Analysis of functional domains present in the N-terminus of the SipB protein

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SipB (593 aa), one of the *Salmonella* invasion proteins (Sips), is secreted via the *Salmonella* pathogenicity island 1 (SPI-1) type III secretion system (T3SS). Here, we report the delineation of several functional regions present in the SipB protein. Our data show that residues 3–8 of the SipB protein are essential for its secretion from the bacterial cell and that the SicA chaperone, which is important to ensure stability of SipB and SipC in the bacterial cytosol, binds to SipB somewhere between amino acids 80 and 100 of the SipB N-terminal region. Interestingly, the N-terminal region (residues 1–160) of SipB (SipB160) cannot be secreted via the SPI-1 T3SS, but fusion of the C-terminal amphipathic region (residues 300–593) to SipB160 can restore secretion via this system.

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INTRODUCTION

Gram-negative bacterial pathogens, including *Salmonella* spp., have evolved sophisticated mechanisms to infect and colonize their hosts. One of these mechanisms requires the virulence-associated type III secretion system (T3SS), which is composed of specialized organelles that translocate bacterial virulence proteins (effectors) from the bacterial cytoplasm directly into the host cell cytoplasm (Cornelis & Van Gijsegem, 2000; Galán & Collmer, 1999; Hueck, 1998; Kimbrough & Miller, 2002; Ramamurthi & Schneewind, 2005). *Salmonella enterica* encodes two T3SSs that play important roles at different stages of the infection cycle (Galán, 2001; Ochman *et al.*, 1996). One of the systems, encoded within *Salmonella* pathogenicity island 1 (SPI-1), mediates the initial interaction of *Salmonella* with the intestinal epithelium. This process allows bacterial entry and stimulation of pro-inflammatory cytokine production (Galan & Curtiss, 1989). The second T3SS is encoded within *Salmonella* pathogenicity island 2 (SPI-2) and is essential for systemic infection (Hensel *et al.*, 1995; Ochman *et al.*, 1996). Some proteins destined to travel the T3SS pathway possess at least two domains that specifically target them to the secretion apparatus (Cheng *et al.*, 1997; Lee & Galan, 2004; Michiels & Cornelis, 1991; Sory *et al.*, 1995). One of the domains is located within the first ~20 aa

of the N-terminal region of the protein, because this region is necessary and sufficient for directing the secretion of hybrid fusion proteins (Cornelis & Van Gijsegem, 2000; Galán & Collmer, 1999; Lloyd *et al.*, 2001). However, there is little similarity among the amino acid sequences of the different proteins that are targeted for secretion. The second domain is located within the first ~140 aa of the effector proteins and serves as a binding site for specific chaperones (Birtalan *et al.*, 2002; Cheng *et al.*, 1997). It has been shown that the secretion and translocation of many effector proteins requires a cognate chaperone (Parsot *et al.*, 2003). These chaperones usually bind to the N-terminal region of the protein and exert various effects on their cognate effector proteins, including enabling their recognition by the T3SS (Birtalan *et al.*, 2002), improving cytosolic stability (Fu & Galan, 1998), prevention of premature interactions (Neyt & Cornelis, 1999), maintenance of the effector protein in a secretion-competent state (Stebbins & Galán, 2001), and transcriptional regulation (Darwin & Miller, 2001). Chaperones associated with the T3SS bind specifically to cognate effector proteins and control their secretion (Cornelis & Van Gijsegem, 2000; Sory *et al.*, 1995; Wattiau *et al.*, 1996).

It is known that *Salmonella* invasion proteins (Sips) are central to the initiation of the entry process. Individual non-polar *sipB/sipC* deletion mutants are entry-deficient because they are unable to deliver other effectors into cultured mammalian cells (Collazo & Galan, 1997). The hydrophobic SipB and SipC effectors form an extracellular complex following their secretion through the SPI-1 T3SS, and are thought to assemble into a plasma-membrane-integral

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Abbreviations: FragC, tetanus toxin fragment C; GST, glutathione S-transferase; LLO, listeriolysin O; rSipB, recombinant SipB; Sip, *Salmonella* invasion protein; SPI, *Salmonella* pathogenicity island; T3SS, type III secretion system.

