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Identification and Characterization of Small-Molecule Inhibitors of Yop Translocation in *Yersinia pseudotuberculosis*▽

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Type three secretion systems (TTSSs) are virulence factors found in many pathogenic Gram-negative species, including the family of pathogenic *Yersinia* spp. *Yersinia pseudotuberculosis* requires the translocation of a group of effector molecules, called Yops, to subvert the innate immune response and establish infection. Polarized transfer of Yops from bacteria to immune cells depends on several factors, including the presence of a functional TTSS, the successful attachment of *Yersinia* to the target cell, and translocon insertion into the target cell membrane. Here we employed a high-throughput screen to identify small molecules that block translocation of Yops into mammalian cells. We identified 6 compounds that inhibited translocation of effectors without affecting synthesis of TTSS components and secreted effectors, assembly of the TTSS, or secretion of effectors. One compound, C20, reduced adherence of *Y. pseudotuberculosis* to target cells. Additionally, the compounds caused leakage of Yops into the supernatant during infection and thus reduced polarized translocation. Furthermore, several molecules, namely, C20, C22, C24, C34, and C38, also inhibited ExoS-mediated cell rounding, suggesting that the compounds target factors that are conserved between *Pseudomonas aeruginosa* and *Y. pseudotuberculosis*. In summary, we have identified 6 compounds that specifically inhibit translocation of Yops into mammalian cells but not Yop synthesis or secretion.

Many pathogenic Gram-negative bacteria express a type three secretion system (TTSS) that translocates effector proteins into the cytosol of their eukaryotic cell targets. Once introduced into host cells, these proteins subvert normal cell functions, e.g., by disrupting innate immune signaling or modulating the phagosomal environment (7, 51, 67, 73). TTSSs are comprised of a base structure, a needle, and a tip/translocon complex (52). The base structure, which spans the inner and outer membranes, shares high structural homology to conserved bacterial flagellar machinery (11). High-resolution microscopy of the base structures of *Shigella* and *Salmonella* revealed that the base consists of several ring structures that surround a hollow cavity (10, 41, 46). The needle is comprised of a small protein that polymerizes to form a hollow tube that starts within the base and protrudes from the bacterial surface (30, 41, 71). Effectors are thought to be translocated through the needle (19, 37, 41, 46), although this has not been demonstrated conclusively for many systems. Many TTSSs secrete effectors into culture supernatants with just the base and needle; however, translocation of effectors into mammalian cells requires three additional components, together called the translocon (28, 31). Two proteins (9, 28, 59) are inserted into the eukaryotic cell membrane to form a pore. The third (53) is critical for proper assembly of the translocon and is localized at the distal end of the needle but is not inserted into the host plasma membrane.

There are three species of *Yersinia* which are pathogenic to humans. *Yersinia pseudotuberculosis* (32) and *Y. enterocolitica* both cause gastroenteritis and lymphadenitis and are commonly transmitted via the fecal-oral route (66). *Y. pestis* is the causative agent of bubonic and pneumonic plague and is commonly transmitted by a flea vector from infected rodents to humans (1, 14). It disseminates through the skin to the lymph nodes, where it causes a bubonic disease. Occasionally, *Y. pestis* disseminates to the lungs of an infected individual, which can lead to a pneumonic transmission from person to person, resulting in a fatal lung infection (42, 66). The TTSS is an essential virulence factor for all three pathogenic *Yersinia* spp. (6, 17, 32, 56). *Yersinia* strains lacking this secretion system can function as live attenuated vaccine strains in mice (6, 61). The critical needle and translocation components of the *Yersinia* TTSS include the needle protein (YscF) (30), the tip protein (LcrV), and the pore-forming proteins (YopB and YopD) (44, 72). The effector proteins translocated by the *Yersinia* TTSS, called Yops, are targeted to neutrophils, macrophages, and dendritic cells, where they inactivate the bactericidal effects of these cells during murine infection (21, 39, 45). Inactivation of the TTSS leads to defective colonization of systemic organs and clearance of the bacteria by the host organism (6, 29, 74).

The process of translocation in *Yersinia* requires close contact between the host cell and the bacterium (8). For the enteric *Yersinia* spp., this contact is mediated by two adhesins, YadA and invasin (8, 36, 82). Both of these molecules bind β1 integrins on the surfaces of target cells (22, 35). In cultured cells, stimulation of β1 integrins by ligands activates Src kinases and RhoA, which in turn enhances translocation of Yops (47). In the absence of Yops, activation of β1 integrins leads to actin rearrangements resulting in bacterial internalization (50). However, in *Yersinia* strains expressing the TTSS and Yops, this process is antagonized by the effector proteins (8). The result is that virulent *Yersinia* adheres tightly to mammalian cells while remaining extracellular.

Since the TTSS is essential for virulence of *Yersinia* and

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli strains		
SY327λpir	Conjugation strain	R. Isberg
Sm10λpir	Conjugation strain	49
Y. pseudotuberculosis strains		
IP2666/pIB1	Wild type	64
IP2666 E-TEM	Amino acids 1 to 100 of YopE + TEM1	This study
IP2666 Δ*yopB*	Deletion of *yopB* (codons 19 to 346)	26
IP2666 Δ*yopB* E-TEM	Δ*yopB* deletion + amino acids 1 to 100 of YopE + TEM1	This study
IP2666 Δ*yscF*	Deletion of *yscF* (codons 2 to 86)	19
IP2666 Δ*lcrV*	Deletion of *lcrV* (codons 19 to 326)	18, 19
IP2666 Δ*yopN*	Deletion of *yopN* (codons 2 to 287)	19
IP2666 Δ*inv*	Deletion of *inv*	21
IP2666 Δ*inv* pYV$^-$	Δ*inv*; virulence plasmid negative	This study
IP2666 Δ*yadA*	Deletion of *yadA*	21
IP2666 Δ*inv* Δ*yadA*	Deletion of *inv* and *yadA*	This study
P. aeruginosa strains		
Pa388	Wild type	80
Pa388 *pscC*	*pscC*::Tn*5Tc*	80
Plasmids		
pSR47S	Gene replacement vector; Kan^r	48
pSR47S-E-TEM	YopE-TEM1; Kan^r	This study
pDS132	Single-copy vector; Cm^r	63
pDS132-YadA	pDS132 expressing YadA; Cm^r	M. Fisher
pCVD442	Suicide vector; Ap^r	20
pCVD442-Δ*yadA*	Suicide vector for deleting *yadA*; Ap^r	21

other Gram-negative pathogens, this system has been a target for development of novel therapeutics (3, 24, 27, 38, 62, 79). Several screens have been designed to identify inhibitors of TTSS synthesis and/or Yop secretion from the bacteria (3, 24, 62). Such inhibitors should also block translocation of effectors into mammalian target cells and therefore abrogate virulence. These screens have led to the identification of several classes of molecules that inhibit not only the TTSS of *Yersinia* but also the TTSSs of other pathogens, such as *Chlamydia*, *Salmonella*, and *Shigella* (5, 33, 57, 58, 77). Here we describe a screen to identify small molecules that block translocation of effectors into mammalian cells. The small molecules that were identified were unique in that they still permitted secretion of Yops from bacteria, but they reduced the polarized translocation of Yops into target cells and caused excessive leakage of Yops into culture supernatants. These compounds may represent novel agents that target effector translocation, an essential process for virulence in *Yersinia* and other TTSS-containing pathogens.

MATERIALS AND METHODS

Strains and bacterial culture conditions. Strains used in this study are listed in Table 1. *Y. pseudotuberculosis* strains were cultured in Luria broth (L broth) at 26°C overnight with aeration. Unless otherwise indicated, strains were diluted 1:40 in 2× YT (16 g tryptone, 10 g yeast extract, 5 g NaCl per liter) containing 5 mM $CaCl_2$ and were incubated at 26°C for 1.5 h with aeration, followed by incubation at 37°C for 1.5 h with aeration to induce synthesis of the TTSS. Compounds were added at a 60 μM final concentration at the shift to 37°C. In some experiments, strains were subcultured 1:40 in secretion medium (2× YT supplemented with 20 mM sodium oxalate and 20 mM $MgCl_2$) and grown as described above.

Escherichia coli and *Pseudomonas aeruginosa* were cultured in L broth at 37°C with aeration overnight. The next morning, *P. aeruginosa* strains were diluted 1:40 in L broth and grown at 37°C for 2 h before the addition of compounds at 60 μM. Cultures were then incubated for an additional 2 h before infection of HEp-2 cells. Overnight cultures of *E. coli* were diluted 1:50 in 2× YT and were grown at 37°C. After a 1.5-h incubation at 37°C, compounds were added to a 60 μM final concentration and then incubated at 37°C for another 1.5 h. Chloramphenicol was used in cultures of *E. coli* at a concentration of 10 μg/ml for maintenance of pDS132 and pDS132-YadA.

To generate the construct encoding the YopE secretion and translocation signals fused to TEM1, the TEM1 fragment was PCR amplified from pBR322, using primers TEM1F and TEM1R (Table 2), and then cloned into the pGEM-T Easy system (Promega). The TEM1 fragment was then digested with NotI and SacI and cloned into pSR47S, generating pSR47S-TEM1. The DNA sequence encoding the first 100 amino acids of YopE was PCR amplified using primers ETEMF and ETEMR, cloned into pGEM-T Easy, and then subcloned into pSR47s-TEM1 by use of BamHI and NotI, creating pSR47s-E-TEM. The pSR47s-E-TEM plasmid was introduced into SY327λpir. The YopE-TEM fusion (E-TEM) was introduced at the IP2666 *yopE* locus by allelic exchange. The E-TEM allele was also introduced into the IP2666 *yopB* strain to generate a Δ*yopB* E-TEM strain. Strains were tested to demonstrate that E-TEM, YopE, and other Yops were secreted normally under secretion-inducing conditions.

