A Small-Molecule Screen Identifies the Antipsychotic Drug Pimozide as an Inhibitor of *Listeria monocytogenes* Infection[▽]

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We developed a screening procedure to identify small-molecule compounds that altered infection by *Listeria monocytogenes* to gain insights into bacterial/host cellular processes required for intracellular pathogenesis. A small-molecule library of 480 compounds with known biological functions was screened, and 21 compounds that altered the *L. monocytogenes* infection of murine bone marrow-derived macrophages (BMM) were identified. The identified compounds affected various cellular functions, such as actin polymerization, kinase/phosphatase activity, calcium signaling, and apoptosis. Pimozide, an FDA-approved drug used to treat severe Tourette's syndrome and schizophrenia, was further examined and shown to decrease the bacterial uptake and vacuole escape of *L. monocytogenes* in BMM. The inhibitory effect of pimozide on internalization was not specific for *L. monocytogenes*, as the phagocytosis of other bacterial species (*Bacillus subtilis*, *Salmonella enterica* serovar Typhimurium, and *Escherichia coli* K12) was significantly inhibited in the presence of pimozide. The invasion and cell-to-cell spread of *L. monocytogenes* during the infection of nonprofessional phagocytic cells also was decreased by pimozide treatment. Although pimozide has been reported to be an antagonist of mammalian cell calcium channels, the infection of BMM in a calcium-free medium did not relieve the inhibitory effects of pimozide on *L. monocytogenes* infection. Our results provide a generalizable screening approach for identifying small-molecule compounds that affect cellular pathways that are required for intracellular bacterial pathogenesis. We also have identified pimozide, a clinically approved antipsychotic drug, as a compound that may be suitable for further development as a therapeutic for intracellular bacterial infections.

Intracellular bacterial pathogens are the cause of numerous diseases and are responsible for tremendous morbidity and mortality worldwide. Traditionally, genetic-based approaches have been used to identify bacterial virulence determinants involved in microbial pathogenesis. While genetic-based approaches have provided insights into the mechanisms of pathogenesis for several bacterial species, the lack of genetic tools for use in many pathogenic systems, coupled with an inability to easily perform the large-scale genetic manipulation of mammalian host cells, has hampered efforts to gain considerable insight into the mechanisms of intracellular bacterial pathogenesis (3). The screening of small-molecule libraries to identify compounds that alter intracellular infection (e.g., chemical genetics) is a powerful approach that holds promise for further elucidating the host cell biological and pathogen-specific mechanisms governing interactions required for intracellular pathogenesis (23). As small molecules can enter and affect multiple pathways within microbes or host cells, numerous insights into the bacterial and host cellular processes required for intracellular infection can be obtained. This approach also has the benefit of potentially identifying compounds that can be further developed as new therapeutics based upon the ability to inhibit bacterial infection within the host (16, 23, 38).

Listeria monocytogenes is a gram-positive, intracellular bacterial pathogen and is a model organism for understanding fundamental aspects of host-pathogen interactions leading to disease and cell-mediated immunity (32). *L. monocytogenes* is a serious threat to immunocompromised individuals, pregnant women, newborns, and the elderly (12, 41). Infections typically occur through the ingestion of contaminated foods such as deli meats, soft cheeses, and unwashed fruits and vegetables (35) and most often results in gastroenteritis or flu-like symptoms, though more severe outcomes include septicemia, meningitis, and death (12, 41). Although antibiotics can be used to successfully treat *L. monocytogenes* infections, case fatality typically is 20 to 30% (39).