Table 1. Strains and plasmids used in this study

Name	Properties	Reference or source
<i>S. typhimurium</i>		
SF530 (χ 3761)	Wild-type	Curtiss <i>et al.</i> (1991)
SL1344	Wild-type	Hensel <i>et al.</i> (1995)
CS401	Wild-type invasive, Str ^r	Ochman <i>et al.</i> (1996)
SF586 (JR501)	<i>hsdSA29 hsdSB121 hsdL6 metE551 trpC2 ilv452 rpL120 galE719 H1-6 H2-c, n, x nml fla-66</i>	Bullas & Ryu (1983)
χ 4370	SL1344, <i>invA::Km</i> , Km ^r	R. Curtis (Seattle)
TK164	CS401, <i>prgH</i> deletion mutant, Str ^r	Sherer <i>et al.</i> (2000)
CAS152	CS401, Δ <i>sspB</i> (<i>sipB</i> , Str ^r)	Sherer <i>et al.</i> (2000)
AT351	<i>flhD::Tn10</i> , Tc ^r	W. D. Hardt (Inst. of Microbiology, ETH Zürich, Switzerland)
M587	<i>fliGHI::Tn10</i> , Tc ^r	W. D. Hardt
YG 101	<i>invA::Km</i> , Km ^r <i>fliGHI::Tn10</i> , Tc ^r	This study
<i>E. coli</i>		
DH5 α	F ⁻ <i>endA1 recA1 hsdR17(r_K⁻ m_K⁺) supE44 thi-1 gvrA96 relA1 ϕ80dlacZΔM15 Δ(<i>lacZYA</i>-Gibco-BRL <i>argF</i>)U169</i>	
BL21 (DE3)	<i>ompT hsdS dcm⁺ Tc^r gal(DE3) endA</i>	Stratagene
Plasmids		
pGEM-T-easy	High-copy-number PCR cloning vector, Ap ^r	Promega
pACYC184	Low-copy-number cloning vector, Tc ^r Cm ^r	New England Biolabs
pET28a (+)	His-tagging expression vector, Km ^r	Novagen
pMW118	Low-copy-number plasmid, Ap ^r	N. Okada (Kitasato University, Tokyo, Japan)
pTETnir15 FragC	Plasmid bearing the gene for tetanus toxin fragment C	A. J. Makoff (Inst. of Psychiatry, London, UK)
pGEX-KG	Expression vector, Ap ^r	Guan & Dixon (1991)
pCMX-M45	Plasmid bearing the genetic information for M45	P. Hearing (State University, NY)
pSB1841	pGEX-KG with 5' 480 bp (amino acids 1–160) of <i>sipB</i> , Ap ^r	This study
pSB1842	pGEX-KG with 5' 90 bp (amino acids 1–30) of <i>sipB</i> , Ap ^r	This study
pSB1843	pGEX-KG with 5' 300 bp (amino acids 1–100) of <i>sipB</i> , Ap ^r	This study
pSB1844	pGEX-KG with 5' 180 bp (amino acids 101–160) of <i>sipB</i> , Ap ^r	This study
pSB1845	pGEX-KG with 3' 279 bp (amino acids 500–593) of <i>sipB</i> , Ap ^r	This study
pSB1846	pGEX-KG with 5' 120 bp (amino acids 1–40) of <i>sipB</i> , Ap ^r	This study
pSB1847	pGEX-KG with 5' 150 bp (amino acids 1–50) of <i>sipB</i> , Ap ^r	This study
pSB1848	pGEX-KG with 5' 210 bp (amino acids 1–70) of <i>sipB</i> , Ap ^r	This study
pSB1849	pGEX-KG with 5' 240 bp (amino acids 1–80) of <i>sipB</i> , Ap ^r	This study
pSB1850	pGEX-KG with full-length <i>sipB</i> (593 aa), Ap ^r	This study
pSB1851	pGEX-KG with <i>sipB</i> $_{\Delta 80-100}$, Ap ^r	This study
pSB1852	pGEX-KG with <i>sipB</i> $_{\Delta 100-140}$, Ap ^r	This study
pHis-SicA	pET28a with structural gene encoding SicA, Km ^r	This study
pSSV160	pGEM-T-easy with <i>lac</i> promoter and sequence encoding N-terminal 160 amino acids of SipB (SipB160), LLO, and M45 epitope tag, Ap ^r	This study
pSSV160-1	Derivatives of pSSV160, in which LLO is deleted, Ap ^r	This study
pSSV160-2	pACYC184 with <i>lac</i> promoter, SipB160, FragC and M45 epitope tag, Cm ^r	This study
pSSV10	Derivative of pSSV160 encoding only 10 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
pSSV7	Derivative of pSSV160 encoding only 7 aa of the SipB N-terminus, Ap ^r	This study
pSSV6	Derivative of pSSV160 encoding only 6 aa of the SipB N-terminus, Ap ^r	This study
pSSV5	Derivative of pSSV160 encoding only 5 aa of the SipB N-terminus, Ap ^r	This study
pSSV4	Derivative of pSSV160 encoding only 4 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,	Derivative of pSSV160, in which the second codon of SipB is deleted, Ap ^r	This study
pSSV Δ 2–3,	Derivative of pSSV160, in which codons 2 and 3 of SipB are deleted, Ap ^r	This study
pSSV Δ 2–5	Derivative of pSSV160, in which codons 2–5 of SipB are deleted, Ap ^r	This study
pSSVN300	pMW118 with native promoter, <i>sicA</i> and region encoding SipB300, Ap ^r	This study
pSSVN160	pMW118 with native promoter, <i>sicA</i> and region encoding SipB160, Ap ^r	This study

Table 1. cont.

Name	Properties	Reference or source
pSSVNΔ161–499	pMW118 inserted with native promoter, <i>sicA</i> and <i>sipB</i> gene encoding a truncated SipB protein from which amino acids 161–499 are deleted, Ap ^r	This study
pSSVNΔ161–299	pMW118 with native promoter, <i>sicA</i> and <i>sipB</i> gene encoding a truncated SipB protein from which amino acids 161–299 are deleted, Ap ^r	This study
pSSVFA3–8	pMW118 with native promoter, <i>sicA</i> , <i>sipB</i> gene encoding a truncated SipB protein from which amino acids 3–8 are deleted, and M45 epitope tag, Ap ^r	This study

structure (translocon) that mediates effector delivery (Hayward & Koronakis, 1999; Scherer *et al.*, 2000). It has previously been reported that the SipB and SipC protein levels are significantly reduced in culture supernatants of an *invE* mutant strain and that InvE interacts with the SipB–SicA and/or SipC–SicA complex, but does not interact with the individual components of these complexes (Kubori & Galan, 2002). This suggests that InvE controls protein secretion or translocation by interacting with a protein complex formed by SipB, SipC and SicA. Despite extensive work with SipB, the region needed for its secretion through the SPI-1 T3SS pathway has not been investigated.

In this study, we show that the 160 amino acid N-terminal region of SipB (SipB160) can be secreted into culture supernatant through the flagella T3SS, not the SPI-1 T3SS. However, when the C-terminus of SipB (residues 300–593) is fused to SipB160, the chimeric protein is targeted to the SPI-1 T3SS. Moreover, we found the minimal SipB amino acid sequence that is necessary and sufficient for mediating secretion of the reporter protein, and a GST pull-down assay also showed that the amino acids 80–100 domain of SipB interacts with its cognate chaperone, SicA.