The *yadA* gene was deleted in the Δ*inv* background by allelic exchange (20). pCVD442-yadAKO (21) was conjugated into IP2666 Δ*inv* as described previously (43).

Tissue culture. HEp-2 cells were maintained in RPMI 1640 (Cellgro) with 5% fetal bovine serum (FBS) at 37°C and 5% CO_2. For all experiments, HEp-2 cells were seeded into either 6-, 24-, or 96-well treated tissue culture plates ~18 h prior to experimentation, at 6×10^5, 2×10^5, or 1.5×10^4 cells per well, respectively. The following standard procedure for HEp-2 cell infections was used for all tissue culture experiments, unless otherwise indicated. *Y. pseudotuberculosis* strains used for infection of HEp-2 cells were gently washed in phosphate-buffered saline (PBS) and resuspended in RPMI with 5% FBS containing

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)
TEM1F	GAGAGAGCGGCCGCCACCCAGAAACGCTGGTG
TEM1R	AGACAGAGCTCGCATGCTGAGTAAACTTGGTCTGACAGT
ETEMF	GGATCCGCATGCGCACTCTCGGCAGACCATC
ETEMR	GGCGGCCGCTAGGACTTGGCATTTGTG

60 μM compound or 0.3% dimethyl sulfoxide (DMSO). This mixture of medium, bacteria, and compound was then used to replace the culture medium on HEp-2 cells. Infection was started by centrifuging the bacteria onto the cells at 290 × *g* for 5 min at room temperature (RT). Plates were moved to 37°C and 5% CO_2 for the remainder of the infection.

High-throughput screen. HEp-2 cells were seeded into 384-well plates at a density of 1×10^4 cells/well in a volume of 25 μl. The CCF2-AM reagent was prepared per the manufacturer's instructions (Invitrogen). Five microliters of the CCF2 mixture was added to each well to yield a final concentration of 1 μg/ml CCF2 per well, and plates were incubated at 30°C for 30 min. After incubation, compounds were transferred to plates by pin transfer. *Y. pseudotuberculosis* IP2666 E-TEM and IP2666 Δ*yopB* E-TEM were grown in 2× YT supplemented with 5 mM $CaCl_2$. *Y. pseudotuberculosis* cells were washed in warm PBS, adjusted to a multiplicity of infection (MOI) of 80:1, and then added to wells containing CCF2-AM and compounds. The plates were incubated at 37°C for 30 min to permit exposure of the bacteria to the compounds before centrifuging them at 290 × *g* for 5 min to initiate the infection. The infection was allowed to proceed for 60 min, 100 μg/ml of gentamicin was added to each well, and the green (520 nm) and blue fluorescence (447 nm) was determined on an EnVision plate reader (Perkin Elmer, Waltham, MA).

For each experiment, a separate control plate was included, with wells that contained only HEp-2 cells to control for background fluorescence signals. In addition, 12 positive controls (wild-type [WT] IP2666 E-TEM) and 12 negative controls (IP2666 Δ*yopB* E-TEM) were included in the last two rows of each plate as plate-specific controls. To determine the values for green and blue fluorescence in each well, first the background green and blue fluorescence was determined by calculating the average green and blue fluorescence in the control plate containing just HEp-2 cells. The background green and blue fluorescence minus 1 standard deviation was then subtracted from the green and blue fluorescence values measured for each well in each plate containing compounds. The ratio of blue to green fluorescence in each well was determined, and the data were sorted by this ratio to identify wells containing compounds that exhibited low ratios (i.e., reduced translocation of E-TEM). For each plate, Z and Z' factors were determined (83). The screen was optimized to yield Z and Z' values between 0.2 and 0.5. When the screen was optimized to yield Z values of >0.5, no hits were detected after screening 20,000 compounds. Potential hits were selected based on the criteria that the ratios fell outside 3 standard deviations for the whole plate and their intrinsic green fluorescence value was within the range of those found for cells infected with the Δ*yopB* E-TEM mutant. Occasionally, we observed Z factors of <0, and in these instances of high variability, there was a very high likelihood of a false-positive result. However, plates with Z factors of <0 were analyzed, and some compounds had a ratio within the range for the Δ*yopB* controls. In these cases, if the adjusted values of blue and green fluorescence fell within the range for the Δ*yopB* control wells, these compounds were included in a preliminary list of potential hits. Of the 100,000 compounds screened, 200 compounds were deemed potential hits based on these criteria. Forty-five compounds were purchased from various companies and tested in a second assay. Libraries were obtained and screened at the ICCB-Longwood screening facility at Harvard University (http://nsrb.med.harvard.edu/about.html). A number of libraries in the collection were screened, including ChemDiv 2, ChemDiv3, ChemDiv4, Maybridge 3, Maybridge 4, and Biomol (http://iccb.med.harvard.edu/screening/compound_libraries/index.htm). Compounds described in this study were purchased from the following manufacturers: C7, C20, C22, and C24 were purchased from TimTec, Inc., and C15, C19, C34, C35, and C38 were purchased from ChemDiv. Compounds were diluted in DMSO to 20 mM stocks and stored at −20°C.

Cell rounding assay. HEp-2 cells were seeded into a 96-well plate at a density of 1.5×10^4 cells/well in a volume of 100 μl. *Y. pseudotuberculosis* was diluted to 1.5×10^6 CFU/ml in RPMI 1640 supplemented with 5% FBS and 60 μM compound or 0.3% DMSO. The cell culture medium was replaced with 100 μl medium containing *Y. pseudotuberculosis* and a compound. After centrifugation, the infection was allowed to proceed for 45 min before imaging. Cells were examined on a Nikon Eclipse TE2000-U microscope (Melville, NY).

Pseudomonas aeruginosa cell rounding assays were performed as described above. Infections were allowed to proceed for 90 min prior to imaging.

FITC-phalloidin staining. HEp-2 cells were seeded into 96-well plates at a density of 5×10^3 cells/well in 100 μl RPMI supplemented with 5% FBS. Compounds were diluted to 60 μM in RPMI with 5% FBS. The medium on the monolayer was replaced with 100 μl of medium with compounds and then incubated at 37°C and 5% CO_2 for 2 h. Cells were fixed in 4% paraformaldehyde for 20 min at RT. The monolayer was washed three times with PBS and then permeabilized by the addition of 0.5% Triton X-100 in PBS for 15 min at RT. Fluorescein isothiocyanate (FITC)-phalloidin was added at a concentration of 130 nM and allowed to incubate at RT for 30 min. After incubation, monolayers were washed three times with PBS, incubated for 1 min with 1.6 μM DAPI (4′,6-diamidino-2-phenylindole), and then washed another three times in PBS. Images were obtained with a Nikon TE2000-U inverted microscope (Molecular Devices, Sunnyvale, CA).

Bacterial growth curves. WT IP2666 was grown overnight at 26°C in L broth with aeration. The cultures were then diluted 1:50 in 2× YT and allowed to incubate with shaking at 26°C for 1 h. At this time, defined as time zero, the optical density at 600 nm (OD_{600}) was measured, and compounds were added to a final concentration of 60 μM. Cultures were returned to 26°C with aeration, and OD_{600} measurements were taken each hour for 7 h.

LDH release assays. HEp-2 cells were seeded into a 96-well plate at a density of 2×10^4 cells/well in 100 μl RPMI supplemented with 5% FBS. Compounds were resuspended in RPMI supplemented with 5% FBS to a final concentration of 60 μM. The medium was replaced with a volume of 100 μl, and cells were incubated at 37°C and 5% CO_2. A 50-μl sample was taken from the wells at 2 h and 24 h for quantification of lactate dehydrogenase (LDH). LDH in supernatants was quantified using a Promega Cytotox 96 nonradioactive cytotoxicity assay kit. Control samples for lysis in the absence of compounds were incubated for 45 min in lysis buffer (supplied in the kit) and then collected at the same time points. The OD_{492} was measured spectrophotometrically on a SpectraMax 5 plate reader (Molecular Devices, Sunnyvale, CA). Each experiment was performed in triplicate and repeated twice.

Indirect immunofluorescence microscopy. *Y. pseudotuberculosis* strains were grown in the presence of compounds as described above. Immunofluorescence was performed as described previously (18). Micrographs were taken with a Nikon TE2000-U inverted microscope with a Photometrics charge-coupled device (CCD) camera at a magnification of ×60, using MetaVue software (Molecular Devices, Sunnyvale, CA). DAPI and Alexa 594 were visualized using Nikon UV-2E/C and G-2E/C filters, respectively. Images were pseudocolored and merged in MetaVue.

Chemical cross-linking. *Y. pseudotuberculosis* was grown in 2× YT supplemented with 5 mM $CaCl_2$ and with 60 μM compound as described for immunofluorescence assay and was subjected to cross-linking with 1 mM BS^3 (a non-membrane-permeating cross-linker) as described previously (2).

Secretion of Yops into culture supernatants. Secretion of Yops into culture supernatants was performed as described previously (19). Briefly, *Y. pseudotuberculosis* was grown in secretion-inducing medium. After 90 min at 37°C in the presence of compounds, supernatants were collected and centrifuged to remove bacteria. The clarified supernatants were precipitated in 10% trichloroacetic acid (TCA) and resuspended in 2× SDS sample buffer. The secreted proteins were separated by SDS-PAGE and visualized by Coomassie blue staining.

