RESEARCH ARTICLE

A New Means To Identify Type 3 Secreted Effectors: Functionally Interchangeable Class IB Chaperones Recognize a Conserved Sequence

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ABSTRACT **Many Gram-negative bacteria utilize specialized secretion systems to inject proteins (effectors) directly into host cells. Little is known regarding how bacteria ensure that only small subsets of the thousands of proteins they encode are recognized as substrates of the secretion systems, limiting their identification through bioinformatic analyses. Many of these proteins require chaperones to direct their secretion. Here, using the newly described protein interaction platform assay, we demonstrate that type 3 secretion system class IB chaperones from one bacterium directly bind their own effectors as well as those from other species. In addition, we observe that expression of class IB homologs from seven species, including pathogens and endosymbionts, mediate the translocation of effectors from *Shigella* directly into host cells, demonstrating that class IB chaperones are often functionally interchangeable. Notably, class IB chaperones bind numerous effectors. However, as previously proposed, they are not promiscuous; rather they recognize a defined sequence that we designate the conserved chaperone-binding domain (CCBD) sequence [$(LMIF)_1XXX(IV)_5XX(IV)_8X(N)_{10}$]. This sequence is the first defined amino acid sequence to be identified for any interspecies bacterial secretion system, i.e., a system that delivers proteins directly into eukaryotic cells. This sequence provides a new means to identify substrates of type III secretion systems. Indeed, using a pattern search algorithm for the CCBD sequence, we have identified the first two probable effectors from an endosymbiont, *Sodalis glossinidius*.**

IMPORTANCE **Many Gram-negative pathogens utilize type 3 secretion systems to deliver tens of effectors into host cells. In order to understand the diverse ways that these organisms cause disease, it is necessary to identify their effectors, many of which require chaperones to be secreted. Here we establish that class IB chaperones are not promiscuous, as previously proposed, but rather recognize a conserved effector sequence. We demonstrate that pattern search algorithms based on this defined sequence can be used to identify previously unknown effectors. Furthermore, we observe that class IB chaperones from at least seven bacterial species are functionally interchangeable. Not only do they bind and mediate the delivery of their own set of effectors into host cells but they also bind to type 3 substrates from other bacteria, suggesting that inhibitors that block chaperone-effector interactions could provide a novel means to effectively treat infections due to Gram-negative pathogens, including organisms resistant to currently available antibiotics.**

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Over 30 species of Gram-negative bacteria, both pathogens and endosymbionts, utilize type 3 secretion systems (T3SSs) to deliver tens of proteins, referred to as effectors, directly into host cells (1). Type 3 effectors target a variety of host cellular processes to promote bacterial spread and survival. While the protein components of these complex secretion machines are highly conserved, each bacterial species delivers its own unique repertoire of effectors into host cells. Although many effectors require chaperones to be secreted, little is known regarding how they are defined as type 3 secreted substrates, limiting their identification through bioinformatic analyses.

Extensive work mapping the regions of effectors required for their secretion has established that two domains play a role, the N-terminal secretion sequence and, in many cases, a downstream chaperone-binding domain (2, 3). The N-terminal secretion sequence is not a specific sequence, but rather, as recent experimental data suggest, an intrinsically structurally disordered region (4). Machine learning algorithms can identify known effectors based on the nature of their N-terminal residues. However, the full utility of these algorithms in identifying new effectors is still unknown (5–7).

Two distinct but structurally related classes of chaperones mediate the secretion of type 3 effectors (8, 9). Class IA chaperones are almost always located within operons adjacent to the genes that encode their one or two cognate effectors. In contrast, class IB chaperones are encoded within large operons surrounded by components of the type 3 secretion machinery rather than effectors. On the basis of the results of structural analyses (10–13), it is

hypothesized that effectors interact with class IA and class IB chaperones via a conserved structural motif, the β-strand motif (11), and that it is likely that the chaperone-effector complex is the signal recognized by the type III secretion apparatus (10, 11). The two best-characterized class IB chaperones are Spa15 from *Shigella flexneri* and InvB from *Salmonella enterica* serovar Typhimurium SPI1 (*Salmonella* pathogenicity island 1) T3SSs. Each has been established to mediate the secretion of multiple effectors, nine in the case of *Shigella* (14–16) and four in the case of *Salmonella* (18, 23, 24). Given their ability to interact with numerous effectors, Spa15 and InvB have been proposed to be promiscuous in their recognition of effectors (19).

Here, we present evidence that class IB chaperones from seven different bacterial species, including pathogens and endosymbionts, are functionally interchangeable. Specifically, class IB chaperones from one species can bind and mediate the type 3 secretion-dependent translocation of effectors from another. These class IB chaperones are not promiscuous, as previously proposed, but rather recognize a defined amino acid sequence motif, which we designate the conserved chaperone-binding domain (CCBD). The CCBD overlaps the previously identified structural β-strand motif, providing evidence that residues of the CCBD sequence directly bind to chaperones. However, the CCBD demonstrates that class IB chaperones recognize a conserved amino acid pattern. The CCBD sequence is the first identified defined amino acid sequence that is common to effectors from any interspecies bacterial secretion system, i.e., one used by bacteria to deliver proteins into eukaryotic cells. Uncovering this sequence not only refines our understanding of how interactions between chaperones and effectors are defined but also provides a new means to identify type 3 substrates from bacteria that encode class IB. Indeed, based on the results of a pattern search algorithm of the *Sodalis glossinidius* genome for proteins that contain the CCBD sequence, we identified the first two likely effectors from an endosymbiont.

RESULTS

Conserved recognition of effectors by *Shigella* and *Salmonella* class IB chaperones. It is well established that class IA chaperone-dependent effectors are recognized as substrates of heterologous T3SSs when their cognate chaperone is also present (20, 21). In contrast, at the start of this study, little was known regarding the behavior of class IB chaperone-dependent effectors in heterologous systems. For example, it was not known whether "promiscuous" chaperones from one system could bind and mediate the secretion of effectors from another. To investigate this possibility, we tested whether class IB chaperones from *Shigella* and *Salmonella* could bind each other's effectors using the *Saccharomyces cerevisiae* yeast-based protein interaction platform (PIP) assay, an assay previously established to be more sensitive than the yeast two-hybrid assay in detecting chaperone-effector interactions (16).

The PIP assay is a visualization system for identifying interacting proteins in living cells. In this assay, one protein is fused to μNS, a reoviral protein that forms inclusions (platforms) when expressed in eukaryotic cells; a second protein is fused to a fluorescent protein (16). When coexpressed in yeast, if the two proteins interact, the fluorescent fusion protein is recruited to the platforms and fluorescent foci are observed. Using the PIP assay, we observed interactions between *Salmonella* InvB and 10 of 23 *Shigella* effectors, the same 10 that interact with *Shigella* Spa15 in the PIP assay (see Fig. S1A in the supplemental material) (16). In all but one case, the majority of yeast cells visualized exhibited fluorescent foci. The exception was green fluorescent protein (GFP)-IpgB1, where the percentage of yeast cells that displayed fluorescent foci with expression of μNS-InvB and GFP-IpgB1 was decreased compared to those expressing μNS-Spa15 and GFP-IpgB1 (34 versus 68%). This observation suggests that IpgB1 interacts more weakly with InvB than Spa15, as recent studies demonstrate that the percentage of yeast displaying fluorescent foci reflect the relative *in vitro* binding affinities of the two proteins for each other (22).