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.

General techniques. DNA cloning was carried out using previously described methods (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).

Plasmid construction. All plasmids used in this study are listed in Table 1. To construct plasmid pSSVN300 we amplified the region encoding the first 300 aa of the N-terminal region of SipB (forward primer CoSPaS, 5'-ggcgtcgacatttcctgacatgaaagatg-3'; reverse primer SipB300R, 5'-ccggagctcttagaattcagccgatttcttt-3') using the *Salmonella* chromosome as a template. The underlined sequences indicate sites for *SalI* and *SacI* in the forward and reverse primers respectively. After the amplified fragments (Pnative and SipB 300 aa) were digested with *SalI* and *SacI*, each fragment was ligated into

pMW118, which had also been digested with *SalI* and *SacI*. To construct pSSVN160, we amplified the region encoding the N-terminal 160 aa of SipB from the *Salmonella* chromosome using sipB-specific primers (forward primer CoSPaS, 5'-ggcgtcgacatttcctgacatgaaagatg-3'; reverse primer SipB160R, 5'-ccggagctcttagcagcgtcataaact-3'). The underlined sequences indicate sites for *SalI* and *SacI*, in the forward and reverse primers respectively. This PCR product also cloned into pMW118 as described above.

To generate SipB (160 aa)-listeriolysin O (LLO)-M45 fusions the following procedure was used. Initially we amplified the *lac* promoter from the pUC119 vector using appropriate primers (forward primer PlacL, 5'-actagtaatacgcacacccgctct-3'; reverse primer PlacR, 5'-gatatccagctgttctctgtgtaa-3'). The underlined sequences indicate sites for *SpeI* and *EcoRV* in the forward and reverse primers respectively. The region encoding the N-terminal 160 aa of SipB was amplified from the *Salmonella* chromosome using appropriate primers (forward primer Lsip, 5'-gatatcatgtgtaaatgacgaagtag-3'; reverse primer Rsip160, 5'-gggtaccgcagcgtcataaact-3'). The underlined sequences indicate sites for *EcoRV* and *KpnI* in the forward and reverse primers respectively. After amplification of the desired fragments, each fragment (Plac and SipB 160 aa) was digested with *EcoRV*, and the fragments were ligated to each other with T4 ligase (Takara). The ligated Plac-SipB160 aa fragments were amplified with primers PlacL and Rsip, then cloned into the pGEM-T Easy vector to generate pSip160. The pSip160 plasmid was digested and purified using the *SacI* site present on the pGEM-T Easy vector. The LLO fragment (amino acids 46–142) was amplified from the *Listeria monocytogenes* chromosome using forward primer LLOL (5'-ggtaccctcgcaagtcctaagcgc-3') and reverse primer LLOR, (5'-ctcgagtggtgtatttctactaattccg-3'). M45 was amplified from the pCMX-M45 plasmid (a gift from P. Hearing, State University of New York, Stony Brook), which included the M45 tag, using appropriate primers (forward primer M45L, 5'-ctcgagaccaccatggatcgagta-3'; reverse primer M45R, 5'-gagctcgttctctcacaagaatcctct-3'). The underlined sequences indicate sites for *KpnI/XhoI*, and *SacI* in the forward and reverse primers respectively. After the amplified fragments (LLO and M45) were digested with the restriction enzymes indicated above, all fragments were ligated to each other with T4 ligase. The ligated SipB-LLO-M45 was then cloned into the digested pSip160, and the cloned construct was named pSSV160 (Plac-Sip160-LLO/M45). This general strategy was used to construct the following plasmids: pSSV160-1 (Plac-Sip160-M45), pSSV10 (SipB_{1–10}-LLO/M45), pSSV8 (SipB_{1–8}-LLO/M45), pSSV7 (SipB_{1–7}-LLO/M45), pSSV6 (SipB_{1–6}-LLO/M45), pSSV5 (SipB_{1–5}-LLO/M45), pSSV4 (SipB_{1–4}-LLO/M45), and pSSV1 (SipB₁-LLO/M45). The pSSVΔ2, Δ2–3, Δ2–4 and Δ2–5, plasmids were derived from pSSV160. To construct plasmid pSSV160-2 (Plac-Sip160-FragC/M45), LLO was replaced with the tetanus toxin fragment C (FragC, first 105 aa). FragC was amplified from pTETnr15 FragC (kindly provided by A. J. Makoff, Department of Psychological Medicine, Institute of Psychiatry, London) using appropriate primers (forward primer FragC L 5'-gccgtgacc atgaaaaccttgattgttggg-3'; reverse primer FragC R, 5'-gccctcgag ctgttcaggtgggaagcaga-3'). The underlined sequences indicate sites for *KpnI* and *XhoI*, respectively.

To construct the truncated plasmid pSSVNΔ161–499, the region encoding amino acids 500–593 of SipB was fused to pSSVN160. The region encoding amino acids 500–593 was amplified from the *Salmonella* chromosome using appropriate primers (forward primer Lsip500, 5'-ggaggtaccaataaccataataaagtggcg-3'; reverse primer Rsip593, 5'-ccgctcgaggcgactctggcgagaataa-3'). The underlined sequences indicate sites for *KpnI* and *XhoI*, in the forward and reverse primers respectively. To construct pSSVNΔ161–299, we amplified amino acids 300–593 of SipB using appropriate primers (forward primer Lsip300, 5'-ggaggtaccaggaagagacgcgcaa-3'; reverse primer Rsip593, 5'-ccgctcgaggcgactctggcgagaataa-3'). The underlined sequences indicate sites for *KpnI* and *XhoI*, in the forward and reverse primers respectively. The amplified fragments were fused to pSSVN160. The DNA sequences of all cloned plasmid constructs were confirmed by DNA sequencing.