Translocation and synthesis assays. Translocation assays were performed essentially as described previously (19). HEp-2 cells were seeded into a six-well plate at 6×10^5 cells per well. *Y. pseudotuberculosis* was diluted to 3×10^7 CFU/ml in RPMI 1640 supplemented with 5% FBS and 60 μM compound or 0.3% DMSO to achieve an MOI of 50:1, and 1 ml of this mixture was placed on the HEp-2 cells and then centrifuged at 290 × *g* to initiate the infection. The infection was allowed to proceed for 45 min at 37°C and 5% CO_2. The tissue culture supernatants were collected to determine the amount of YopE leakage (see below). HEp-2 cells were washed two times with cold PBS and then lysed with eukaryotic lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 μM phenylmethylsulfonyl fluoride [PMSF], 10 μM leupeptin, 1 μM pepstatin) for 20 min at 4°C. Lysates were fractionated by centrifugation, and both the soluble fraction (HEp-2 cell cytosol) and the insoluble fraction (*Y. pseudotuberculosis* and HEp-2 cell nuclei and membranes) were electrophoresed in a 12.5% Tris-glycine-polyacrylamide gel. The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with anti-YopE (1:10,000), anti-β-actin (1:10,000), or anti-S2 (1:10,000) antibody. Secondary antibodies, i.e., anti-mouse–horseradish peroxidase (HRP) or anti-rabbit–HRP, were used at 1:10,000. Blots were developed using chemiluminescence (Perkin Elmer Western Lightning instruction manual). Images of blots were obtained on a UMax Astra6700 scanner (Techville, Dallas, TX) and were quantified by densitometry using ImageJ (National Institutes of Health). YopE detected in the soluble fraction (translocated YopE) was normalized to the amount of S2 protein detected in the insoluble fraction (equivalent to bacterial cell number). This value was then normalized to the amount of actin protein in the insoluble fraction (equivalent to HEp-2 cell number) to control for sample loading. Percent translocation was determined by comparing the level of YopE detected in the presence of compounds to that in the DMSO control.

Total bacterial YopE protein was measured by normalizing the amount of YopE detected in the insoluble fraction (containing intact bacteria) to the amount of S2 protein detected in the same fraction. The percent YopE synthesis

was determined by comparing the amount of YopE protein detected in the presence of compounds to that in the DMSO control. The experiment was repeated three times.

YopE leakage determination. Cell culture supernatants collected from the translocation assay described above were centrifuged at 16,000 × *g* for 2 min to remove intact *Y. pseudotuberculosis* and HEp-2 cells. Eight hundred microliters of clarified supernatant was mixed with YopE antibody and then added to protein A beads and an equal volume of TNET buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 8.0, and 1% Triton X-100). The samples were incubated with rotation at 4°C overnight. Beads were washed three times in TNET and then boiled in sample buffer lacking reducing agent to release protein. The samples were subjected to 12.5% SDS-PAGE, transferred to PVDF membranes, and probed for YopE. Clean-Blot HRP reagent (Thermoscientific) was used as the secondary antibody. Proteins were visualized using chemiluminescence. The amount of leaked YopE was normalized to the amount of S2 detected in the insoluble fraction collected in the translocation and synthesis assay described above. The ratio of leaked YopE was arbitrarily set to a value of 1 for the DMSO control, and each infection in the presence of compound was compared to it. The experiment was repeated three times.

Statistical analysis. Differences in levels of translocation of Yop leakage in the presence of compounds compared to that in DMSO were determined by a paired *t* test.

Adherence assays. HEp-2 cells were seeded into 96-well plates at a density of 2×10^4 cells/well in 100 μl RPMI. *Y. pseudotuberculosis* was diluted to 2×10^6 CFU/ml in RPMI containing 60 μM compound or 0.3% DMSO. One hundred microliters of the bacteria-compound mixture was added to each well. The bacteria were spun onto the cells at 290 × *g* for 5 min and incubated at 37°C for 30 min, and then the wells were washed vigorously with ice-cold PBS to remove any unbound *Y. pseudotuberculosis*. Both *Y. pseudotuberculosis* and HEp-2 cells were fixed in 4% paraformaldehyde for 60 min and washed three times with PBS. An enzyme-linked immunosorbent assay (ELISA) was performed by incubating the wells with a 1:1,000 dilution of polyclonal rabbit anti-*Yersinia* antibody in 1% bovine serum albumin (BSA) in PBS at RT for 1.5 h with gentle shaking, and then the cells were washed three times in PBS and incubated with a 1:10,000 dilution of anti-rabbit–HRP in 1% BSA in PBS for 1.5 h with shaking. The HRP activity was visualized with the TMB ELISA reagent (Thermoscientific) and was measured spectrophotometrically by determining the OD_{450}. The binding of *E. coli* expressing YadA to HEp-2 cells was determined as described above, with the modification that a 1:1,000 dilution of anti-LamB (a gift of Andrew Wright) was used to detect *E. coli*. Each experiment was done in triplicate and repeated two times. The averages and standard errors are shown for one experiment.

YadA autoagglutination assay. *Y. pseudotuberculosis* was grown at 26°C overnight in L broth in the presence of 60 μM C20 or 0.3% DMSO. A 200-μl inoculum from the overnight culture was introduced into 2 ml warm RPMI. The cultures were incubated statically for 3 h at 37°C and 5% CO_2 in the presence of 60 μM C20 or 0.3% DMSO. The top 100 μl of the culture was removed, and the OD_{600} was measured. The cultures then were vortexed, and the OD_{600} was read again. The ratio of the OD_{600} of the settled culture to that of the vortexed culture was determined, and the ratio for IP2666 in 0.3% DSMO was set to 100%. The Δ*yadA* strain in 0.3% DMSO was set to 0% autoagglutination. Each experiment was done in triplicate and repeated two times. The averages and standard errors are shown for one experiment.

Hemolysis of SRBCs. Hemolysis assays were conducted as described previously (19). Briefly, *Y. pseudotuberculosis* was grown in secretion medium and supplemented with 60 μM compound. *Y. pseudotuberculosis* was pelleted at 16,000 × *g* for 2 min and then resuspended to a concentration of 1×10^9 CFU/ml. Sheep red blood cells (SRBCs; Innovative Research, Southfield, MI) were washed three times in cold 1× PBS and resuspended to 1×10^9 cells/ml in warm RPMI. The SRBCs and *Y. pseudotuberculosis* were mixed at an MOI of 1:1 in the presence of 60 μM C20 or 0.3% DMSO in a round-bottomed 96-well plate and then were pelleted at 2,000 × *g* for 7 min to bring *Y. pseudotuberculosis* into contact with SRBCs. The infection was allowed to proceed for 3 h at 37°C and 5% CO_2. After the incubation, RPMI was removed from SRBCs and replaced with cold 1× PBS containing 100 μg/ml gentamicin and 60 μM C20 or 0.3% DMSO. The SRBCs were incubated overnight at room temperature. The next morning, the SRBCs were gently resuspended and then pelleted at 2,000 × *g*, and the OD_{545} of the supernatant was determined on a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA). The percent lysis of SRBCs in the presence of each adhesin mutant or the C20 compound was normalized to the percent lysis of SRBCs by WT *Y. pseudotuberculosis* in 0.3% DMSO (set to 100%). The experiment was done in triplicate and repeated three times.

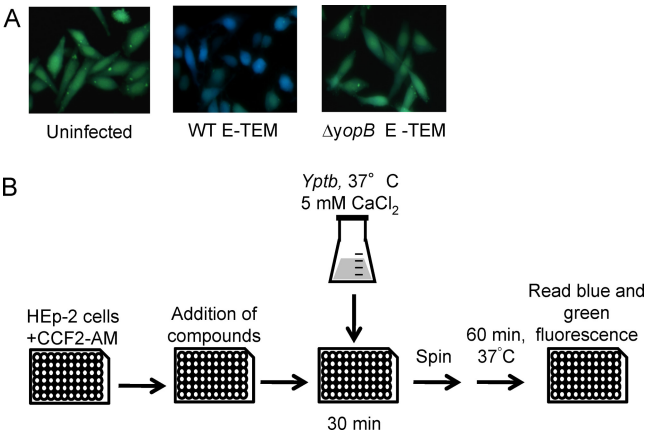

FIG. 1. HTS for small-molecule inhibitors of Yop translocation. (A) Fluorescence micrographs of HEp-2 cells loaded with CCF2-AM. (Left to right) Uninfected cells, HEp-2 cells infected with the WT E-TEM strain, and cells infected with the Δ*yopB* E-TEM strain. (B) Schematic of HTS. HEp-2 cells were loaded with CCF2-AM. Compounds were introduced by pin transfer, and *Y. pseudotuberculosis* (*Yptb*) was added at an MOI of 80:1. The compounds and *Y. pseudotuberculosis* were incubated together for 30 min before centrifugation to bring *Y. pseudotuberculosis* into contact with HEp-2 cells. After 60 min, the raw fluorescence values at 447 nm and 520 nm were determined on a plate reader.

RESULTS

HTS to identify small-molecule inhibitors of Yop translocation. We designed a high-throughput screen (HTS) to identify small molecules that would prevent or reduce translocation of Yops into mammalian cells, a critical facet of *Yersinia* virulence. We used a fluorescence-based system to monitor translocation of a chimeric protein into HEp-2 cells. The chimeric protein, E-TEM, is composed of the first 100 amino acids of YopE, which contains its secretion and translocation signals that are required to direct it into mammalian cells (70), fused to a fragment of β-lactamase (TEM1) (16). A recombinant *Y. pseudotuberculosis* strain expressing E-TEM (WT E-TEM) was used to infect HEp-2 cells treated with the non-membrane-permeating, nonfluorescent dye CCF2-AM (84). CCF2-AM is comprised of fluorescein conjugated to coumarin by a lactam ring. Outside the mammalian cell, the dye is nonfluorescent, but once it is taken up by the cell, it is modified by esterases and becomes trapped. Modified CCF2-AM fluoresces green when excited at 409 nm due to fluorescence resonance energy transfer (FRET) from the coumarin to the fluorescein ring (Fig. 1A, uninfected). If the lactam ring between the fluorophores is cleaved by TEM1, the FRET is lost and the coumarin fluoresces blue (Fig. 1A, WT E-TEM), indicating that E-TEM has been translocated into the host cell. If *Y. pseudotuberculosis* is unable to translocate E-TEM into host cells, CCF2-AM will remain uncleaved and the cells will fluoresce green (Fig. 1A, Δ*yopB* E-TEM). A measure of green-to-blue conversion can be obtained on a fluorescence plate reader, and this assay is therefore amenable to a high-throughput approach.

