Identification of New Secreted Effectors in *Salmonella enterica* Serovar Typhimurium

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A common theme in bacterial pathogenesis is the secretion of bacterial products that modify cellular functions to overcome host defenses. Gram-negative bacterial pathogens use type III secretion systems (TTSSs) to inject effector proteins into host cells. The genes encoding the structural components of the type III secretion apparatus are conserved among bacterial species and can be identified by sequence homology. In contrast, the sequences of secreted effector proteins are less conserved and are therefore difficult to identify. A strategy was developed to identify virulence factors secreted by *Salmonella enterica* serovar Typhimurium into the host cell cytoplasm. We constructed a transposon, which we refer to as mini-Tn5-cycler, to generate translational fusions between *Salmonella* chromosomal genes and a fragment of the calmodulin-dependent adenylate cyclase gene derived from *Bordetella pertussis* (*cyaA'*). In-frame fusions to bacterial proteins that are secreted into the eukaryotic cell cytoplasm were identified by high levels of cyclic AMP in infected cells. The assay was sufficiently sensitive that a single secreted fusion could be identified among several hundred that were not secreted. This approach identified three new effectors as well as seven that have been previously characterized. A deletion of one of the new effectors, *steA* (*Salmonella* translocated effector A), attenuated virulence. In addition, SteA localizes to the *trans*-Golgi network in both transfected and infected cells. This approach has identified new secreted effector proteins in *Salmonella* and will likely be useful for other organisms, even those in which genetic manipulation is more difficult.

Pathogenic bacteria interact with host cells to create unique niches for replication and dissemination. Bacterial pathogens modify their host cells via the expression of exotoxins, proteases, and several other factors that are required for virulence. To alter the host cell, bacterial virulence factors must reach a host target. The ability of bacterial proteins to gain access to the host cell cytoplasm is often a critical step in pathogenesis. There are several defined mechanisms by which this secretion and subsequent uptake can take place. Bacterial proteins can be auto-transported, they can pass through the general secretory pathway, or most important from the standpoint of virulence, they can be secreted by one of several specialized mechanisms found in pathogenic bacteria. Many gram-negative bacterial pathogens encode type III secretion systems (TTSSs), syringe-like macromolecular complexes, to directly inject proteins into the host cell (8, 14, 22, 49). The structural genes encoding the TTSS “needle complex” are conserved among bacterial pathogens and appear to have been acquired through horizontal gene transfer. This high degree of homology has facilitated their identification through genome sequencing and analysis. In contrast, the secreted effector proteins (EPs) are often species specific, lack a consensus secretion signal, and have been difficult to identify.

Salmonella enterica serovar Typhimurium encodes two TTSSs on separate pathogenicity islands. *Salmonella* pathogenicity island 1 (SPI-1) encodes a TTSS that is responsible for mediating the intestinal phase of *Salmonella* infection (13, 52).

The SPI-1 TTSS is highly expressed during late log phase in media that are relatively rich and contain high levels of salt, conditions that are thought to simulate the environment in the small intestine (2). SopE, SipA, SptP, and AvrA are effector proteins secreted via the SPI-1 TTSS, and they promote the invasion of epithelial cells and enhance inflammation (7, 13, 15, 38, 51, 52).

A second TTSS, encoded by *Salmonella* pathogenicity island 2 (SPI-2), is essential for the systemic phase of infection (48). This secretion system is expressed under nutrient-starved conditions (including low magnesium and low pH) that may mimic the intracellular environment encountered by *Salmonella* (6, 10, 28, 32). The expression of the structural components of the secretion apparatus and many of its secreted proteins is controlled by a two-component regulatory system encoded within SPI-2 by the *ssrA/B* genes (20, 33, 35, 37, 50). Many phenotypes in infected cells have been associated with this TTSS. These phenotypes include delayed macrophage cytotoxicity, avoidance of oxidative burst, and altered inducible nitric oxide synthase (iNOS) localization (4, 45, 46, 48). However, the secreted virulence factors responsible for producing these phenotypes have yet to be identified. Further elucidation of EPs in *S. enterica* serovar Typhimurium may reveal the mechanisms responsible for these and other phenotypes.

An extremely useful technique has been developed to investigate the secretion of EPs. Sory et al. used the amino-terminal adenylate cyclase domain of the hemolysin/adenylate cyclase toxin (CyaA) from *Bordetella pertussis* as a tool to demonstrate type III secretion of EPs in *Yersinia enterocolitica* (27, 40). The adenylate cyclase domain is contained within the first 400 amino acids of CyaA and is called CyaA'. CyaA' activity is entirely dependent on host cell calmodulin and is thus inactive

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within the bacterial cell. Adenylate cyclase activity is therefore only observed when *CyaA'* is translocated into host cells as part of a translational fusion to a secreted EP. The secretion of fusion proteins can thereby be easily monitored by measuring the levels of cyclic AMP (cAMP) in infected cells.

For this study, we adapted the reporter system developed by Sory et al. (40) for use in the construction of a EZ::TN (Epicenter) (17)-derived transposon called mini-Tn5-cycler. Mini-Tn5-cycler mutagenesis was used to introduce translational fusions to *CyaA'*, thereby identifying secreted effectors by assaying cAMP levels in infected cells. The technique is sensitive because the assay detects secreted fusions even if they constitute <0.5% of the bacteria used to infect cells. The method is versatile, requiring only electroporation of a transposon/transposase complex into the target organism and no other genetic manipulation. Using this method, we identified three previously uncharacterized *S. enterica* serovar Typhimurium secreted effectors. One of these localizes to the *trans*-Golgi network (TGN) and is required for the colonization of mouse spleens following intraperitoneal infection.

MATERIALS AND METHODS

Bacterial strains, tissue culture, and growth conditions. The strains and plasmids used for this study are listed in Table 1. *Salmonella enterica* serovar Typhimurium strain 14028s was used as the wild-type (WT) strain. Bacteria were grown at 37°C in Luria-Bertani broth (LB). Kanamycin was used at 60 µg ml⁻¹. Chloramphenicol was used at 30 µg ml⁻¹. Carbenicillin was used at 100 µg ml⁻¹. Tetracycline was used at 20 µg ml⁻¹. HeLa cells and J774 macrophages were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, sodium pyruvate, and nonessential amino acids and grown at 37°C with 5% CO₂. All P22 transductions were performed as previously described (30). Transductants were streaked for isolation on LB agar containing 10 mM EGTA and then confirmed for smooth lipopolysaccharide and lack of pseudolysogeny by cross-streaking transductants against P22 on Evan's blue uranine plates.

Construction of *S. enterica* serovar Typhimurium mutant strains. An *ssaK::cat* (MJW1301) strain was constructed by first cloning the *ssaK* open reading frame (ORF) using PCR and then introducing a chloramphenicol resistance cassette into the *SpeI* site of the gene. This construct was moved into the suicide vector pKAS32 (39), and then the disrupted *ssaK* gene was reintroduced into strain 14028s as previously described (50). The construction of *invA::cat* is described elsewhere (45) and was transduced from SR-11 × 3014 into 14028s using P22 phage, resulting in strain MJW1835. Δ *srp*, Δ *steA*, Δ *steB*, and Δ *steC* strains were constructed using the λ -red PCR-based gene deletion method (9) and were verified by PCR. All PCR primer sequences can be obtained upon request.

