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Targeting Type III Secretion in *Yersinia pestis*▽†

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***Yersinia pestis*, the causative agent of plague, utilizes a plasmid-encoded type III secretion system (T3SS) to aid it with its resistance to host defenses. This system injects a set of effector proteins known as Yops (*Yersinia* outer proteins) into the cytosol of host cells that come into contact with the bacteria. T3SS is absolutely required for the virulence of *Y. pestis*, making it a potential target for new therapeutics. Using a novel and simple high-throughput screening method, we examined a diverse collection of chemical libraries for small molecules that inhibit type III secretion in *Y. pestis*. The primary screening of 70,966 compounds and mixtures yielded 421 presumptive inhibitors. We selected eight of these for further analysis in secondary assays. Four of the eight compounds effectively inhibited Yop secretion at micromolar concentrations. Interestingly, we observed differential inhibition among Yop species with some compounds. The compounds did not inhibit bacterial growth at the concentrations used in the inhibition assays. Three compounds protected HeLa cells from type III secretion-dependent cytotoxicity. Of the eight compounds examined in secondary assays, four show good promise as leads for structure-activity relationship studies. They are a diverse group, with each having a chemical scaffold not only distinct from each other but also distinct from previously described candidate type III secretion inhibitors.**

Yersinia pestis, a gram-negative bacterium in the family *Enterobacteriaceae*, is the causative agent of plague (10). A hallmark of this devastating disease is the rapid development of systemic infection following the deposition of a small inoculum into the skin via the bite of an infected flea (9). This fulminant course is dependent on the ability of the bacteria to overwhelm host defenses, a feat which they accomplish by a combination of stealth and active suppression targeting innate immune mechanisms. The type III secretion (T3S) system (T3SS) of *Y. pestis* is crucial to the suppression arm of this strategy. This system injects a set of effector proteins directly into the cytoplasm host cells in contact with the bacteria. These act to derange key defensive functions, including phagocytosis and cytokine production (11, 12).

Although several other adaptations also play key roles in virulence, the loss of T3S is sufficient to render *Y. pestis* completely avirulent, even when the bacteria are introduced directly into the bloodstream (39). T3SS is shared by *Y. pseudotuberculosis* and *Y. enterocolitica*, which are also pathogenic for humans, and is encoded by a 70-kb plasmid, termed pCD1 in most *Y. pestis* strains (3, 11, 17). The major injected *Yersinia* outer proteins (Yops), usually designated YopH, YopE, YopM, YopJ, YopO, and YopT, target the cytoskeleton, proinflammatory signaling pathways, and probably other cell functions as well. T3SS involves at least 39 other proteins that play supporting roles in regulation, secretion, and translocation. Some notable members of this cast include the secreted translocon proteins YopB and YopD, which collaborate to form a pore in the host cell membrane; YscF, which forms the shaft of a needle-like channel through which the secreted proteins are thought to travel (16, 34); LcrV, which sits at the tip of this needle and which may serve as the interface with the YopB-YopD pore (26); and a set of chaperones (SycD, SycE, SycH, SycT, and SycN) that assist with the translocation of specific Yops (11, 36, 38, 40). The energy driving the secretion process is provided by the ATPase YscN (4).

T3SSs are also present in many other gram-negative pathogens. Examples include *Salmonella* spp., *Shigella* spp., *Pseudomonas aeruginosa*, enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli*, and *Chlamydia* spp. Many details regarding their workings remain to be determined, but there is no doubt that they are both common and play important roles in bacterial virulence (11, 18). For these reasons, they constitute a potential target for novel therapeutics. Several attempts have been to identify small-molecule T3SS inhibitors, with some success (1, 19, 22, 23).

The regulation of the T3SS function in the yersinae is interesting and unusual, offering distinct advantages for in vitro manipulation and the development of screening techniques. At temperatures below about 34°C, the expression of many of the proteins required for assembly of the functional T3SS apparatus, known as the injectisome, is repressed. Above this temperature the apparatus is formed, but under physiological conditions it secretes little or no protein unless contact is made with cell membranes. Such contact both triggers secretion by an as yet undetermined mechanism and results in the increased expression of many Yop genes (5, 32, 33, 37).

In vitro, high levels of secretion and Yop gene expression can be induced by a lack of Ca^{2+} ions in the medium during incubation at 37°C. However, when secretion is induced in this way, the bacteria are unable to grow (25). The increased transcription of *yopE* under these conditions was the basis of the technique used by Kauppi et al. to screen a library of 9,400

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TABLE 1. Bacterial strains and plasmid used[a]