To construct plasmids encoding glutathione S-transferase (GST)–SipB fusion proteins, PCR-generated DNA fragments carrying parts of *sipB* were cloned into the *Bam*HI and *Xho*I sites of pGEX-KG (Guan & Dixon, 1991), yielding pSB1841 (GST–SipB_{1–160}), pSB1842 (GST–SipB_{1–30}), pSB1843 (GST–SipB_{1–100}), pSB1844 (GST–SipB_{101–160}), pSB1845 (GST–SipB_{500–593}), pSB1846 (GST–SipB_{1–40}), pSB1847 (GST–SipB_{1–50}), pSB1848 (GST–SipB_{1–70}), pSB1849 (GST–SipB_{1–80}), and pSB1850 (GST–SipB_{1–593}). To construct pSB1851 (GST–SipB_{Δ80–100}) and pSB1852 (GST–SipB_{Δ100–140}), the region encoding the amino acids 80–100 of SipB was amplified from the *Salmonella* chromosome using appropriate primers (forward primer SipB1L, 5'-ccgggcatcggttaaatgacgaagt-3'; reverse primer SipB80R, 5'-ggaagatctggagagttttcccgggcg-3'; reverse primer SipB100R, 5'-ggaagatctcgaaacatcgcccgtag-3'). The underlined sequences indicate sites for *Bam*HI and *Bgl*II. The fragments encoding SipB amino acids 101–593 and SipB amino acids 141–593 were amplified from the *Salmonella* chromosome with appropriate primers (forward primer SipB101L, 5'-ggaagatctgtctcaactgagctcg-3' or SipB141L, 5'-ggaagatctgtctatgaagcagtatcaa-3' respectively with reverse primer SipB593R, 5'-ggcctcgagttagtcgagctctggcg-3'). The underlined sequences indicate sites for *Bgl*II and *Xho*I. The amplified fragments (SipB_{1–80}, SipB_{1–100}, SipB_{101–593} and SipB_{141–593}) were digested with *Bgl*II and ligated with T4 ligase. The ligated SipB_{Δ80–100} and SipB_{Δ100–140} were amplified with primers SipB1L and SipB593R and then cloned into the pGem-T Easy vector to give pSipB_{Δ80–100} and pSipB_{Δ100–140}. pSipB_{Δ80–100} and pSipB_{Δ100–140} were digested with *Bam*HI and *Xho*I. Each fragment was ligated with T4 ligase to pGEX-KG which had also been digested with *Bam*HI and *Xho*I. To construct pHis–SicA, a PCR-generated DNA fragment carrying *sicA* was cloned into the *Eco*RI and *Xho*I sites of pET28a(+) (Novagen).

GST–recombinant SipB protein (GST–rSipBs) purification and GST pull-down assay. The pSB1841 and pGEX-KG plasmids were transformed into *E. coli* BL21 (DE3) cells, and expression of protein was induced at mid-exponential phase by the addition of 1 mM IPTG. GST and GST–fusion proteins were purified by affinity chromatography using glutathione–Sepharose beads and used in pull-down assays. For the pull-down assay, 40 ml L-broth or L-broth containing 0.3 M NaCl was inoculated with 0.8 ml of an overnight culture of *E. coli* or *S. enterica* serovar Typhimurium, respectively. The cultures were grown with rotation at 200 r.p.m. at 37 °C, and harvested when they reached OD₆₀₀ 0.9. Bacterial cells were washed once by low-speed centrifugation with chilled PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4) and cell pellets were resuspended in PBS containing 1 mM PMSF and 5 mM EDTA. The cell suspension was subjected to ultrasonication and the unbroken cells and cell wall fragments were pelleted by centrifugation at 15 000 g for 30 min and discarded. The prepared cytosolic proteins were then pre-absorbed for 4 h at 4 °C with glutathione–Sepharose beads in order to remove non-specifically bound proteins. Pre-absorbed lysates were then mixed with 30 µl slurry of pre-swollen

glutathione–Sepharose beads in the presence of 0.1 % NP-40, 1 mM PMSF and equal amounts of GST–rSipB (purified from *E. coli* BL-21) in a final volume of 1 ml. The mixture was incubated for 3 h at 4 °C with gentle rocking, and proteins bound to the beads were recovered by centrifugation at 500 g for 4 min. The beads were washed four times in 0.7 ml PBS containing 0.1 % NP-40, then proteins were released from the beads by boiling in Laemmli buffer, and then separated by SDS-PAGE along with pre- and post-incubation (unbound fraction) samples. The proteins separated by SDS-PAGE were visualized by silver staining or detected using anti-His monoclonal antibody.

Preparation of culture supernatant proteins and Western blotting. Culture supernatant proteins were prepared as follows. Bacterial supernatants (10 ml) were passed through a 0.45 µm syringe filter to remove bacteria. Protein in the bacteria-free medium was precipitated to 10 % (v/v) by the addition of cold trichloroacetic acid, and incubated on ice for 2 h. The protein was collected by centrifugation at 4 °C, 10 000 g for 20 min. 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 and 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 monoclonal antibody against the M45 tag (a kind gift from P. Hearing, State University of New York, Stony Brook) and a polyclonal anti-SipB antibody, followed by incubation with a horseradish peroxidase-labelled anti-mouse and anti-rabbit antibody. Blots were developed using a chemiluminescence detection kit.

Antibody preparation. GST–SipB160 that had been purified as described above was digested with thrombin (Roche), and the digested SipB160 protein was separated. The antiserum was prepared by injection of rabbits with 150 µg of the protein dispersed in complete Freund's adjuvant. Two and four weeks later, rabbits were immunized again with 50 µg of the protein dispersed in incomplete Freund's adjuvant.