Construction of mini-Tn5-cycler transposon and mutagenesis using mini-Tn5-cycler transposomes. The mini-Tn5-cycler transposon was constructed from the DICE II transposon (11). pDICE II was digested with *EcoRI* and *XbaI* and religated. A *BamHI* site downstream of the kanamycin cassette was then removed using a Quick Change site-directed mutagenesis kit (Stratagene). The *cyaA'* gene was PCR amplified from pMJW1753 and then cloned into the *NdeI* and *BamHI* sites. The resulting plasmid, pCycler, contains the completed mini-Tn5-cycler transposon. Mini-Tn5-cycler transposon/transposase complexes were prepared as previously described (17). Transposon/transposase complexes were electroporated into *Salmonella* using the following electroporation conditions. Overnight cultures of *Salmonella* were diluted 1:100 in LB and grown at 37°C for 3 h with aeration. The culture was then pelleted and washed three times with ice-cold deionized water. Following the washes, the pellet was resuspended in 1/500 the original culture volume in ice-cold 10% glycerol. One to 3 µl of transposon/transposase complex was added to 70 µl of electrocompetent cells, which were transferred into 1-mm-gap electroporation cuvettes (BTX). For electroporation, an Electro Cell manipulator 600 (BTX) was used with the following settings: resistance, 2.5 kV; capacitance timing, 25 µF; resistance timing, 129 Ω; and charging voltage, 1.70 kV.

Creation of *srfH-cyaA'*, *steA-cyaA'*, *steB-cyaA'*, and *steC-cyaA'* fusions. The *srfH* ORF was PCR amplified and cloned into pBluescript. This construct was mutagenized with the transposon in vitro, and in-frame fusions to *srfH* were identified by PCR and sequencing. In vitro mutagenesis of *srfH* was performed using

an EZ::TN kit (Epicenter). The in-frame fusion was then cloned into the suicide vector pKAS32 and used for allelic exchange, as previously described (39), to generate the chrom-*srfH-cyaA'* strain MJW1883. To generate pMJW1753, *cyaA'* (bp 4 to 1233) was PCR amplified from a clinical isolate of *B. pertussis* and cloned into pWSK29 (47) under *lacp* control, with a GGG 5' extension to recreate the *SmaI* site. Cloning into this site creates a glycine linker. The *srfH* ORF and promoter were PCR amplified and cloned into the *SmaI* site of pMJW1753 to generate *psrfH-cyaA'*. As a control, *cyaA'* was fused to the carboxy-terminal end of the β -galactosidase alpha peptide, generating *placZ-cyaA'*. The full-length *steA-cyaA'*, *steB-cyaA'*, and *steC-cyaA'* fusions were generated using the λ -red recombination system (9). To generate PCR products for recombination, forward primers contained 40 bp from the carboxy terminus of the gene being targeted at the 5' end plus the sequence 5'-CTGTCTCTTATACATCTCA-3', and reverse primers contained 40 bp downstream of the gene being targeted plus the sequence 5'-CTGTCTCTTATACATCTGGT-3'. Primers containing overhanging 5' sequences specific for *steA*, *steB*, and *steC* were then used to amplify the mini-Tn5-cycler transposon using PCR. The PCR products were digested with *DpnI*, dialyzed, and then electroporated into 14028s/pKD46.

Screening for translocated proteins. Libraries of ~5,000 mini-Tn5-cycler insertions were made. Libraries were diluted in LB to approximately 500 to 1,000 CFU/ml based on optical density readings at 600 nm. One hundred microliters of diluted library was grown in each well of a 96-well plate. Each well was then used to infect J774 cells (using SPI-2 conditions) or HeLa cells (using SPI-1 conditions) seeded in 96-well plates. If infection resulted in at least a 10-fold increase in cAMP levels, then the pool of mini-Tn5-cycler insertions from the 96-well plate was diluted and plated to isolate individual colonies. One hundred fifty to 300 colonies (three times the original pool size) were isolated using toothpicks, patched, and numbered. Numbered colonies were grouped into pools of 10 and then used to reinfect J774 or HeLa cells. If infection resulted in increased cAMP, then the colonies from that group of 10 mini-Tn5-cycler insertions were retested individually. Individual colonies with adenylate cyclase activity were transduced using P22 and then retested. Isolates that maintained adenylate cyclase activity following transduction were processed for sequencing.

Bacterial infection of cultured cells and ELISAs. Unless otherwise stated, J774 or HeLa cells were plated in 96-well plates at $\sim 2 \times 10^4$ cells/well and incubated overnight at 37°C with 5% CO₂. For the infection of J774 cells under SPI-2 conditions, stationary-phase bacteria were added at a multiplicity of infection (MOI) of 250. Bacteria were centrifuged onto the cell monolayer at $200 \times g$ for 5 min and then incubated at 37°C with 5% CO₂ for 1 h. The cell culture was then washed twice with phosphate-buffered saline (PBS), DMEM supplemented with 100 µg ml⁻¹ gentamicin was added, and the culture was incubated for another hour. After 1 h, the culture was washed twice with PBS, overlaid with DMEM containing 10 µg ml⁻¹ gentamicin, and incubated for another 7 to 9 h. For SPI-1-dependent infections of J774 and HeLa cells, stationary-phase cultures of 14028s were diluted 1:33 in LB and grown with aeration at 37°C for 3 h. Bacteria were then added to J774 cells at an MOI of 50, centrifuged onto the monolayer at $200 \times g$ for 5 min, and incubated for 1 h. HeLa cells were infected at an MOI of 150, centrifuged at $200 \times g$ for 5 min, and incubated for 1.5 to 2 h. Following infections, cells were washed once with PBS and then lysed with 0.1 M HCl. The level of cAMP in the lysates was determined using a direct cAMP enzyme-linked immunosorbent assay (ELISA) kit (Assay Designs) according to the manufacturer's instructions. In all cases, the MOI refers to the amount of bacteria initially added to host cells. The actual number of bacteria entering host cells was between 1 and 5% of the initial inoculum.

Sequencing of mini-Tn5-cycler insertion sites and sequence analysis. Chromosomal DNA was prepared from isolated mini-Tn5-cycler mutants as previously described (1). Chromosomal DNA was digested with *EcoRI* and cloned into the *EcoRI* site of pACYC184. Plasmids containing chromosomal inserts were electroporated into GeneHogs competent cells (Invitrogen), and insertions harboring chromosomal fragments with mini-Tn5-cycler were selected on LB agar supplemented with kanamycin. Plasmids from kanamycin-resistant colonies were then purified using a QIAprep spin miniprep kit (QIAGEN). The DNA sequence of the fusion junction was obtained using the primer 5' GTTGACCA GCGGAACATCAATGTG 3', which is complementary to bp 166 to 190 of the 5' end of mini-Tn5-cycler. Sequence analysis was performed using MacVector 7.1.1 software and the NCBI BLAST server at <http://www.ncbi.nlm.nih.gov/BLAST/>.

Competitive infection studies. Competitive infections were based on a protocol described by Ho et al. (21). Each strain was grown overnight in LB at 37°C with aeration. The bacteria were pelleted, resuspended in PBS, and diluted in PBS to approximately 2,000 to 20,000 CFU/ml. Each test strain was mixed 1:1 with the reference strain MA6054, and 100 µl of the mixture was injected intraperitoneally into 6- to 8-week-old female BALB/c mice. Three days after

