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Targeting Bacterial Virulence: Inhibitors of Type III Secretion in *Yersinia*

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Summary

Agents that target bacterial virulence without detrimental effect on bacterial growth are useful chemical probes for studies of virulence and potential candidates for drug development. Several gram-negative pathogens employ type III secretion to evade the innate immune response of the host. Screening of a chemical library with a luciferase reporter gene assay in viable *Yersinia pseudotuberculosis* furnished several compounds that inhibit the reporter gene signal expressed from the *yopE* promoter and effector protein secretion at concentrations with no or modest effect on bacterial growth. The selectivity patterns observed for inhibition of various reporter gene strains indicate that the compounds target the type III secretion machinery at different levels. Identification of this set of inhibitors illustrates the approach of utilizing cell-based assays to identify compounds that affect complex bacterial virulence systems.

Introduction

Recent studies have revealed that various pathogenic bacteria use related virulence systems, findings that contradict the long-held paradigm that each bacterium has a unique mode of action. These findings have implications for development of novel antibacterial agents as well as research aimed at understanding bacterial virulence. The plasmid-encoded Ysc (*Yersinia* secretion) type III secretion (TTS) system of *Yersinia* represents the archetype of one of these common virulence systems in which the bacterium adheres to a eukaryotic cell and injects a set of bacterial effector proteins, Yops (*Yersinia* outer proteins), into the lumen of the target cell, resulting in inhibition of the innate immune response [1, 2]. The genus *Yersinia* includes eleven known species, of which *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are pathogenic to humans. *Y. pestis*, the causative agent of plague, is one of the most virulent bacteria known to man [3]. Bacterial virulence systems, such as the processes of protein secretion across the bacterial membranes and translocation into the target cell, represent attractive points of attack for development of novel antibacterial agents [4–6]. The TTS apparatus is essential for the bacteria to evade the immune defense, and it is likely that agents that inhibit secretion can result in an antibacterial response without actually killing the bacteria. This chemical attenuation will enable the host to clear the infection, and we hypothesize that the selective pressure for resistance will be dramatically less than for conventional antibiotics since the cost of mutating a specific virulence attribute is probably very high. Compounds that target TTS can also be employed as chemical probes to further study the secretion and its regulation using chemical genetics [7, 8]. The approach of using small molecules to study cellular microbiology holds great promise, but only a few examples can be found in the literature [9]. Moreover, several mammalian pathogens, including *Yersinia* species, *Salmonella* species, *Shigella flexneri*, *Pseudomonas aeruginosa*, enteropathogenic *Escherichia coli*, *Chlamydia* species, and also plant pathogens like *Xanthomonas campestris*, *Erwinia* species, *Pseudomonas syringae*, and *Ralstonia solanacearum*, employ closely related TTS systems that are crucial for virulence [10, 11]. The most ancient TTS system is the machinery involved in assembly of flagella [10]. It was recently reported that the flagellar type III export apparatus in *Y. enterocolitica* also functions as a protein secretion system [12]. This important observation was corroborated by the finding that the YplA protein of *Y. enterocolitica* in fact is secreted by the Ysc, the chromosomally encoded Ysa (*Yersinia* secretion apparatus), and the flagellar TTS systems in *Y. enterocolitica* [13]. Interestingly, some components of TTS systems in different species are interchangeable, suggesting evolutionary conservation and that data generated with one specific TTS system might be valid also for others [14]. The relevance of *Yersinia* TTS-based research is further underscored by the fact that multiresistance strains have been found in *Y. pestis* [15, 16], and that *Y. pestis* is a potential weapon in biological warfare and bioterrorism [17, 18].

The components of the plasmid-encoded Ysc type III virulence system, i.e., the effector proteins (Yops) and proteins involved in regulation and secretion, are encoded by a ~70 kb virulence plasmid [19]. Although many functions of the *Yersinia* TTS and its regulation are poorly understood, results from different laboratories have contributed to provide a broad picture of secretion-related events [2, 10, 11]. When the bacterium enters the host, the temperature shift to 37°C induces a pathogenic switch in which ~20 Ysc proteins that form the secretion apparatus spanning the bacterial inner and outer membranes are produced. This temperature shift also results in expression of chaperones, Sycs (specific yop chaperones). Expression of the Ysc proteins, Sycs and Yops, is regulated by the temperature-induced activator protein LcrF [20]. The negative element, LcrQ, on the other hand, suppresses full expression of the Yops until the bacterium is directly in contact with a eukaryotic cell [21]. Upon this intimate contact with the eukaryotic target cell, LcrQ is secreted, resulting in elevated Yop production [21]. The cognate Sycs then bind to the newly

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produced Yops and deliver them to the Ysc apparatus. In parallel, a poorly understood chain of events results in formation of a pore in the eukaryotic cell membrane. The Yops are secreted through the Ysc machinery and then likely translocated through the pore into the cytoplasm of the eukaryotic cell [2]. In the eukaryotic cell, six different Yops, YopE, YopH, YpkA, YopJ, YopM, and YopT, specifically inactivate innate immune reactions [1].

To enable identification of novel antibacterial agents and chemical probes for chemical genetics, we developed a whole-cell bacterial reporter gene assay based on *Y. pseudotuberculosis*. This assay can be used to identify compounds that target known or unknown components directly or indirectly affecting the Ysc TTS machinery. The assay relies on monitoring expression from the *yopE* promoter. It has been employed to screen a ~9,400 compound library, yielding several molecules that inhibit TTS at concentrations that do not prevent bacterial growth.