To screen for small molecules that block translocation, HEp-2 cells were seeded into 384-well plates and incubated with CCF2-AM (see the screening schematic in Fig. 1B). For each 384-well plate, one row of cells was infected with WT

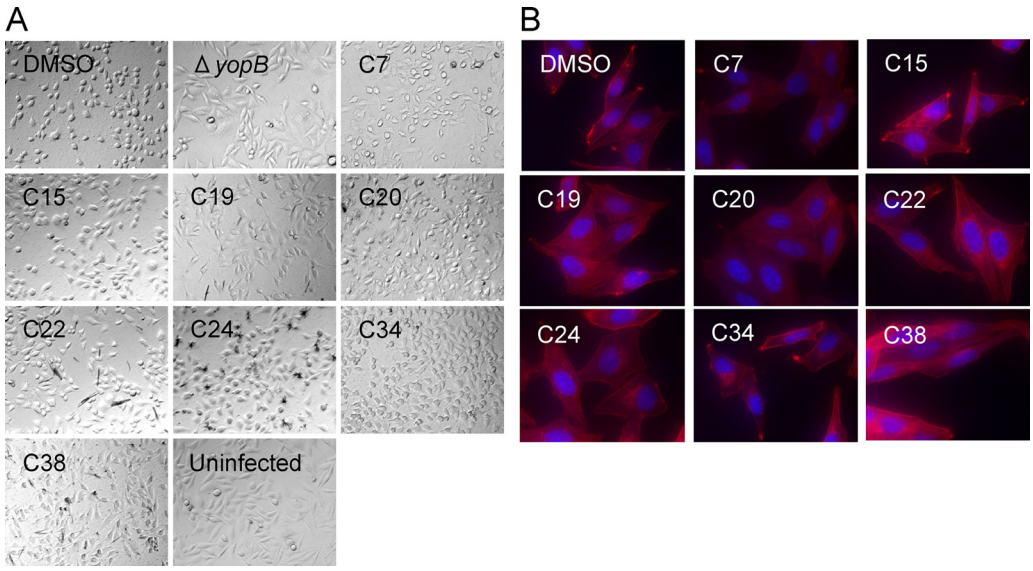

FIG. 2. YopE-mediated cell rounding was reduced after exposure of bacteria and HEP-2 cells to compounds. (A) *Y. pseudotuberculosis* was grown at 37°C in the presence of Ca^{2+} and compounds. HEp-2 cells were infected with WT IP2666 or IP2666 Δ*yopB* at an MOI of 10:1 in the presence of 60 μM compound (indicated in each panel). Images were taken at 45 min postinfection. (B) HEp-2 cells were cultured for 2 h in the presence of 60 μM compound in the absence of *Y. pseudotuberculosis*. Actin was visualized with FITC conjugated to phalloidin, and DAPI was used to visualize nuclei.

E-TEM but not exposed to compounds, which served as a positive control for translocation. Another row was infected with the Δ*yopB* E-TEM strain, which secreted Yops and E-TEM but was unable to translocate them, serving as a negative control. The remaining wells received compounds in concentrations ranging from 20 to 60 μM, as well as WT E-TEM. The *Y. pseudotuberculosis* cells were grown under conditions where the TTSS was expressed and primed for Yop secretion but the Yops were not secreted (see Materials and Methods). WT E-TEM was incubated with the compounds for 30 min prior to centrifugation of *Y. pseudotuberculosis* onto the monolayer to permit the exposure of *Y. pseudotuberculosis* to compounds prior to contact with cells and initiation of infection. Sixty minutes after the initiation of infection, the levels of both green and blue fluorescence in each well were determined by a plate reader. Raw values for blue and green intensities were adjusted to exclude background fluorescence, and the ratios of blue to green fluorescence were calculated (see Materials and Methods). Failure to translocate the E-TEM construct in the presence of compounds resulted in low blue/green fluorescence ratios.

Approximately 100,000 compounds were screened and ranked based on their ratios of blue to green fluorescence. Most wells infected with WT E-TEM and exposed to compounds exhibited ratios that grouped together with the positive controls. Compounds that led to a low blue/green ratio were further analyzed to determine if the low ratio was due to aberrantly high green fluorescence caused by autofluorescence in the wells. Only compounds with low blue/green ratios and green values that fell within the range of the typical observed values for the plate were considered for further analysis.

Roughly 200 of the wells exposed to compounds yielded low blue/green ratios. Of these, we acquired and screened the top 45 compounds in a second assay that did not rely on TEM1 activity or CCF2-AM fluorescence but was dependent on translocation of an effector, YopE, into HEp-2 cells. YopE, a Rho-GTPase activating protein (Rho-GAP), disrupts signaling by RhoA, Rac1, and RhoG (7, 50, 78), leading to rounding of cells and detachment from the tissue culture plate, a phenotype that is easily visualized by light microscopy. The cells infected with WT *Y. pseudotuberculosis* in the presence of DMSO alone (Fig. 2A, DMSO) showed a rounded phenotype, consistent with high levels of translocation of YopE. Cells infected with *Y. pseudotuberculosis* that was incapable of translocating YopE (Fig. 2A, Δ*yopB*) remained flat, similar to the uninfected cells. Of the 45 compounds tested, 13 inhibited cell rounding at 60 μM (Fig. 2A and data not shown), suggesting that the molecules prevented normal levels of YopE translocation. One of the compounds that diminished cell rounding could not be obtained in large enough quantities for subsequent characterization. We also investigated whether the compounds caused perturbations of the actin cytoskeleton in HEp-2 cells, which may influence translocation of Yops into target cells. After a 2-h exposure to just the compounds, actin stress fibers were detected by FITC-rhodamine. Those compounds that inhibited *Y. pseudotuberculosis*-mediated cell rounding did not appear to disrupt the actin cytoskeleton in HEp-2 cells (Fig. 2B).

Eight compounds do not cause significant toxicity to HEp-2 cells or *Y. pseudotuberculosis*. The 12 compounds remaining after the above tests were next evaluated for toxicity to either *Y. pseudotuberculosis* or HEp-2 cells. To determine if the compounds inhibited bacterial growth, we grew *Y. pseudotuberculosis* at 26°C for 7 h in the presence of a 60 μM concentration of each compound. Nine of the compounds had no effect on bacterial growth under these conditions (Fig. 3A), while three proved to be antibacterial (data not shown). The nine nonantibacterial compounds were assessed for the ability to cause

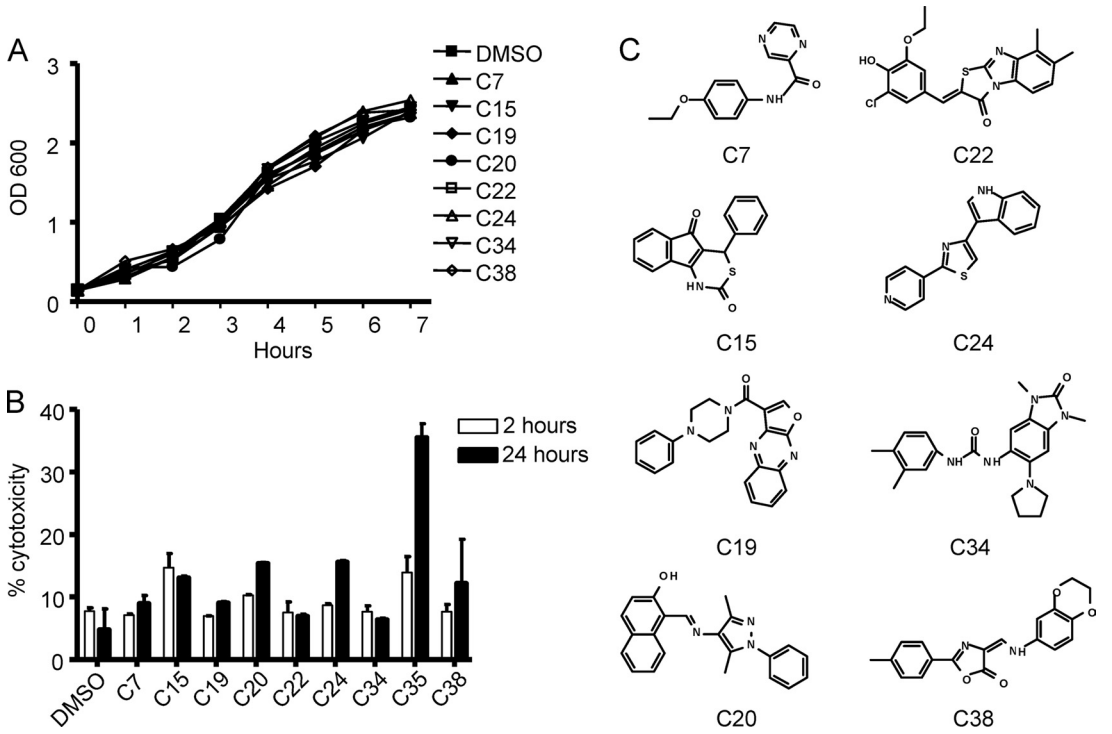

FIG. 3. Compounds are not toxic to *Y. pseudotuberculosis* and have limited toxicity to HEp-2 cells. (A) Growth of *Y. pseudotuberculosis* was not inhibited by incubation with compounds. *Y. pseudotuberculosis* was grown in the presence of 60 μM compound or 0.3% DMSO for 7 h, and the OD_{600} was recorded each hour. ■, DMSO; ▲, C7; ▼, C15; ◆, C19; ●, C20; □, C22; △, C24; ▽, C34; ◇, C38. (B) LDH release from HEp-2 cells in the presence of 60 μM compound. The amount of LDH released into the supernatants was determined at 2 h and 24 h. The means and standard deviations from one representative experiment are plotted. (C) Structures of compounds that inhibited cell rounding of HEp-2 cells at a concentration of 60 μM and were not antibacterial or cytotoxic to HEp-2 cells.

damage to epithelial cells by monitoring the release of LDH, a cytoplasmic enzyme whose presence in cell supernatants indicates membrane damage. HEp-2 cells were incubated with a 60 μM concentration of each compound in the absence of *Y. pseudotuberculosis*. Tissue culture supernatants were collected after 2 or 24 h and assessed for LDH levels. Two compounds, C15 and C35, caused elevated LDH release at 2 h (Fig. 3B). C15 did not cause further membrane damage between 2 and 24 h of exposure. In contrast, C35 had LDH release levels above baseline at 2 h, and this effect was exacerbated at 24 h (Fig. 3B). Compounds C20 and C24 caused slightly elevated LDH release at 24 h. Because of the high levels of toxicity caused by C35 at both time points, it was excluded from further characterization. In all subsequent experiments with the remaining 8 compounds, HEp-2 cells were exposed to compounds for 2 h or less. The structures of the 8 compounds characterized further are shown in Fig. 3C.