TABLE 1. Strains and plasmids used for this study

Strain or plasmid	Genotype	Source or reference
Strains		
14028s	WT <i>S. enterica</i> serovar typhimurium	ATCC
HH100	14028s <i>steC</i> ::mini-Tn5-cycler fusion at amino acid 447	This work
HH101, 104	14028s <i>steA</i> ::mini-Tn5-cycler fusion at amino acid 174	This work
HH102, 105, 106, 109, 110, 111–114, 116–123, 126	14028s <i>slrP</i> ::mini-Tn5-cycler fusion at amino acid 123	This work
HH107	14028s <i>steC</i> ::mini-Tn5-cycler fusion at amino acid 428	This work
HH108	14028s <i>pipB2</i> ::mini-Tn5-cycler fusion at amino acid 176	This work
HH115	14028s <i>sptP</i> ::mini-Tn5-cycler fusion at amino acid 307	This work
HH124, 125	14028s <i>pipB2</i> ::mini-Tn5-cycler fusion at amino acid 305	This work
HH127	14028s <i>pipB2</i> ::mini-Tn5-cycler fusion at amino acid 165	This work
HH128, 131	14028s <i>srhH</i> ::mini-Tn5-cycler fusion at amino acid 149	This work
HH129	14028s <i>steC</i> ::mini-Tn5-cycler fusion at amino acid 448	This work
HH130	14028s <i>steC</i> ::mini-Tn5-cycler fusion at amino acid 369	This work
HH132	14028s <i>steB</i> ::mini-Tn5-cycler fusion at amino acid 137	This work
HH133, 134	14028s <i>pipB2</i> ::mini-Tn5-cycler fusion at amino acid 241	This work
HH135, 138	14028s <i>pipB2</i> ::mini-Tn5-cycler fusion at amino acid 145	This work
HH136	14028s <i>sseJ</i> ::mini-Tn5-cycler fusion at amino acid 168	This work
HH137	14028s <i>avrA</i> ::mini-Tn5-cycler fusion at amino acid 252	This work
HH139	14028s <i>sseJ</i> ::mini-Tn5-cycler fusion at amino acid 368	This work
HH140, 141, 142	14028s <i>sipA</i> ::mini-Tn5-cycler fusion at amino acid 511	This work
HH200	MJW1835 <i>steC</i> ::mini-Tn5-cycler fusion at amino acid 447	This work
HH201	MJW1835 <i>steA</i> ::mini-Tn5-cycler fusion at amino acid 174	This work
HH202	MJW1835 <i>slrP</i> ::mini-Tn5-cycler fusion at amino acid 123	This work
HH208	MJW1835 <i>pipB2</i> ::mini-Tn5-cycler fusion at amino acid 176	This work
HH215	MJW1835 <i>sptP</i> ::mini-Tn5-cycler fusion at amino acid 307	This work
HH228	MJW1835 <i>srhH</i> ::mini-Tn5-cycler fusion at amino acid 149	This work
HH232	MJW1835 <i>steB</i> ::mini-Tn5-cycler fusion at amino acid 137	This work
HH237	MJW1835 <i>avrA</i> ::mini-Tn5-cycler fusion at amino acid 252	This work
HH239	MJW1835 <i>sseJ</i> ::mini-Tn5-cycler fusion at amino acid 368	This work
HH240	MJW1835 <i>sipA</i> ::mini-Tn5-cycler fusion at amino acid 511	This work
HH300	MJW1301 <i>steC</i> ::mini-Tn5-cycler fusion at amino acid 447	This work
HH301	MJW1301 <i>steA</i> ::mini-Tn5-cycler fusion at amino acid 174	This work
HH302	MJW1301 <i>slrP</i> ::mini-Tn5-cycler fusion at amino acid 123	This work
HH308	MJW1301 <i>pipB2</i> ::mini-Tn5-cycler fusion at amino acid 176	This work
HH315	MJW1301 <i>sptP</i> ::mini-Tn5-cycler fusion at amino acid 307	This work
HH328	MJW1301 <i>srhH</i> ::mini-Tn5-cycler fusion at amino acid 149	This work
HH332	MJW1301 <i>steB</i> ::mini-Tn5-cycler fusion at amino acid 137	This work
HH337	MJW1301 <i>avrA</i> ::mini-Tn5-cycler fusion at amino acid 252	This work
HH339	MJW1301 <i>sseJ</i> ::mini-Tn5-cycler fusion at amino acid 368	This work
HH340	MJW1301 <i>sipA</i> ::mini-Tn5-cycler fusion at amino acid 511	This work
MA6054	14028s <i>ara-907 araD 901</i> ::MudJ	21
MJW1883	14028s <i>srhH</i> ::mini-Tn5-cycler chromosomal <i>srhH-cyaA'</i> fusion at amino acid 145	This work
MJW1301	14028s <i>ssaK</i> :: <i>cat</i>	This work
MJW1835	14028s <i>invA</i> :: <i>cat</i>	This work
Δ <i>slrP</i>	14028s Δ <i>slrP</i>	This work
Δ <i>steA</i>	14028s Δ <i>steA</i> :: <i>kan</i>	This work
Δ <i>steB</i>	14028s Δ <i>steB</i> :: <i>kan</i>	This work
Δ <i>steC</i>	14028s Δ <i>steC</i> :: <i>kan</i>	This work
SteA-HA/14028s	14028s with chromosomal double-HA-tagged SteA	This work
Plasmids		
pMJW1753	pWSK29 + <i>cyaA'</i>	This work
pMJW1791	pMJW1753 + <i>lacZ</i> (<i>placZ-cyaA'</i>)	This work
pMJW1810	pMJW1753 + <i>srhH</i> (<i>psrhH-cyaA'</i>)	This work
pMini-Tn5-cycler	pCRScript + mini-Tn5-cycler	This work
pEGFP-N1	EGFP transfection vector	Clontech
pSteA-EGFP	pEGFP-N1 + <i>steA</i>	This work
p <i>steA</i>	pWKS30 + <i>steA</i>	This work
pNFB15	Template vector for making double HA tag with a kanamycin resistance cassette	Lionello Bossi

injection, the mice were sacrificed, and their spleens were harvested and homogenized. Spleen suspensions were diluted and plated on LB plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; 40 μ g/ml) and arabinose (1 mM). The reference strain MA6054 has arabinose-inducible β -galacto-

sidase activity and can be easily distinguished from the test strains when plated on LB agar with X-Gal and arabinose. The competitive index (CI) was then calculated using the following equation: (percentage of test strain recovered/percentage of reference strain recovered)/(percentage of test strain inoculated/

percentage of reference strain inoculated). Student's *t* test was performed to analyze the CIs. Complementation of $\Delta steA$ was achieved by cloning the entire *steA* ORF and 62 bp upstream of the start codon into the low-copy-number expression vector pWKS30. The resulting plasmid, *psteA*, was electroporated into the $\Delta steA$ strain.