Strain or plasmid	Genotype	Key properties	Reference
Y. pestis strains[b]			
JG401	KIM Δ*pgm*/pMT1$^+$, pCD1$^-$, pCD1K$^+$, pPCP1$^-$/pML001	Luminant, T3S$^+$	31
JG406	KIM Δ*pgm*/pMT1$^+$, pCD1$^-$, pPCP1$^-$/pML001	Luminant, T3S$^-$	31
JG153	KIM Δ*pgm*/pMT1$^+$, pCD1$^+$, pPCP1$^-$	T3S$^+$	31
JG154	KIM Δ*pgm*/pMT1$^+$, pCD1$^-$, pPCP1$^-$	T3S$^-$	31
E. coli strains			
β2155	*thrB1004 pro thi strA hsdS lacZ*ΔM15 (F′ *lacZ*ΔM15 *lacI^q traD36 proA$^+$ proB$^+$*) Δ*dapA*::*erm pir*::*RP4*	Supports replication and mobilization of pFD1	14
KC14	JPN15/pMAR7 Δ*tir*::*cat*	Tir$^-$	8
KC21	JPN15/pMAR7 ΔLEE::*cat*	T3S$^-$	27
Plasmids			
pML001	pBR322 derivative containing *luxABCD*	Confers luminescence	31
pCD1K	pCD1 Y0093::*magellan3*	T3S$^+$	31
pMM85	pHSG576 *yopE*::*bla*	YopE β-lactamase	24
pKC17	pK184 HA-*tir$_{EPEC}$ cesT*	Expresses HA-Tir	8
pFD1	pGP704 *magellan3*, *Himar1* transposase	Suicide vector for delivery of *magellan3*	14

[a] Superscript minus signs indicate negativity for the plasmid, protein, or secretion; and superscript plus signs indicate positivity.
[b] All *Y. pestis* strains were avirulent.

compounds for their ability to inhibit T3SS in *Y. pseudotuberculosis* (22). We took advantage of the inhibition of growth that accompanies the secretion induced by low levels of calcium to devise an alternative screening technique. Here, we describe this method and report on the initial characterization of a novel set of inhibitors of *Yersinia* T3S, identified through the screening of 70,966 compounds and mixtures from 13 small-molecule and extract libraries.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *Y. pestis* strains used in this study were derivatives of *Y. pestis* strain KIM. The strains and the plasmids used are listed in Table 1. Plasmid pMM85 (YopE-Bla) (24) was the gift of Olaf Schneewind (University of Chicago).

Y. pestis and *Y. pseudotuberculosis* were cultured in TB broth (1% [wt/vol] Bacto tryptose [BD Bioscience], 0.5% [wt/vol] NaCl, 0.3% [wt/vol] beef extract paste [BD Bioscience]) and brain heart infusion (BHI; BD Bioscience) broth, as indicated. For high-throughput screening, *Y. pestis* was grown in TB broth for 20 h at 26°C and diluted to 1×10^4 bacteria/ml, the compounds to be screened were added, and the bacterial cultures were then shifted to 37°C and incubated for 18 h. To monitor the cultures for the secretion of Yops, *Yersinia* strains were grown in BHI broth at 30°C overnight, diluted to an optical density at 600 nm (OD_{600}) of 0.04 in fresh BHI broth, and incubated at 30°C to an OD_{600} of 0.2. The culture was then shifted to 37°C, and incubation was continued for an additional 2 h. EPEC strains were cultured in LB medium at 37°C, unless indicated otherwise.

Compounds and antibodies. The compounds and extract libraries used in the screening were provided by the NIH-supported National Screening Laboratory at Harvard Medical School. Larger quantities of the test compounds were obtained from commercial venders. Compounds 1, 2, and 3 were purchased from TimTec Inc.; compound 3 diproprionate was purchased from Sigma-Aldrich; and compound 4 and INP0007 were purchased from ChemBridge Corporation. Mouse monoclonal anti-YopM antibody (1A1), rabbit polyclonal anti-YopD antibody (5331), and rabbit polyclonal anti-YopH antibody (G5788) were gifts from Susan Straley (University of Kentucky). A rabbit polyclonal anti-YopE antibody was the gift of Olaf Schneewind (University of Chicago). Mouse anti-hemagglutinin (anti-HA) monoclonal antibody HA.11 was purchased from Covance, and rabbit anti-OmpA antibody was kindly provided by Carol Kumamoto (University of Massachusetts Medical School).

Construction of *Y. pestis* strain JG401. Random mutagenesis of pCD1 with the transposon *magellen3* (35), which confers kanamycin resistance, was used to create pCD1 (Y0093::*magellen3*). Mutagenesis was performed with *Y. pestis* by using the suicide delivery plasmid pFD1 (35) in donor strain β2155 (14). For brevity, plasmid pCD1 (Y0093::*magellen3*) is hereafter referred to as pCD1K. Plasmid pML001 carries the *lux* operon from *Photorhabdus luminescens*. Both pCD1K and pML001 were introduced into *Y. pestis* JG154 by electroporation to generate JG401. JG406 is a spontaneous segregant of JG401 lacking pCD1K and was isolated by selection for growth at 37°C (31). The luminescence of the bacterial cultures was measured with a Packard Picolite luminometer (United Technologies) and an EnVision plate reader (Perkin-Elmer).

HTS. High-throughput screening (HTS) was conducted with luminescent *Y. pestis* strain JG401. Thirty microliters of JG401 culture (1×10^4 bacteria/ml) was added into each well of 384-well cell culture plates by a liquid-handling robot (a μFill plate dispenser with a Bio-Stack apparatus, both of which were from Bio-Tek Instruments). One hundred nanoliters of screening compound (5 mg/ml in dimethyl sulfoxide [DMSO]) was applied to each well of the assay plates by an Epson compound transfer robot with Epson standard-volume pin arrays. The plates were incubated overnight at 37°C, and the relative luminescence was measured by a plate-reading luminometer equipped with a 40-plate automatic loader (EnVision plate reader; Perkin-Elmer). The dwell time per well was set at 0.1 s. Statistical analysis of the primary screening results was carried out as described previously (31).