In a complementary set of PIP assays, *Salmonella* InvB and *Shigella* Spa15 each interacted with three *Salmonella* effectors that require InvB for their efficient secretion by the *Salmonella* T3SS (SopA, SopE1, and SopE2) (see Fig. S1B in the supplemental material) (18, 23, 24). However, the fourth *Salmonella* InvB-dependent effector, SipA, bound only InvB. Neither InvB nor Spa15 interacted with any of the 13 *Shigella* effectors previously established to not require Spa15 for their secretion, including those that bind class IA chaperones, demonstrating that these class IB chaperones exhibit some specificity in their interactions (Fig. S1C) (16).

Complementation of secretion of *Shigella* Spa15-dependent effectors by *Salmonella* InvB. To determine whether the interactions detected in the PIP assay were physiologically relevant, we tested whether expression of the *Salmonella* InvB chaperone would restore secretion of *Shigella* Spa15-dependent effectors from a strain lacking Spa15 (*Shigella* Δ*spa15* mutant). Complementation with InvB restored secretion to essentially wild-type levels for eight of the nine *Shigella* Spa15-dependent effectors (Fig. 1A), all but IpgB1, the effector that exhibited decreased interaction with InvB in the PIP assay. Similarly, secretion of the three *Salmonella* InvB-dependent effectors that bind Spa15 (SopA, SopE1, and SopE2) from the *Shigella* Δ*spa15* mutant was only observed with complementation by either Spa15 or InvB (Fig. 1B). SipA, the only *Salmonella* InvB effector that interacted exclusively with InvB in the PIP assay, was secreted at relatively low levels from both the wild-type *Shigella* and the *Shigella* Δ*spa15* mutant, suggesting that it can be secreted in the absence of a chaperone. Complementation of the *Shigella* Δ*spa15* mutant strain with *Salmonella* InvB, but not *Shigella* Spa15, results in increased levels of secreted SipA (Fig. 1B). Together, the results of these studies demonstrate that the class IB chaperones from *Salmonella* and *Shigella* are nearly functionally interchangeable. These are the first class I chaperones yet to be identified to bind and promote the secretion of each other's effectors.

For these experiments, epitope-tagged versions of each effector were expressed under the control of a weakened version of the isopropyl-β-d-thiogalactopyranoside (IPTG)-inducible *trc* promoter from a low-copy-number plasmid (pBR ori) (25). This allowed us to ensure that the effectors were expressed at close to physiologic levels when type 3 secretion was induced (16), since at least in the case of *Shigella* OspF, this system results in similar levels of expression as observed under native conditions (data not shown). Similarly, for the complementation experiments, Spa15 and InvB were expressed under the control of a weakened *trc* promoter from another low-copy-number plasmid (pSC101 ori). We observed no differences in the levels of secretion of each of the 9 Spa15-dependent effectors from wild-type *Shigella* versus *Shigella*

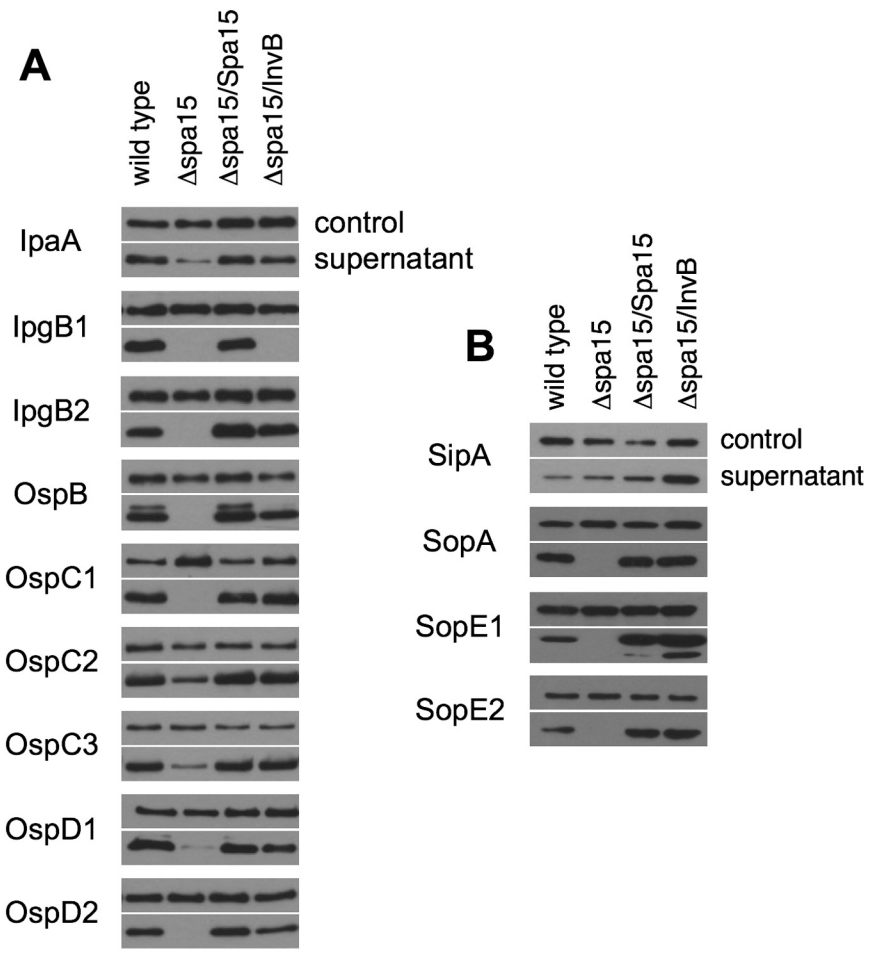

FIG 1 *Shigella* and *Salmonella* class IB chaperones are functionally interchangeable. *S. flexneri* strains (wild type, Δ*spa15* mutant, and the Δ*spa15* mutant complemented with *Shigella* Spa15 or *Salmonella* InvB) and expressing the designated FLAG-tagged *Shigella* (A) or *Salmonella* (B) effectors were grown under conditions that induce T3SS. The supernatant proteins were precipitated with TCA, separated by SDS-PAGE, and immunoblotted with anti-FLAG and anti-IcsA antibodies. IcsA is an autotransporter and secreted by a mechanism other than type 3 secretion and serves as a loading control. The blots shown are representative of at least three experiments.

Δ*spa15* mutant complemented with a plasmid expressing Spa15, suggesting that the phenotypes observed are not due to Spa15 or InvB overexpression. Thus, *Salmonella* InvB and *Shigella* Spa15 are able to recognize and mediate the secretion of a subset of each other's effectors.