Expression of SteA-EGFP and SteA-HA in HeLa cells and visualization by microscopy. To make SteA-enhanced green fluorescent protein (SteA-EGFP), *steA* was PCR amplified and cloned into pEGFP-N1 (Clontech). The resulting plasmid, pSteA-EGFP, and pEGFP-N1 were purified using a QIAGEN EndoFree Maxi kit. HeLa cells were grown to ~25 to 50% confluency on Lab-Tek II chambered cover glass (Nalge Nunc International) and were transfected for 24 h using FuGENE 6 transfection reagent (Roche). Bodipy-TR-ceramide (Molecular Probes) was used to stain the Golgi network in live cells following the manufacturer's recommendations. A chromosomal SteA-hemagglutinin (SteA-HA) fusion was constructed using the λ -red recombination system as described by Uzzau et al. (44). To make a double-HA-tagged SteA, the plasmid pNFB15 (received from Lionello Bossi) was used as a template for PCR using the following primer pair: 5' CGACATAAAAGCTCGCTACCATAACTATTTGGA CAATTATTATCCGTATGATGTGCCGGA 3' and 5' CTGATTTCCTAACAA AACTGGCTAAACATAAAGCGCTTTTACACCTGCAGATCATCGAGCT 3'. The PCR product generated from these primers was introduced into 14028s/pKD46 via electroporation, and transformants were selected on LB agar containing kanamycin. The SteA-HA fusion was verified by PCR and Western blotting. SPI-1 conditions (described above) were used to infect confluent HeLa cells on cover glass in six-well plates with SteA-HA-expressing 14028s and WT 14028s, using an MOI of 100. Bacteria were centrifuged onto the cell monolayer, and the infection was allowed to proceed at 37°C for 20 min. After this incubation, the cells were washed three times with PBS, and DMEM supplemented with 100 μ g ml⁻¹ gentamicin was added for 1 hour and then replaced with DMEM supplemented with 10 μ g ml⁻¹ gentamicin for the remainder of the 4-hour infection. Bodipy-TR-ceramide (Molecular Probes) was used to stain the TGN, and then the cells were fixed in 4% paraformaldehyde for 20 min. A mouse anti-HA monoclonal antibody (Covance) was used at a 1:100 dilution, and an Alexa Fluor 488-conjugated goat anti-mouse (Molecular Probes) secondary antibody was used at a 1:1,000 dilution. The DNA stain DRAQ5 (Alexis Biochemicals) was used at a 1:1,000 dilution to visualize host cell nuclei and bacteria. A 60 $\times$ oil-immersion, 1.4-numerical-aperture objective lens was used along with standard filter sets for EGFP and Alexa Fluor 488 (488 nm), Texas Red (568 nm), and DRAQ5 (685 nm) visualization. *z* sections (0.2 μ m) were captured at a resolution of 1,024 by 1,024 pixels. Images were acquired by Aurelie Snyder of the OHSU-MMI Research Core Facility (<http://www.ohsu.edu/core>) with an Applied Precision DeltaVision image restoration system. This includes an API chassis with a precision motorized XYZ stage, a Nikon TE200 inverted fluorescence microscope with standard filter sets, halogen illumination with an API light homogenizer, a CH350L camera (500 kHz, 12-bit, 2 Mp, KAF 1400 GL, 1,317 $\times$ 1,035, liquid cooled), and DeltaVision software. Deconvolution using the iterative constrained algorithm of Sedat and Agard and additional image processing were performed on an SGI Octane workstation. Images were processed for deconvolution using Softworx (Applied Precision) image processing software.

RESULTS

Construction of mini-Tn5-cycler transposon. The mini-Tn5-cycler transposon (shown in Fig. 1A) is a modified EZ::TN (Epicenter)-based transposon. One advantage of this transposon is that stable transposon/transposase complexes can be prepared that can then be introduced to recipient bacteria by direct transformation of chemically competent or electrocompetent bacteria (17). The transposition reaction requires magnesium ions supplied from the recipient cell cytoplasm to complete the reaction, resulting in insertions in the recipient DNA. Alternatively, the complete reaction may be carried out in vitro, and the recombinant DNA can then be introduced directly into the desired bacterium. This last method of transposition allows for the generation of DNA insertions within genes of bacteria that are not usually amenable to such genetic manipulation, and this procedure can be further extended to yeast and mammalian cells. Thus, this construct can be utilized in many pathogenic organisms, making it an important tool for

the identification of secreted virulence factors. The basis for the identification of secreted *Salmonella* virulence factors is that the mini-Tn5-cycler transposon contains a promoterless *cyaA'* gene, oriented to allow the construction of translational fusions with external genes.

Functional analysis of mini-Tn5-cycler mutagenesis. To confirm that mini-Tn5-cycler transposition could result in functional *cyaA'* gene fusions, *srfH* (also called *sseI*), an *S. enterica* serovar Typhimurium gene encoding an effector secreted by the SPI-2 TTSS (12, 33, 50), was cloned into a suicide vector and mutagenized with mini-Tn5-cycler in vitro (Fig. 1B). An in-frame chromosomal *srfH::mini-Tn5-cycler* allele was created. This strain was used to infect J774 macrophages under growth conditions in which the SPI-1 TTSS is repressed and the SPI-2 TTSS is induced (45). The level of cAMP in the infected cells was then measured by ELISA. Using an input MOI of ~1, which results in <5% of cells being infected, we observed a >30-fold increase in host cell cAMP over the background levels when J774 macrophages were infected with *srfH::mini-Tn5-cycler* (Fig. 1C). Background levels of cAMP were detected in cells infected with either WT 14028s or a strain expressing a β -galactosidase-*cyaA'* (*placZ-cyaA'*) in-frame fusion from a low-copy-number vector (Fig. 1C). Approximately 160-fold higher levels of cAMP were observed if a *srfH-cyaA'* fusion was expressed from a low-copy-number plasmid vector (*psrfH-cyaA'*) (Fig. 1C). Secretion of the SrfH-CyaA' fusion protein did not appear to significantly increase the level of macrophage cell death during the course of an 8-h assay (data not shown). We wished to establish if a mixed infection containing a minority of the hybrid fusion-expressing bacteria and a majority of bacteria that do not express *cyaA'* could be used. This would allow us to screen large pools of mutagenized bacteria rather than having to screen the bacteria one by one, which is an impossible task. For control experiments, we used a mixed infection containing *srfH::mini-Tn5-cycler* at various ratios with the parent strain. The dilution of *srfH::mini-Tn5-cycler* with a 200-fold excess of wild-type 14028s cells still resulted in a 10-fold increase in cAMP levels in infected J774 cells (data not shown). These results demonstrate that a single in-frame fusion to a secreted EP can be detected among 200 proteins that do not express *cyaA'*. To make the assay even more sensitive, we tried varying the input MOI and found that even an MOI of 500 bacteria per cell was tolerated and further increased the detected cAMP levels.

Library construction and analysis. The strategy used to identify secreted effectors is shown in Fig. 2. Mini-Tn5-cycler transposon/transposase complexes were electroporated into *S. enterica* serovar Typhimurium strain 14028s to create libraries containing approximately 5,000 independent insertions. These bacteria were mixed together, the number of bacteria was determined by measuring the optical density, and the bacteria were then diluted into wells of a 96-well microtiter dish so that the wells contained pools of 50 to 100 bacteria. These pools were either grown overnight to stationary phase and used to infect J774 macrophages for 8 to 10 h at an input MOI of ~250 or grown to logarithmic phase and used to infect HeLa cells for 2 h at an input MOI of ~150. Following infection, cells were lysed with 0.1 M HCl, and the concentration of intracellular cAMP was determined. The bacteria corresponding to any well showing at least a 10-fold increase in cAMP above background

FIG. 1. Schematic representation of the mini-Tn5-cycler transposon (A) and mutagenesis of *srhH* (B). IE and OE are the modified Tn5 transposon ends (also called mosaic ends [17]). *cyaA'* is the promoterless 400 amino acids of the amino terminus of *cyaA* from *Bordetella bronchiseptica* (27, 40). KAN represents the Tn903 aminoglycoside phosphotransferase. To test our system, *srhH* was cloned into pBluescript (B). The purified plasmid was mutagenized in vitro using mini-Tn5-cycler transposon/transposase complexes (transposons) by the addition of 5 mM magnesium. Plasmids containing insertions were selected on LB-kanamycin plates, and in-frame insertions in *srhH* were identified by PCR and sequencing. An in-frame fusion of *srhH-cyaA'* at the codon encoding amino acid 145 was then cloned into the suicide vector pKas32. The suicide vector containing an in-frame insertion of mini-Tn5-cycler was then electroporated into 14028s. Crossover events were selected on LB-kanamycin, and a double crossover was identified by PCR. (C) Secretion by *srhH::mini-Tn5-cycler*. Stationary-phase cultures of *srhH::mini-Tn5-cycler*, a mutant expressing a fusion from a low-copy-number plasmid (*psrH-cyaA'*), or a mutant expressing a fusion of *CyaA'* to the β -galactosidase alpha peptide (*placZ-cyaA'*) were used to infect J774 macrophages for 8 h at an MOI of ~ 1 . Infected cells were lysed with 0.1 M HCl, and the concentration of cAMP (pmol/ml) in the lysates was measured by ELISA.

levels were replated for the isolation of individual colonies. From these colonies, smaller and smaller pools were constructed until individual positive clones were obtained. The transposon in each positive clone was P22 transduced to a new background, retested, and processed for DNA sequencing to identify the transposon-*Salmonella*-chromosome junction.