Assay of effector protein secretion. Overnight cultures of strains JG153 and JG154 were grown as indicated above in TB broth at 30°C and were used to inoculate BHI broth to an OD_{600} of 0.04. The cultures were then grown at 30°C in glass tubes (18 by 150 mm) and aerated by rotation on a Rollordrum apparatus (New Brunswick). When the OD_{600} reached 0.2, the compounds under test were dissolved in DMSO and were then added at the indicated concentrations. The final concentration of DMSO in all cultures except those with compound 3 diproprionate, including the controls, was 0.3%; in experiments with compound 3 diproprionate, no DMSO was used. The cultures were then shifted to 37°C to induce T3S and were incubated for an additional 2 to 3 h. The culture supernatants were collected following centrifugation ($3,200 \times g$, 10 min). Proteins were precipitated from 2 ml of supernatant with 10% (final concentration) trichloroacetic acid (TCA), washed twice with 1 ml of 1:1 (vol/vol) ethanol-ether, and dissolved in 30 μl water. Ten microliters of this solution, which was mixed with sample buffer, was used to load a single lane for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent preparation of Western blots for YopM, YopH, and YopD. Development of the Western blots was stopped before the positive Yop controls reached saturation, and the intensity and area of each band were quantified by video densitometry.

Effects of compounds on bacterial growth. Mid-log-phase cultures were diluted to an OD_{600} of 0.05 in TB broth containing 2.5 mM $CaCl_2$. Two hundred microliters of the culture was added to each well of 96-well tissue culture plates (Falcon Microtest, catalog number 353072; Becton Dickinson). A total of 0.6 μl of 20 mM test compounds was added to the wells (final concentration, 60 μM). The 20 mM stocks of compounds 1 to 4 were prepared in DMSO, and the compound 3 diproprionate stocks were prepared in water. The cultures were

incubated in a Spectra Max 250 plate reader (Molecular Devices) at 37°C with continuous agitation (the automix function was activated). The OD_{600} of the 200-μl culture volume was measured at 6-min intervals for 24 h, and the values were corrected to the standard OD_{600} (with a cuvette with a 1-cm path length) by using equation 1. This equation was derived by measuring the OD_{600} values of 10 samples of various densities in both a standard cuvette with a 1-cm path length and the 96-well plate. The growth rates were calculated from the exponential portion of the resulting growth curves.

$$OD_{600\,(200\,\mu l)} = 0.201 \times OD_{600} - 0.003 \quad (1)$$

where R^2 is equal to 0.999 for $1.6 \geq OD_{600} \geq 0.06$.

Cytotoxicities of test compounds. HeLa cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. A total of 1×10^5 HeLa cells/well were seeded in a 24-well tissue culture plate in DMEM with 3% heat-inactivated FBS 1 day prior to the assay. The test compounds were applied, and the cells were incubated at 37°C for 3 h. The level of disruption of the cell monolayer was recorded as the percentage of cell detachment from the culture plate. The amount of lactate dehydrogenase (LDH) released from the HeLa cell cultures was measured with a cytotoxicity detection kit (Roche), according to the manufacturer's protocol.

T3S-mediated cytotoxic response. HeLa cell monolayers grown in DMEM supplemented with 3% heat-inactivated FBS and 24 μM HEPES (pH 7.4) were infected with *Y. pestis*, and the test compounds were immediately added. The infected cultures were then incubated at 37°C with 5% CO_2. HeLa cells were infected with pCD1-positive *Y. pestis* strains, as indicated above. The cytotoxic response was initially observed as the rounding up of the cells. The level of inhibition of this response was obtained by determining the percentage of the HeLa cells that showed morphological changes among all treated cells. Strain JG154 was used as a T3S-negative control.

Translocation of a YopE-Bla fusion protein into HeLa cells. HeLa cells were maintained as described above. One day prior to infection, 3.5×10^4 cells were seeded in each well of 24-well plates. Two hours before infection, the cells were washed with phosphate-buffered saline and 350 μl of DMEM with 24 μM HEPES was added to each well. HeLa cells were infected with mid-log-phase YopE::Bla-expressing *Y. pestis* strains [either JG153(pMM85) or JG154(pMM85), as indicated] at a multiplicity of infection of 10 for 3 h. The β-lactamase substrate CCF2-AM (Invitrogen) was added to live cells at the concentration indicated by the manufacturer. The cells were incubated at room temperature for 1 to 2 h and were then analyzed by fluorescence microscopy with a Nikon Eclipse E800 microscope with a β-lactamase filter set (Chroma).

Tir secretion in EPEC culture. Assays for determination of EPEC T3S were performed as described previously (15). Briefly, KC14(pKC17) (8) or KC21(pKC17) (8, 27) cultures were grown in LB medium at 37°C for approximately 8 h, diluted 1:500 into prewarmed DMEM-HEPES, and grown in a 5% CO_2 atmosphere overnight. Overnight cultures were further diluted 1:50 into 5 ml of prewarmed DMEM-HEPES and grown in a 5% CO_2 atmosphere at 37°C for 2 h. Test compound dissolved in DMSO or DMSO alone was added to the cultures, and incubation was continued for an additional 8 h. To separate the bacteria from the secreted proteins, the cultures were centrifuged at $3,300 \times g$ for 10 min and the supernatant was passed through a 0.45-μm-pore size low-protein-binding filter. The bacterial pellet was resuspended in 50 μl of loading buffer, boiled for 10 min, and subjected to SDS-PAGE; and immunoblots were prepared. The secreted proteins were precipitated with 10% cold TCA overnight at 4°C, and immunoblots were prepared as described above.