Identification of a conserved CCBD sequence. The observation that Spa15 and InvB recognize the same subset of effectors suggested that these chaperones are discriminate in their recognition of effectors and led us to search for a conserved chaperone-binding domain (CCBD) sequence. Previous studies had failed to recognize a sequence common to all type 3 effectors. However, when we restricted our alignment studies to the N-terminal 70 residues of the 13 *Shigella* and *Salmonella* effectors that interact with Spa15 and/or InvB, we identified a shared sequence, $(LMIF)_1XXX(IV)_5XX(IV)_8X(N)_{10}$ (Fig. 2A). Interestingly, the only effector that does not contain isoleucine or valine at position 5 is *Salmonella* SipA, the effector that interacts exclusively with *Salmonella* InvB. This consensus sequence is not found in any of the 11 *Shigella* effectors that do not require Spa15 for their secretion (16) or in any *Salmonella* effectors, other than those that interact with InvB. This sequence is the first defined amino acid sequence that has been identified that is shared by secreted effectors of any interspecies bacterial secretion system, and we designate it the conserved chaperone-binding domain sequence.

Site-directed mutagenesis studies confirmed a role for the CCBD sequence in mediating chaperone-effector interactions. The conversion of all four of the conserved residues (positions 1, 5, 8, and 10) of the CCBD sequence of *Shigella* OspC1 or OspD1 to alanines completely abolished their secretion via the *Shigella* T3SS (Fig. 2B) and resulted in loss of interaction of OspD1 and Spa15 in the PIP (Fig. 2C) and yeast two-hybrid (Y2H) protein-protein interaction assays (see Table S1 in the supplemental material). We also examined whether single mutations at position 1, 5, 8, or 10 of the CCBD sequence would disrupt secretion and/or chaperone binding. For these studies, each residue was mutated to glycine to disrupt the hydrophobic contacts predicted to mediate chaperone-effector interaction in accordance with the corresponding positions in the SipA-InvB crystal structure. Point mutations at positions 1 and 10, the less conserved residues, resulted in a mild-to-moderate decrease in secretion, while mutation of either position 5 or 8, positions invariantly present as isoleucines or valines, essentially disrupted all secretion (Fig. 2D). In each case, the single point mutations disrupted interactions between Spa15 and OspD1 in the PIP and Y2H assays (Table S1) with the magnitude of loss paralleling the observed loss in secretion (Fig. 2C). Since decreased interactions in the PIP and Y2H assays are both associated with decreased binding affinities determined *in vitro* (22, 26), these observations suggest that these residues of the CCBD sequence directly bind class IB chaperones, as observed in the SipA-InvB cocrystal structure (11).

Interactions between the CCBD and class IB chaperones determine substrate specificity. We next investigated whether interactions between the CCBD and class IB chaperones are sufficient to define chaperone substrate specificity. To address this question, we exploited our earlier observations that *Salmonella* InvB binds and complements the secretion of all of the *Shigella* CCBD-containing effectors except for IpgB1 (Fig. 1). IpgB1 shares a high degree of structural homology with IpgB2 (27), another *Shigella* CCBD-containing effector, suggesting that the unstructured N-terminal regions of these proteins dictate whether InvB recognizes them as effectors. Indeed, by swapping the first 47 residues of IpgB1 with the first 50 residues of IpgB2 (the respective regions of the effectors located upstream of their conserved struc-

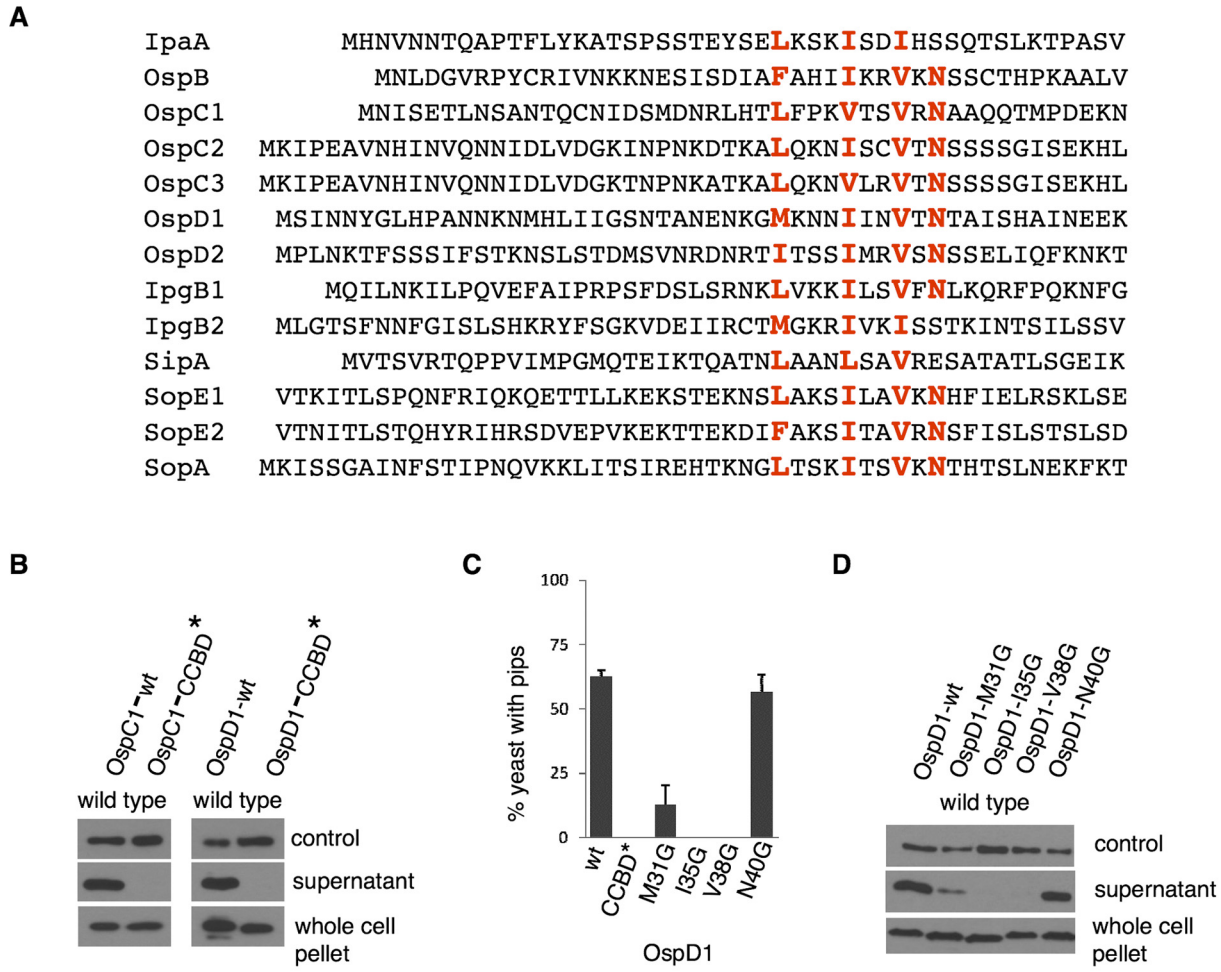

FIG 2 Identification of the conserved chaperone-binding domain, a sequence common to *Shigella* and *Salmonella* class IB-dependent effectors. (A) Multiple-sequence alignment of effectors whose secretion is promoted by InvB or Spa15. The CCBD sequence is shown in large red letters. (B to D) Wild-type *Shigella* expressing epitope-tagged alleles of wild-type (wt) and mutant alleles of OspC1 and OspD1 were grown under conditions that induce T3SS. The residues at positions 1, 5, 8, and 10 in CCBD* were changed to alanines. The supernatant and pellet fractions were immunoblotted with anti-FLAG antibody to assess for secretion and protein stability, respectively. The supernatant fractions were also immunoblotted with anti-IcsA antibody as a loading control. All blots shown are representative of at least three experiments. (C) Yeast coexpressing μNS-Spa15 and the designated GFP-OspD1 alleles were visualized 4 to 5 hours after induction of each protein in the PIP assay. The percentage of yeast expressing fluorescent foci (pips) was determined by counting 100 cells. The values shown are representative of two independent experiments done in triplicate.