Six libraries were generated from independent electroporation reactions containing a total of $\sim 30,000$ insertions. The majority of these were screened for *cyaA'* secretion in infected J774 macrophages. After screening these insertions, we iden-

tified a total of 23 positive signals, of which 17 were fusions to the known secreted effector *slrP*. Sequence analysis demonstrated that all *slrP* insertions had occurred at the same nucleotide position, although at least five of these were independent isolates. This suggested the presence of a Tn5 transpositional "hot spot." To avoid this hot spot, six additional libraries, each containing approximately 5,000 insertions, were constructed in a Δ *slrP* background. Sixteen positive fusions were identified from a screen of 25,000 insertions in this Δ *slrP* background. In addition to our screens with the J774 macrophage cell line, a

FIG. 2. Strategy for identifying effectors. Libraries of approximately 5,000 insertions were generated by electroporating mini-Tn5-cycler transposon/transposase complexes into either 14028s or a Δ *slrP* derivative. These colonies were diluted in LB broth to approximately 500 to 1,000 bacteria/ml (based on optical density readings at 600 nm), and 100- μ l aliquots of the diluted library were grown to either stationary phase or late log phase. These cultures were then used to infect J774 cells or HeLa cells seeded in 96-well plates. After 8 to 10 h of infection for J774 cells or 2 h for HeLa cells, the medium was removed, and 0.1 M HCl was added to lyse the cells. The level of cAMP in each well was then measured using an ELISA. Any pool from which infected cells showed at least 10 times higher levels of cAMP than the background level was isolated and screened further by repooling into smaller groups (10 or 1) until individual bacteria containing the cognate *CyaA'* fusion were obtained.

single library of 5,000 insertions in the Δ *slrP* background was screened in HeLa cells. Three clones were identified from this pool. Each contained a *cyaA'* fusion to *sipA*, which encodes a previously characterized effector (23). Sequence analysis of each of these *sipA* insertions demonstrated that they were identical and likely to be siblings. In summary, for every 5,000 mini-Tn5-cycler insertions screened, three or four positive fusions were identified.

In total, we isolated 42 positive clones, each of which con-

tained an in-frame insertion in either a gene encoding a known EP or an ORF encoding a protein of unknown function. Following DNA sequencing of all 42 clones, we found fusions to 10 different ORFs, of which 7 had been previously identified to encode secreted effectors. Three of the fusions were to unknown ORFs that presumably encode new effectors. Table 2 lists the genes isolated in our screen, along with a short description of each gene's reported function, the number of times each gene was isolated, and the number of unique insertion

TABLE 2. List of genes isolated in screens^a

Gene	No. of times isolated	No. of unique insertion sites	No. of independent isolates	Known/predicted function of gene product	Reference(s)
<i>slrP</i>	17	1	5	Type III secreted protein involved in murine virulence	42
<i>pipB2</i>	8	5	5	Type III secreted protein, SsrB regulated	25
<i>steC</i> (STM1698)	5	4	5	Putative inner membrane protein, SsrB regulated	31, Rue and Heffron (unpublished)
<i>sipA</i> ^b	3	1	1	Type III secreted protein—neutrophil migration, inflammation, actin polymerization	15, 38, 51
<i>sseJ</i>	2	2	2	Type III secreted protein, SsrB regulated	33
<i>steA</i> (STM1583)	2	1	1	Putative cytoplasmic protein	31
<i>srfH</i>	2	1	1	Type III secreted protein, affects macrophage motility, SsrB regulated	32, 50, M. Wormley and F. Heffron (unpublished data)
<i>sptP</i>	1	1	1	Type III secreted protein with phosphatase activity	24
<i>steB</i> (STM1629)	1	1	1	Putative dipicolinate reductase	31
<i>avrA</i>	1	1	1	Type III secreted protein, NFκB localization	7

^a Genes were isolated by mini-Tn5-cycler mutagenesis and ELISA of J774 cells or HeLa cells

^b Identified from infection of HeLa cells.

sites and independent isolates. The genes identified were *sipA* (29), *slrP* (33, 42), *pipB2* (25), *sptP* (24), *sseJ* (18), *srhH* (18, 21), *avrA* (7, 19), and *Salmonella enterica* serovar Typhimurium LT2 reference numbers STM1583, STM1629, and STM1698 (31). We refer to these last genes as *Salmonella* translocated effectors (*ste*) *steA* (STM1583), *steB* (STM1629), and *steC* (STM1698). Interestingly, there were five unique insertions in *pipB2* and four unique insertions in *steC* (Table 2).

An intact TTSS is required for secretion of the newly identified EP. The fact that seven of the identified genes encode known effectors strongly suggested that our approach was working, but it was necessary to confirm that the newly identified ORFs were also secreted via a type III secretion apparatus. For these experiments, we utilized both genetic mutants defective in needle complex assembly and growth conditions that either induce or repress expression of the two *Salmonella* type III secretion systems. Each fusion was transduced into both an *invA::cat* mutation that renders the bacteria defective for SPI-1 TTSS-dependent secretion and an *ssaK::cat* mutant defective for SPI-2 TTSS-dependent secretion. The 10 unique mini-Tn5-cycler fusions were tested under conditions that allow expression of the SPI-1 TTSS (41). Strains harboring *cyaA'* fusions were grown to late log phase and used to infect J774 macrophage-like cells for 1 h. As shown in Fig. 3A, there was a significant increase in cAMP for J774 cells infected with the SipA-, SptP-, AvrA-, SlrP-, SteA-, and SteB-CyaA' fusions. The secretion of these fusions was dependent on an intact SPI-1- but not SPI-2-encoded TTSS. Secretion of the remaining four fusions (*SseJ*, *SrhH*, *PipB2*, and *SteC*) could not be detected under SPI-1-inducing conditions (Fig. 3A). Similar results were observed following infection of HeLa cells (data not shown).

Next, strains harboring each *cyaA'* fusion in either a WT, *invA::cat*, or *ssaK::cat* background were grown to stationary phase in order to repress SPI-1 and induce SPI-2 expression. These cultures were used to infect J774 macrophages for 8 h at an input MOI of ~250. As shown in Fig. 3B, with the exception of SipA-CyaA', every fusion that we tested resulted in a significant increase in host cell cAMP which was dependent on an intact SPI-2 TTSS. Similar results were found when we infected the dendritic cell line JAWS II (data not shown).

We focused on the characterization of the three newly identified secreted effectors. We constructed *cyaA'* fusions to full-length copies of *steA*, *steB*, and *steC* to rule out aberrant secretion by the flagella or some as yet uncharacterized mechanism. As before, we tested the full-length CyaA' fusions to SteA, SteB, and SteC in either the WT, *invA::cat*, or *ssaK::cat* background for secretion into infected host cells. The same conditions were used as before to induce either the SPI-1 TTSS or the SPI-2 TTSS, and the secretion profiles of the full-length fusion proteins were found to be identical to those of the original fusions (Fig. 4).