RESULTS

Secretion mediated by the N-terminus of SipB is not dependent on the SPI-1 T3SS

Previous research has shown that wild-type SipB is secreted into the culture supernatant via the SPI-1 T3SS (Wattiau *et al.*, 1996). In agreement with this, our data show that the SipB protein can be secreted into culture supernatant in the wild-type strain and the *flhGHI* strain, which is defective for flagella type III secretion (Fig. 1a) (Stecher *et al.*, 2004). However, the *invA* and *flhGHI/invA* double mutant strains, which are defective for type III secretion (Galan *et al.*, 1992), cannot mediate the secretion of SipB (Fig. 1a).

It has been demonstrated that the second domain of effector proteins, contained within the first ~140 aa, serves as a binding site for specific chaperones (Birtalan *et al.*, 2002; Cheng *et al.*, 1997) and that the secretion and translocation of many effector proteins requires a cognate chaperone (Parsot *et al.*, 2003; Lee & Galan, 2004). However, it has not previously been determined whether a chaperone-binding site that mediates secretion through

Fig. 1. The N-terminal region of SipB mediates SPI-1 T3SS-independent secretion. (a) Expression and secretion of wild-type SipB protein in the wild-type, $\Delta sipB$, *invA::Km*, *fliGHI::Tn10* and *invA::Km/fliGHI::Tn10* double mutant. SipB was detected with a polyclonal anti-SipB160 antibody. (b) Expression and secretion of SipB160 (encoded by pSSVN160) in the wild-type, $\Delta sipB$, *invA::Km*, *fliGHI::Tn10* and *invA::Km/fliGHI::Tn10* double mutant. (c) The C-terminal region of SipB is necessary for SPI-1 T3SS-mediated secretion. The truncated SipB protein (pSSVN $\Delta 161-299$) was expressed in wild-type, $\Delta sipB$, *invA::Km*, *fliGHI::Tn10* and *invA::Km/fliGHI::Tn10* double mutant. The SipB variants and DnaK were detected with a polyclonal anti-SipB160 antibody and a monoclonal anti-DnaK antibody, respectively. DnaK was used as a control for contamination by cytosolic proteins (b and c, lower panels). For whole-bacteria lysates, an equivalent of 100 μ l bacterial culture was loaded on the gel. For supernatants, an equivalent of 500 μ l culture was used.

the SPI-1 T3SS is present in the N-terminus of SipB. Therefore, we reasoned that, if the 160 N-terminal amino acid residues of SipB (SipB160) have chaperone-binding sites for mediating secretion through the SPI-1 T3SS, SipB160 would not be secreted into culture supernatant in *invA* or *prgH* mutant strains, since these strains lack the SPI-1 T3SS-mediated secretion of the wild-type SipB protein. To examine the validity of this hypothesis, we amplified a DNA fragment encoding SipB160, cloned it into the low copy plasmid pMW118, and named this

construct pSSVN160 (for a more detailed description, see Table 1). pSSVN160 was introduced into wild-type, $\Delta sipB$, *invA*, *fliGHI* and *fliGHI/invA* bacterial strains. Whole-cell lysates and culture supernatants of the resulting strains were examined by Western blotting for the expression and secretion of the hybrid proteins. Interestingly, we found that SipB160 can be secreted into culture supernatant in the *invA* mutant (Fig. 1b). However, in *fliGHI* and *fliGHI/invA* strains, SipB160 cannot be secreted into culture supernatant (Fig. 1b). It is known that the disruption of

flhDC genes, which are responsible for expression of the entire flagella regulon, is known to have polar effects upon the expression of SPI-1 T3SS (Eichelberg & Galán, 2000). To rule out this possibility, we used the *fliGHI* mutant, which is a flagellin-deficient strain. The non-flagellated *fliGHI* strain can export SPI-1 effector proteins, but not flagellar subunits, into the culture supernatant (Stecher *et al.*, 2004).

To investigate whether the region between amino acids 160 and 300 of SipB is responsible for secretion through SPI-1 T3SS, we also constructed a plasmid expressing the first 300 amino acid residues of the SipB N-terminal region (SipB300) and named this construct pSSVN300. This construct was introduced into wild-type, *fliGHI* or *invA* bacterial strains. Like SipB160, the SipB300 protein was also secreted into culture supernatant in the *invA* mutant, but not in the *fliGHI* strain (data not shown).

The C-terminal region of SipB is necessary for SPI-1 T3SS-mediated secretion

It has been reported that amphipathic α -helices on certain proteins are involved in interaction with other proteins. For instance (1) the amphipathic α -helix is one of the predicted characteristics of substrate-specific flagellar and virulence type III export chaperones, and the amphipathic domain of a chaperone is used for binding with its cognate effector (Bennett & Hughes, 2000; Cornelis & Van Gijsegem, 2000); and (2) the effector protein, YopD, which is essential for translocation in *Yersinia pseudotuberculosis*, contains a predicted amphipathic domain located near its C-terminus which is known to mediate an interaction with its cognate chaperone, LcrH, and is also critical for *Yersinia* virulence (Tengel *et al.*, 2002; Francis & Wolf-Watz, 1998). In addition, McGhie *et al.* (2002) showed that a C-terminal domain of SipB is predicted to include an extended amphipathic α -helix (residues 526–593). If the SPI-1 T3SS-mediated secretion of the SipB protein is associated with its amphipathic C-terminus, we would expect that the SipB160 protein with its C-terminus added would not be secreted, or would be secreted at reduced levels, in the *invA* mutant bacterial strain, when compared to wild-type secretion. To investigate the relevance of the C-terminal region in the SPI-1 T3SS-mediated secretion of SipB, we constructed a plasmid (pSSVN Δ 161–499) which encodes a truncated SipB protein devoid of its central region (for a more detailed description, see Table 1). We introduced pSSV Δ 161–499 into wild-type, *fliGHI* and *invA* mutant bacterial strains. In contrast to the secretion of the SipB160 protein from the *invA* mutant, the SipB Δ 161–499 protein was secreted into culture supernatant in wild-type and *fliGHI* strains in a similar way to the wild-type SipB protein. However, the secretion of the truncated SipB Δ 161–499 protein was significantly reduced in the *invA* mutant (data not shown). The presence of proteins in the culture supernatants was not the result of bacterial lysis or non-specific leakage, since DnaK protein was