Numerous genetic-based studies have led to a thorough understanding of the cell biology of *L. monocytogenes* infection and the characterization of several bacterial virulence determinants (10, 32). *L. monocytogenes* can readily infect both professional phagocytic and nonprofessional phagocytic host cells. Following internalization, bacteria initially reside within a membrane-bound vacuole, which is rapidly lysed to allow *L. monocytogenes* access to the host cell cytosol. Vacuolar lysis is mediated by a secreted pore-forming cytolysin, listeriolysin O (LLO), and a phosphatidylinositol-specific phospholipase C (PI-PLC) (13, 33). Once in the cytosol, bacteria express ActA, an *L. monocytogenes* surface protein that polymerizes host actin to facilitate actin-based motility (26). Bacteria subsequently spread to additional host cells by forming pseudopod-like projections that are internalized by neighboring cells, resulting in the formation of double-membrane vacuoles surrounding bacteria in secondary host cells. Bacteria subsequently escape the secondary vacuole using LLO and a bacterium-encoded broad-range phospholipase C (PC-PLC) (2). While several bacterial and a few host cell determinants involved in intracellular infection have been identified, little is

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known about the specific mechanisms governing host-pathogen interactions during *L. monocytogenes* infection.

High-throughput screens of small-molecule libraries have been conducted previously to gain insights into bacterial physiology and have identified compounds that inhibit bacterial growth, metabolism, or development (8, 16, 22, 44). These screens often have used high-throughput plate reader-based assays that have the ability to rapidly screen thousands of compounds. However, very few small-molecule screening studies have been conducted using a host-pathogen infection system (23, 25, 36). Using *L. monocytogenes* as a model intracellular bacterial pathogen, we have developed a small-molecule screening procedure to identify compounds that alter the intracellular infection of host cells. The screening procedure allows the exposure of both bacteria and host cells to the small-molecule compounds to facilitate the disruption of host-pathogen interactions. While similar small-molecule screens have been described previously for pathogens such as hepatitis C virus (25), the protozoan parasite *Toxoplasma gondii* (5), and the bacterial pathogen *Pseudomonas aeruginosa* (27), our screening procedure differs significantly in that the infection of primary mammalian host cells (e.g., murine-derived macrophages) was used to gain a more relevant account of conditions that are encountered during the in vivo infection of the host. Our approach also utilizes automated microscopy and the subsequent visual analysis of images to more precisely discern alterations in intracellular infection following exposure to the compound libraries (1, 6).

To our knowledge, this study represents the first small-molecule screen in which primary mammalian host cells were used to examine infection by a microbial pathogen. We screened a library of 480 compounds containing a diverse range of known biological activities. In addition to the identification of 21 compounds that significantly affected *L. monocytogenes* infection, we further characterized one compound, pimozide, which inhibited multiple stages of intracellular infection by *L. monocytogenes*. Pimozide, a diphenylbutylpiperidine, is an FDA-approved compound that has neuroleptic properties and has been used clinically to treat severe Tourette's syndrome and schizophrenia (40). Using a variety of established assays for *L. monocytogenes* infection, we determined that pimozide significantly decreased the efficiency of macrophages to phagocytose *L. monocytogenes* and other bacterial species and also decreased the vacuole escape and cell-to-cell spread of *L. monocytogenes*.

MATERIALS AND METHODS

Bacterial strains. Wild-type *L. monocytogenes* strain 10403S was grown in brain heart infusion (BHI) medium (Difco, Detroit, MI) at 30°C without shaking prior to infection of host cells. *L. monocytogenes* 10403S expressing green fluorescent protein (*L. monocytogenes*-GFP) (strain DH-L1252) was used to infect host cells for screening and vacuole escape assays. *Escherichia coli* K12 strain MM294 was grown at 37°C shaking in Luria-Bertani (LB) medium (Difco). Similarly, *Bacillus subtilis* strain 3610 and *Salmonella enterica* serovar Typhimurium strain IR715 were grown in LB at 37°C shaking.

Cell culture. Murine bone marrow-derived macrophages (BMM) were prepared as previously described (4). Briefly, femurs and tibias were removed from 6- to 8-week-old BALB/c mice (Jackson Laboratories, Bar Harbor, ME), and bone marrow cells were flushed from the femurs with complete Dulbecco's modified Eagle's medium (DMEM; Mediatech, Herndon, VA) supplemented with 7.5% fetal bovine serum (FBS; HyClone, Logan, UT), 2 mM glutamine, and 100 μg/ml penicillin and streptomycin (P/S) and was washed once with DMEM.