tural domains), we switched their substrate specificity, as InvB now recognizes IpgB1, but not IpgB2, as a type 3 substrate (Fig. 3A and B). This substrate specificity is not determined by the N-terminal secretion signal, as additional swaps established that substrate specificity maps to regions that encompass the CCBD sequences of the two proteins (Fig. 3A and B). The switch in recognition of the IpgB1/IpgB2 hybrids likely represents differences in their binding affinities, as the ability of InvB to complement the secretion of these proteins from the *Shigella* Δ*spa15* mutant correlates with its capacity to bind the proteins in the PIP assay (Fig. 3C). All of the hybrid effectors were secreted by the *Shigella* Δ*spa15* mutant in the presence of Spa15 (Fig. 3B). These observations suggest that the CCBD is a major determinant in defining interactions between effectors and class IB chaperones.

The CCBD sequence is unique to effectors that bind class IB chaperones. Notably, at least in the case of SipA, whose crystal structure in complex with InvB has been solved, the CCBD domain overlaps with the β-strand motif, a region characterized by three hydrophobic residues (ψ) separated by 1 to 4 variable residues ($\psi X_{2-4}\psi X_{1-2}\psi$) (11). This structural motif is found in effectors that bind class IA and IB chaperones. Unlike the CCBD sequence, there is no sequence conservation of residues in the β-strand motif other than their hydrophobic nature. Over 35 type 3 effectors have been proposed to contain the structural β-strand motif, including those dependent on Spa15 or InvB for their secretion (11). We randomly selected and tested whether nine of these effectors (*Salmonella* SifA, SifB, SigD, SptP, and SseJ, *Escherichia coli* EspH, Map, and Tir, and *Yersinia pseudotuberculosis* YopH) interact with Spa15 or InvB in the PIP assay (see Fig. S2A in the supplemental material). This set included six effectors (SigD, SptP, EspH, Map, Tir, and YopH) previously shown to bind class IA chaperones. None of these effectors interacted with Spa15 or InvB. Similarly, we observed no interactions in the PIP assay between the nine *Shigella* CCBD-containing effectors and two atyp-

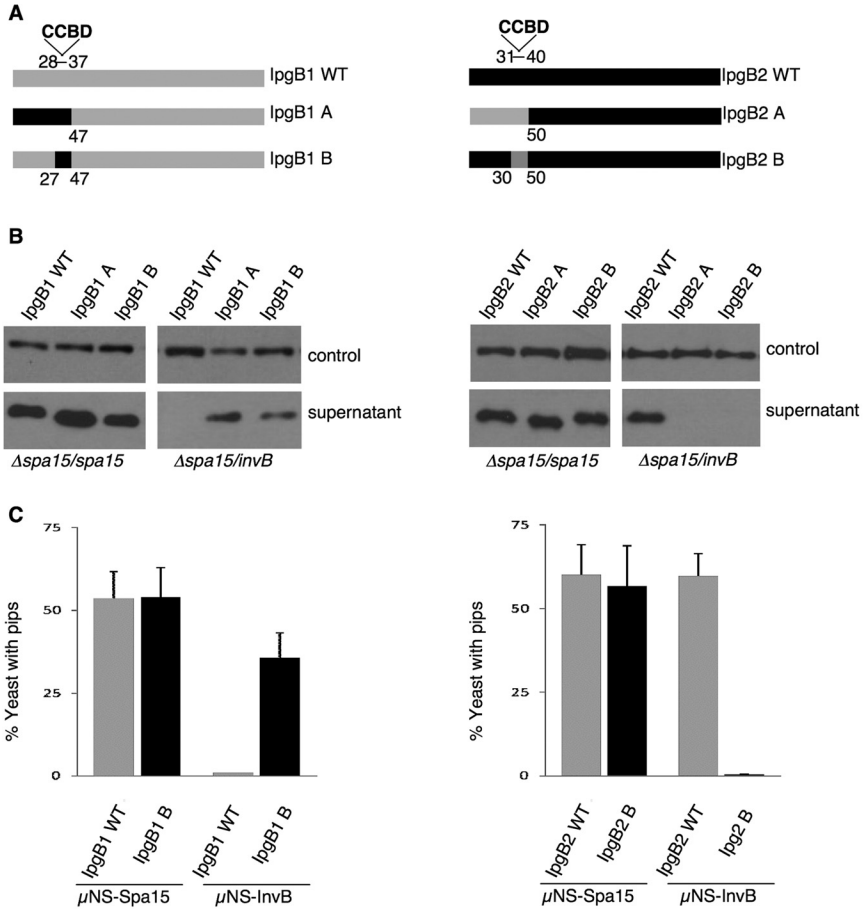

FIG 3 The CCBD sequence defines chaperone-effector interactions. (A) Schematic of IpgB1/IpgB2 hybrid proteins used in this study. (B) *Shigella* Δ*spa15* mutant complemented with Spa15 or InvB and expressing epitope-tagged alleles of the designated IpgB1 or IpgB2 hybrid proteins grown under conditions that induce T3SS. The supernatant fractions were immunoblotted with anti-FLAG antibody to assess for secretion. The supernatant fractions were also immunoblotted with anti-IcsA antibody as a loading control. (C) Yeast coexpressing μNS-Spa15 or μNS-InvB and the designated IpgB1/IpgB2 alleles were visualized 4 to 5 hours after induction of each protein in the PIP assay. The percent of yeast expressing fluorescent foci (pips) was determined by counting 100 cells. The values shown are representative of two independent experiments done in triplicate.

ical class IA chaperones that have been established to bind multiple effectors, CesT from enterohemorrhagic *E. coli* and SrcA from the *Salmonella* SPI2 T3SS (Fig. S2C) (28, 29). Therefore, we hypothesize that the amino acid sequence of the CCBD defines the specificity of interactions of class IB chaperones with their cognate effectors.

Conserved recognition of CCBD-containing effectors by class IB chaperones in distantly related species. We next investigated whether the recognition of CCBD-containing effectors is restricted to closely related pathogens like *Shigella* and *Salmonella* or a feature common to all class IB chaperones. Towards this goal, we tested the ability of seven additional class IB chaperones, four from human pathogens (*Proteus mirabilis* [30]), *Burkholderia dolosa, Burkholderia mallei* [31] and *Yersinia enterocolitica* [32]) and three from endosymbionts (*Sodalis glossinidius* [33, 34] and *Hamiltonella defensa* [35]) to bind to *Shigella* CCBD-containing effectors. *Sodalis glossinidius*, a tsetse fly endosymbiont, contains three T3SSs, two of which (SSR1 and SSR2) encode InvB homologs. Similarly, *Y. enterocolitica* encodes two T3SSs, the well-studied Ysc T3SS and the relatively poorly characterized Ysa T3SS. Only the latter system encodes a class IB chaperone.