Needles and LcrV are present on the surface of *Y. pseudotuberculosis* in the presence of compounds. Inhibition of cell rounding in the presence of the tested molecules could be due to the inability of *Y. pseudotuberculosis* to form a functional TTSS needle, which is essential for the translocation of YopE (28, 31). To assess whether the TTSS was assembled on the surface of *Y. pseudotuberculosis*, we investigated whether the compounds disrupted needle architecture. First, we assayed for the presence of YscF on the surface of *Y. pseudotuberculosis* by immunofluorescence with anti-YscF antibody. Surface-localized YscF appeared as dots that surrounded the bacterium (Fig. 4A, DMSO/α-YscF panel). Staining with anti-YscF antibody revealed that YscF was associated with *Y. pseudotuberculosis* after growth in all compounds tested (Fig. 4A). YscF staining was reduced in C24- and C38-treated bacteria, but the significance of this was unclear.

To determine if the compounds affected the structure of the needle, chemical cross-linking analysis was performed on bacteria grown in the presence of compounds. BS^3, a non-membrane-permeating cross-linker, covalently links YscF lysine residues between neighboring YscF molecules in the assembled needle, resulting in a characteristic laddering pattern observed by Western analysis with YscF antibody (19, 25) (Fig. 4B, DMSO + BS^3). None of the compounds led to major changes in the cross-linking pattern of the YscF polymer, indicating that the general structure of the needle was not significantly affected by the compounds. There was, however, a slight difference in the structure of the needles formed in the presence of C34. The cross-link of two YscF monomers (as indicated by the asterisk in Fig. 4B) was apparent in *Y. pseudotuberculosis* exposed to DMSO only. In the presence of C34, the cross-link of two monomers was consistently weaker. C15 and C20 also appeared to have a weaker dimer cross-link, but this was not reproducible. Together, the immunofluorescence and cross-linking data indicate that YscF was polymerized on the surface and formed needles that were not detectably different in the

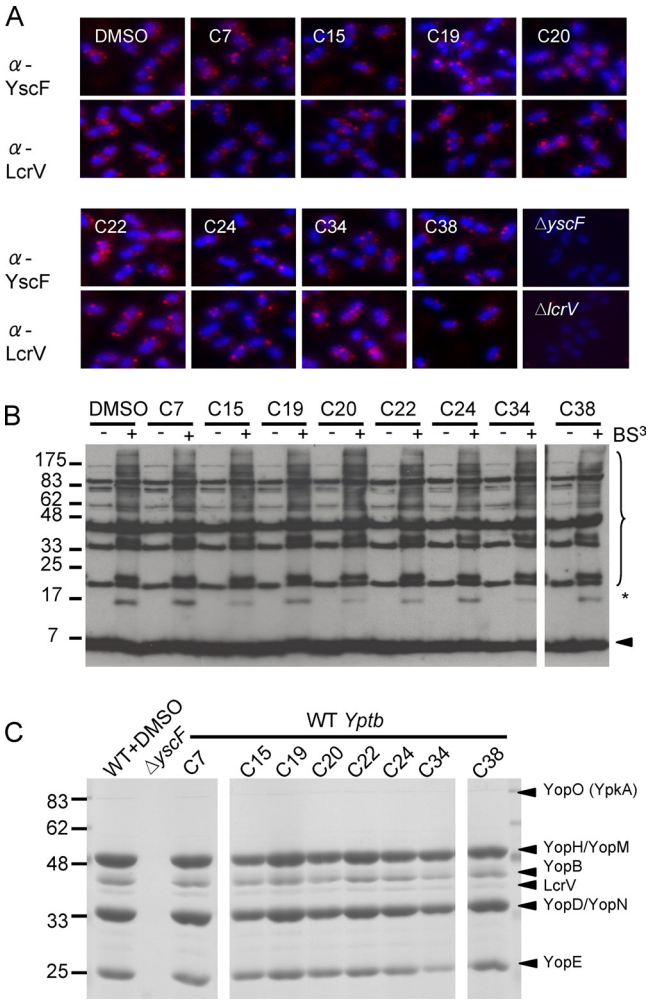

FIG. 4. Localization and assembly of extracellular YscF and LcrV after exposure to compounds. (A) *Y. pseudotuberculosis* was grown in 2× YT supplemented with 5 mM $CaCl_2$ at 37°C with a 60 μM concentration of the indicated compound or with DMSO for 1.5 h. *Y. pseudotuberculosis* was mounted and fixed on coverslips, labeled with anti-YscF or anti-LcrV antibody, and then visualized with Alexa Fluor 594-conjugated anti-rabbit antibody (red stain). Coverslips were counterstained with DAPI (blue stain). Images were pseudocolored and merged in MetaVue. (B) *Y. pseudotuberculosis*, grown in 3 mM $CaCl_2$ with 60 μM compound or 0.3% DMSO, was treated with 1 mM BS^3 or water. *Y. pseudotuberculosis* was solubilized, and Western blot analysis was performed with anti-YscF antibody. The asterisk shows YscF dimers, the arrowhead denotes YscF monomers, and the brace indicates high-molecular-weight YscF polymers. (C) Cultures of *Y. pseudotuberculosis* (*Yptb*) were grown in secretion medium (see Materials and Methods) with 60 μM compound. Yop secretion was detected by precipitation of cultured supernatants in 10% TCA. Proteins were separated by SDS-PAGE and stained with Coomassie blue to detect secreted Yops. The protein concentration was normalized to the OD, and equivalent amounts were loaded in each lane.

presence of compounds from needles formed in the absence of compounds.

LcrV has been observed at the tip of the needle, and this localization is likely required for efficient pore formation and subsequent translocation (12, 13, 53). Therefore, destabilization of LcrV at the tip could lead to reduced translocation. The association of LcrV with the exterior of *Y. pseudotuberculosis* was tested by immunofluorescence with LcrV antibodies (Fig. 4A, DMSO/α-LcrV panel). Similar to anti-YscF staining, surface-localized LcrV appeared as dots surrounding the bacterium. LcrV associated with *Y. pseudotuberculosis* in the presence of a 60 μM concentration of each compound (Fig. 4A), although the overall levels of fluorescence for *Y. pseudotuberculosis* grown in C24 and C38 were slightly reduced, consistent with lower levels of YscF staining. These data suggest that although there may be some differences in the architecture of the needles in the presence of C34, the defects in translocation caused by the compounds were not due to an inability of *Y. pseudotuberculosis* to form needles or an inability of LcrV to localize on the surface.

Secretion of Yops by *Y. pseudotuberculosis in vitro* is not inhibited by compounds. To evaluate the ability of the compounds to block secretion, *Y. pseudotuberculosis* were grown at 37°C in the absence of calcium, which permits high levels of Yop secretion into culture supernatants (81). A strain lacking YscF (Fig. 4C, lane Δ*yscF*) was incapable of secreting Yops and served as a negative control for secretion. The ability to secrete Yops was not impeded by the compounds (Fig. 4C), with the exception of C34, which consistently secreted lower, but detectable, levels of Yops. These results, combined with the immunofluorescence and cross-linking data, suggest that these compounds do not disrupt the ability of *Y. pseudotuberculosis* to form a Yop secretion-competent TTSS.

Compounds reduce translocation of YopE into HEp-2 cells without altering synthesis of Yops. We next evaluated whether the ability of the compounds to reduce cell rounding was due to a defect in translocation of YopE, the effector responsible for this phenotype (7). HEp-2 cells were infected in the presence of compounds or DMSO, and after 45 min, the amount of YopE translocated into HEp-2 cells was determined by Western analysis. Translocated YopE protein levels were normalized to both the total amount of bacteria (Fig. 5A, α-S2) and the total amount of HEp-2 cells loaded (Fig. 5A, α-β-actin). The *yopB* and *yscF* mutants lack necessary components for translocation and secretion, respectively, and as expected, they were defective in translocation (Fig. 5A, lanes Δ*yopB* and Δ*yscF*). A strain carrying a deletion of the regulatory protein YopN, which hypersecretes Yops into culture supernatants, also hypertranslocated Yops into target cells compared to cells infected with the WT (Fig. 5A, lane Δ*yopN*). Analysis revealed that 6 of the 8 compounds significantly reduced YopE translocation into the cytosol of HEp-2 cells (Fig. 5A), consistent with the defects in cell rounding (Fig. 2A). C7 did not inhibit translocation of YopE into HEp-2 cells, supporting the observation that the cell rounding defect was weak, and therefore the difference in translocation of YopE may not be strong enough to detect in this assay. C34 also did not inhibit translocation of YopE but was a potent inhibitor of cell rounding. This suggests that C34 may target other factors that influence cell rounding but do not interfere with translocation.