Cells were cultured in 100-mm-diameter petri dishes for 3 days in BMM medium (DMEM supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/ml P/S, 55 μM β-mercaptoethanol [β-ME], and 30% L929 cell supernatant). On day 3, fresh BMM medium was added to the cultures. On day 6, BMM cells were harvested by the removal of medium and the addition of cell-harvesting medium (phosphate-buffered saline [PBS], 0.7 mM EDTA, 3% FBS). Recovered BMM were washed with PBS and plated at an appropriate density for 18 to 24 h prior to infection experiments.

The murine-derived professional phagocytic cell lines J774 and RAW 264.7 were cultured in DMEM supplemented with 7.5% FBS, 2 mM glutamine, 1 mM sodium pyruvate, and 100 μg/ml P/S. The murine-derived L2 fibroblast cell line was grown in RPMI medium (Mediatech) supplemented with 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 μg/ml P/S, and 55 μM β-ME (RPMI-10). For intracellular growth, gentamicin protection, vacuole escape, and cell-to-cell spread assays, host cell infections were performed in antibiotic-free medium. All cell cultures were maintained at 37°C in a 5% CO$_2$-air atmosphere.

Small-molecule screen. BMM were seeded at 1×10^4 cells per well in 40 μl of medium in black-walled, clear-bottom 384-well plates (Corning-Costar, Corning, NY). Twenty-four hours later, the compound library was added by automated pin transfer (100 nl of each compound/well; final concentration, ~12.5 μg/ml), and the cells were incubated with the compounds for 2 h. BMM were infected at a multiplicity of infection (MOI) of 1:2 by addition of *L. monocytogenes*-GFP in 10 μl medium using an automated plate dispenser. After an additional incubation for 1 h, 10 μl medium containing 5 μg/ml gentamicin was added to each well to kill extracellular bacteria. Infected BMM were incubated for an additional 14 h followed by being washed with PBS three times. Cells were fixed with 3.2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 15 min at room temperature (RT; 18 to 25°C). Plates were washed with PBS and then stained for 10 min at RT with Hoechst 33342 (Invitrogen, Carlsbad, CA) diluted 1:5,000 in 1% bovine serum albumin (BSA) in TBS-TX (25 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100). Fluorescence microscopy images were acquired at two positions per well using a cellWoRx high-content cell analysis system (Applied Precision, Issaquah, WA) and analyzed with MetaMorph image analysis software (Molecular Devices, Downington, PA). The Biomol ICCB Known Bioactives compound library (Biomol, Plymouth Meeting, PA) containing 480 compounds (http://iccb.med.harvard.edu/screening/compound_libraries/bioactives_biomol_med.htm) was screened three times in duplicate at the National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (NSRB) (http://nsrb.med.harvard.edu) at Harvard Medical School (Boston, MA).

Host cell toxicity assay. BMM were seeded at 1×10^4 cells/well in solid white 384-well plates. Twenty-four hours later the compound library was added by automated pin transfer, and BMM were incubated for 17 h. The CellTiterGlo (Promega, Madison, WI) luminescent cell viability assay reagent was added to each well per the manufacturer's protocol, and the plates were placed on an orbital shaker for 2 min to facilitate host cell lysis. Plates then were incubated for 10 min at RT. The luminescence in each well was detected using an EnVision plate reader (Perkin Elmer, Waltham, MA).