not detected in the culture supernatants (data not shown). Therefore, we next constructed a plasmid (pSSVN Δ 161–299) which had an expanded C-terminal region. (for a more detailed description, see Table 1). We also introduced pSSV Δ 161–299 into wild-type, Δ *sipB*, *invA*, *fliGHI* and *invA/fliGHI* double mutant. As shown in Fig. 1(c), SipB Δ 161–299 protein was secreted into culture supernatant in wild-type, Δ *sipB* and *fliGHI* strains and, as expected, in the *invA* strain, SipB Δ 161–299 protein was not secreted into culture supernatants, like native SipB.

The secretion signal of SipB is present between amino acids 3 and 8 of the N-terminus

To confirm that the 160 aa of the N-terminus of SipB can mediate secretion of reporter protein and expression by the constitutive promoter, we fused SipB160 to amino acids 46–142 of LLO (97 aa), which lacks its secretion signal and membrane-lysing activity. The resulting constructs were tagged at their C-termini with the 3.3 kDa M45 epitope to allow detection by Western blotting with an anti-M45 antibody. The DNA fragment encoding SipB160–LLO–M45 was fused to the *lac* promoter and cloned into the plasmid (see Methods). We introduced the constructs pSSV160, pSSV160-1 and pSSV1 (see Table 1 for detailed description) into the wild-type bacterial strain and found that the first 160 N-terminal residues of SipB are indeed able to mediate the secretion of both LLO–M45 (pSSV160) and M45 (pSSV160-1) (Fig. 2a). In contrast, the construct without the N-terminal region of SipB (pSSV1) did not cause secretion of the LLO reporter protein (Fig. 2a). The presence of SipB160–LLO–M45 or SipB160–M45 in culture supernatants did not result from cell leakage, because OmpR could not be detected in the same culture supernatant preparations (Fig. 2a, lower panels).

Next, we wanted to investigate whether the N-terminal region of SipB can mediate secretion of another reporter protein. We therefore cloned a DNA fragment encoding SipB160–FragC–M45 (FragC consists of the first 105 aa of tetanus toxin fragment C, which are encoded by nucleotides 1–315) into pACYC184. The resulting construct was named pSSV160-2 (see Table 1 for detailed description). The expression and secretion of SipB160–FragC–M45 (pSSV160-2) showed a similar pattern to that revealed by SipB160–LLO–M45 (pSSV160) (data not shown) indicating that the 160 N-terminal amino acids of SipB can secrete reporter proteins using its own secretion signal.

To further define the secretion signal of SipB, we constructed a series of chimeric proteins composed of increasingly smaller segments of the N-terminus of SipB fused to LLO (see Table 1). A chimeric protein containing the first 10 N-terminal residues of SipB fused to LLO (pSSV10) was efficiently secreted by *S. typhimurium* (Fig. 2b). In contrast, the first one (pSSV1) or four amino acids (pSSV4) of SipB were unable to mediate the secretion of LLO (Fig. 2b). To further refine our knowledge of the

minimal sequence necessary for mediating secretion, we generated fusion vectors which included the first 8 (pSSV8), 7 (pSSV7), 6 (pSSV6), or 5 (pSSV5) N-terminal residues. As shown in Fig. 2(c), the first five or six amino acids of SipB were not sufficient for mediating the secretion of LLO. This indicates that the secretion signal of SipB is encoded within its first seven amino acids. However, the secretion level of LLO-M45 which included the first seven N-terminal amino acids was markedly reduced compared to the protein that included the first eight N-terminal amino acids (Fig. 2c).

Fig. 2. Secretion signal of SipB. (a) Secretion of hybrid SipB160/LLO/M45 fusion proteins into culture supernatants by *S. typhimurium*. The N-terminal amino acids of SipB were fused to LLO lacking its secretion signal, and the C-terminal ends of these hybrid proteins were tagged with an M45 epitope. These plasmid constructs were derived from plasmid pGEM-T-easy. No, bacterial cells contained no vector. (b) The first 160 (pSSV160, SipB160-LLO-M45), 10 (pSSV10, SipB10-LLO-M45), 4 (pSSV4, SipB4-LLO-M45), or 1 (pSSV1, SipB1-LLO-M45) amino acids of SipB were fused to LLO and expressed in wild-type *S. typhimurium*. (c) The first 4 (pSSV4), 5 (pSSV5), 6 (pSSV6), 7 (pSSV7), 8 (pSSV8) or 10 (pSSV10) amino acids of SipB were fused to LLO and expressed in wild-type *S. typhimurium*. For whole-bacteria lysates, an equivalent of 100 µl bacterial culture was loaded on the gel. For supernatants, an equivalent of 500 µl culture was used. OmpR was used as a control for contamination with cytosolic proteins.

We next delineated the N-terminal boundary of the SipB secretion signal by constructing derivatives of pSSV160 in which the amino acids at the N-terminus of SipB were progressively deleted (see Table 1). Deletion of the second amino acid (SipBA2-LLO) did not affect the secretion of the LLO reporter protein, although there was a reduction in the amount of secretion (data not shown). In contrast, deletion of amino acids 2–3 (SipBA2–3-LLO) or 2–5 (SipBA2–5-LLO) abolished the secretion of the SipB-LLO chimeric proteins (data not shown). We noted that deletion of amino acids 2–4 (SipBA2–4-LLO), 2–6 (SipBA2–6-LLO), or 2–7 (SipBA2–7-LLO) prevented secretion of the SipB-LLO chimeric proteins (data not shown).

