-conjugated anti-mouse IgG secondary antibody. As shown in Fig. 2(a), IFA showed that the M45 molecules were exposed to the cell surface by recombinant proteins harbouring the N-terminal 160 aa of SipB, but not by fusion proteins lacking this fragment. To further confirm the surface localization of the recombinant SipB160, we carried out a proteinase K assay followed by Western blotting with anti-M45 antibody. Treatment of the recombinant SipB160-expressing cells with proteinase K resulted in degradation of the recombinant protein (Fig. 2b, upper panel). To examine this digestion in more detail, we carried out time-dependent proteinase K treatment (Fig. 2b, lower panel), and found that the recombinant SipB160 protein was partially degraded after 20 min, and completely degraded after 90 min. These data confirm that M45 molecules were displayed on the surface of bacteria through localization by SipB160.

Fig. 2. The N-terminal 160 aa of SipB mediate its localization to the bacterial cell surface. (a) IFA of bacterial cell surfaces. Wild-type *S. typhimurium* cells containing pSSV1 or pSSV160 were grown in LB broth containing 0.3 M NaCl to OD₆₀₀ ~0.9, stained with 1.5 g ml⁻¹ Hoechst 33342 dye (blue signal), and then incubated with an anti-M45 primary mAb followed by (Fc-specific) FITC-conjugated anti-mouse IgG (green signal). (b) Proteinase K susceptibility assay of recombinant SipB160-LLO-M45 fusion proteins. Cells harbouring pSSV1 (lanes 1, 3 and 4) or pSSV160 (lanes 2, 5 and 6) were grown in LB broth containing 0.3 M NaCl to OD₆₀₀ ~0.9, and incubated with (+) or without (-) proteinase K (200 µg ml⁻¹) (upper panel). Time-dependent susceptibility was assayed with 20 µg proteinase K ml⁻¹ (lower panel). Outer-membrane proteins were prepared from proteinase K-treated (or untreated) cells, as described in Methods. Recombinant SipB proteins were detected by Western blotting with anti-M45 mAbs.

The N-terminal 100–140 aa of SipB are necessary for its outer-membrane localization

To further delineate which of the N-terminal 160 aa were responsible for the outer-membrane localization of SipB, outer-membrane fractions, supernatants and cell lysates were prepared from strains expressing various mutant SipB proteins (pSSV1, -8, -15, -30; see Table 1). Although all tested strains could secrete M45 (Fig. 3a), only the strain

Fig. 3. N-terminal amino acids 100–140 of SipB are essential for its localization to the outer membrane. (a, b) Cells containing pSSV1, pSSV8, pSSV15, pSSV30 and pSSV160 were grown in LB broth containing 0.3 M NaCl to $OD_{600} \sim 0.9$, and culture supernatant proteins, whole-cell lysates and outer-membrane proteins were prepared as described in Methods. Expression and secretion of the recombinant SipB proteins were detected by Western blotting with anti-M45 mAbs. (c) Cells harbouring pSSV1, pSSV160, pSSV72, pSSV100, pSSV120 and pSSV140 were grown in LB broth to $OD_{600} \sim 0.9$, whole-cell lysates and outer-membrane proteins were prepared, and the recombinant SipB proteins were detected by Western blotting with anti-M45 mAbs. (d) Whole-cell lysates, supernatants and outer-membrane proteins from cells containing pSSV160 (lanes 2, 4 and 6) and pSSV Δ_{2-30} -LLO (lanes 1, 3 and 5) were prepared, and recombinant SipB proteins were detected by Western blotting with anti-M45 mAbs. OmpW was used as an outer-membrane marker and DnaK was used as a control for cytosolic protein contamination.

containing parental pSSV160 directed M45 to the outer membrane (Fig. 3b). To further clarify the region necessary for localization to the outer membrane, we generated fusion vectors that included the first 72 (pSSV72), 100 (pSSV100), 120 (pSSV120) and 140 (pSSV140) N-terminal residues. As shown in Fig. 3(c), pSSV120 and pSSV140 could mediate localization to the outer membrane, but pSSV100 could not, indicating that the fragment between N-terminal amino acids 100 and 140 of SipB was essential to the outer-membrane localization of SipB.