Intracellular growth assay. The intracellular growth of bacteria was analyzed as previously described (20), with some modifications. BMM or L2 cells were seeded 24 h prior to infection at 4×10^5 BMM/well or 2×10^5 L2 cells/well in a 24-well plate. Cells were washed with PBS and pretreated for 2 h with medium containing 18.1 μM pimozide or an equivalent volume of dimethylsulfoxide (DMSO). Wild-type *L. monocytogenes* 10403S was grown for 14 to 16 h in BHI medium at 30°C without shaking. The bacterial culture was washed once with PBS and used for the infection of host cells at the indicated MOI in the presence of 18.1 μM pimozide or DMSO. At 1 h postinfection, monolayers were washed with PBS, and medium containing 5 μg/ml (for BMM) or 50 μg/ml (for L2 cells) gentamicin with 18.1 μM pimozide or DMSO was added. The number of intracellular bacteria was determined at the indicated time points by removing the medium from the wells and lysing the host cells in 500 μl of 0.1% Triton X-100 in PBS. Dilutions of the host cell lysates were plated on LB agar and grown 16 to 20 h at 37°C to enumerate the total number of intracellular bacteria.

Gentamicin protection assays. Gentamicin protection assays were performed as previously described (18), with modifications. BMM, J774 cells, or RAW 264.7 cells were seeded at 4×10^5 cells/well in a 24-well plate. After 16 h, the cells were washed with PBS and pretreated for 2 h with medium containing 18.1 μM pimozide or an equivalent volume of DMSO. The indicated bacterial strain that had been grown for 14 to 16 h was washed and used to infect the monolayer at the indicated MOI in the presence of 18.1 μM pimozide or DMSO. Thirty minutes postinfection, cells were washed with PBS, and medium containing 50 μg/ml gentamicin with 18.1 μM pimozide or DMSO was added. Ninety minutes postinfection, medium was removed and the cells were lysed in 500 μl of 0.1%

Triton X-100 in PBS. Dilutions of host cell lysates were plated on LB agar and grown for 16 to 20 h at 37°C to enumerate the total number of intracellular bacteria.

For calcium-free experiments, BMM were washed with PBS and calcium-free medium (145 mM NaCl, 4.5 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, 10 mM glucose, 10 mM HEPES; final solution pH of 7.35) containing 18.1 μM of pimozide or an equal volume of DMSO was added, and cells were incubated for 30 min prior to infection. Following preincubation, BMM were infected with wild-type *L. monocytogenes* 10403S as described and incubated for 15 min. Cells then were washed with PBS and calcium-free medium containing 50 μg/ml gentamicin, and 18.1 μM pimozide or DMSO was added. Forty-five minutes postinfection, medium was removed and cells were lysed in 500 μl of 0.1% Triton X-100 in PBS. Dilutions of host cell lysates were plated on LB agar and grown for 16 to 20 h at 37°C to enumerate the total number of intracellular bacteria.

Hemolytic activity assay. Wild-type *L. monocytogenes* strain 10403S was grown for 14 to 16 h in BHI broth at 30°C without shaking. The culture was diluted 1:20 in BHI medium containing either 18.1 μM pimozide or an equivalent volume of DMSO and grown shaking at 37°C until the optical density at 600 nm (OD_{600}) was ~1.5. The determination of hemolytic activity contained in culture supernatants was performed as described previously (33), with slight modifications. Briefly, bacterial cultures were centrifuged and supernatants were diluted and incubated with sheep red blood cells (sRBCs) for 30 min at 37°C. Samples were centrifuged (13,000 × g) for 1 min. Next, 100 μl of the supernatant was transferred to a 96-well flat-bottom microtiter plate (Nalge Nunc International, Rochester, NY), and the absorbance at 541 nm for each well was measured in a VERSAmax microplate reader using SoftMax Pro v1.2 software (Molecular Devices, Downingtown, PA). Hemolytic units (HU) were defined as the reciprocal of the dilution of culture supernatant that yielded 50% lysis of sRBCs.