RESULTS

Basis of the screening method. As noted above, our screening technique is based on peculiarities of the physiology of *Y. pestis* that are not well understood. The combination of low concentrations of calcium ions (<1 mM) and incubation at temperatures above 34°C strongly induces the expression of T3SS and results in the secretion of the Yop effector proteins directly into the surrounding medium (12, 25, 42). Note that this is distinct from the targeted secretion accomplished by this system during cell contact, in which effector proteins are directed into the cells at physiological Ca^{2+} concentrations (~2.5 mM). The low-calcium-concentration-dependent induction of T3S is also accompanied by the inhibition of bacterial growth. In certain media with very low sodium ion concentrations, this growth inhibition is weak or absent (6, 7), suggesting that the leakage of Na^+ into the cells through the active section apparatus may be the key factor in growth inhibition. Whatever its true cause, we reasoned that at least some inhibitors of T3S would prevent this growth inhibition and could be detected on this basis.

To implement the screening, we constructed a specialized *Y. pestis* strain. This strain was rendered avirulent both by a large spontaneous chromosomal deletion that removed the genes needed for iron acquisition during infection and by the elimination of native plasmid pPCP1, which encodes an outer membrane protease (Pla) also required for full virulence. This combination of lesions has been shown to result in avirulence in a primate model of pneumonic plague (41). This strain was also made luminescent through addition of the *lux* operon from *Photobacterium luminescens*, carried by plasmid pML001. T3SS in *Y. pestis* is encoded by a 70-kb plasmid designated pCD1. Either segregation of this plasmid or internal deletions which compromise the T3SS function, both of which occur at appreciable frequencies, would give rise to false-positive results in a T3S inhibition assay. To circumvent this problem, we modified pCD1 by insertion of a version of a transposon mariner carrying a kanamycin resistance determinant, *magellen3* (35). In addition to providing a means for imposing selection against segregation, we reasoned that the insertion of *magellen3* at some locations within pCD1 might reduce the rate of spontaneous deletions affecting T3SS expression, either through tight linkage with the sequences most subject to spontaneous deletion or by some other means. Accordingly, we screened random *magellen3* insertion mutants of pCD1 for their frequency of colony formation on medium with a low calcium concentration supplemented with kanamycin at 37°C: only bacteria with defects in T3S can form colonies on this medium. One mutant that showed markedly reduced plating efficiencies ($<10^{-8}$) had an insertion in the putative helicase gene, Y0093. Due to its high degree of stability, pCD1(Y0093::*magellen3*) (i.e., pCD1K) was used in the screening strain, and that strain was designated JG401.

Screening. The HTS assay, illustrated in Fig. 1A, is extremely simple to perform. *Y. pestis* strain JG401 suspended in TB broth containing 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid to sequester low levels of intrinsic Ca^{2+} was distributed into 384-well plates, followed by pin transfer of the compounds to be screened. After overnight incubation at 37°C, the luminescence of each well was measured. Elevated luminescence implies growth and, hence, the presumptive inhibition of T3S.

We screened 10 commercially available small-molecule libraries and three plant extract libraries, listed in Table S1 in the supplemental material. They contained 69,093 individual compounds and 1,873 plant extracts. Measurements of luminescence were interpreted relative to the luminescence of negative controls, to which no compound was added, and positive controls supplemented with $CaCl_2$ (final concentration, 2.5 mM) rather than a test compound.

HTS data analysis. We performed statistical analysis as described previously (31). We chose a twofold elevation of luminescence relative to that for the controls receiving no test

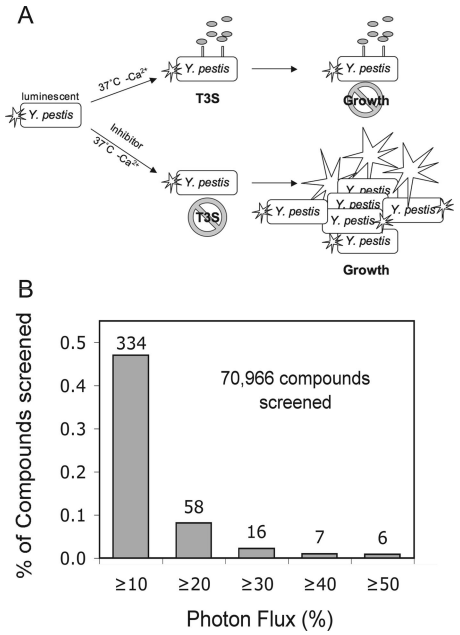

FIG. 1. HTS. (A) The HTS strategy. In *Y. pestis*, T3S is induced and growth is concomitantly inhibited during incubation at 37°C in medium lacking Ca^{2+}. Some classes of small molecules that block T3S are expected to relieve this T3S-mediated growth inhibition. Measurement of the luminescence of the bacteria provides a sensitive method of measuring bacterial growth and, hence, of identifying presumptive T3S inhibitors. (B) Distribution of photon flux among primary hits from the HTS. Data from the screening of 70,966 compounds in duplicate were collected and analyzed. The active compounds (see Materials and Methods for details) were categorized into strong (100% ≥ photon flux ≥ 50%), medium (50% > photon flux ≥ 25%), and weak (25% > photon flux ≥ 10%) hits and numbered 6 (0.01%), 40 (0.06%), and 375 (0.54%) compounds, respectively.