To increase the throughput of our protein interaction studies, we developed an automated microscopy-based version of the PIP assay. Automating the PIP assay decreased the time needed for image acquisition and analysis allowing for the rapid analysis of hundreds of yeast cells for the formation of fluorescent foci (pips) (Fig. 4A). One issue we encountered with the automated PIP assay was that the segmentation software used had difficulty identifying fluorescent foci in yeast that express weakly fluorescent GFP fusion proteins, specifically GFP-OspB, GFP-OspC3, and GFP-OspD2. To account for differences in GFP intensities (Fig. 4B), for each effector protein, we normalized the number of yeast that exhibited fluorescent foci with each homolog to those observed with *Shigella* Spa15.

As summarized in Fig. 4B (raw data shown in Fig. S3A in the supplemental material), all of the class IB chaperones, except those from the *Sodalis* SSR1 and *B. mallei* T3SSs, interacted with *Shigella* CCBD-containing effectors. The lack of detection of interactions of the *Sodalis* SSR1 and *B. mallei* homologs with effectors was not due to their inability to form platforms in yeast, as fluorescent foci were observed when each of these chaperones was simultaneously fused to a cyan fluorescent protein-μNS fusion protein (Fig. S3B).

Functionally interchangeability as a general feature of class IB chaperones. To confirm that the chaperone-effector interactions detected in the PIP assay were physiologically relevant, we investigated whether each of the class IB homologs could complement translocation of the nine *Shigella* CCBD-containing effectors using the well-established TEM-1 β-lactamase reporter assay (36). Briefly, in this assay, HeLa cells are preloaded with CCF4/AM, a fluorescence resonance energy transfer (FRET)-based dye, that emits green fluorescence. When translocated into host cells, the TEM-1 β-lactamase fusion proteins cleave the substrate, disrupting FRET and resulting in cells that emit blue fluorescence (Fig. 5A). The translocation efficiency of each effector corresponds to the percentage of cells that fluoresce blue.

To facilitate our studies, we adapted the TEM-1 β-lactamase reporter assay to monitor the translocation of *Shigella* effectors into HeLa cells in a 96-well format. Effector–TEM-1 fusions were expressed under the control a weakened *trc* IPTG-inducible promoter from a low-copy-number plasmid (pBR ori). After the expression of effectors was induced, HeLa cells were infected with *Shigella* at a multiplicity of infection (MOI) of 100 for 2 h. The cells were examined and the numbers of green and blue cells were

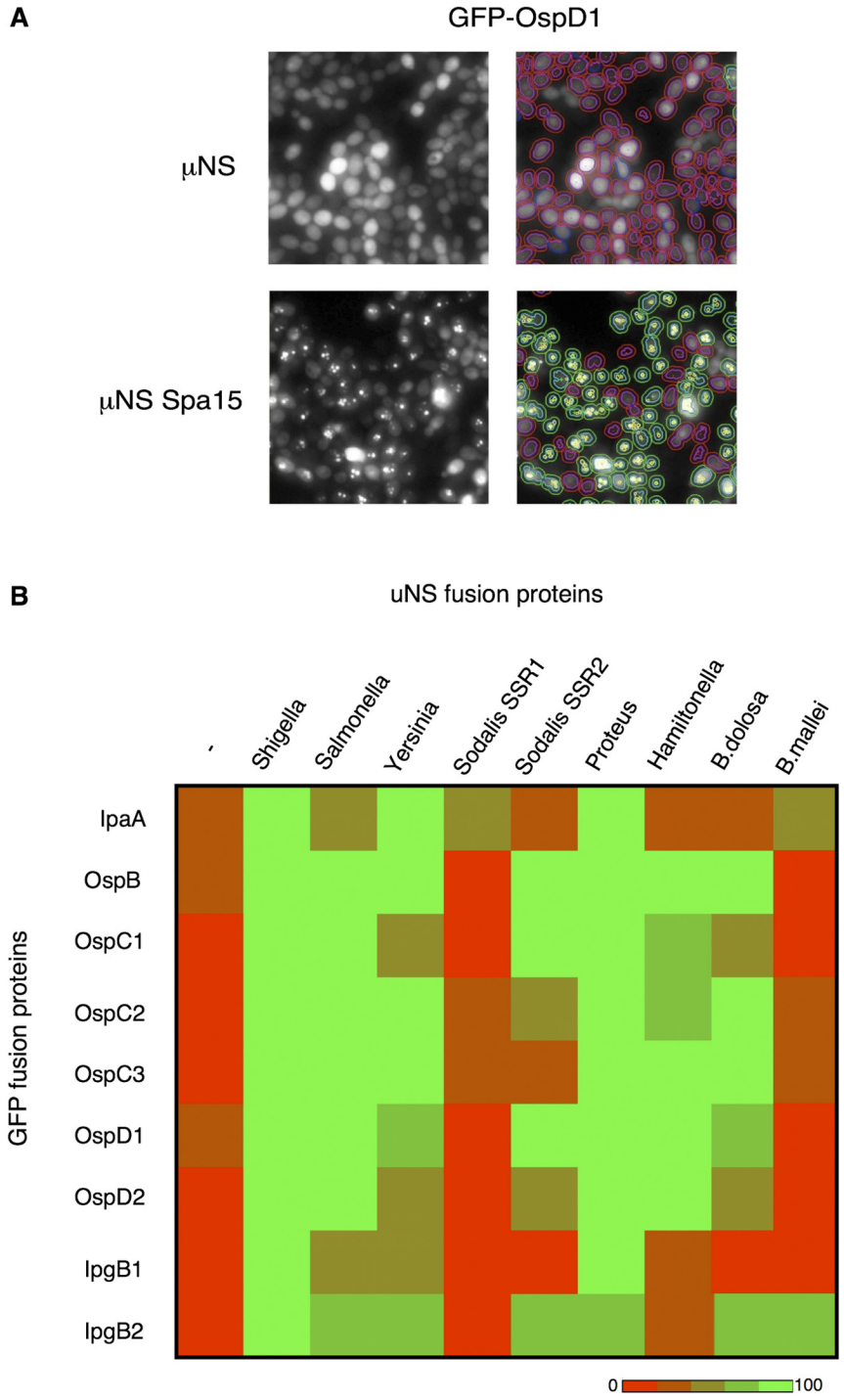

FIG 4 Summary of interactions of class IB chaperones with CCBD-containing effectors obtained by automated microscopy. (A) Images of yeast expressing GFP-OspD1 in the presence of either μNS (negative control) or μNS-Spa15 (positive control) obtained via automated microscopy. The images in the left column are unprocessed, while the images in the right column represent pip-positive (green) and pip-negative (red) yeast as determined by the IN Cell Workstation segmentation software. (B) The heatmap shown (45) represents relative number of yeast with fluorescent foci (pips) visualized with coexpression of the designated μNS-class IB chaperone and GFP-effector fusion protein pairs. For each GFP fusion protein, the absolute percentage of yeast expressing fluorescent foci was normalized to the absolute percentage of yeast expressing fluorescent foci when the GFP-effector fusion protein was coexpressed with μNS-Spa15. The data are representative of 3 independent experiments done in duplicate. At least 6,000 cells were quantified per condition.

counted. These conditions were used for our high-throughput studies given that at the 2-h time point we observed similar levels of translocation of all nine effectors into host cells with wild-type *Shigella* and the *Shigella* Δ*spa15* mutant complemented with Spa15 (Fig. S4A in the supplemental material).