A decrease in translocation could result from several defects, including defects in expression of Yops, sensing cell contact to trigger Yop translocation, and adherence of *Y. pseudotuberculosis* to target cells, leading to faulty pore formation. We tested whether the compounds reduced the level of Yop synthesis by assessing the total levels of YopE in *Y. pseudotuberculosis* during infection by Western analysis (Fig.

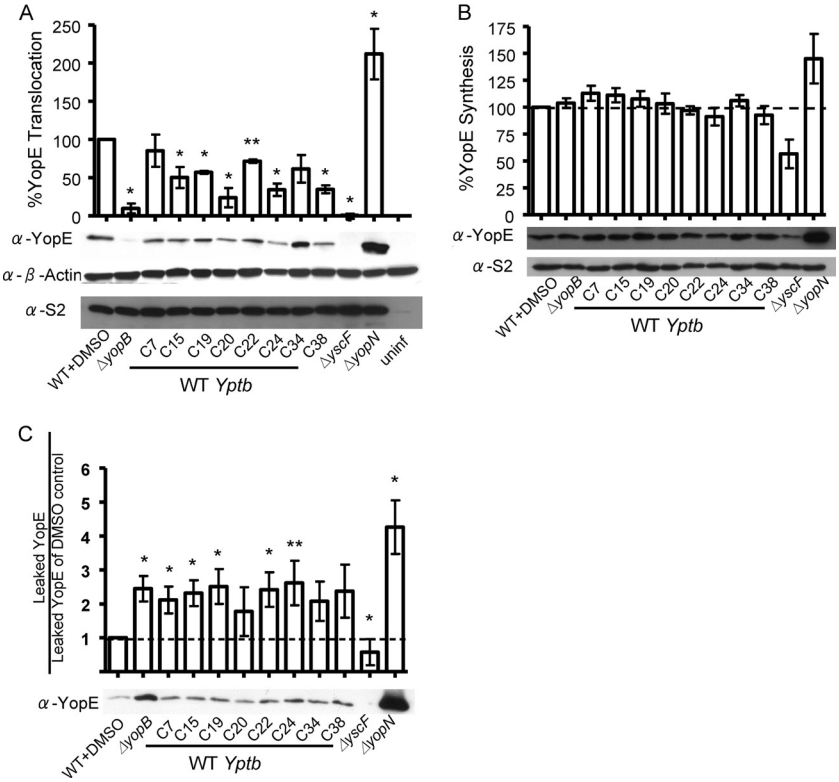

FIG. 5. Translocation of YopE into HEp-2 cells reveals a translocation defect caused by compounds. *Y. pseudotuberculosis* (*Yptb*) was grown in 2× YT in the presence of 60 μM compound or 0.3% DMSO. *Y. pseudotuberculosis* was then used to infect HEp-2 cells at an MOI of 50:1 in the presence of 60 μM compound. After 1 h, the tissue culture supernatants were collected and the HEp-2 cells were lysed, whereupon the soluble fraction from HEp-2 cells and the insoluble fraction from the wells were collected. (A) HEp-2 cells were lysed, and cytosol fractions were separated by SDS-PAGE and probed with antiserum to YopE. The percent translocation was determined by measuring the amount of YopE in the soluble fraction and normalizing it to the amount of S2 protein in the insoluble fraction. This ratio was then normalized to the amount of actin as a loading control. The amount of YopE translocated by WT *Y. pseudotuberculosis* in 0.3% DMSO was set to 100% translocation. (B) The insoluble fraction was separated by SDS-PAGE, and Western blot analysis was performed with anti-YopE and anti-S2 antibodies. The levels of YopE were normalized to the level of S2 (percent YopE synthesis). The amount of YopE in the DMSO-treated sample was set to 100%. (C) Tissue culture supernatants were collected, and bacteria were removed by centrifugation. Anti-YopE antibody was used to immunoprecipitate YopE protein. The proteins collected were separated by SDS-PAGE and subjected to Western blot analysis with antiserum to YopE. The amount of leaked YopE was determined by normalizing YopE protein levels from the immunoprecipitated fraction to S2 levels from the insoluble fraction. The ratios for compound-containing wells to DMSO controls were graphed. For all panels, the means and standard deviations for three independent experiments are plotted. Representative Western blots are also shown. *, $P \leq 0.05$; **, $P \leq 0.06$.

5B). As expected (19), the *yscF* mutant synthesized fewer Yops, due to either downregulation or a failure to upregulate synthesis after cell contact because it cannot secrete Yops, a phenotype seen previously for this mutant (19), while the *yopN* mutant produced higher levels of YopE (19). The levels of YopE during infection in the presence of compounds were comparable to those in the DMSO control for all compounds tested, indicating that the failure to translocate Yops was not due to a defect in Yop synthesis.

All compounds cause increased leakage of Yops into culture supernatants during infection of HEp-2 cells. Lowered levels of translocated YopE could be caused by an inability to efficiently transfer YopE in a polarized manner into HEp-2 cells. It has been demonstrated previously that some mutants in the TTSS with low levels of translocation leak excessive Yops into culture supernatants during infection (19). To assess whether these compounds led to inefficient transfer of YopE into host cells, we assayed for the presence of YopE in the medium of infected HEp-2 cells (Fig. 5C). Infection with the *Y. pseudotuberculosis* Δ*yopB* mutant led to an excess of YopE leaked into supernatants, and as anticipated, higher levels of YopE were detected in supernatants of HEp-2 cells infected with the Δ*yopN* strain (19). Infection with WT *Y. pseudotuberculosis* in the presence of C7, C15, C19, C22, and C24 caused significant leakage of Yops into the culture supernatants compared to that in DMSO-treated controls. The remaining compounds, C20, C34, and C38, also consistently caused elevated levels of Yop leakage, though this trend was not statistically significant. These results suggest that the compounds interfere with the transfer of Yops into host cells. Alternatively, the compounds could cause aberrant secretion of Yops into tissue culture supernatants, independent of cell contact. To test this possibility, we grew *Y. pseudotuberculosis* strains under conditions that are nonpermissive for Yop secretion in the presence of the compounds and found that none of the compounds induced Yop secretion in the absence of HEp-2 cells (data not shown). These results indicate that the compounds interfere with the efficient polarized translocation of YopE, resulting in excessive

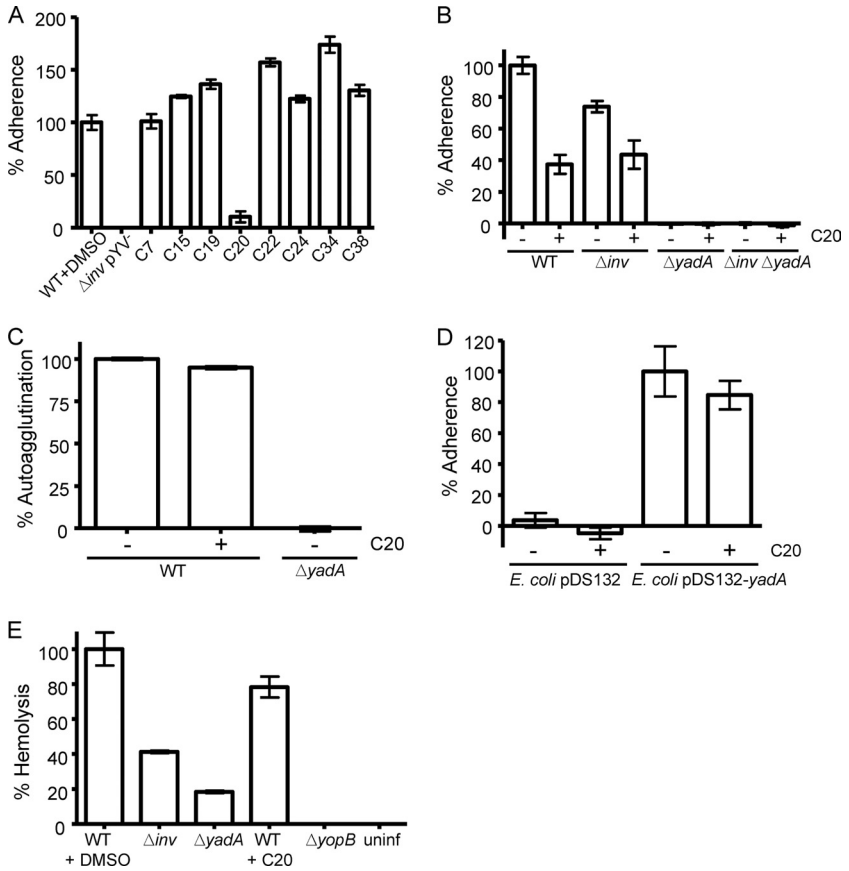

FIG. 6. Adherence of *Y. pseudotuberculosis* to HEp-2 cells is reduced by C20. (A) Adherence of *Y. pseudotuberculosis* to HEp-2 cells in the presence of compounds was determined by ELISA. WT *Y. pseudotuberculosis* was incubated with 60 μM compound. HEp-2 cells were infected in the presence of compounds with *Y. pseudotuberculosis* at an MOI of 10:1 for 30 min at 37°C. HEp-2 cells were washed to remove any unbound *Y. pseudotuberculosis*, fixed with 4% paraformaldehdye, and then probed with antisera to *Yersinia*. Anti-rabbit–HRP was used to detect anti-*Yersinia* antibody. The HRP activity was visualized with the TMB ELISA reagent, and the DMSO control was set to 100% adherence. The means and standard deviations for one representative experiment are plotted. (B) WT *Y. pseudotuberculosis* or adherence mutants were grown in 2× YT supplemented with 5 mM Ca^{2+} with C20 or 0.3% DMSO and were used to infect HEp-2 cells. The percent adherence was determined by ELISA as described above. The percent adherence of WT *Y. pseudotuberculosis* grown in 0.3% DMSO was set to 100%. The means and standard deviations for one representative experiment are plotted. (C) Overnight cultures of *Y. pseudotuberculosis* were inoculated into RPMI and allowed to incubate statically at 37°C for 3 h in the presence or absence of C20. Autoagglutination was measured as described in Materials and Methods. The percent autoagglutination of WT IP2666 in 0.3% DMSO was set to 100%. The means and standard deviations for one representative experiment are shown. (D) *E. coli* carrying pDS132 or pDS132-*yadA* was cultured in 60 μM C20 or in 0.3% DMSO and was used to infect HEp-2 cells in the presence of C20 or DMSO. Adherence of *E. coli* to HEp-2 cells was determined by ELISA as described for panel A, using antiserum to the *E. coli* phage λ receptor, LamB. The means and standard deviations for one representative experiment are plotted. (E) Hemolysis of SRBCs by *Y. pseudotuberculosis* adherence mutants or WT *Y. pseudotuberculosis* grown in the presence of 60 μM C20. *Y. pseudotuberculosis* was grown in secretion medium in the presence of either 0.3% DMSO or C20 and was used to infect SRBCs at an MOI of 1:1. The percent hemolysis was measured by the amount of hemoglobin released from the SRBCs (see Materials and Methods). Hemolysis by WT *Y. pseudotuberculosis* in 0.3% DMSO was set to 100%. The means and standard deviations for one representative experiment are shown.