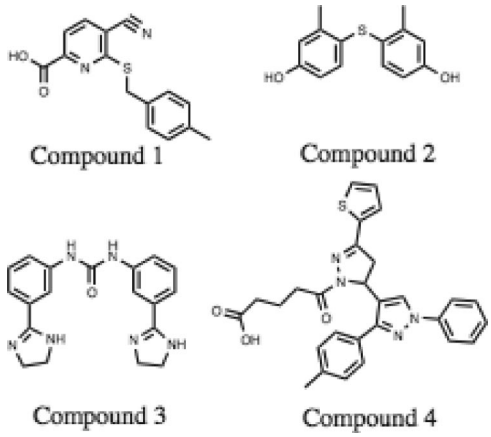

FIG. 2. Lead compounds and *Y. pestis* growth. Chemical structures of compounds 1 to 4: compound 1, 5-cyano-6-(4-methylbenzylthio)picolinic acid (CAS number 329057-04-7); compound 2, 4,4′-thiobis(3-methylphenol) (CAS number 3530-35-6); compound 3, 1,3-bis[3-(4,5-dihydro-1*H*-imidazol-2-yl)phenyl]urea (CAS number 27885-92-3); compound 4, 5-oxo-5-[5-(1-phenyl-3-*p*-tolyl-1*H*-pyrazol-4-yl)-3-(thiophen-2-yl)-4,5-dihydro-1*H*-pyrazol-1-yl]pentanoic acid (CAS number 378757-83-6). Compound 3 diproprionate (propanoic acid, compound 3 with *N*,*N*′-bis[3-(4,5-dihydro-1*H*-imidazol-2-yl) phenyl]urea [2:1]) (CAS number 55750-06-6) was used due to its relatively high degree of solubility in water.

compound as the threshold for the indication of presumptive inhibition. The effects of the compounds on luminescence are summarized as a distribution in Fig. 1B. A total of 421 compounds and extracts were identified as primary HTS hits. On the basis of the intensity of the inhibition, the novelty of the chemical structures, commercial availability, and the correlation of our results with the results of other screens for unrelated activities performed at the National Screening Laboratory, we selected eight strong and moderate hits from the primary hits for further detailed study. Below, we report on the results of follow-up studies with these eight compounds which gave moderate or strong signals in the preliminary assay.

Four of the eight compounds are T3S inhibitors. In the primary screening, we observed that the selected compounds were able to promote *Y. pestis* growth and/or luminescence at 37°C in the absence of Ca^{2+}. To determine the validity of our assumption that some compounds with this effect would be bona fide T3S inhibitors, direct assays of T3S were performed. Three T3S substrates, YopH, YopM, and YopD, were chosen as secretion indicators to evaluate the compounds. Both YopH and YopM are delivered to the cytoplasm of target cells and play important roles in virulence. YopD is delivered to the cytoplasmic membrane of host cells, where, in combination with YopB, it forms pore-like structures that are required for the translocation of other proteins across the membrane (11, 12). The effects of the eight selected compounds on YopM, YopH, and YopD secretion were determined in assays without target cells, in which secretion was induced by shifting bacterial cultures in medium with a low concentration of Ca^{2+} from 26°C to 37°C (for details, see Materials and Methods). To detect the secreted proteins, culture supernatants were subjected to SDS-PAGE, followed by Western blotting with antibodies specific for each of the Yops. Four of the compounds, designated compounds 1 to 4, showed a concentration-dependent inhibition of secretion. The other four compounds did not inhibit secretion (data not shown). The structures of compounds 1 to 4 are shown in Fig. 2, and their effects in secretion assays are shown in Fig. 3. Interestingly, differential inhibition of secretion among the Yop species was observed with compounds 1 and 3. Compound 1 inhibited YopH and YopM secretion with a 50% inhibitory concentration (IC_{50}) of approximately 10 μM, but for YopD secretion, it was clear that inhibition was very weak (Fig. 3A). Compound 3 inhibited YopH secretion with an IC_{50} of 15 μM; at about 15 μM, its level of inhibition of YopD approached 50%, but the level of inhibition decreased at higher concentrations. Its inhibition of YopM secretion was barely observable (Fig. 3C). The IC_{50} of compound 2 was about 10 μM for all three Yops.

Compounds 1 to 4 do not inhibit *Y. pestis* growth. The primary screening assay is dependent on increased luminescence, which is used as a surrogate for increased growth. One advantage of this approach is the ability to reject compounds that act indirectly on the inhibition of secretion via their toxic effects that result in growth inhibition. In order to confirm that the selected compounds do not negatively affect the growth of *Y. pestis*, we monitored the OD_{600}s of the bacterial cultures in the presence of the compounds (60 μM) under conditions in which T3S is not induced. None of the four compounds showed an