As summarized in Fig. 6B and shown in Fig. S4A in the supplemental material, all of the class IB chaperones, except those from the *Sodalis* SSR1 and *B. mallei* T3SSs, complemented the translocation of CCBD-containing effectors from the *Shigella* Δ*spa15* mutant into host cells. Lack of translocation by the *Sodalis* SSR1 and *B. mallei* chaperones was not due to lack of expression or decreased stability of these proteins, as both effectors are present at levels similar to Spa15 in *Shigella* (Fig. S4B). Rather, as neither interact with CCBD-containing effectors in the PIP assay, it is likely that these homologs do not complement secretion because they do not bind the effectors. Interestingly, *Sodalis* SSR2, like *Salmonella* InvB, poorly complemented the translocation of IpgB1. Consistent with this observation, the *Sodalis* SSR2 homolog, like *Salmonella* InvB, complements the secretion into the media of all the *Shigella* CCBD-containing effectors, except for IpgB1 (Fig. S5). Taken together, the PIP and translocation assay results suggest that, with the exception of homologs from the *Sodalis* SSR1 and *B. mallei* T3SSs, the majority of class IB chaperones are functionally interchangeable and recognize a conserved amino acid domain, the CCBD sequence.

Identification of the first effectors from an endosymbiont based on a pattern search algorithm for the CCBD sequence. While the proteins that comprise T3SSs, including class IB chaperones, are highly conserved, each bacterial species delivers its own unique set of effectors into host cells. Although class IB chaperones can be identified based on homology searches, with the exceptions of *Shigella* Spa15 and *Salmonella* InvB, none of their cognate effectors are known. Since most of the homologs bind and mediate the translocation of *Shigella* CCBD-containing effectors, we reasoned that we could identify their native effectors by searching their respective genomes for open reading frames (ORFs) that contain the CCBD sequence. We focused our initial studies on *Sodalis glossinidius*, partic-

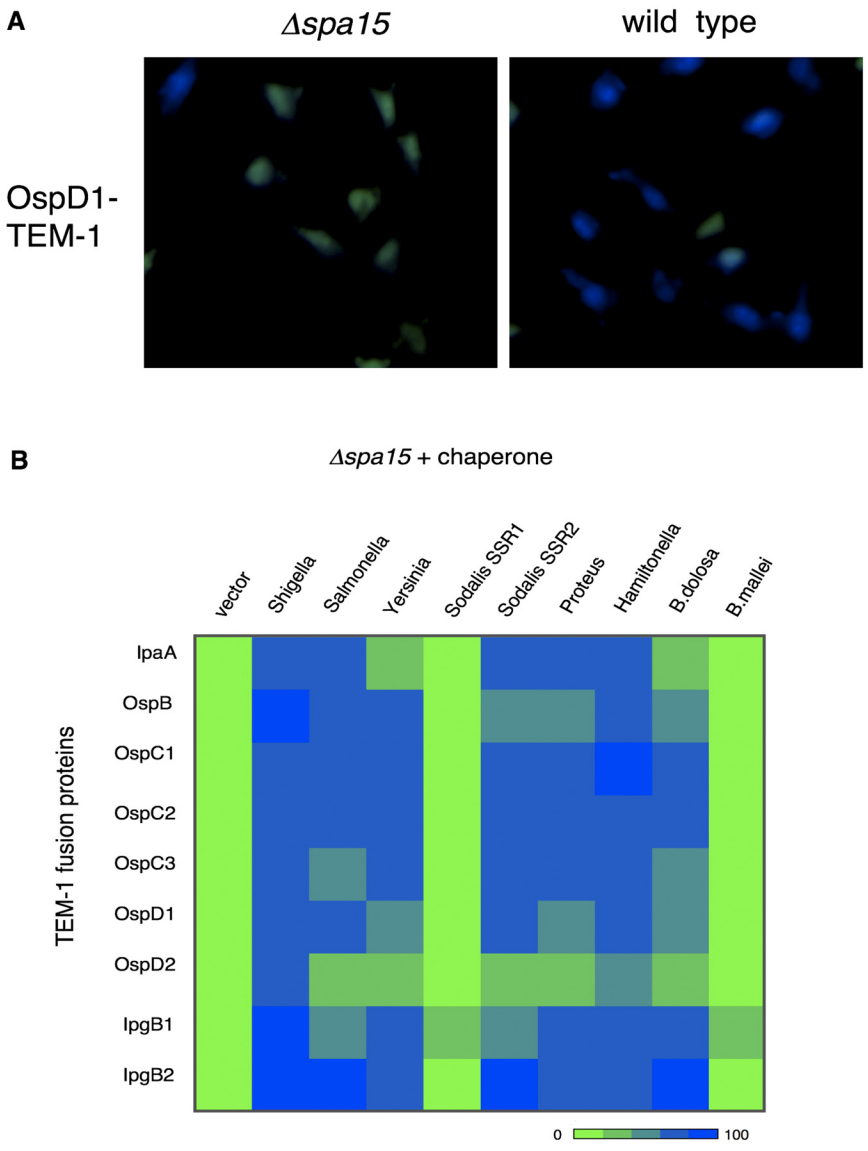

FIG 5 Functional interchangeability of class IB chaperones in a type 3 translocation assay. (A) Images of HeLa cells preloaded with CCF4/AM infected with wild-type (WT) *Shigella* or *Shigella* Δ*spa15* mutant expressing OspD1–TEM-1. (B) The heatmap shown (45) is representative of the percent blue cells observed when the HeLa cells were infected with *Shigella* Δ*spa15* mutant strain that carries plasmids that express the designated class IB chaperone with each of the effector–TEM-1 fusion proteins. Translocation was quantified by measuring the percentage of cells that fluoresce blue (cleaved CCF4/AM). Data are representative of two independent experiments done in triplicate and at least 600 cells were counted for each sample.

ularly given that no effectors from an endosymbiont have yet been identified. Using the Bioinformatics Toolkit pattern search algorithm (37) for the CCBD sequence $[(LMIF)_1XXX(IV)_5XX(IV)_8X(N)_{10}]$ in the first 25 to 45 N-terminal residues of annotated ORFs (a region chosen based on the location of the CCBD sequence in all 13 *Shigella* and *Salmonella* Spa15/InvB-dependent effectors), we identified 13 *Sodalis* proteins, 9 of which are annotated as housekeeping proteins. We focused our studies on the remaining proteins that were annotated as hypothetical ORFs (see Fig. S6A in the supplemental material).

Two of these four *Sodalis glossinidius* proteins, SG0576 and SG0764, were stably expressed in *Shigella*, and both of these were secreted into the media by wild-type *Shigella* under conditions that induce type 3 secretion (Fig. 6A). The secretion of both was impaired or absent from the *Shigella* Δ*spa15* mutant, and as predicted based on the results of binding and translocation studies, expression of the class IB homolog from the *Sodalis* SSR2, but not the SSR1 T3SS, restored secretion of both proteins from the *Shigella* Δ*spa15* mutant (Fig. 6A). Neither protein was secreted from a *Shigella* strain that does not form a functional T3SS due to loss of expression of the structural protein MxiM (*Shigella* Δ*mxiM* mutant) (see Fig. S6B in the supplemental material) (38). Last, SG0576 and SG0764 were both translocated into host cells via the *Shigella* T3SS in a class IB chaperone-dependent manner (Fig. 6B). Together, these observations strongly suggest that these proteins are substrates of the *Sodalis* SSR2 T3SS. Thus, we have identified the first type 3 effectors from an endosymbiont.