Assessment of vacuolar escape. Determination of vacuolar escape frequency was performed as described previously (28). BMM seeded on glass coverslips for 24 h were incubated in medium containing 18.1 μM pimozide or an equivalent volume of DMSO for 2 h. *L. monocytogenes*-GFP grown for 14 to 16 h in BHI medium at 30°C without shaking was diluted 1:20 in BHI medium and grown at 37°C with shaking until the OD_{600} was ~0.5. Coverslips were infected with *L. monocytogenes*-GFP at an MOI of 10:1 for 30 min and washed with PBS, and medium containing 50 μg/ml gentamicin with 18.1 μM pimozide or an equivalent volume of DMSO was added. At 90 min postinfection, the coverslips were washed with PBS and fixed with 3.2% paraformaldehyde for 14 to 16 h at 4°C. The BMM were permeabilized and blocked with 1% BSA in TBS-TX for 15 min at RT. Host cell F actin was stained with Texas Red-phalloidin (Invitrogen, Carlsbad, CA) at a 1:200 dilution for 30 min at RT. Coverslips were washed with TBS-TX and then stained with Hoechst 33342 (Invitrogen). Coverslips were mounted and analyzed by fluorescence microscopy. Images were acquired using MetaMorph imaging software (Molecular Devices). For each acquisition, a Z series was obtained, and the collected Z stack was merged into one plane using the stack arithmetic: maximum command of MetaMorph. Appropriate colors were assigned to each fluorescent image (blue for Hoechst, green for GFP, and red for Texas Red). The color images were autoscaled and overlaid using the overlay command of MetaMorph. To quantify vacuole escape, at least 100 bacteria per coverslip were examined, and the percentage of bacteria associated with F actin was determined.

Plaquing assay. The analysis of plaque formation in L2 cell monolayers was performed as previously described (21). Twenty-four hours prior to infection, 2×10^6 L2 cells were seeded in each well of a 6-well plate. L2 cells were preincubated for 2 h in medium containing 18 μM pimozide or an equivalent volume of DMSO. A culture of wild-type *L. monocytogenes* grown for 14 to 16 h was washed with PBS and used to infect L2 cells at an MOI of 1:5. At 1 h postinfection, monolayers were washed three times with PBS and a 0.7% agarose-medium overlay in DMEM–5% FBS containing 30 μg/ml gentamicin, and 18.1 μM pimozide or an equivalent volume of DMSO was added. At 72 h postinfection, a second agarose-medium overlay containing 6% neutral red, 30 μg/ml gentamicin, and 18.1 μM pimozide or an equivalent volume of DMSO was added. At 96 h postinfection, plates were scanned to digital images, and the diameters of 10 plaques per well were determined using Canvas software (ACD Systems of America, Victoria, Canada).

Statistical analysis. Paired, two-tailed Student's *t* tests were calculated using Prism software (Graphpad, San Diego, CA). At least three independent experiments were included in each analysis. A *P* value of < 0.05 was considered significant.

RESULTS

Small-molecule screen to identify compounds affecting *L. monocytogenes* infection. Numerous aspects of intracellular infection by *L. monocytogenes* have been characterized using classical genetic-based approaches in conjunction with in vitro infection models (10, 41). Nonetheless, the mechanisms governing many cellular processes required for intracellular infection are poorly understood. To gain further insight into the host-pathogen interactions required for productive intracellular infection, we developed a screening assay to identify small-molecule compounds that altered the infection of *L. monocytogenes* in BMM (see Materials and Methods). We utilized the Known Bioactives Library produced by Biomol Corporation for the NSRB at Harvard Medical School. This library contains 480 diverse compounds with defined biological activity. Compounds resulting in increased or decreased GFP fluorescence were identified as candidate molecules that altered the ability of *L. monocytogenes*-GFP to invade, survive, or replicate within primary host cells. In addition, compounds within the library corresponding to known antibiotics were discarded from further analysis.