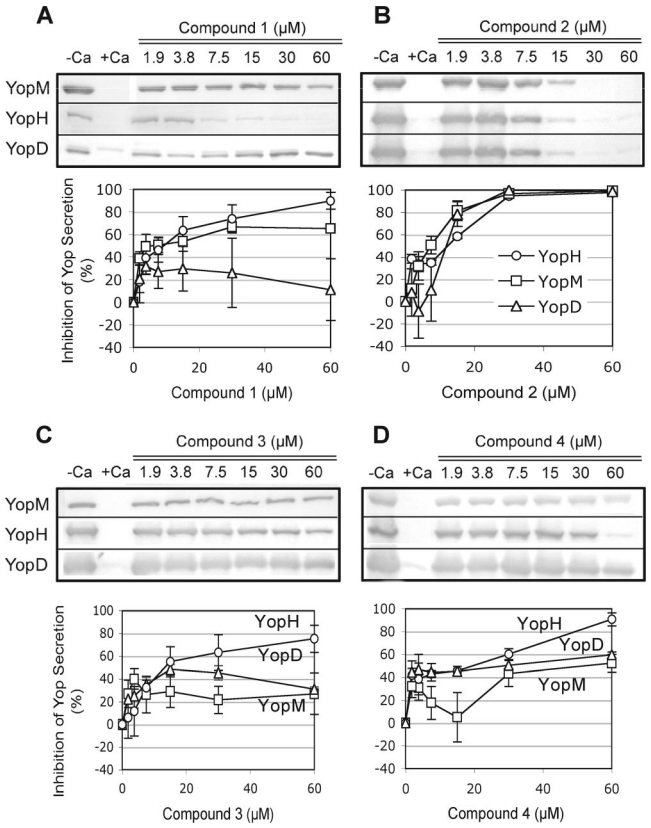

FIG. 3. Effects of selected compounds on Yop secretion. Representative Western blots of YopM, YopH, and YopD in *Y. pestis* culture supernatants 2 h after shifting of the incubation temperature from 30°C to 37°C to induce T3S for each compound are shown. Yop secretion was quantified by densitometry, as described in Materials and Methods. The means and standard deviations from at least three independent experiments are plotted. (A) Compound 1; (B) compound 2; (C) compound 3; (D) compound 4.

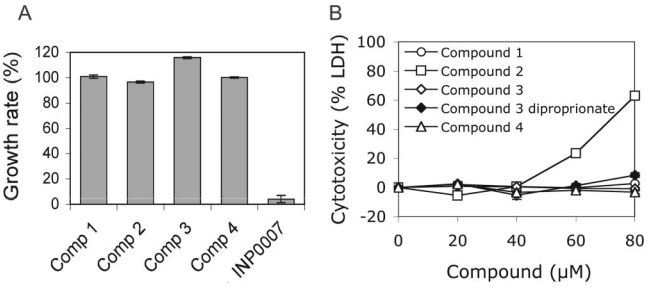

FIG. 4. Effects of compounds on *Y. pestis* growth and cytotoxicities of the compounds for HeLa cells. (A) Effects of compounds on the growth rate of *Y. pestis*. The exponential rate of growth of *Y. pestis* JG153 in the presence of 60 μM of the indicated compounds is reported as a percentage of the rate observed in the absence of added compound. The cultures contained TB broth with 2.5 mM Ca^{2+} and were incubated at 37°C with aeration. Means and standard deviations from triplicate experiments are shown. Comp, compound. (B) Cytotoxicities of compounds 1 to 4, as determined by the LDH assay. HeLa cells were treated with up to 60 μM of compounds 1 to 4 (including compound 3 as the diproprionate salt) at 37°C for 2 to 3 h. Aliquots of the cell culture supernatants were sampled. The amounts of LDH released by 0.1% Triton X-100-treated cells and untreated normal cells were taken as LDH_{high} and LDH_{low} respectively. Percent cytotoxicity was calculated as [observed LDH − LDH_{low})/(LDH_{high} − LDH_{low})] × 100. Error bars represent the standard deviations of the means from three independent assays.

inhibitory effect on the growth of *Y. pestis* at the concentrations tested (Fig. 4A). To our surprise, the previously identified T3S inhibitor INP0007 (compound 1 in reference 30) completely inhibited growth at 60 μM (Fig. 4A).

Direct cytotoxicity. The toxicities of compounds 1 to 4 for HeLa cells were assessed in two ways: observing the effects on cell morphology (rounding, detachment) and obtaining quantitative measurements of LDH release. Compound 2 caused cell rounding and detachment, while the other compounds did not when they were used within the range of concentrations used in the T3S inhibition assays (data not shown). Consistent with this observation, compound 2 also caused concentration-dependent LDH release, while compounds 1, 3, and 4 also had no effect in this assay (Fig. 4B).

Compounds 1, 3, and 4 inhibit T3S-mediated cytotoxicity. One result of the delivery of effector proteins by the *Y. pestis* system to cells in culture is a cytotoxic reaction characterized by cytoskeletal dysfunction, resulting in cell rounding and detachment (11). To determine if the compounds could inhibit the delivery of effector proteins to HeLa cells, we examined their ability to block this effect. Because compound 2 directly caused similar effects (see above), only compounds 1, 3, and 4 were tested in this assay. All three compounds protected infected HeLa cells from T3S-induced morphological changes. The protection afforded by all three compounds was concentration dependent (Fig. 5B). Although we did not measure the rates of growth of the *Y. pestis* organisms in the medium supplemented with the inhibitors used in these experiments, this medium supplemented with the inhibitors at 60 μM supported the substantial growth of *Y. pestis* in overnight cultures.