***steA* is required for efficient colonization of mouse spleens.** To determine if *steA*, *steB*, or *steC* plays a role in a mouse infection model, competitive infections were performed. Deletions of *steA*, *steB*, and *steC* were generated using the λ -red recombination system (9), and the competitive index of each strain was determined (Table 3). Neither the $\Delta steB$ nor $\Delta steC$ strain had a competitive index statistically different from that of the control wild-type strain. However, the $\Delta steA$ strain had

FIG. 3. SPI-1 TTSS (A)- and SPI-2 TTSS (B)-dependent secretion of *cyaA'* fusions into J774 cells. Levels of cAMP were measured within the macrophage-like cell line J774 following infection with the 10 *cyaA'* fusions listed in Table 2. Three different backgrounds were used for this experiment. These were WT (gray bars), *invA::cat* (black bars), and *ssaK::cat* (white bars). Bacteria were grown to late log phase and used to infect J774 cells at an input MOI of ~50 for 1 hour (A) or were grown to stationary phase and used to infect J774 cells for 8 h at an input MOI of ~250 (B). The cells were then lysed with 0.1 M HCl, and an ELISA (Assay Designs) was used to quantitate the cAMP levels. The cAMP concentration (in pmol/ml) was measured in triplicate samples, and the error bars represent 1 standard error of the mean.

an approximately threefold competitive disadvantage for mouse spleen colonization. Expressing *steA* from its native promoter in a low-copy-number vector (*psteA*) complemented this competitive defect.

SteA localizes to the Golgi network in host epithelial cells.

Because of its potential role as a virulence factor, we further characterized *steA*. HeLa cells were transfected with an expression vector expressing either EGFP alone or a translational fusion of SteA to EGFP. As shown in Fig. 5, cells transfected with the EGFP expression vector alone displayed uniform fluorescence throughout the cell. In contrast, EGFP fluorescence was concentrated in perinuclear regions in cells transfected with a plasmid expressing the SteA-EGFP fusion protein. To further define this perinuclear compartment, transfected cells were costained with Bodipy-TR-ceramide, a dye that targets the Golgi network. In Fig. 5C, SteA-EGFP is shown to extensively colocalize with Bodipy-TR-ceramide. This suggests that SteA localizes to the TGN when it is expressed in host cells.

The subcellular localization of SteA translocated by the bacteria was also investigated. SteA-HA/14028s, a double-HA-

FIG. 4. SPI-1 TTSS- and SPI-2 TTSS-dependent secretion of full-length CyaA' fusions. Levels of cAMP were measured within the macrophage-like cell line J774 following infection with SteA-CyaA', SteB-CyaA', and SteC-CyaA'. Three different backgrounds were used for this experiment. These were WT (gray bars), *invA::cat* (black bars), and *ssaK::cat* (white bars). Bacteria were grown to late log phase and used to infect J774 cells at an input MOI of ~50 for 1 hour (SPI-1 conditions) or were grown to stationary phase and used to infect J774 cells for 8 h at an input MOI of ~250 (SPI-2 conditions). The cells were then lysed with 0.1 M HCl, and an ELISA (Assay Designs) was used to quantitate the cAMP levels. The cAMP concentration (in pmol/ml) was measured in triplicate samples, and the error bars represent 1 standard error of the mean.

tagged SteA fusion-expressing strain, was used to infect HeLa cells for 4 hours under SPI-1-inducing conditions. Alexa Fluor 488-conjugated antibodies were used to visualize SteA-HA by fluorescence microscopy, and Bodipy-TR-ceramide was again used to visualize the TGN. In many infected cells, little to no SteA-HA-specific fluorescence was seen, possibly due to low expression levels of SteA. In addition, most of the SteA-HA-specific fluorescence that was observed was found only in proximity to bacteria in infected cells. However, in a few isolated cells containing large numbers of bacteria, broader SteA-HA-specific staining could be seen (Fig. 6B). In these cases, it was possible to see SteA-specific staining that was not directly adjacent to bacteria. As shown in Fig. 6D, in a cell with extensive SteA-HA-specific staining, SteA-HA colocalized with Bodipy-TR-ceramide. This staining was specific, as it was never observed in cells infected with WT 14028s (Fig. 6F). These data, along with the data from transfected cells, strongly suggest that secreted SteA localizes to the TGN.

DISCUSSION

This report describes a novel strategy for the identification of secreted effector proteins. In this work, three previously unidentified effectors, SteA, SteB, and SteC, were found. Using a competitive infection model, we show that one of these effectors, SteA, is required for *Salmonella* to colonize the mouse spleen. SteA was also shown to localize to the trans-Golgi network within both transfected and infected epithelial cells. Evidence of the power of this approach is demonstrated by the identification of seven known secreted effectors in the same screen.

At least four strategies have been used to identify secreted EPs in *Salmonella* and other pathogens. Guttman et al. described a de novo method of screening using wilting of plant leaves as an easily observed phenotype. However, their method is limited to certain plant pathogens such as *Pseudomonas syringae* (18). Luo and Isberg used selection and screening to identify type IV secreted proteins in *Legionella pneumophila* (29). Their method requires the identification of secreted proteins based on interbacterial transfer and thus could not be applied to the type III secreted effectors we have found. Tu et al. constructed a mini-Tn5cyaA' transposon similar to ours but identified only surface-exposed proteins in *Bordetella bronchiseptica* (43). Our mini-Tn5-cycler screen employed a more sensitive enzymatic assay and relied on the infected host cell to supply calmodulin. In our assay, we only identified translocated effectors, as evidenced by the fact that an intact secretion apparatus was required for each of the 10 EPs found. Of the 60,000 mutants we screened, 42 produced detectable adenylate cyclase activity in infected cells, and each encoded an in-frame fusion to a secreted effector protein.

TABLE 3. Competitive infections using Δ steA, Δ steB, Δ steC, and steA complemented strains^a

Genotype	Median CI	No. of mice	P value
14028s	1.026	10	>0.05
Δ steA	0.306	10	<0.05
psteA/ Δ steA	1.261	10	>0.05
Δ steC	2.497	10	>0.05
Δ steB	1.071	10	>0.05

^a All strains were competed against MA6054. BALB/c mice were injected intraperitoneally, and then spleens were harvested and recovered CFUs were used to determine the CI as described in Materials and Methods. Student's *t* test was used for statistical analysis of the data, and the resulting *P* values are shown.

FIG. 5. A SteA-EGFP fusion expressed in HeLa cells colocalizes with the TGN. HeLa cells were transfected for 24 h with pEGFP (bottom panels) or pSteA-EGFP (top panels), and Bodipy-TR-ceramide (red) was used to stain the TGN. The images shown are 0.2- μ m z sections captured using deconvolution microscopy. EGFP fluorescence images are shown in panels A and D, and Bodipy-TR-ceramide fluorescence images are shown in panels B and E. Panels C and F show mergers of panels A and B and panels D and E, respectively. In panels C and F, the areas where EGFP and Bodipy-TR-ceramide fluorescence overlap are yellow.

We wondered if it is possible to calculate the total number of effectors encoded by *Salmonella* based on the sample we examined. Assuming that insertion is random, there are several other factors that will reduce the chance of identifying any given effector. First, there is a one-in-six chance of an insertion occurring in the correct orientation and reading frame of any given gene. Second, the target area must be only a portion of a given gene because sequences that are essential for secretion or binding to a chaperone will be excluded. Third, however sensitive the assay is, the level of expression must be above a given threshold of detection. These caveats make it difficult to extrapolate from the number of effectors identified in our screen but do imply that there are many as yet undetected effectors. In addition, we have only examined specific conditions and cell types. More EPs might be identified if other cell types are used and if the infection time is varied. For example, SseK2, a recently identified effector in *S. enterica* serovar Typhimurium, is secreted only after 21 h of infection (26). SseK2 and possibly other effectors secreted at later time points would only have been detected if we had lengthened the infection time. One additional limitation that we observed stemmed from the existence of transpositional hot spots resulting in the

repeated isolation of mini-Tn5-cycler fusions to *slrP*. In fact, many of the identified genes were only found after the deletion of *slrP*. Presumably, a systematic deletion of effectors that are uncovered in the screen could be used to detect additional new genes. Additionally, some genes encoding EPs are simply not amenable to mini-Tn5-cycler mutagenesis, including any that are targeted to vesicles that do not contain calmodulin as well as those with extremely small targets for transposition.