Results and Discussion

In Vitro Regulation of Secretion and Reporter Gene Constructs

With the aim to develop an assay that can detect compounds that affect both known and unknown targets involved in the Ysc TTS system, we focused on a transcriptional reporter gene assay in viable bacteria. Regulation of this TTS machinery in *Y. pseudotuberculosis* is relatively well understood, and its comparatively low virulence in humans made it a suitable candidate for assay development. Interestingly, the expression and secretion of Yops has been found to be Ca^{2+} dependent in vitro. At 26°C, *Yersinia* grow both in the presence and absence of Ca^{2+}, whereas millimolar concentration of Ca^{2+} is required for growth at 37°C. In Ca^{2+}-depleted medium at 37°C, a metabolic downshift is observed and growth is halted after a few generations [22]. This is accompanied by LcrQ secretion and subsequent strong expression and secretion of the Yops in absence of eukaryotic cells [23]. The role of this Ca^{2+} dependence in vivo has, however, not been clarified. The simplified regulatory model for Syc and Yop expression given in Figures 1A and 1B was used to design reporter gene constructs for screening in the absence of eukaryotic target cells. For a transcriptional reporter system, we employed the luciferase gene from *Vibrio Harveyi* [24]. In the presence of *n*-decanal, this enzyme oxidates flavine mononucleotide, resulting in light emission at 490 nm. The light signal can be detected with a light-sensitive charge-coupled device (CCD) camera, thus enabling efficient monitoring of protein expression. Following a previously reported strategy, the luciferase-encoding hybrid gene, *luxAB*, was cloned to be under the control of the effector protein *yopE* promoter on the virulence plasmid in *Y. pseudotuberculosis* serotype III (YPIII) [25]. All strains employed in this study are based on YPIII, and in the following paragraphs the strains are labeled only with the name of the virulence plasmid. The construct was introduced in both wild-type bacteria (pIB102) [26] and a nonvirulent strain (pIB29) [27] which lacks the gene for the effector protein YopH [27]. The

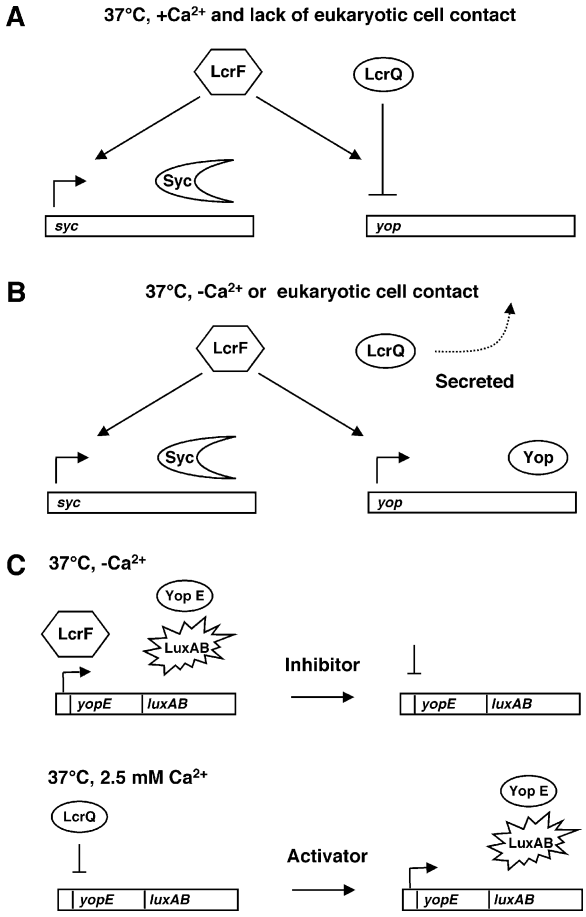

Figure 1. The Current Model for Regulation of Yop and Syc Expression Involving Temperature and Ca^{2+} Dependence via the Positive Element LcrF and the Negative Element LcrQ in Wild-Type *Y. pseudotuberculosis* Harboring the Virulence Plasmid pIB102

(A) At 37°C, LcrF is a positive regulator of Syc and Yop expression, but at this stage Yop expression is suppressed by the negative regulator LcrQ.

(B) Upon eukaryotic cell contact or Ca^{2+} depletion at 37°C, LcrQ is secreted, resulting in Yop expression.

(C) Schematic representation of reporter gene constructs for identification of inhibitors or activators of *Yersinia* TTS in the absence of eukaryotic target cells.

resulting strains pIB102EL [25] and pIB29EL were found to exhibit secretion properties, including in vitro temperature and Ca^{2+} regulation, identical to wild-type *Y. pseudotuberculosis* (data not shown). Thus, using a bacterium with the *luxAB* reporter gene construct under the control of a native virulence gene promoter, it is possible to monitor processes regulating secretion-specific transcription. In addition, it is possible to screen for inhibitors or activators in the absence of eukaryotic cells by manipulating the temperature and the Ca^{2+} concentration as schematically shown in Figure 1C. This cloning strategy was also used to prepare an additional strain in which *luxAB* was cloned downstream of the promoter for YerA, the specific YopE chaperone. In the resulting wild-type (pIB102AL) strain, the luciferase expression is dependent on temperature but independent of the Ca^{2+} concentration [28]. Notably, the *luxAB* reporter gene was