DISCUSSION

Many Gram-negative bacteria utilize T3SSs to deliver tens of proteins directly into host cells during the course of an infection. The identification of effectors remains challenging, even in the postgenomics era, in part given their lack of a defined searchable conserved secretion signal. Here, we demonstrate that class IB chaperones are not promiscuous, as previously proposed, but rather recognize a defined amino acid sequence, which we designate the conserved chaperone-binding domain. This CCBD sequence is recognized by class IB chaperones from both endosymbionts and animal pathogens. Seven of the nine class IB homologs studied here not only bound CCBD-containing effectors but were functionally interchangeable in the context of the *Shigella* T3SS. Notably, by using a pattern search algorithm to screen for proteins that contain the CCBD sequence, we successfully identified the first putative effectors from an endosymbiont, *Sodalis glossinidius*.

Candidate effectors have traditionally been identified from bacterial genome sequences by searching for proteins that share sequence similarity with type 3 effectors or mammalian proteins as well as by focusing on those encoded by genes within pathogenicity islands or exhibit a distinctive GC content. Recently, several groups have developed machine learning algorithms to identify candidate effectors based on their N-terminal 15 to 20 residues, the secretion signal. Curiously, using available Web-based tools for two of these algorithms, we found that the SIEVE (39) and

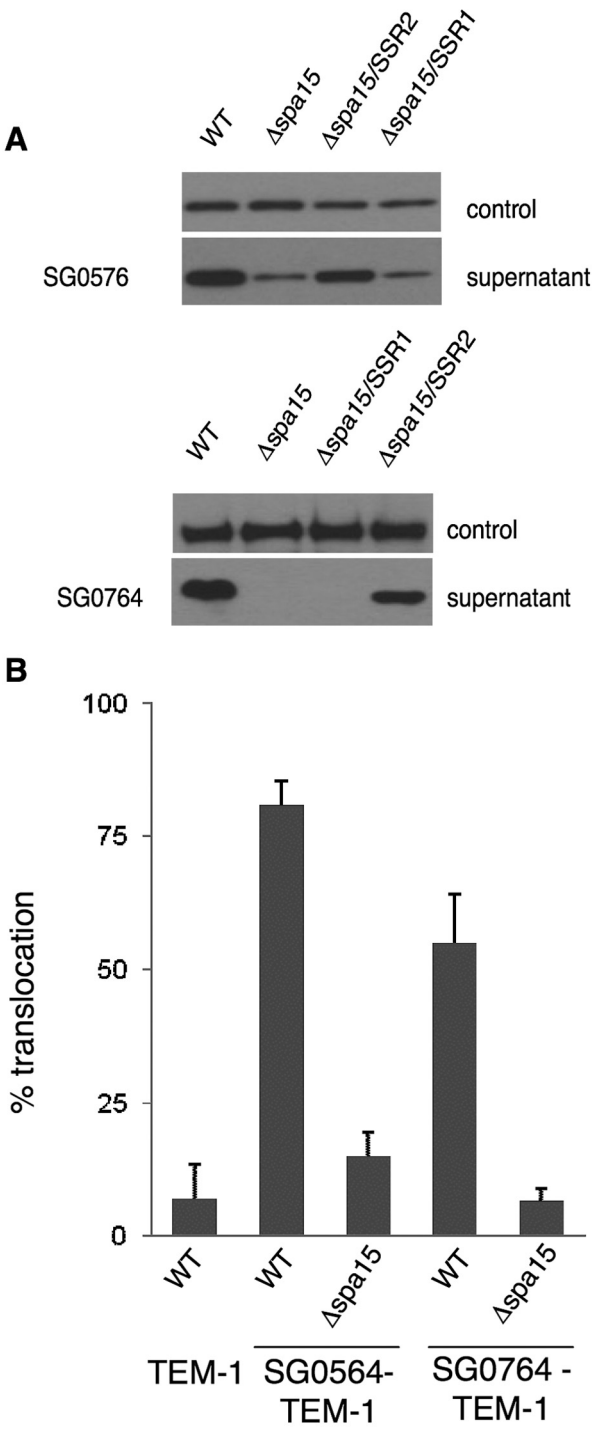

FIG 6 SG0567 and SG0764 are the first identified effectors from an endosymbiont. (A) Wild-type *Shigella* or the *Shigella* Δ*spa15* mutant complemented with the Spa15 homologs from the *Sodalis* SSR1 or SSR2 T3SSs and expressing FLAG-tagged *Sodalis* candidate effectors were grown under conditions that induce T3SS. The supernatant proteins were immunoblotted with anti-FLAG and anti-IcsA antibodies. The blots shown are representative of at least three experiments. (B) HeLa cells preloaded with CCF4/AM were infected with wild-type *Shigella* or the *Shigella* Δ*spa15* mutant expressing the designated protein fused to TEM-1 for 2 hours. The cells were fixed, and the percentage of blue cells were determined. Values are representative of three independent experiments done in triplicate, and at least 600 cells were counted for each sample (means plus standard errors of the means [SEM] [error bars] are shown).

Effective T3 (5) algorithms identify only about half of the 15 *Shigella, Salmonella*, and *Sodalis* CCBD-containing effectors, suggesting that CCBD-based searches will lead to the identification of effectors missed by the machine learning algorithms.

While the conserved residues of the CCBD are clearly important, they are not sufficient to define effectors, as this sequence is found in bacterial housekeeping proteins, which are unlikely to be secreted. This is not surprising, as additional determinants play a role in defining effectors. For example, our pattern search algorithms for the CCBD sequence motif do not select for proteins that encode an N-terminal secretion signal. In addition, it is possible, that the variable residues of the CCBD sequence, the residues indicated as X in the consensus sequence, play a role in defining an effector. Curiously, these residues in the verified effectors are enriched for nucleophilic and charged residues. This is a particularly interesting finding, as recent work suggests that the three-dimensional structure of the chaperone-effector complex defines the secretion signal (10, 40). Last, binding to the chaperone does not ensure that a protein is secreted via the T3SS, as prior to being secreted, the effectors are unfolded by an T3SS-associated ATPase that presumably enables them to be transported through a ~20-angstrom channel into host cells. Indeed, heterologous proteins with high intrinsic stability that have been engineered to encode amino-terminal secretion signals are not secreted (41). Future experimental work and bioinformatic analyses designed to address the possibilities outlined above will likely result in refinements that improve the specificity of pattern search algorithms for the detection of effectors.