Next, we made the construct pSSVFA3–8 (see Table 1 for a more detailed description) expressing a SipB protein that lacked only amino acids 3–8 (SipBFA3–8), in order to confirm whether the region between amino acids 3 and 8 is essential for the secretion of SipB. In strains containing pSSVFA3–8 (*sipB* deletion mutant), the truncated SipBFA3–8 was expressed efficiently in the cytosol, but SipBFA3–8 was not secreted into culture supernatant (data not shown). These results indicate that amino acids 3–8 of the N-terminal region of SipB are essential for its secretion.

The domain encoding amino acids 80–100 of the SipB protein interacts with the SicA chaperone

In this study, we considered that certain chaperones could bind to the N-terminus of SipB in order for SPI-1 T3SS-dependent secretion to occur, before we recognized that SPI-1 T3SS-independent secretion of SipB160 occurs. To identify the proteins that interact with the SipB protein, a PCR-generated DNA fragment encoding amino acids 1–160 of *sipB* was cloned into the GST fusion vector pGEX-KG, and the resulting GST-SipB160 fusion protein was expressed in *E. coli* BL21, purified, and bound to glutathione-Sepharose beads. The GST-SipB160 proteins on the beads were then incubated with proteins from

cytosolic fractions of *S. typhimurium* and the bound proteins were separated by SDS-PAGE before being subjected to silver staining. As a result, we found that the GST-SipB160 protein interacts with one protein (approximate size, 18 kDa) in the cytosolic fraction (data not shown). However, we could not detect proteins with a similar size to GST-SipB160 since this 43 kDa protein masked proteins of similar sizes. The interacting protein from the cytosol fraction was identified as SicA by MALDI-TOF mass spectrometry and peptide mass fingerprinting. Previously, Tucker & Galan (2000) reported that SicA can bind to both SipB and SipC, and suggested that this binding prevents the premature association and degradation of SipB and SipC. Despite intense investigation, however, the SicA-binding site on SipB has not yet been identified. To analyse whether SicA binds any region of SipB160 or whether it can interact with the C-terminus of SipB, we constructed several GST-recombinant SipB proteins (GST-rSipBs) as described in Methods. Fig. 3(a) (upper panel) shows that the GST-rSipBs were sufficiently expressed at similar levels in the *E. coli* cytosol. We also introduced plasmids encoding His-SicA into *E. coli* and prepared whole-cell lysates which included SicA. We then examined the ability of this protein to interact with GST-rSipBs in a GST pull-down assay. As shown in Fig. 3(a) (lower panel, lanes 4 and 5), beads coated with GST-SipB₁₋₁₀₀ and GST-SipB₁₋₁₆₀ were able to pull down SicA. In contrast, GST-SipB₁₋₃₀, SipB₁₀₁₋₁₆₀, and SipB₅₀₀₋₅₉₃ were unable to pull down SicA (Fig. 3a lower panel; lanes 3, 6 and 7). To further refine the region of SipB that interacts with SicA, more GST-rSipB proteins were tested in pull-down assays. As shown in Fig. 3(b), GST-SipB₁₋₈₀ does not interact with SicA, indicating that SicA interacts with SipB somewhere between amino acids 80 and 100 of the SipB N-terminal domain but not with the C-terminus of SipB. To confirm that the region between amino acids 80 and 100 of the SipB N-terminal domain is essential for binding to SicA, we constructed plasmids encoding SipB variants in which amino acids 80–100 and 100–140 were deleted (GST-SipB_{Δ80-100} and GST-SipB_{Δ100-140}, respectively). As shown in Fig. 3(c), full-length SipB (GST-SipB) and the GST-SipB_{Δ100-140} proteins can interact with SicA (lower panel, lanes 3 and 4), but GST-SipB_{Δ80-100} protein cannot bind to SicA (lower panel, lane 2), indicating that SicA only recognizes the amino acid 80–100 region of SipB and not other regions. Fig. 3(c) (upper panel) also shows that all the GST-fused proteins (GST-SipB_{Δ80-100}, GST-SipB_{Δ100-140} and GST-SipB) were similarly expressed in *E. coli*.

DISCUSSION

In this study, we sought to define the functional domains present in SipB. We have shown that residues 3–8 of the SipB protein constitute the essential region for secretion of SipB from the bacterial cell and that the SicA chaperone, which is important in stabilizing SipB (and SipC) in the

bacterial cytosol, binds to SipB somewhere between amino acids 80 and 100 of its N-terminal domain. We also showed that the C-terminal region of SipB may be needed for secretion through the SPI-1 T3SS.

The SicA homologue in *Shigella flexneri*, known as IpgC, binds to the SipB homologue IpaB somewhere between residues 58 and 72 of the N-terminus of IpaB. This interaction is required to maintain the stability of IpaB (Page *et al.*, 2001). It was also suggested that SicA is capable of binding both SipB and SipC and acts as a partitioning factor for SipB and SipC, in order to prevent their premature association and degradation (Tucker & Galan, 2000). Based on this information, we constructed the GST-SipB160 fusion plasmid and found that the binding to its cognate chaperone, SicA, occurs somewhere between residues 80 and 100 of the N-terminal domain of SipB (Fig. 3). In this study, we also found that GST-_{Δ100-140} and GST-SipB proteins are expressed well in both *E. coli* and *S. typhimurium*, but the expression of GST-SipB_{Δ80-100} can only be readily detected in *E. coli*, and not in *S. typhimurium* (data not shown). Our findings suggest that in *S. typhimurium*, SicA may recognize the region between amino acids 80 and 100 of SipB and confers stability to SipB by inhibiting its premature interaction with SipC.