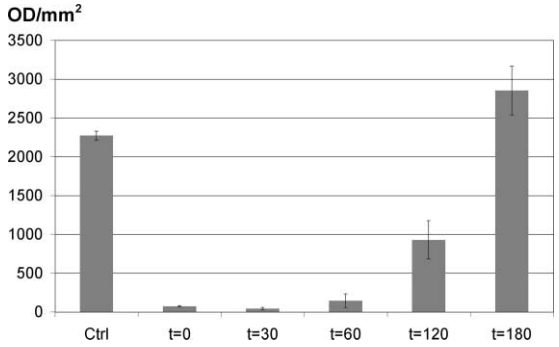

Figure 2. Inhibition of the Reporter Gene Signal by a Monoclonal Anti-YopB Antibody

Inhibition of the luciferase reporter gene light signal in the strain pIB29EL by a mouse monoclonal anti-YopB antibody added at the indicated time-points (t in minutes) during the assay process; n = 4.

transcriptionally fused downstream of the structural gene, generating a new operon structure. Thus, all strains employed in this study exhibit wild-type phenotype with respect to regulation and virulence.

Assay Optimization: Scope and Limitations

A typical procedure involved diluting an overnight bacterial culture and dispensing it into 96-well plates. After an initial incubation at room temperature that allows the culture to attain stable growth, the plate is shifted to 37°C. In the absence of Ca^{2+}, growth is suppressed and Yop secretion is activated. In the presence of Ca^{2+}, Yop secretion remains low and the bacteria continue to grow. The plate is then shifted back to room temperature, and, finally, *n*-decanal is added and light emission is measured. The assay conditions, including initial bacterial density, incubation times, etc., have been optimized (data not shown) to give a robust and reliable system primarily for identification of Yop secretion inhibitors (see Experimental Procedures).

Since the assay is based on a transcriptional readout, compounds perturbing processes unrelated to TTS systems have the potential to result in reduction of light emission. To address this issue, a number of well-studied antibiotics with known modes of actions were evaluated. Carbenicillin, streptomycin, nalidixic acid, and polymyxin B all resulted in a dose-dependent inhibition of light emission with IC_{50} values of 15, 4, 15, and 0.15 μM, respectively. These data clearly illustrate that the assay, despite having only a short growth period (<3 hr), can efficiently detect bacteriocidal agents acting on a variety of bacterial targets.

The primary goal of this investigation was, however, to use this assay to screen for novel agents specifically targeting the Ysc TTS system. We therefore evaluated a panel of antibodies targeting a set of relevant *Yersinia* proteins that putatively are present on the bacterial surface and directly or indirectly regulate the secretion. Interestingly, a monoclonal antibody against YopB, one of the putative pore-forming proteins [2], could completely inhibit or reduce light emission when added to the assay solution at time points up to 2 hr after start of the experiment (Figure 2). After the shift to 37°C, the secretion system is activated, but increased Yop expression is not detectable until after approximately 1 hr, indicating that addition of the Yop-neutralizing antibody results in an immediate inhibitory response. Thus, agents that specifically target one component of the TTS machinery can result in a complete suppression of Yop expression.

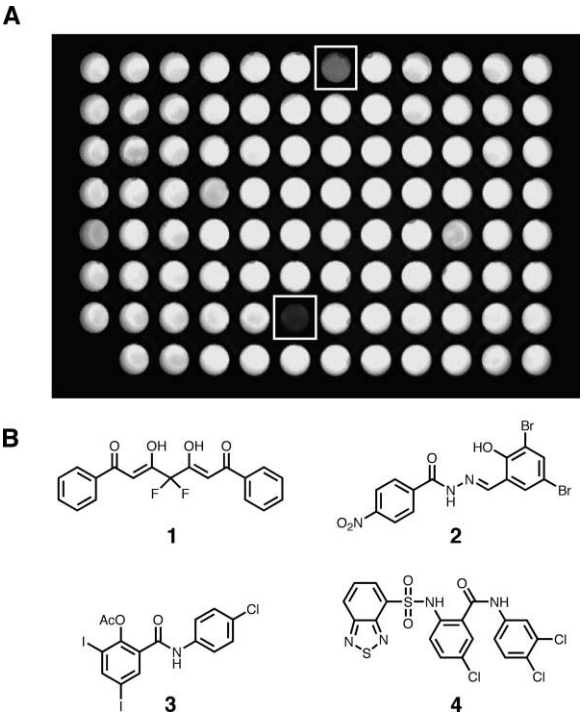

Figure 3. Screening and Identification of Potential Type III Secretion Inhibitors

(A) Screening for inhibitors of TTS. The photo shows a typical result from the library screening campaign. One compound per well is screened at 20 μg/ml in columns 2–11. The dark well in the lower left corner contains 10 μM streptomycin as positive control. Remaining wells in columns 1 and 12 are used as controls. The white squares indicate potential screening hits.

(B) Structures of the identified inhibitors **1–4**.

In conclusion, the assay can be used to screen for agents targeting different bacterial functions, including compounds that specifically interfere with the Ysc TTS system. Since the screening is performed with viable bacteria, issues such as membrane permeability will be addressed directly. Moreover, the assay is well suited for high-throughput screening of large compound collections.