Our technique can be used to identify secreted type III EPs from a wide range of pathogens and possibly proteins secreted by other mechanisms. CyaA' has been used to demonstrate type IV secretion (5), and in *B. pertussis*, CyaA is secreted via a type I secretion system (16). Finally, there are many genetically intractable organisms for which the isolation of a large number of transposon insertions is simply not possible, even by electroporation of transposon complexes. In these cases, it may be possible to express a gene library from a plasmid in a genetically tractable host that also expresses the complete structural apparatus for secretion, thereby making it amenable to mini-Tn5-cycler mutagenesis.

Three new secreted EPs were identified in the screen, namely, SteA, SteB, and SteC. The genes encoding all three of

FIG. 6. Secreted SteA colocalizes with the TGN in infected HeLa cells. HeLa cells were infected with SteA-HA/14028s (top panels) or with WT 14028s (bottom panels) for 4 hours at an MOI of 100 under SPI-1-inducing conditions. HA-specific antibodies were used to visualize HA-tagged SteA (green). Bodipy-TR-ceramide (red) was used to stain the TGN, and the DNA stain DRAQ5 (blue) was used to visualize host cell nuclei and bacteria. DRAQ5 fluorescence images are shown in panels A and E, HA tag-specific fluorescence images are shown in panels B and F, and Bodipy-TR-ceramide fluorescence images are shown in panels C and G. Panels D and H show mergers of the images from panels A to C and E to G, respectively.

these proteins have low GC contents (*steA* GC content, 43%; *steB* GC content, 41.9%; and *steC* GC content, 38%), suggesting horizontal acquisition, which is common for virulence-associated genes. The $\Delta steA$ strain was found to have a competitive defect in colonization of the mouse spleen, whereas *steB* and *steC* did not appear to play a significant role in this model. This competitive defect suggests that *steA* is required either for passage of the bacteria from the peritoneal cavity into the spleen, for survival and replication within host cells, or for avoiding host immune defenses. Interestingly, SteA localizes to the Golgi network in transfected and infected HeLa cells. SseG, another EP in *S. enterica* serovar Typhimurium, has also been shown to localize to the Golgi network (36). The presence of SseG was found to be important for the association of *Salmonella*-containing vacuoles with the Golgi network. Furthermore, the association of *Salmonella*-containing vacuoles with the Golgi network was required for normal bacterial replication within HeLa cells. We are investigating whether SteA plays a similar role to that of SseG in infected cells. The coding sequence of *steA* is 94% conserved in *Salmonella enterica* serovar Typhi strains TY2 and CT18 and 95% conserved in *Salmonella enterica* serovar Paratyphi strain ATCC 9150. This conservation suggests that *steA* may be important for virulence in human infections as well. In a recent paper by Morgan et al., STM1698 (the ORF encoding SteC) was identified as the gene for a colonization factor specific for the chick infection model (34). The coding sequence of *steC* is 93% conserved in *Salmonella enterica* serovar Paratyphi strain ATCC 9150 and *Salmo-*

nella enterica serovar Typhi strains TY2 and CT18, again suggesting a possible role in human infection. Of the three newly described proteins, only SteB has significant homology to a bacterial protein from a different species: it shares 40% amino acid identity to a protein in the tropical pathogen *Chromobacterium violaceum*. This pathogen is found in water and soil throughout tropical South America and causes septicemia with metastatic abscesses with a 64% fatality rate. *C. violaceum* contains genes encoding a TTSS, suggesting that the homology may be meaningful (3). SteB (STM1629) is encoded in a genetic island in close proximity to the gene for another secreted protein, SseJ (STM1631). STM1630, the ORF immediately downstream of *steB*, is required for virulence in both the calf and chick infection models (34).

Interestingly, five of the EPs identified were secreted by both the SPI-1 and SPI-2 TTSSs (SptP, SlrP, AvrA, SteA, and SteB), whereas SipA was observed to be secreted only via the SPI-1 TTSS. Since these five proteins are secreted by both TTSSs, they may function in both the intestinal and systemic phases of infection. Four of the identified proteins, SseJ, SrfH, PipB2, and SteC, were only secreted via the SPI-2 TTSS. These results raise two possibilities that are not exclusive, either that these effectors are only expressed under one condition or that they cannot be secreted through the alternative needle complex. The expression of all four of these genes is regulated by SsrB (33, 50; J. Rue and F. Heffron, unpublished data). These data suggest that proteins secreted exclusively by the SPI-2 TTSS are regulated by SsrB, while proteins secreted by both the

SPI-1 and SPI-2 TTSSs are regulated by an unknown mechanism. The observed secretion patterns may be a result of a SPI-1 or SPI-2 TTSS-specific signal in the RNA messages or amino acid sequences of these proteins. Alternatively, TTSS specificity may be determined by either the regulation of expression of the EPs themselves or the regulation of expression of the chaperones required for their secretion.

While the mini-Tn5-cycler transposon may allow the identification of a large number of new EPs, identifying these proteins is only the first step in the further study of EPs. Many years have been spent studying secreted bacterial EPs, but the functions of only a few have been fully elucidated. Several more *S. enterica* serovar Typhimurium EPs are thought to exist because the cognate EPs for many observed pathogenic phenotypes remain a mystery. This report provides the initial step in expanding our knowledge of the repertoire of secreted EPs in *Salmonella* and potentially many other bacterial pathogens.