Wild-type SipB is known to be secreted into culture supernatant via the SPI-1 T3SS pathway (Hersh *et al.*, 1999; Tucker & Galan, 2000). Secretion of effector proteins through the T3SS normally requires cognate chaperones. These chaperones bind regions within the first ~140 aa of at least some of the effector proteins (Cheng *et al.*, 1997; Stebbins & Galán, 2001). Recently, in *S. typhimurium*, it was found that the first 35 aa of SptP could mediate secretion independently of SPI-1 T3SS, without the chaperone binding domain. However, SptP containing its chaperone-binding domain (amino acids 35–139) was secreted through its cognate SPI-1 T3SS (Lee & Galan, 2004). In the present study, we found that the secretion mediated by the 160 N-terminal amino acid region of SipB occurs independently of SPI-1 T3SS. This suggests that the N-terminal fragment of SipB may not be enough for secretion through the SPI-1 T3SS.

Components of the virulence and flagella export machineries (type III export system) are homologous, and their supramolecular structures are quite similar (Hueck, 1998). Recent studies of flagella type III export proteins have demonstrated that export of these structural components is mediated by cytosolic substrate-specific chaperones. The chaperones bind to the C-terminal amphipathic domains of their substrates (Auvray *et al.*, 2001; Bennett *et al.*, 2001; Fraser *et al.*, 1999). Given that a C-terminal domain (residues 526–593) of SipB was predicted to be an extended amphipathic α -helix (Hayward *et al.*, 2000; McGhie *et al.*, 2002), we suspected that SPI-1 T3SS-dependent secretion of wild-type SipB may be due to an unidentified chaperone that interacts with the C-terminus of SipB. To investigate this possibility, we constructed a truncated SipB containing

Fig. 3. SicA interacts with the SipB N-terminal region but not the C-terminal region. (a) Expression of various recombinant SipB proteins fused to GST (GST-rSipBs) was determined by Western blotting with anti-GST antibodies (upper panel). Whole-cell lysates containing plasmids with His-tagged *sicA* were extracted from *E. coli* and subjected to GST-rSipBs pull-down assays as described in Methods. (b) Amino acids 80–100 of the N-terminal region of SipB interact with SicA. Plasmids encoding GST-rSipBs were constructed and pull-down assays were carried out as described in Methods. (c) GST-SipB $_{\Delta 80-100}$, GST-SipB $_{\Delta 100-140}$ and GST-SipB (wild-type) proteins were expressed in *E. coli* and their expression determined by immunoblotting with anti-GST antibodies (upper panel). Upper bands in lanes 2 and 4 (upper panel) are the estimated sizes of GST-SipB $_{\Delta 80-100}$ and GST-SipB (wild-type). The presence of His-SicA bound to the GST-fused SipB proteins after pull-down was detected by immunoblotting with monoclonal antibodies specific to the His-tag (b; and a and c lower panels).

both the N-terminal domain (residues 1–160) and the C-terminal amphipathic domain (residues 500–593). In contrast to the SPI-1 T3SS-independent secretion of SipB160, secretion of the truncated SipB (SipB $_{\Delta 161-499}$)

was partially dependent on SPI-1 T3SS (data not shown). However, when we expanded the C-terminal region (residues 300–593), we showed that secretion of the truncated SipB (SipB $_{\Delta 161-299}$) was also entirely abolished

in the *invA* mutant as for native SipB, so it appears that not only the C-terminal amphipathic domain may be required for its secretion through SPI-1 T3SS, but also another region present in the wild-type SipB protein.

Recent studies suggest that there may be another mechanism for SPI-1 T3SS-dependent secretion of the truncated SipB. Kubori & Galan (2002) reported that SipB and SipC levels are significantly reduced in culture supernatants of the *invE* mutant strain. They also showed that InvE interacts with the SipB–SicA and/or SipC–SicA complex, but does not interact with the individual components of this complex. This suggests that InvE controls protein secretion or translocation by interacting with a protein complex formed by SipB, SipC and SicA. In this study, we showed that, although the 160 N-terminal amino acids of SipB can interact with SicA, the SipB160 protein was secreted independently of SPI-1 T3SS and the wild-type SipB protein was targeted for secretion through the SPI-1 T3SS. Our results and other recent findings suggest that SipB160 may not be secreted through the SPI-1 T3SS because it is unable to form a stable complex with SicA and SipC, or because InvE may not recognize the complex formed by SipB160 and SicA. However, to investigate the prospects for T3SS-independent secretion of SipB160, we must specifically present data supporting such possibilities. In this paper, we were not able to suggest a mechanism describing the specific direction of SipB to the SPI-1 T3SS machinery. However, the finding that the N-terminal region of SipB is required for SPI-1 T3SS-independent secretion, and that the C-terminal region is associated with SPI-1 T3SS secretion, offers insight into the regulation of the T3SS process.

In this study, we used the LLO sequence, lacking its secretion signal, and fused it to the M45 reporter. It is known that when using *Salmonella* as a carrier of heterologous antigens for vaccination, translocation of the antigen into the host cytosol induces strong T-cell priming (Igwe *et al.*, 2002; Rüssmann *et al.*, 2001, 1998). Therefore, along with our findings for the SipB secretion signal, we suspected that the first 160 N-terminal amino acids of SipB could be used to mediate the translocation of LLO into the host cytosol and to induce a T-cell response to *Listeria*. However, as shown in this report, the first 160 aa of SipB could not be secreted through the SPI-1 T3SS. Therefore, we did not conduct further studies regarding the translocation of SipB160 into the host cytosol or the induction of T-cell priming against LLO. We showed that the secretion signal of SipB could mediate secretion of some reporters (LLO, FragC, M45, etc.). We also suggest that the secretion signal of SipB may be used for the secretion of some B-cell antigens because secretion of heterologous antigens from live *Salmonella* vectors enhances the immune response to a foreign protein (Galen & Levine, 2001).

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