Screening for Secretion Inhibitors

The assay was used to screen a compound collection consisting of ~9,400 unique substances (ChemBridge, DiverSet F) for inhibition of the *luxAB* reporter signal using the nonvirulent strain pIB29EL (Figure 3A). The compounds were screened in duplicates at a final concentration of 20 μg/ml. The complete screening campaign gave ~30 compounds that caused at least 40% inhibition (duplicate average) of the luciferase light signal. For 26 of these compounds, dose-response rela-

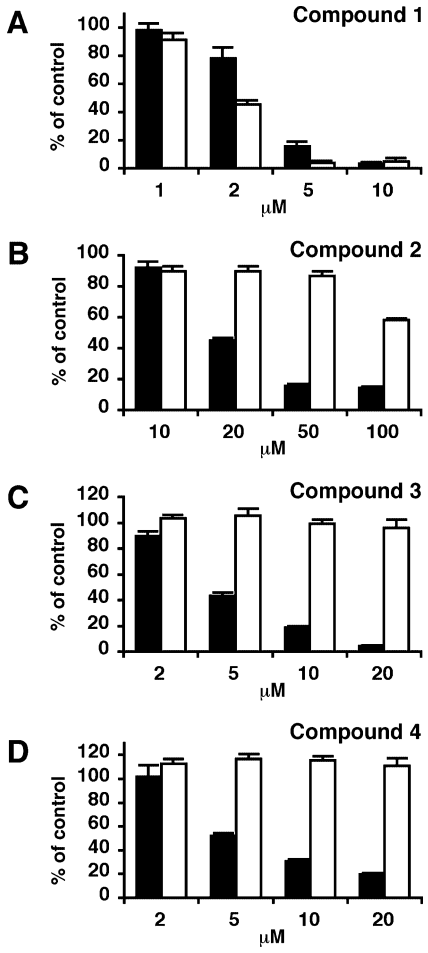

Figure 4. Initial Hit Characterization

Effect on luciferase light emission (black bars) and bacterial growth (white bars) for the nonvirulent screening strain pIB29EL in the presence of different concentrations of compounds **1–4**; n = 4.

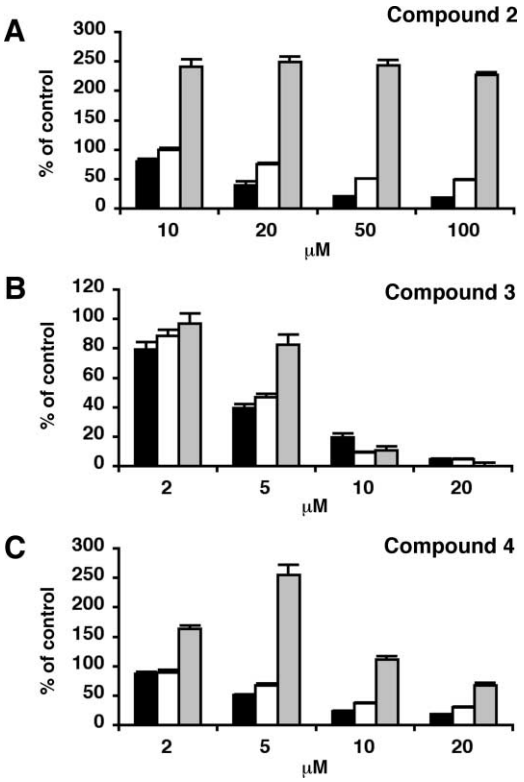

Figure 5. Characterization of Screening Hits Using Strains with the Reporter Gene Downstream of Different Virulence Promoters

Effect on luciferase light emission in different wild-type bacteria (p102EL, black bars; pIB102AL, white bars; pIB102FL, gray bars) in the presence of different concentrations of compounds **2–4**; n = 4.

tionships were established and inhibition of bacterial growth was examined. All experiments based on the luciferase readout were carried out in quadruplicate in the same manner as the primary screen (see Experimental Procedures). Of the 26 compounds, 12 were found to have an IC_{50} for inhibition of the luciferase light signal of 50 μM or less (data not shown). In terms of growth inhibition, the compounds could be divided into two groups. In the first group, data for inhibition of growth paralleled inhibition of light emission as shown for compound **1** (Figures 3B and 4A). Compounds **2–4** (Figure 3B) are representative of the second group in which no or modest inhibition of growth was observed at concentrations that caused major reduction of the luciferase light signal (Figures 4B–4D). The inhibition of light emission observed for substance **1** is most likely a result of the growth inhibition rather than a direct interference with the Ysc TTS system. Although not of primary interest for this investigation, such compounds are still valuable. To our knowledge, data regarding antibacterial effect of **1** cannot be found in the literature. Compounds **2–4**, on the other hand, show a selectivity for inhibition of the luciferase light signal over growth inhibition, indicating that these substances possibly act on targets directly or indirectly regulating expression of the Yops but not affecting growth (Figures 4B–4D).