Compound 3 inhibits translocation of a YopE::Bla fusion protein to HeLa cells. The T3S-dependent morphological change induced in mammalian cells is primarily caused by the effector protein YopE, a GTPase-activating protein affecting actin polymerization (11). Thus, inhibition of these morphological changes implies the inhibition of YopE translocation or activity. A more direct assay for T3S effector translocation based on the use of effector::β-lactamase fusion proteins and the cell-permeant florescent β-lactamase substrate CCF2-AM has been devised (24). Using this method, we tested the abilities of compounds 1, 3, and 4 to block the translocation of a YopE::Bla fusion from *Y. pestis* to HeLa cells. At 80 μM, compound 3 (diproprionate salt) completely inhibited translocation in this assay, and at 40 μM, it yielded an estimated 50% inhibition (Fig. 6). Compounds 1 and 4 showed no obvious effect in this assay. The latter result is somewhat surprising, as both of these compounds inhibited T3S-mediated cytotoxicity (Fig. 5B), a phenomenon dependent on Yop translocation. One possible explanation for this result is that the cytotoxicty reaction requires higher levels of Yop translocation to yield a positive result than does the YopE::Bla-based fluorescent substrate assay, at least under the conditions used in our experiments. It is also possible that the YopE::Bla fusion protein is less sensitive to inhibition than native Yops.

Compounds 1 and 3 also inhibit T3S in EPEC. To determine if the inhibitory effects of compounds 1 to 4 might extend to T3SSs other than the T3SS of the yersiniae, we tested their

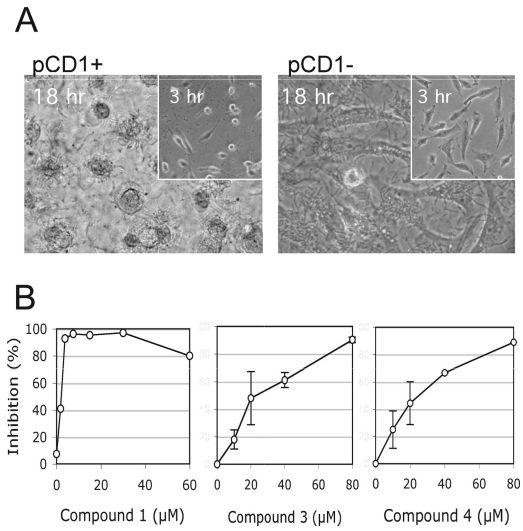

FIG. 5. Lead compounds and cytotoxicity. The effects of the compounds on the T3S-mediated cytotoxic response of HeLa cells exposed to *Y. pestis* were determined. (A) HeLa cells exposed to *Y. pestis* JG153 (with pCD1 [pCD1$^+$], positive control) (left) and JG154 (without pCD1 [pCD1$^-$], negative control) (right). The images were taken at 3 h and 18 h postinfection for low-magnification and high-magnification images, respectively. (B) Inhibition observed following addition of different concentrations of compound 1, compound 3 (diproprionate), and compound 4 to HeLa cells exposed to *Y. pestis*. The morphological changes in the HeLa cells were scored at 3 h postinfection. Percent inhibition was calculated as {1 − [(observed number of rounded cells − number of rounded cells without cytotoxicity)/(number of rounded cells with cytotoxicity − number of rounded cells without cytotoxicity)]} × 100. Error bars represent the standard deviations of the means from three independent assays.

ability to block T3S in EPEC. Unlike the *Y. pestis* system, the T3SS of EPEC secretes effectors into the medium during growth at 37°C without special treatment. In strain KC14 (8), in the presence of compounds 1 and 3, the level of secretion of the EPEC effector Tir was significantly reduced (Fig. 7). Compounds 2 and 4 were tested at concentrations up to 60 μM (data not shown), but no inhibition was observed. KC21 (27) was used as a T3S-negative control strain.

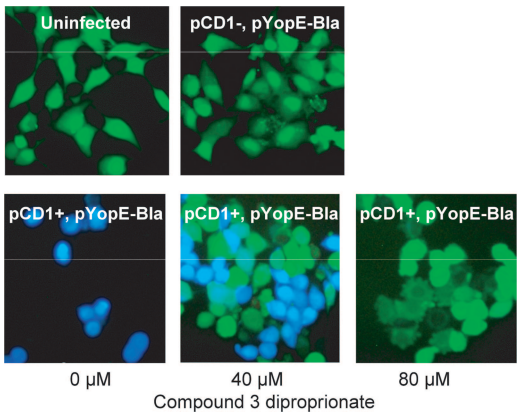

FIG. 6. Compound 3 diproprionate inhibits YopE-Bla translocation to HeLa cells. HeLa cells were infected with JG153(pMM85), which expresses YopE-Bla, and were then incubated at 37°C for 3 h. Compound 3 diproprionate was added to the cell culture at the time of infection. CCF2-AM was added, and live cells were visualized by fluorescence microscopy. pCD1$^-$, cells negative for pCD1; pCD1$^+$, cells positive for pCD1; Bla, β-lactamase.