In summary, our current work demonstrates that by exploiting the functional interchangeability of class IB chaperones and their conserved recognition of the CCBD sequence motif we have developed a new experimental genome-mining means for the identification of previously unknown effectors. Notably, while it is relatively straightforward to monitor the secretion and translocation of *Shigella* effectors, with the exception of the *Salmonella* SPI T3SS, *in vitro* conditions that induce other T3SS genes that encode class IB chaperones are currently unknown. However, as demonstrated with *Sodalis* CCBD-containing effectors, candidate effectors from bacteria that encode functionally interchangeable class IB chaperones can be screened for those that are recognized as substrates of the *Shigella* T3SS. Once likely effectors of these relatively poorly characterized T3SSs are identified, these proteins can be used as a readout to identify *in vitro* conditions that induce their respective native T3SSs. Furthermore, our discovery that class IB chaperones recognize a conserved sequence provides a new means to pursue the development of novel antimicrobial agents using rationale drug design approaches.

MATERIALS AND METHODS

Plasmids. All of the plasmids involving *S. flexneri* and many of the *S.* Typhimurium ORFs have been previously described (16, 42). The remaining bacterial and yeast expression plasmids were created via Gateway (Invitrogen) site-specific recombination (16).

Basically, the ORF encoding each effector and each chaperone was amplified from genomic DNA (gDNA) by nested PCR such that it is flanked by attB sites. In addition to an attB site, a Shine-Dalgarno sequence was introduced upstream of each ORF. We created open (lacking a stop codon) and closed versions of each ORF when carboxy fusions were desired. Mutant alleles of the effectors were created by sewing PCR. The amplified genes were then introduced into pDNR223 and/or pDNR221 to create Gateway entry vectors via BP reactions (Invitrogen). Each insert

was sequence verified and subsequently transferred to a variety of Gateway destination vectors via LR reactions (Invitrogen).

For the μNS fusion proteins in the PIP assays, the chaperones and effectors were introduced into pAG416GAL-μNS-ccdB, pAG415GAL-μNS-ccdB, and/or pAG415GAL-CFP-μNS-ccdB. For the GFP fusion proteins, the chaperones and the *Salmonella* effectors, with the exception of SipA, were introduced into pAG413GAL-GFP-ccdB and pBY011 (43). Conventional cloning was used created GFP-SipA (amino acids 1 to 254) in pRS313.

To generate FLAG tag fusions for the secretion assays, the wild-type, mutant, and hybrid effectors were introduced into pDSW206-FLAG-ccdB (ColE1 ori, ampicillin resistance) (16, 25), and the chaperones were introduced into pNG162-ccdB (p204 promoter [IPTG-inducible], pSC101 ori, spectinomycin resistance) (16, 44).

To generate TEM-1 fusions for the translocation assay, the *Shigella* and *Sodalis* effectors were introduced into pDSW206-TEM-1-ccdB (ColE1 ori, ampicillin resistance) (a gift from John Leong and Loranne Magoun, University of Massachusetts Medical School)

Finally, for chaperone stability, chaperones were tagged with Myc at their N termini and introduced onto the pNG162-ccdB plasmid.

All oligonucleotide primers used in the constructs described here are described in Table S2 in the supplemental material.

Conventional (manual) PIP assays. Yeast assays were preformed as previously described (16). Yeast cells were visualized 4 to 6 h after galactose induction using a Nikon TE300 microscope with Chroma Technology filters and a 100× objective. Images were captured digitally using a black-and-white Sensys charge-coupled-device (CCD) camera and IP LAB software (Scanalytics).

High-content image analysis (automated PIP assays). The yeast cells were grown in 96-well microtiter plates (Nunc) as previously described (16) and transferred into 96-well microtiter plates (BD). Each experimental condition was performed in duplicate on a plate. Three sets of identical plates were examined, bringing the total number of replicates to six per condition. The cells were imaged at a magnification of ×40 in each well using the IN Cell Analyzer 1000 (GE Healthcare, Piscataway, NJ). Briefly, the IN Cell Analyzer 1000 is an epifluorescence microscope with fully automated image acquisition capabilities, including a motorized stage, motorized filter wheel, and computer-controlled CCD camera. Three fields were preselected to avoid sampling bias throughout the plate. Exposure parameters were empirically optimized for GFP fluorescence to ensure that images fall within the linear range of exposure. Following exposure optimization, images were collected in selected wells and stored for analysis. Images were analyzed using IN Cell Workstation Software (GE Healthcare). Cells were segmented using the nucleus "Top Hat" algorithm and a 1-μm cell collar. Cell detection was determined by endogenous GFP expression within the yeast. Foci or pip were identified as an organelle between 0.2 μm and 2.0 μm with a mean organelle intensity/cell intensity ratio above 1.5. Output parameters included the following: cell count, nucleus area (whole yeast cell), nucleus intensity (whole yeast cell intensity), organelle count, organelle intensity, organelle intensity/cell intensity, percent pip formation, and total number of pip formation. Cells were classified as pip positive if cells expressed ≥1 organelle; otherwise, cells were classified as pip negative. Cells not expressing GFP were not analyzed.

Secretion and chaperone stability assays. The pDSW206-based plasmids encoding each of the IPTG-inducible FLAG-tagged effectors were transformed into the designated *Shigella* strains. Complementation experiments were performed with a *Shigella* Δ*spa15* mutant strain, where homologous chaperones were expressed under an IPTG-inducible pNG162 vector. Congo red type 3 secretion assays were conducted as previously described. Basically, the total cell and supernatant fractions were separated by two centrifugations (each centrifugation at 20,000 × *g* for 2 min). The cell pellet of the first centrifugation was taken as the total cell fraction. Proteins in the supernatant of the second centrifugation were precipitated with trichloroacetic acid (TCA) (10% [vol/vol]). Protein content of the pellet and supernatant fraction were assessed by Western blotting with anti-FLAG antibody (Sigma). For loading control, membranes were probed with anti-IcsA (an autotransporter that is cleaved and released into the media once it reaches the surface of *Shigella*). We have previously established that lysis is not an issue using this protocol, as we rarely, if ever, detect evidence of cytoplasmic proteins (16) or unprocessed IcsA.

High-throughput TEM-1-based translocation assay. Translocation of effectors into HeLa cells was performed as previously described (36) with minor modifications. Basically, bacteria were grown overnight in tryptic soy broth medium (BD Scientific) in a 96-well plate. In the morning, the cultures were back diluted (1/100), and 1.0 mM IPTG was added when the bacterial cultures reached an optical density at 600 nm (OD_{600}) of 0.6. Thirty minutes after induction, bacteria were centrifuged, and the pellet was resuspended in phosphate-buffered saline (PBS). HeLa cells (1.5×10^4 cells/well, in 96-well black plates with clear bottom [Costar]) preloaded with CCF4/AM according to the manufacturer's instructions (Invitrogen) were infected at an MOI of 100. The plates were centrifuged for 10 min to promote contact of the bacteria with HeLa cells. IPTG (0.1 mM) was added to the medium, and the plates were incubated at 37°C for 2 h. Subsequently, the infected cells were fixed with paraformaldehyde, and the percentage of effector translocated was assessed via fluorescence microscopy (Nikon TE300 microscope with Chroma Technology filters and a 40× objective) by determining the percentage of cells that fluoresce blue.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00243-11/-/DCSupplemental.

Figure S1, PDF file, 0.2 MB.
Figure S2, TIFF file, 0.9 MB.
Figure S3, TIFF file, 0.9 MB.
Figure S4, TIFF file, 1 MB.
Figure S5, TIFF file, 0.2 MB.
Figure S6, TIFF file, 0.3 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0.1 MB.

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