leakage of Yops into culture supernatants during infection. In addition, these results suggest that *Y. pseudotuberculosis* retains the ability to sense cells and to trigger the process of polarized translocation of Yops from *Y. pseudotuberculosis* into target cells.

C20 reduces adherence of *Y. pseudotuberculosis* to HEp-2 cells. Adherence of *Y. pseudotuberculosis* to host cells is essential for the translocation of Yops. To test if compounds reduced adherence of *Y. pseudotuberculosis* to HEp-2 cells, we infected monolayers of HEp-2 cells with *Y. pseudotuberculosis* in the presence of compounds. Adherent *Y. pseudotuberculosis* can be detected with sera raised against whole *Yersinia* and the amount of bound *Y. pseudotuberculosis* determined by ELISA. One compound, C20, significantly reduced binding of *Y. pseudotuberculosis* to HEp-2 cells, to 15 to 20% of the levels of the DMSO-treated control (Fig. 6A). Most compounds did not interfere with binding, suggesting that the translocation defect caused by the remainder of the compounds was not due to inefficient adherence of *Y. pseudotuberculosis* to target cells.

Two well-described proteins, YadA and invasin, contribute to adherence of *Y. pseudotuberculosis* and to delivery of Yops into HEp-2 cells (8). We asked if C20 interfered with YadA- or invasin-mediated binding. If C20 blocked adherence through either YadA or invasin, then deletion of its target should prevent further interference of adherence mediated by other factors. Deletion of invasin led to a small decrease in adherence

of *Y. pseudotuberculosis* to HEp-2 cells but did not block the ability of C20 to further inhibit binding (Fig. 6B), indicating that the effect of C20 is not mediated through invasin. In the absence of YadA, binding of *Y. pseudotuberculosis* to HEp-2 cells was not detected above background levels (Fig. 6B, Δ*yadA* and Δ*yadA* Δ*inv*), and therefore we could not assess whether C20 influenced binding via this adhesin by this assay.

Since the presence of YadA was critical for adherence of *Y. pseudotuberculosis* to HEp-2 cells (69), we assayed if YadA-mediated autoagglutination was affected by C20. *Yersinia* cells expressing YadA protein can clump in dense cultures through the interaction of the YadA moieties on neighboring cells. This interaction can be measured as a change in the OD between a settled culture and one that has been dispersed. The autoagglutination activity of *Y. pseudotuberculosis* was unaffected by C20 (Fig. 6C), indicating that C20 did not inhibit YadA-mediated autoagglutination. We could not rule out, however, that C20 may disrupt the YadA-receptor interaction occurring at the *Y. pseudotuberculosis*-HEp-2 cell interface. To test if C20 interfered with the binding of YadA-expressing bacteria that do not express other *Yersinia* adhesins, YadA was expressed in *E. coli*. *E. coli* harboring either pDS132-*yadA* or pDS132 alone was cultured in the presence or absence of C20, and the amount of bound *E. coli* was assessed by ELISA. *E. coli* expressing YadA bound to HEp-2 cells, while *E. coli* expressing vector alone did not (Fig. 6D). Incubation with C20 did not block the ability of *E. coli* expressing YadA to adhere to HEp-2 cells, suggesting that YadA was not the target of C20.

Translocon assembly and insertion of pore-forming proteins are essential for adequate translocation of Yops. The failure to properly adhere to cells may result in defective translocon insertion into the host cell plasma membrane, leading to defective translocation. To test whether adherence was required for pore formation, the pore-forming ability of *Y. pseudotuberculosis* strains with adherence defects was evaluated. SRBCs were infected with WT *Y. pseudotuberculosis* or with strains lacking invasin or YadA, and pore formation was detected by the release of hemoglobin (19, 28). The percent hemolysis of SRBCs infected with the Δ*inv* mutant was reduced to 60% of the WT control level, and that with the Δ*yadA* mutant was reduced 80% (Fig. 6E), indicating that adherence to SRBCs is critical for insertion of the pore. SRBCs infected with *Y. pseudotuberculosis* in the presence of C20 exhibited slightly reduced leakage of hemoglobin. This is consistent with the finding that C20 reduced adherence of *Y. pseudotuberculosis* to mammalian target cells, though the reduction in hemolysis was not as dramatic as that with the adhesin mutants. These data suggest that the requirement for adherence to HEp-2 cells and the requirement for adherence and/or translocon insertion are different between HEp-2 cells and SRBCs and that C20 interferes with factors that vary between the two cell types.

Some compounds diminish cell rounding caused by translocation of *Pseudomonas* effectors. To determine whether the compounds reduced the translocation of effectors by other bacteria with closely related TTSSs, the compounds were evaluated to determine whether they inhibited translocation of ExoS from *Pseudomonas aeruginosa* (75). ExoS, like YopE, has a Rho-GAP activity that disrupts the actin cytoskeleton in cells targeted by *Pseudomonas*, leading to rounding of cells (40). The *P. aeruginosa* strain Pa388 (80) was used to infect HEp-2 cells in the presence of compounds, and the degree of cell rounding was observed by light microscopy (Fig. 7). HEp-2 cells infected with a *pscC* mutant which lacks an essential component of the TTSS remained flat, similar to uninfected cells (Fig. 7, *pscC* panel). Incubation with C7, C15, and C19 had little or no effect on ExoS-dependent cell rounding (data not shown), while C20, C22, C24, C34, and C38 all reduced cell rounding. These data demonstrate that several small molecules reduce translocation of TTSS effectors from other bacteria and suggest that these molecules target common features required for translocation. In contrast, C7, C15, and C19 may target *Y. pseudotuberculosis*-specific mechanisms.

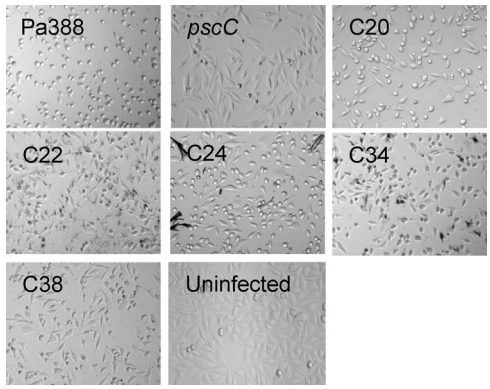

FIG. 7. *Pseudomonas aeruginosa* ExoS-dependent cell rounding was blocked by C20, C22, C24, C34, and C38. Cultures of WT *P. aeruginosa* (Pa388) or a translocation-defective mutant (Pa388 *pscC*) were grown at 37°C in the presence of 60 μM compound or 0.3% DMSO. The cultures were used to infect HEp-2 cells at an MOI of 10:1 in the presence of 60 μM compound. The infection was allowed to proceed for 90 min at 37°C before imaging. The experiment was repeated twice, and representative micrographs are shown.

DISCUSSION

HTSs that target virulence factors have been used to identify novel small molecules that could potentially be used as anti-infectives against pathogenic species of bacteria (65). Small-molecule anti-infectives are different from traditional antibiotics because they target factors important for the virulence of these organisms but not for viability. Thus, the target may avoid the rapid selective pressure that occurs with many other antibiotics, though this has not been demonstrated. Several molecules that inhibit virulence factors have been identified in HTSs, and some are effective in infection models (24, 34). For instance, virstatin (34) is an inhibitor of *Vibrio cholerae* ToxT, a transcriptional regulator of cholera toxin and toxin-coregulated pilus. Virstatin reduces the bacterial burden in mice infected with *V. cholerae*, without deleterious effects on bacterial growth. In another example, two compounds have been identified that inhibit intracellular trafficking of Shiga toxin, ricin, and diphtheria toxin during specific stages of toxin translocation (68), leading to the diminished activity of these proteins. Additional HTSs seeking to abrogate quorum sensing (QS) (55) have led to the discovery of a specific homoserine lactone mimic whose activity leads to downregulation of specific virulence factors in *Pseudomonas* (54).