To examine whether the outer-membrane localization of SipB160 was dependent on its secretion, we used pSSV Δ_{2-30} , a derivative of pSSV160 that lacks the secretion signal of SipB (Kim *et al.*, 2007). As shown in Fig. 3(d), recombinant SipB lacking a secretion signal could not be localized to the outer membrane. Furthermore, native SipB was not localized to the outer membrane in an *invA* mutant strain lacking SPI-1-dependent T3SS (Fig. 1b). Taken together, these findings suggest that SipB localizes to the outer membrane following its secretion outside the cell.

To further elucidate the residues responsible for the outer-membrane localization of SipB, we first generated a template plasmid for alanine mutagenesis. We cloned a full-length *sipB* gene into a low-copy plasmid pMW118 (pSM), and introduced the pSM plasmid into the $\Delta sipB$ strain. Preparation and analysis of culture supernatants, cell lysates and outer-membrane fractions from the $\Delta sipB$ mutant strain containing plasmid pSM confirmed that, consistent with wild-type SipB, recombinant SipB was expressed in the cytosol, secreted into the culture supernatant, and localized on the bacterial outer membrane (Fig. 4). We then used an oligo cassette mutagenesis approach (see Methods) to construct eight variant plasmids in which blocks of 4 aa were replaced with alanine, stepwise from amino acids 111 to 142. Since a secondary-structure prediction of the N-terminal 160 aa of SipB revealed that amino acids 111–118 represented a

Fig. 4. N-terminal amino acids 135–138 of SipB are indispensable for its localization to the bacterial outer membrane. (a) Whole-cell lysates and culture supernatant proteins were prepared from wild-type cells (lane 1), $\Delta sipB$ cells (lane 2), and $\Delta sipB$ cells containing pSA7 (lane 3) or pSM (lane 4). SipB and DnaK (cytosolic contamination control, lower panel) were detected with polyclonal anti-SipB160 and monoclonal anti-DnaK antibodies, respectively. (b) Expression of SipB protein in the outer-membrane fractions of wild-type cells (lane 1), $\Delta sipB$ cells (lane 2), and $\Delta sipB$ cells containing pSA7 (lane 3) or pSM (lane 4). Outer-membrane fractions were prepared by sucrose density-gradient fractionation as described in Methods. For a more detailed comparison, fractions 1 and 2, 3 and 4, and 5 and 6 were pooled and resolved by SDS-PAGE, and the blots were detected with polyclonal anti-SipB160, polyclonal anti-OmpW, and monoclonal anti-DnaK antibodies, respectively. OmpW was used as an outer-membrane marker (middle panel) and DnaK was used to control for contamination of cytosolic proteins (lower panel).

putative coiled-coil domain (J net secondary structure prediction; <http://www.compbio.dundee.ac.uk/~www-jpred/>), we hypothesized that the putative coiled-coil domain of SipB (residues 111–118) was associated with the interaction with the bacterial cell surface, and first selected the region between amino acids 111 and 142 (pSA1–pSA8; see Table 1) for alanine substitution mutagenesis. SipB_{Ala135–138} (pSA7) and wild-type SipB (pSM) were both expressed and secreted to culture supernatants at wild-type levels, whereas the levels of SipB_{Ala135–138} in the outer-membrane fraction were dramatically reduced (Fig. 4b), demonstrating that amino acids 135–138 of SipB are required for SipB localization in the outer membrane.

DISCUSSION

Although numerous studies have examined the function of T3SS secretion proteins and translocons, the interaction between the translocon and the bacterial cell surface is less well understood. It was recently reported that IpaB and IpaD, the translocon proteins of *Shigella*, can be localized on the bacterial cell surface (Olive *et al.*, 2007; Veenendaal *et al.*, 2007). However, the surface localization of PopB and PopD of *Pseudomonas*, BopB and BopD of *Bordetella*, and BipB and BipD of *Burkholderia* have not yet been characterized. In the case of *Salmonella*, the surface localization of SipD has not been characterized before.