Further Characterization of Screening Hits

A more detailed investigation was carried out for many of the 26 compounds, although emphasis was placed on compounds **2–4**. As a first step, the compounds were assayed using the wild-type control strain, pIB102AL, with *luxAB* downstream of the promoter for YerA, the chaperone for YopE. As seen in Figures 5A and 5C, compounds **2** and **4** showed a somewhat stronger inhibitory effect on the wild-type strain pIB102EL with *luxAB* under control of the *yopE* promoter than on pIB102AL. Compound **3**, on the other hand, had no selectivity for either of these two strains, as illustrated in Figure 5B. In order to zoom in on potential targets and to further support a selective mode of action for compounds **2–4**, one additional wild-type strain with the *luxAB* gene downstream from the *lcrF* promoter (pIB102FL) was used. Based on the regulatory model in Figures 1A and 1B, compounds that inhibit the signal from both the *yopE* and the *yerA* promoters could in fact target gene expression of the positive regulator LcrF, since the *yopE* and *yerA* promoters are both positively regulated by LcrF. Importantly, in contrast to the *yopE* promoter, the *yerA* promoter is not regulated by the negative Ca^{2+} control loop (cf. Figures 1A and 1B). Consistent with this

regulatory model, compound **3** was found to inhibit the reporter gene signal in all three strains, pIB102EL, pIB102AL, and pIB102FL, to more or less the same extent (Figure 5B). These results suggest that compound **3** targets LcrF directly or, more likely, acts on regulatory elements upstream of LcrF, thus explaining the block of expression of YerA and YopE. Compounds **2** and **4**, on the other hand, were found to enhance the expression from the *lcrF* promoter in pIB102FL at concentrations that reduce expression of YopE (Figures 5A and 5C). For compound **2**, this increase in expression from the *lcrF* promoter was dose independent in the concentration range investigated (Figure 5A). Compound **4**, on the other hand, produced a peak around 5 μM and a modest inhibition above 10 μM (Figure 5C). The selectivity patterns observed for compound **2** and **4** suggest that these compounds might target Yop secretion or the Ca^{2+}-dependent regulation involving the negative LcrQ loop rather than the temperature-dependent LcrF activating cascade (cf. Figures 1A and 1B). Under the assay conditions, LcrF is expressed at elevated levels, and currently we cannot explain why LcrF expression is stimulated. However, this observation is potentially important since the mechanisms that regulate LcrF are poorly understood.

Western analysis was performed in order to establish inhibition of actual Yop secretion in the presence of compounds **2–4**. The wild-type strain pIB102EL was grown at ambient temperature (~21°C) in the presence or absence of different compound concentrations for 1 hr and then shifted to 37°C to allow induction and secretion of the Yops. After 2.5 hr at 37°C, the bacteria were removed by centrifugation, and the protein content in the supernatant, i.e., the surrounding media, was examined by Western analysis using a polyclonal serum active against all secreted Yops. As seen in Figures 6A–6C, compounds **2–4** efficiently inhibited Yop secretion in a dose-dependent manner, and the IC_{50} values for inhibition of the luciferase readout (cf. Figures 4B–4D) match the IC_{50} values for inhibition of Yop secretion (cf. Figures 6A–6C). These experiments demonstrate that an observed inhibition of actual Yop secretion corresponds to an inhibition of Yop expression.

Interestingly, several of the proteins that constitute the Ysc TTS system for Yop secretion have homologs in the secretion system used for assembly of the flagellum [10]. Preferably, chemical probes for investigation of the regulation and function of the Ysc TTS should be selective for the Yop secretion pathway, while candidates for drug development might benefit from inhibiting both processes. *Y. psedotuberculosis* flagella assembly mutants have been found to be avirulent in a mouse infection model (H.W.-W. et al., unpublished results), and motility was found to be required to initiate invasion by *Y. enterecolitica* [29]. Recent studies with *Y. enterocolitica* show that the flagellar, Ysc, and the chromosomally encoded Ysa TTS machineries all are capable of secretion of extracellular proteins, and thus certain proteins can be secreted by more than one system [13, 30]. Thus, compounds that target multiple TTS systems can provide valuable insights in common themes in TTS within a certain bacterium. In order to study selectivity for inhibition of Yop TTS over TTS flagellum assembly,

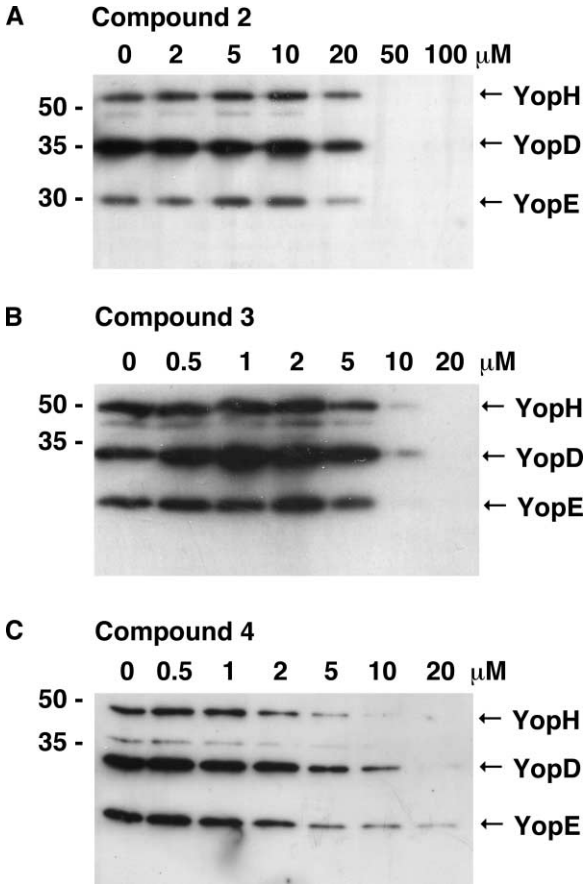

Figure 6. Inhibition of Yop Secretion

Compounds **2–4** inhibit Yop secretion. Yop secretion from the wild-type strain pIB102EL in presence of different concentrations of compounds **2–4** was investigated by Western analysis.