In addition to these general virulence factor inhibitor

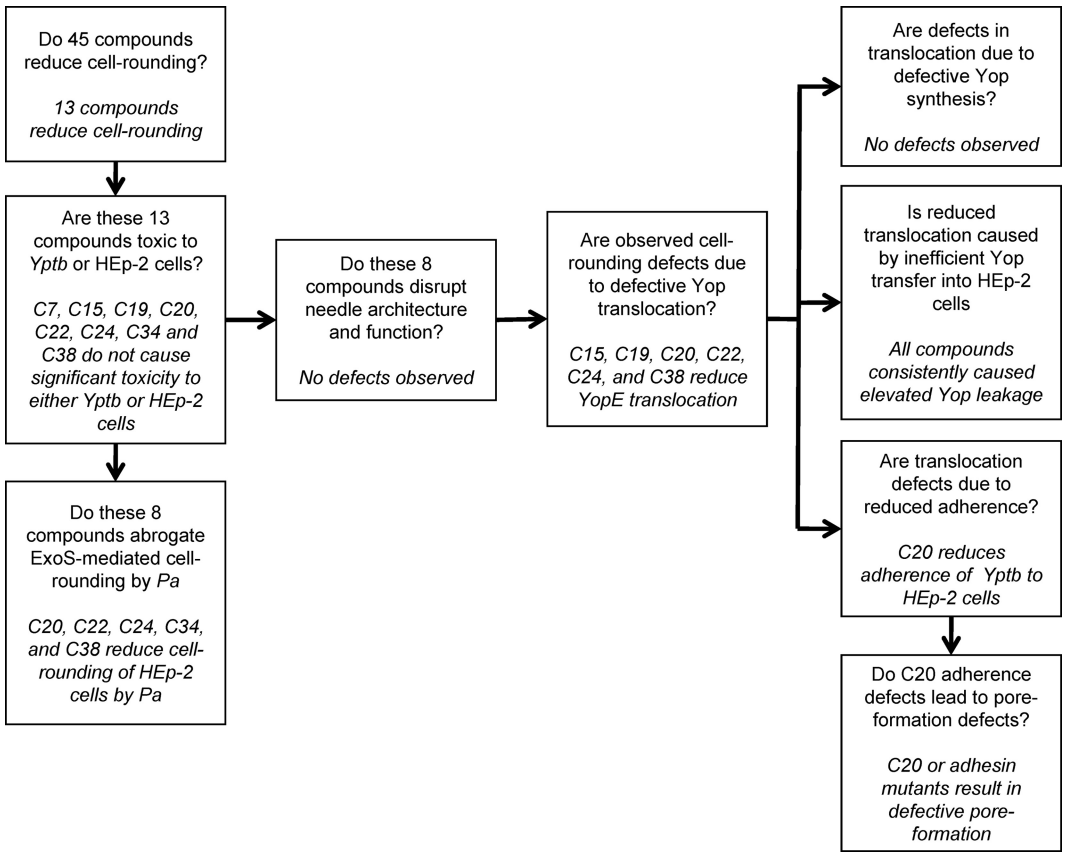

FIG. 8. Summary of results for compounds identified in the screen for small-molecule inhibitors of Yop translocation. *Yptb*, *Y. pseudotuberculosis*; *Pa*, *P. aeruginosa*.

screens, several HTSs have identified inhibitors of bacterial protein secretion systems important for virulence, including the type III (3, 24, 27, 38, 62) and type IV (15) secretion systems. Inhibition of these protein secretion systems may block the ability of pathogenic organisms to deliver many virulence factors, and such inhibitors may thus be potent anti-infectives. The previous HTSs involving TTSSs have identified molecules that inhibit the transcription or secretion of proteins from bacteria. Translocation of effector proteins into host cells is also critically important for virulence of organisms that rely on protein secretion systems and thus may be an important target for the identification of novel classes of inhibitors. Here we present a screen where we identified 13 compounds that appeared to inhibit translocation of the E-TEM fusion protein into mammalian cells (Fig. 8). Secondary assays revealed that six of the molecules specifically interfered with translocation of effectors without blocking protein synthesis or secretion of effectors *in vitro* or causing toxicity to *Y. pseudotuberculosis* or HEp-2 cells (Fig. 8). They were structurally distinct, yet all were small, planar, and hydrophobic. Given this similarity, it is possible that these molecules disrupt hydrophobic interactions occurring at the membrane between *Y. pseudotuberculosis* and the target cell. However, since these compounds are chemically different from each other, each molecule could target different factors important in translocation.

Events that are critical for Yop translocation but not for secretion include the abilities to sense cell contact, form pores (28), adhere to host cells (8), and activate host cell signal transduction cascades (23, 47). Our results showing that Yops flux through the base and needle after host cell contact but are leaked into the extracellular space rather than translocated into host cells strongly support the idea that many of these compounds act at the interface between the TTSS and the host cell (Fig. 8). Moreover, our results suggest that the compounds do not prevent sensing of host cells, since secretion is triggered upon cell contact. Likewise, since LcrV remains situated on the tip of the TTSS when compounds are present, the compounds do not appear to disrupt its association with needles (Fig. 8). However, excessive leakage of Yops may result from a failure of YopB, YopD, and LcrV to form a functional pore through their presumed interaction, a failure of YopB and/or YopD to properly insert into membranes, or a disruption of one or more host factors required for adequate pore formation or translocation.

Another key requirement for translocation, but not for secretion, is adherence of *Y. pseudotuberculosis* to host cells (8, 47). The fact that C20 interferes with bacterial adherence likely contributes to the observed translocation defect (Fig. 8). C20 also had a small but reproducible defect in SRBC hemolysis, which is consistent with the result that C20 disrupts adherence, leading to inefficient translocon insertion and effector translocation. However, C20 did not appear to interfere with YadA or

invasin function. C20 may interfere with another adhesin; it may alter other bacterial membrane properties, such as lipopolysaccharide (LPS), which in turn reduces adherence; or it may reduce/modify host cell receptors or host membrane characteristics that are necessary for tight interactions between bacteria and host cells. In fact, the observation that C20 also reduces translocation by *P. aeruginosa* suggests that C20 targets a similar factor or mechanism that is conserved or required between both organisms (Fig. 8).

The finding that C20, C22, C24, C34, and C38 all demonstrate diminished ExoS-mediated cell rounding in HEp-2 cells suggests that these compounds may target host cell factors or that they target conserved bacterial elements between the closely related TTSSs of *Yersinia* and *P. aeruginosa*, including the homologous translocon components. There is a precedent for regulation of translocation by host cell factors (4, 47). Upon interaction of *Yersinia* adhesins with host cell integrins, Src kinase activation leads to enhanced translocation (47). Additionally, bacterial contact with lipid rafts has been implicated in triggering of type three secretion in *Shigella flexneri* (76). It is possible that C22, C24, C34, and C38 alter one or more of these factors. Conversely, the observation that several other compounds, C7, C15, and C19, have no discernible effects on *P. aeruginosa*-mediated cell rounding suggests that these compounds target mechanisms specific to *Y. pseudotuberculosis*. C34 caused slight differences in the structure of the needle, slightly reduced levels of secretion, and dramatic inhibition of cell rounding but no reduction in translocated YopE. These results suggest that C34 has multiple targets, including a host factor that reduces the cell rounding activity caused by YopE, or, conversely, that C34 directly inhibits YopE activity.

Finally, one or more of these compounds may act on the host cell membrane and/or host cell factors required for translocation. Our LDH data indicated that one compound did indeed affect membrane permeability after extensive incubation, but most of the compounds had no discernible effect on LDH release. Similarly, none of the compounds appeared to disrupt actin filaments, suggesting that RhoA was still active and that active RhoA is critical for Yop translocation (47). However, one or more of these compounds could target host cell factors or membranes critical for translocation and have only subtle effects on cell shape or membrane permeability. In fact, a recent screen with *Legionella* identified 22 compounds that reduced translocation by type IV secretion into J774 cells (15). These compounds target various host cell processes, including cytoskeleton proteins and proteins involved in cytoskeleton dynamics, as well as surface proteins that may be involved in binding and internalization of *Legionella*, but they did not all have gross effects on cell morphology. Identification of the targets of the molecules identified in this work may reveal that a number of them affect eukaryotic factors which are important for translocation.

To date, there have been several studies designed to identify inhibitors of TTSS-mediated effector secretion in various types of bacteria, including *Y. pseudotuberculosis*, *Y. pestis*, *Chlamydia*, *Salmonella enterica* serovar Typhimurium, *P. aeruginosa*, and enteropathogenic *E. coli* (EPEC) (3, 5, 24, 33, 38, 57, 58, 60, 62, 77). The best-studied inhibitors were first identified by Kauppi et al., in a screen that monitored YopE promoter activity (38). This family of molecules appears to inhibit type III secretion in a variety of pathogens, including *Chlamydia* (5, 57), *Shigella* (77), and *Salmonella* (33, 58). Another study identified molecules that permitted *Y. pseudotuberculosis* growth under Yop-inducing conditions, which are normally restrictive to growth (62). Several of these molecules inhibited both Yop secretion and secretion of type III secretion-related proteins from EPEC (62). Two additional studies screened for molecules that reduced effector secretion from EPEC (27) and *Salmonella* (24). In the EPEC study, one compound inhibited expression of type III secretion-related proteins but not the expression of other, non-type-III secretion proteins. The *Salmonella* study also identified many compounds that inhibited protein expression. One of the compounds identified, however, did not inhibit protein expression or growth of bacteria but reduced secretion of effectors from several TTSSs and interfered with assembly of the needle complex (24). While these molecules are all potentially powerful molecules, and some reduce virulence in infection model systems, the protein targets of these molecules have not been identified.

In the screen reported here, we did not identify molecules that interfered with assembly of the TTSS. We postulate that this bias arose from the fact that we grew the *Y. pseudotuberculosis* under conditions where the TTSS was preassembled prior to exposure to compounds and cells. However, despite the fact that we observed this bias, the screen could potentially have identified compounds that destabilized the base, needle, or LcrV at the tip. Interestingly, no molecules were identified that disrupted the preformed structure, suggesting that such compounds could be extremely rare.

The process of translocation is critical to the virulence of *Yersinia* and other pathogenic bacteria that rely on a TTSS. In our screen, we identified several compounds that have an inhibitory effect on translocation in both *Yersinia* and *Pseudomonas*. Further study of these molecules has the potential to elucidate some of the complex interactions between bacteria and host cells that are required for translocation, and they could also function as inhibitors of the virulence of these organisms.

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