We identified several phenotypes following the infection of BMM in the presence of the small-molecule compounds. The majority of the identified compounds that altered intracellular infection resulted in decreased GFP fluorescence (designated the down phenotype), with the following characteristics: decreased GFP fluorescence due to fewer BMM being infected, but within the infected cells the GFP fluorescence was as robust as that of cells in control wells; in some instances very few to no BMM were infected; and in some wells fewer host cells remained following the screen, as evidenced by decreased Hoechst staining. We also observed two additional phenotypes, characterized by increased GFP fluorescence (designated the up phenotypes). The intensity of GFP fluorescence per infected host cell was more robust, consistent with an increase in the intracellular replication rate or increased bacterial uptake. Nonetheless, in some instances the number of infected BMM per well was less than that of control wells, suggesting a possible defect in the cell-to-cell spread of *L. monocytogenes* resulting in increased bacterial numbers within the initial BMM infected. One well displayed an additional unique phenotype of punctate GFP fluorescence (designated the spot phenotype). Increased GFP fluorescence was observed in punctate locations within infected BMM, which previously has been shown to be consistent with bacterial replication within vacuoles (1). We theorized that some of the compounds are toxic to BMM. Therefore, the host cell toxicity of the identified compounds was assessed using CellTiterGlo, which quantifies ATP as an indicator of metabolic activity. The CellTiterGlo toxicity assay allowed the initial list of identified compounds to be condensed from 13% of the bioactive small-molecules screened to 4.4% of the total compounds in the library (21 out of 480).

We next classified the 21 identified small-molecule compounds that altered *L. monocytogenes* infection into four categories: three distinct categories based upon known biological activity and one additional miscellaneous group (Table 1). One subgroup of compounds comprised known inhibitors of actin polymerization (cytochalasin D and latrunculin B). The iden-

TABLE 1. Classification of compounds affecting
L. monocytogenes infection[a]

Compound and category	Known activity[b]
Actin disruption	
Cytochalasin D**	F actin capper
Latrunculin B	Actin polymerization inhibitor
Kinase/phosphatase inhibitor	
AG-370*	PDGF receptor kinase inhibitor
AG-879	NGF receptor kinase inhibitor
Okadaic acid**	PP1 and PP2A phosphatase inhibitor
SP-600125	JNK kinase inhibitor
Calcium pathway modulation	
Mastoparan	Inhibits calcineurin, PI breakdown, activates GTPases
Flunarizine	Calcium channel inhibitor
Pimozide	Calcium channel inhibitor
SKF-96365	Calcium channel inhibitor
W7	Calmodulin antagonist
2,5-Ditertbutylhydroquinone	ER calcium ATPase inhibitor
Wortmannin	PI3 kinase inhibitor
GF-109203X	Protein kinase C inhibitor
U73122	PLC inhibitor
Other	
BW-B 70C	5-Lipoxygenase inhibitor
Prazocin	Adrenoreceptor agonist
HA14–1	Bcl-2 ligand; induces apoptosis
2-Methoxyantimycin A3	Bcl-2/Bcl-xl ligand; induces apoptosis
Hoescht 33342	DNA minor groove binder
Erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride HCl**	PDE2 inhibitor; adenosine deaminase inhibitor

[a] The 21 compounds identified from the Known Bioactives library that affected *L. monocytogenes* infection of BMM were categorized into four groups based upon known activity. All identified compounds resulted in decreased GFP fluorescence, except for those of the spots phenotype (*) and those for which fewer cells were infected but greater fluorescence per infected cell was seen (**). Only compounds that did not result in host cell toxicity are indicated.