bacterial motility and Yop transcription in the presence of compounds **2–4** were investigated. The experiments were carried out in six-well plates with Ca^{2+}-depleted migration agar containing different concentrations of compounds **2–4**. A small volume of a pIB102EL culture was dispensed in the center of each well, and the plates were incubated at 22°C for 18 hr. During the incubation, motile bacteria will form a circular zone of bacteria spreading from the center of the well. The plates were then shifted to 37°C for 3 hr to allow onset of Yop expression and secretion. *n*-decanal was subsequently added to each well, and light emission resulting from Yop and luciferase expression was recorded with the light-sensitive camera and subsequently quantified. The plates were photographed in visible light, and the diameters of the motility zones were measured. DMSO alone proved to have little or no effect on motility but caused up to 45% inhibition of the *luxAB* reporter gene signal (Figures 7A–7F). A dose-dependent inhibition of both motility (Figure 7A) and the reporter gene signal (Figure 7B) was observed for compound **2**. However, the experiment does not address if compound **2** shows a quantifiable selectivity for any of the two TTS systems. Compound **3**, on the other hand, had virtually no effect on motility (Figure 7C) at concentrations causing strong or

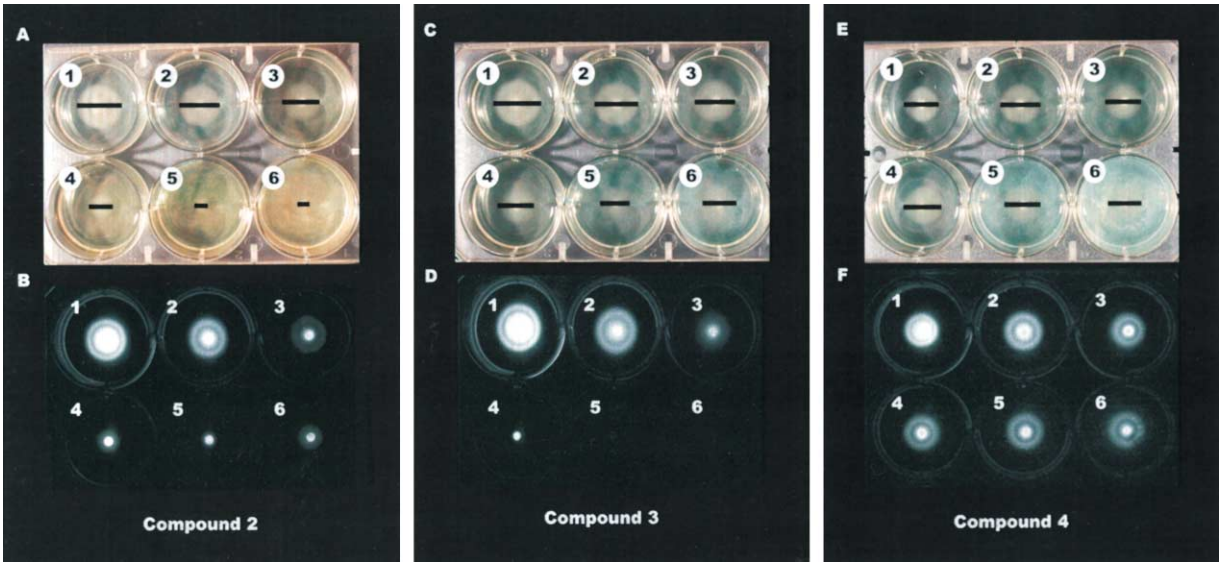

Figure 7. Investigation of Selectivity for Inhibition of Yop Type III Secretion over Flagellar Type III Secretion and Motility

Investigation of flagellum mediated motility (in [A], [C], and [E], the black bars indicate diameter of the zones of motility) and luciferase light emission as a result of transcription from the *yopE* promoter (B, D, and F) in the presence of increasing concentrations (wells 3–6) of compounds **2–4**. In (A)–(F), well 1, no additives; well 2, DMSO control. In (A) and (B), wells 3–6, 5, 10, 20, and 50 μM, respectively, of **2**. In (C) and (D), wells 3–6, 2, 5, 10, and 20 μM, respectively, of **3**. In (E) and (F), wells 3–6: 5, 10, 20, and 50 μM, respectively, of **4**.

complete inhibition of the reporter signal (Figure 7D). These results indicate that the TTS system responsible for flagellum assembly is fully functional and that bacterial growth is unaffected. Motility was not affected by compound **4** (Figure 7E) while a ~45% dose-independent inhibition of the reporter gene signal was observed (Figure 7F). This dose independence might be explained by the fact that a precipitate formed in the wells containing higher concentrations of compound **4** (Figure 7E, wells 5 and 6). Yop secretion is effectively inhibited by compound **4** (Figure 6C), but the effect on motility at higher concentrations is unclear due to the limited solubility. Compound **3**, on the other hand, is highly selective and targets the regulation and secretion of Yops without effect on flagellum assembly and motility. Our results obtained with different reporter gene strains (Figure 5B) suggest that compound **3** acts on a target upstream of the temperature-triggered activator LcrF. The Ysa and flagellar TTS system are active at low temperature, and the lack of inhibition of motility further supports that compound **3** targets regulation of the Ysc TTS system in a highly selective mode. Compound **2**, on the other hand, inhibits both processes, possibly by inhibition of closely related proteins involved in secretion of Yops and construction of the flagellum.