It was recently suggested that the cell-surface localization of *Shigella* IpaB, which is homologous to SipB, could be dependent on the presence of IpaD, and furthermore that an interaction between IpaB and IpaD is required for insertion of the translocation pore into the host-cell membrane (Johnson *et al.*, 2006; Veenendaal *et al.*, 2007). In *Salmonella*, *sipD* mutants have been shown to be deficient for invasion of epithelial cells (Kaniga *et al.*, 1995; Collazo & Galan, 1997), even though these *sipD* mutants have increased secretion of Sip proteins (Kaniga *et al.*, 1995). Here, we showed that SipB could be localized to the bacterial outer membrane of a $\Delta sipD$ strain (Fig. 1). Therefore, we suggest that unlike IpaD (Veenendaal *et al.*, 2007), SipD does not participate in localizing SipB to the bacterial cell surface.

SipB in *S. typhimurium* has been shown to have a few discrete functional domains, including a large C-terminal domain that contains two α -helical transmembrane regions essential to its integration into the host-cell plasma membrane (McGhie *et al.*, 2002). However, although numerous reports have focused on translocons, no earlier study has investigated whether IpaB and SipB have any specific regions required for bacterial surface localization. Here, we used deletion analysis to identify regions essential to the surface localization of SipB. Our results revealed that the N-terminal 160 aa of SipB (SipB160) were sufficient to direct outer-membrane localization. More detailed analysis identified the region between amino acids 100 and 140 as

being essential for the outer-membrane localization of SipB160. Construction of plasmids encoding alanine-substituted full-length SipB and their expression in the *sipB* deletion strain revealed that construct pSA7, encoding SipB_{Ala135-138}, exhibited reduced outer-membrane localization. These findings collectively indicated that the N-terminal amino acids 100–140 of SipB are essential to its surface localization, and further suggested that SipB160 and wild-type SipB are localized to the same region of the *Salmonella* cell surface.

Many proteins secreted through T3SS components have been predicted to share a common coiled-coil structure (Pallen *et al.*, 1997; Delahay & Frankel, 2002). The coiled-coil proteins are reported to interact with themselves and/or each other (Delahay & Frankel, 2002). Therefore, we hypothesized that residues 111–118 of SipB are required for an interaction with the bacterial cell surface. However, our results revealed that N-terminal residues 135–138 were indispensable for the surface localization of SipB. To further characterize residues 135–138, we aligned the amino acid sequences of BipB, IpaB and SipB. Interestingly, the amino acid 135 (E, Glu) in SipB (corresponding to residues 152 in BipB and 129 in IpaB) was identical in all three proteins, and a high sequence homology was found among residues 135–138 (data not shown). Glutamic acid is a negatively charged amino acid typically found on the surface of proteins. Thus, residue 135 may play a role in the binding of SipB to the outer membrane by interacting with proteins or other molecules.

This study revealed that a recombinant SipB protein (pSSV_{Δ2-30}) lacking a secretion signal could not be localized to the outer membrane, and *invA* mutant cells failing to secrete SipB also failed to show its localization to the outer membrane, suggesting that SipB is localized to the outer membrane after its secretion outside the cell. Our findings of the secretion-dependent localization of SipB to the outer membrane are consistent with an earlier report that showed that an *invJ* mutant, which was defective in secretion of SipB protein to the culture supernatant, also failed to show SipB localization to the bacterial cell surface (Collazo & Galan, 1996). Although the SPI-2-encoded translocon (SseB, SseC and SseD) is structurally different from that involving SPI-1, the former is also known to be localized to the outer surface of the bacterial cell after secretion *in vitro* (Beuzon *et al.*, 1999; Klein & Jones, 2001; Nikolaus *et al.*, 2001). Therefore, it seems as though the secretion of translocons such as SipB is indispensable for their localization to the bacterial cell surface.

Although future work will be required to elucidate the mechanism(s) underlying the specific targeting of SipB to the bacterial cell surface, our novel finding that the N-terminal region of SipB is required for its localization to the surface of *Salmonella* may provide new insights into the presence of translocons on the bacterial cell surface.

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