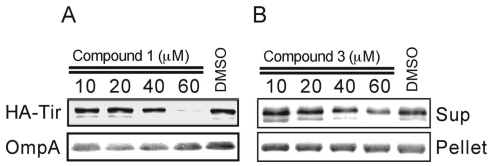

FIG. 7. Compounds 1 and 3 inhibit T3S by EPEC cells. Bacterial cultures treated with increasing concentrations of the indicated compounds were centrifuged, and the resulting pellets were lysed. Immunoblots prepared from these samples were stained with anti-OmpA antibody (OmpA is an *E. coli* outer membrane protein) to provide a measure of bacterial density. Immunoblots stained with anti-HA were prepared from TCA precipitates of the culture supernatant to provide a measure of secreted HA-tagged Tir, a T3S substrate in EPEC. (A) Compound 1; (B) compound 3 diproprionate salt. On the basis of estimates derived from densitometry of the immunoblots, 60 μM compound 1 blocks Tir secretion by 96%, while 60 μM compound 3 reduces Tir secretion by 81%.

DISCUSSION

The HTS assay based on relieving the growth inhibition associated with the in vitro activation of T3S secretion in *Y. pestis* described above has proved to be both effective and convenient. The rate of false-positive results was low, in the sense that the screening of 70,966 compounds yield only 431 positive hits (0.6%), among which strong positive hits were observed at a rate of 0.01%. Z' values (43), a statistical measure of assay quality, between 0.5 and 1 are generally regarded as excellent for HTS methods. In our HTS assay, the Z' value for random assay plates averaged 0.7 (31). The assay clearly yields bona fide inhibitors. Addition of the test compounds to the bacterial suspension in the appropriate medium is the only preincubation manipulation required, and luminometry is the only postincubation step. The use of a luminescent *Y. pestis* derivative allows a reliable means of detection of modest growth at low bacterial densities and avoids interference by common artifacts (e.g., bubbles) that afflict direct measurements of growth by determination of the OD. This method is strongly biased against compounds that inhibit bacterial growth, an effect that would yield false-positive results by some other approaches. It also selects for compounds that act on the bacteria rather than on target cells, as the latter are not present in the assay. The chief disadvantage of this approach is that it may fail to detect some classes of inhibitors because they do not relieve growth inhibition. For example, compounds that interfere at the level of formation of the connection of the secretion apparatus with target cells would probably not be detected.

Several other techniques have been used to screen for T3S inhibitors. These include enzyme-linked immunosorbent assay-based detection of proteins secreted from EPEC (19), inhibition of sheep erythrocyte lysis by EPEC (1), and inhibition of induction of a *yopE*::*luxAB* fusion in *Y. pseudotuberculosis*. The erythrocyte lysis assay probably depends on pore formation by T3S translocon proteins (EspB and EpsD in EPEC, which are paralogues of *Yersinia* YopB and YopD, respectively), while

the *yopE*::*luxAB* fusion assay depends on the observation that the induction of T3S in the yersiniae is accompanied by substantial increases in the levels of transcription of effector genes, including *yopE* (22). Each of these approaches has yielded some potential inhibitors. The *yopE*::*luxAB* fusion method has been used most extensively, because, like our method, it is well suited to HTS. Initial experiments by the use of this method yielded 4 lead compounds from the screening of 9,400 compounds from the ChemBridge DiverSet F library. Initial structure-activity relationship studies (13, 21, 30) have been performed with two of these compounds, a salicylaldehyde compound (previously designated INP0007 [20]) and a 2-arylsulfonylamino-benzanilide, by using activity assays based on whole bacteria, because the targets of these compounds are not established. The activities of INP0007 and selected analogs against both *Salmonella enterica* serovar Typhimurium have been tested in vitro and in a tissue culture model (20, 29), and the activities of these compounds against *Chlamydia trachomatis* have been tested in tissue culture (2, 28). The results of these studies are consistent with the inhibition of T3S in these species.

The inhibitory compounds identified in our assay are a diverse group and in all cases are distinct from those discussed above. One interesting feature of the inhibition data is the differential action of the inhibitors on the secretion of different Yop species. Only compound 2 acted effectively against all three Yop species examined, while each of the other compounds inhibited YopH secretion more effectively than they inhibited the secretion of YopD and YopM. Moreover, the relative activities against the secretion of YopD and YopM also varied among the compounds. These differences suggest that the compounds differ in their mechanisms of action. The abilities of compounds 1 and 3, but not those of compounds 2 and 4, to inhibit the secretion of Tir by EPEC provides additional evidence that the mechanisms of action are diverse. Like INP0007 and related compounds, the novel scaffolds of compounds 1 and 3 clearly indicate that they have spectra of activity that extend beyond the yersiniae. This confirms the utility of the *Yersinia* system as a general platform for identifying T3S inhibitors.

Compound 2 was remarkable in comparisons with the other T3S inhibitors described here and elsewhere for its low IC_{50} and its ability to cause very complete inhibition. While the toxicity of this compound for mammalian cells is unfortunate, studies of its mechanism of action against T3SSs may prove useful in the development of improved inhibitors. It is also possible that modifications of this compound may sufficiently reduce its toxicity to provide a useful therapeutic index.

One puzzling observation is that INP0007 inhibited the growth of both *Y. pestis* and *Y. pseudotuberculosis*, with stronger inhibition against the former being observed. This conflicts with the results of Kauppi et al. (22). We cannot explain this inconsistency and can only suggest that it may be due to differences in the growth medium used or may be due to the presence of a minor but potent growth inhibitor in our commercial preparation. Minor contaminants were observed by mass spectroscopy (see Fig. S1 in the supplemental material), but the effect of this compound on bacterial growth is an issue that should be revisited.

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