The bacterial targets in *Y. pseudotuberculosis* for compounds **1-4** are unknown at this stage. The compounds are synthetic derivatives that can be prepared in five or less chemical steps utilizing commercially available starting materials. These short synthetic sequences are well suited for synthesis of second-generation libraries, e.g., by parallel solution synthesis and establishment of structure-activity relationships. Several of the hits from the primary screening belong to a class of acylated salicylaldehyde hydrazones, of which compound **2** is the most potent. Compounds belonging to this class of hydrazones and their corresponding metal complexes have been prepared and evaluated for biological activity including antibacterial activity (see [31–33] for some examples), and cytotoxicity against tumor cell lines has been reported ([34] and references therein). Compound **3**, clioxanide, is a known antihelmintic *O*-acetyl salicylanilide [35], and antimicrobial effects have been reported for various salicylanilides [36]. Compounds similar to **3** but lacking the acetyl group are weakly acidic compounds known to induce uncoupling of oxidative phosphorylation, resulting in inhibition of ATP synthesis [37]. Recent studies also describe that salicylanilides closely related to **3** inhibit bacterial two-component systems [38, 39], and it is possible that compound **3** acts on a *Yersinia* two-component system that affects the Ysc TTS. This is a potentially interesting observation since no role of a two-component system has been implicated in regulation and secretion of Yops. Compound **4** and related derivatives have been found to display in vivo antihelmintic activity [40] and show low or no toxic effect in vivo [41].

In conclusion, we have employed a robust reporter gene assay in viable bacteria to identify inhibitors of Ysc type III secretion in *Yersinia*. Several compounds were identified that inhibit secretion in a direct or indirect manner at concentrations at which no or weak inhibition of bacterial growth was observed. Moreover, the selectivity patterns observed for these compounds indicate that they inhibit different pathways regulating Yop expression and secretion. The compounds have a selective mode of action and have different effects on the flagellum assembly and motility. We now plan to employ these compounds in in vitro and in vivo experiments to further study TTS in *Yersinia* and other gram-negative

bacteria. This set of chemical probes will hopefully aid the elucidation of the molecular events leading to Yop translocation and inhibition of the innate immune response. Importantly, these inhibitors also constitute examples of antibacterial agents with a novel mode of action, i.e., inhibition of virulence. The bacterial targets of these inhibitors are unknown, and our efforts will focus on target identification and optimization with the goal to increase selectivity and potency. Identification of this set of inhibitors illustrates the approach of utilizing cell-based assays to identify compounds that affect complex virulence systems in a direct or indirect manner.

Significance

As a result of changes in human lifestyle and the remarkable capacity of bacteria to develop resistance, bacterial infections are again considered to be a major threat to human health. One strategy to overcome bacterial resistance is to develop antibacterial agents that target virulence mechanisms, i.e., events that enable the bacterium to enter the host, disarm the host's defense, multiply, and finally spread within the host or to a new host. Various pathogenic bacteria use related virulence systems, of which the Ysc type III secretion (TTS) system of *Yersinia* represents one of the archetypes. During infection, *Yersinia* bacteria adhere to immune cells, e.g., macrophages, and use the TTS machinery to make a pore in the membrane of the target cell and subsequently inject a set of effector proteins that inhibit the innate immune response. In the present study, a luciferase reporter gene system in *Yersinia pseudotuberculosis* was used to screen a chemical library consisting of ~9,400 compounds in order to identify molecules that target known or unknown components directly or indirectly involved in TTS. The screening process furnished compounds that inhibit the reporter gene signal expressed from the *yopE* promoter and effector protein secretion in a dose-dependent manner ($IC_{50} < 50$ μM) at concentrations with no or modest effect on bacterial growth. Three of the inhibitors were investigated in detail, and the selectivity patterns observed for inhibition of various reporter gene strains indicate that the compounds target the TTS machinery at different levels. Since these compounds inhibit TTS without detrimental effect on growth, they are valuable chemical tools for investigation of TTS-dependent virulence. Furthermore, these inhibitors may apply less pressure for development of resistance than conventional antibiotics. Importantly, several components of different TTS systems are interchangeable, and data generated with *Yersinia* are likely to be valid also for other gram-negative bacteria.

Experimental Procedures

Bacterial Strains and Growth Conditions

Y. pseudotuberculosis serotype III strains with the *luxAB* contruct were prepared from either the nonvirulent YopH mutant *Y. pseudotuberculosis* pIB29 [27] or the wild-type *Y. pseudotuberculosis* pIB102 [26] by constructing *yopE-luxAB*, *yerA-luxAB*, and *lcrF-luxAB* operon fusions essentially as described previously [25]. In the *lcrF* construct, the negative self-regulatory C-terminal helix-loop-helix region has been deleted. The resulting strains, pIB29EL (*yopE-luxAB*), pIB29AL (*yerA-luxAB*), pIB102EL [25] (*yopE-luxAB*, wild-type), pIB102AL (*yerA-luxAB*, wild-type), and pIB102FL (*lcrF-luxAB*, wild-type), were grown at room temperature on LB plates containing chloramphenicol (Sigma) at a final concentration of 50 μg/ml. From plates not older than 1 week, bacteria for experiments were grown in liquid brain/heart infusion (BHI) broth (Oxoid; Unipath Ltd., Basington, UK) containing 2.5 mM $CaCl_2$ or 20 mM $MgCl_2$ and 5 mM EGTA for Ca^{2+} depletion and 19 μg/ml chloramphenicol.