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REFERENCES

- Ausubel, F. M. 1987. Current protocols in molecular biology. Greene Publishing Associates, Brooklyn, N.Y.
- Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee. 1996. Co-ordinate regulation of *Salmonella* typhimurium invasion genes by environmental and regulatory factors is mediated by control of *hilA* expression. *Mol. Microbiol.* **22**:703–714.
- Brazilian National Genome Project Consortium. 2003. The complete genome sequence of *Chromobacterium violaceum* reveals remarkable and exploitable bacterial adaptability. *Proc. Natl. Acad. Sci. USA* **100**:11660–11665.
- Chakravorty, D., I. Hansen-Wester, and M. Hensel. 2002. *Salmonella* pathogenicity island 2 mediates protection of intracellular *Salmonella* from reactive nitrogen intermediates. *J. Exp. Med.* **195**:1155–1166.
- Chen, J., K. S. de Felipe, M. Clarke, H. Lu, O. R. Anderson, G. Segal, and H. A. Shuman. 2004. *Legionella* effectors that promote nonlytic release from protozoa. *Science* **303**:1358–1361.
- Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow. 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. *Mol. Microbiol.* **30**:175–188.
- Collier-Hyams, L. S., H. Zeng, J. Sun, A. D. Tomlinson, Z. Q. Bao, H. Chen, J. L. Madara, K. Orth, and A. S. Neish. 2002. Cutting edge: *Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF- κ B pathway. *J. Immunol.* **169**:2846–2850.
- Cornelis, G. R., and F. Van Gijsegem. 2000. Assembly and function of type III secretory systems. *Annu. Rev. Microbiol.* **54**:735–774.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Deiwick, J., T. Nikolaus, S. Erdogan, and M. Hensel. 1999. Environmental regulation of *Salmonella* pathogenicity island 2 gene expression. *Mol. Microbiol.* **31**:1759–1773.
- Ellefson, D., A. W. van der Velden, D. Parker, and F. Heffron. 2000. Identification of bacterial class I accessible proteins by disseminated insertion of class I epitopes. *Methods Enzymol.* **326**:516–527.
- Figuerola-Bossi, N., and L. Bossi. 1999. Inducible prophages contribute to *Salmonella* virulence in mice. *Mol. Microbiol.* **33**:167–176.
- Galan, J. E. 2001. *Salmonella* interactions with host cells: type III secretion at work. *Annu. Rev. Cell Dev. Biol.* **17**:53–86.
- Galan, J. E., and A. Collmer. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**:1322–1328.
- Galkin, V. E., A. Orlova, M. S. VanLoock, D. Zhou, J. E. Galan, and E. H. Egelman. 2002. The bacterial protein SipA polymerizes G-actin and mimics muscle nebulin. *Nat. Struct. Biol.* **9**:518–521.
- Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann, and A. Danchin. 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. *EMBO J.* **7**:3997–4004.
- Goryshin, I. Y., J. Jendrisak, L. M. Hoffman, R. Meis, and W. S. Reznikoff. 2000. Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. *Nat. Biotechnol.* **18**:97–100.
- Guttman, D. S., B. A. Vinatzer, S. F. Sarkar, M. V. Ranall, G. Kettler, and J. T. Greenberg. 2002. A functional screen for the type III (Hrp) secretome of the plant pathogen *Pseudomonas syringae*. *Science* **295**:1722–1726.
- Hardt, W. D., and J. E. Galan. 1997. A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. *Proc. Natl. Acad. Sci. USA* **94**:9887–9892.
- Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. *Science* **269**:400–403.
- Ho, T. D., N. Figuerola-Bossi, M. Wang, S. Uzzau, L. Bossi, and J. M. Slauch. 2002. Identification of GtgE, a novel virulence factor encoded on the Gifsy-2 bacteriophage of *Salmonella enterica* serovar Typhimurium. *J. Bacteriol.* **184**:5234–5239.
- Hueck, C. J. 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol. Mol. Biol. Rev.* **62**:379–433.
- Kaniga, K., D. Trollinger, and J. E. Galan. 1995. Identification of two targets of the type III protein secretion system encoded by the *inv* and *spa* loci of *Salmonella typhimurium* that have homology to the *Shigella* IpaD and IpaA proteins. *J. Bacteriol.* **177**:7078–7085.
- Kaniga, K., J. Uralil, J. B. Bliska, and J. E. Galan. 1996. A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen *Salmonella typhimurium*. *Mol. Microbiol.* **21**:633–641.
- Knodler, L. A., B. A. Vallance, M. Hensel, D. Jackel, B. B. Finlay, and O. Steele-Mortimer. 2003. *Salmonella* type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes. *Mol. Microbiol.* **49**:685–704.
- Kujat Choy, S. L., E. C. Boyle, O. Gal-Mor, D. L. Goode, Y. Valdez, B. A. Vallance, and B. B. Finlay. 2004. SseK1 and SseK2 are novel translocated proteins of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **72**:5115–5125.
- Ladant, D., and A. Ullmann. 1999. *Bordetella pertussis* adenylate cyclase: a toxin with multiple talents. *Trends Microbiol.* **7**:172–176.
- Lee, A. K., C. S. Detweiler, and S. Falkow. 2000. OmpR regulates the two-component system SsrA-SsrB in *Salmonella* pathogenicity island 2. *J. Bacteriol.* **182**:771–781.
- Luo, Z. Q., and R. R. Isberg. 2004. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. *Proc. Natl. Acad. Sci. USA* **101**:841–846.
- Maloy, S. R., V. J. Stewart, and R. K. Taylor. 1996. Genetic analysis of pathogenic bacteria: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainville, N.Y.
- McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson. 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature* **413**:852–856.
- Miao, E. A., J. A. Freeman, and S. I. Miller. 2002. Transcription of the SsrAB regulon is repressed by alkaline pH and is independent of PhoPQ and magnesium concentration. *J. Bacteriol.* **184**:1493–1497.
- Miao, E. A., and S. I. Miller. 2000. A conserved amino acid sequence directing intracellular type III secretion by *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **97**:7539–7544.
- Morgan, E., J. D. Campbell, S. C. Rowe, J. Bispham, M. P. Stevens, A. J. Bowen, P. A. Barrow, D. J. Maskell, and T. S. Wallis. 2004. Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* **54**:994–1010.
- Ochman, H., F. C. Soncini, F. Solomon, and E. A. Groisman. 1996. Identification of a pathogenicity island required for *Salmonella* survival in host cells. *Proc. Natl. Acad. Sci. USA* **93**:7800–7804.
- Salcedo, S. P., and D. W. Holden. 2003. SseG, a virulence protein that targets *Salmonella* to the Golgi network. *EMBO J.* **22**:5003–5014.
- Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden. 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **93**:2593–2597.
- Silva, M., C. Song, W. J. Nadeau, J. B. Matthews, and B. A. McCormick. 2004. *Salmonella typhimurium* SipA-induced neutrophil transepithelial migration: involvement of a PKC- α -dependent signal transduction pathway. *Am. J. Physiol. Gastrointest. Liver Physiol.* **286**:G1024–G1031.
- Skorupski, K., and R. K. Taylor. 1996. Positive selection vectors for allelic exchange. *Gene* **169**:47–52.
- Sory, M. P., A. Boland, I. Lambermont, and G. R. Cornelis. 1995. Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. *Proc. Natl. Acad. Sci. USA* **92**:11998–12002.
- Steele-Mortimer, O., S. Meresse, J. P. Gorvel, B. H. Toh, and B. B. Finlay.

1999. Biogenesis of *Salmonella typhimurium*-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cell Microbiol.* **1**:33–49.
42. **Tsolis, R. M., S. M. Townsend, E. A. Miao, S. I. Miller, T. A. Ficht, L. G. Adams, and A. J. Baumler.** 1999. Identification of a putative *Salmonella enterica* serotype Typhimurium host range factor with homology to IpaH and YopM by signature-tagged mutagenesis. *Infect. Immun.* **67**:6385–6393.
 43. **Tu, X., I. Nisan, J. F. Miller, E. Hanski, and I. Rosenshine.** 2001. Construction of mini-Tn5cyaA' and its utilization for the identification of genes encoding surface-exposed and secreted proteins in *Bordetella bronchiseptica*. *FEMS Microbiol. Lett.* **205**:119–123.
 44. **Uzzau, S., N. Figueroa-Bossi, S. Rubino, and L. Bossi.** 2001. Epitope tagging of chromosomal genes in *Salmonella*. *Proc. Natl. Acad. Sci. USA* **98**:15264–15269.
 45. **van der Velden, A. W., S. W. Lindgren, M. J. Worley, and F. Heffron.** 2000. *Salmonella* pathogenicity island 1-independent induction of apoptosis in infected macrophages by *Salmonella enterica* serotype Typhimurium. *Infect. Immun.* **68**:5702–5709.
 46. **Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni, and F. C. Fang.** 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* **287**:1655–1658.
 47. **Wang, R. F., and S. R. Kushner.** 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**:195–199.
 48. **Waterman, S. R., and D. W. Holden.** 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. *Cell Microbiol.* **5**:501–511.
 49. **Winstanley, C., and C. A. Hart.** 2001. Type III secretion systems and pathogenicity islands. *J. Med. Microbiol.* **50**:116–126.
 50. **Worley, M. J., K. H. Ching, and F. Heffron.** 2000. *Salmonella* SsrB activates a global regulon of horizontally acquired genes. *Mol. Microbiol.* **36**:749–761.
 51. **Zhang, S., R. L. Santos, R. M. Tsolis, S. Stender, W. D. Hardt, A. J. Baumler, and L. G. Adams.** 2002. The *Salmonella enterica* serotype Typhimurium effector proteins SipA, SopA, SopB, SopD, and SopE2 act in concert to induce diarrhea in calves. *Infect. Immun.* **70**:3843–3855.
 52. **Zhou, D., and J. Galan.** 2001. *Salmonella* entry into host cells: the work in concert of type III secreted effector proteins. *Microbes Infect.* **3**:1293–1298.

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