Compounds, Antibiotics, and Antibodies

The chemical library that consists of 9,400 unique compounds in 96-well plate format was purchased from ChemBridge (Diverset F). The compounds were dissolved in DMSO to give a stock solution of 2 mg/ml. For compounds further characterized in the described experiments, additional 5 or 10 mg samples were purchased from ChemBridge. Streptomycin (Sigma), carbenicillin (Sigma), polymyxin B (mixture of polymyxin B_1 and B_2, Fluka), and nalidixic acid (Sigma) were dissolved in water. Antibodies against the different *Yersinia* proteins were available from other projects at the department of Molecular Biology, Umeå University.

General Screening and Assay Conditions

An overnight culture grown at room temperature in BHI medium containing 20 mM $MgCl_2$ and 5 mM EGTA for Ca^{2+} depletion was diluted to $OD_{600} = 0.15–0.25$ (10 mm path length). In parallel, the compounds to be tested were dispensed into the wells of a white 96-well plate (FluoroNunc, Nunc) containing 50 μl of medium per well. To each well, 50 μl of the bacterial solution was added. For compounds dissolved in DMSO, the final DMSO concentration was kept below 2%. The plate was incubated on an orbital shaker at room temperature (~21°C) for 1 hr. The temperature was then shifted to 37°C, and incubation with shaking was continued for 2 hr. Subsequently, the temperature was shifted back to room temperature, and the plate was incubated for 2 hr without shaking. Finally, 100 μl freshly made *n*-decanal emulsion (Sigma; 10 μl/100 ml water, emulsified by vigorous shaking) was added to each well, and the light emission was measured within 1–3 min after addition. Light emission was recorded with a light-sensitive charge-coupled device (CCD) camera, DIANA chemoilluminescence detection module (Raytest, Isotopenmeβgeräte, GmbH). The intensity of the light signals was quantified using the computer program TINA (version 2.0). Primary screening of the compound library was carried out in duplicate with a final concentration of 20 μg/ml. Other experiments were carried out in quadruplicate with modifications as indicated in the text and figure legends. For antibody experiments, 5 μl monoclonal mouse ascites solution was used per well. Results were typically reproduced in at least three independent experiments.

Growth Inhibition Experiments

Growth inhibition was measured by growing bacteria at 37°C in the presence of different compound concentrations in 96-well plates containing 100 μl bacterial culture in BHI medium with 2.5 mM $CaCl_2$ per well. The experiments were carried out in a Molecular Device Spectramax 340 plate reader with continuous shaking at 37°C and periodic determination of absorption at the wavelength 600 nm. Experiments were carried out in triplicate or quadruplicate, and the results were typically reproduced in at least three independent experiments. Growth inhibition was quantified based on growth rates observed in the presence and absence of compound over a period of time (typically 3–4 hr) with approximately linear growth.

Western Analysis of Protein Secretion

An overnight culture grown at room temperature in BHI medium containing 20 mM $MgCl_2$ and 5 mM EGTA for Ca^{2+} depletion was diluted to $OD_{600} = 0.15–0.25$. In parallel, the compounds to be tested were dispensed into the wells of a 96-well plate containing 50 μl of medium per well. To each well, 50 μl of the bacterial solution was added. The plate was incubated on an orbital shaker at room temperature for 1h. The temperature was then shifted to 37°C, and incubation with orbital shaking was continued for 2.5 hr. Subsequently, the cultures were transferred to microcentrifuge tubes. After

brief centrifugation, the content of supernatants was investigated by standard Western analysis (12% SDS-PAGE) using a polyclonal total anti-Yop serum from rabbit. The level of protein secretion was estimated by photographing the Western membrane with the light-sensitive camera followed by quantification of the light intensity from the YopE bands (see general screening and assay conditions section).

Motility Agar Assay

Motility was measured as the movement of bacterial cells through semisolid motility agar (Luria broth containing 0.25% Bactoagar). Around 10^7 *Y. pseudotuberculosis* YPIII pIB102EL bacteria in a 2.5 μl drop were placed at the center of each well in a 6-well plate (Falcon Multiwell) containing 5 ml motility agar supplemented with 5 mM EGTA, 20 mM $MgCl_2$, and different concentrations of test substance in 0.1 ml DMSO per well. The plates were incubated at 22°C for 18 hr to allow for the bacteria to grow and to move out from the center of the plate. To induce Yop and LuxAB expression, plates were shifted to 37°C (at this temperature flagellum production stops) and incubation was continued for 3 hr. By placing 5 μl of *n*-decanal in the lid over each well to expose the bacteria to decanal fumes, light emission from the bacteria was induced. Emitted light was monitored and quantified using the CCD camera and software as described in the general assay and screening conditions section. Immediately after measurement of light emission, a picture was taken in visible light to document the bacterial migration. Migration was then quantified directly from the picture as the diameter of the zone covered by bacteria.

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