[b] PDGF, platelet-derived growth factor; NGF, nerve growth factor; PP1, protein phosphatase 1; PP2, protein phosphatase 2; JNK, c-Jun N-terminal kinase; PI, phosphoinositide; ER, endoplasmic reticulum, PI3, phosphoinositide 3-kinase; PLC, phospholipase C; PDE2, phosphodiesterase 2.

tification of these compounds in the screen was not surprising, as it is well established that the phagocytosis of bacteria requires the modulation of the actin cytoskeleton and that *L. monocytogenes* further polymerizes host cell actin to move within the cytosol. Therefore, the inhibition of actin polymerization can result in both defects in bacterial uptake and/or bacterial motility and spread to neighboring cells. The phenotypes we observed upon the treatment of BMM with compounds in the actin disruption subgroup were consistent with those of previous reports on their effects on *L. monocytogenes* infection (7). The second subgroup of compounds included inhibitors of kinase/phosphatase activities. Many tyrosine kinase pathways are involved in the reorganization of the actin cytoskeleton, and receptor kinase signaling pathways also can control actin polymerization at the plasma membrane by the activation of Src family kinases, which in turn activate WASP proteins that transduce signals to the actin cytoskeleton (34). It is probable that receptor kinase signaling pathways can act in

concert with Src signals, enhancing actin reorganization activated by Cdc42/Rac GTPases (30, 31). While currently there are no data to support the involvement of tyrosine kinase activity during the uptake of *L. monocytogenes* into professional phagocytes, *L. monocytogenes* uses a host tyrosine kinase receptor to mediate internalization into nonprofessional phagocytic cells. The *L. monocytogenes* InlB protein binds the cell membrane receptor c-Met, which undergoes autophosphorylation and creates docking sites for the adaptor proteins Gab1, Cbl, and Shc, leading to the recruitment of phosphatidylinositol 3-kinase (PI3K), which results in actin ruffling involved in bacterial invasion (17, 31, 37). The largest subgroup of identified small molecules comprised compounds that modulate calcium signaling. These molecules represented 43% of the compounds that affected *L. monocytogenes* infection (Table 1). Calcium is a necessary component of many cellular signaling pathways, including phagocytosis, the activation of immune factors, and macrophage apoptosis (29). Furthermore, during *L. monocytogenes* infection, protein kinase C activation has been shown to lead to calcium release that modulates bacterial uptake and escape from the vacuole (43). The remaining six compounds were grouped into a separate category (other) based on no known conserved functions. These compounds included inducers of apoptosis, a lipoxygenase and an adenosine deaminase inhibitor, an adrenoreceptor agonist, and Hoechst 33342.

Pimozide decreases intracellular infection by *L. monocytogenes*. We chose pimozide, a diphenylbutylpiperidine with neuroleptic properties, for further study. Pimozide is an FDA-approved drug currently used to treat severe Tourette's syndrome and schizophrenia via its ability to block dopaminergic receptors on neurons (40). The ability of pimozide to inhibit the potassium-induced calcium-dependent contractions of rat vas deferens has been described, but the mechanism of pimozide inhibition of calcium channels has not been thoroughly studied (14). The presence of pimozide during the *L. monocytogenes* infection of BMM resulted in a down phenotype in which fewer BMM were infected. The infected host cells had similar or decreased levels of GFP fluorescence compared to those of BMM infected in the presence of the solvent (DMSO) alone (Fig. 1A). However, no significant differences were observed in bacterial growth rates when *L. monocytogenes* was grown in BHI broth in the presence of pimozide or DMSO (Fig. 1B). This result indicated that pimozide was affecting an aspect(s) of intracellular infection by *L. monocytogenes* and not simply decreasing the inherent ability of bacteria to replicate. Since the extracellular growth of *L. monocytogenes* was not altered by pimozide, we further examined bacterial replication within BMM to discern the stage(s) of intracellular infection that was affected by pimozide treatment. During the infection of BMM in the presence or absence of pimozide, the rate of intracellular bacterial replication was similar. Nonetheless, pimozide treatment resulted in an approximately 1-log reduction in the number of intracellular bacteria over the 10-h time period examined (Fig. 1C). These data suggest that pimozide treatment decreases the efficiency of an early aspect of intracellular infection, such as bacterial uptake by BMM and/or escape from the phagocytic vacuole.

Internalization of bacteria by professional phagocytic cells is decreased by pimozide. The results shown in Fig. 1 indicated
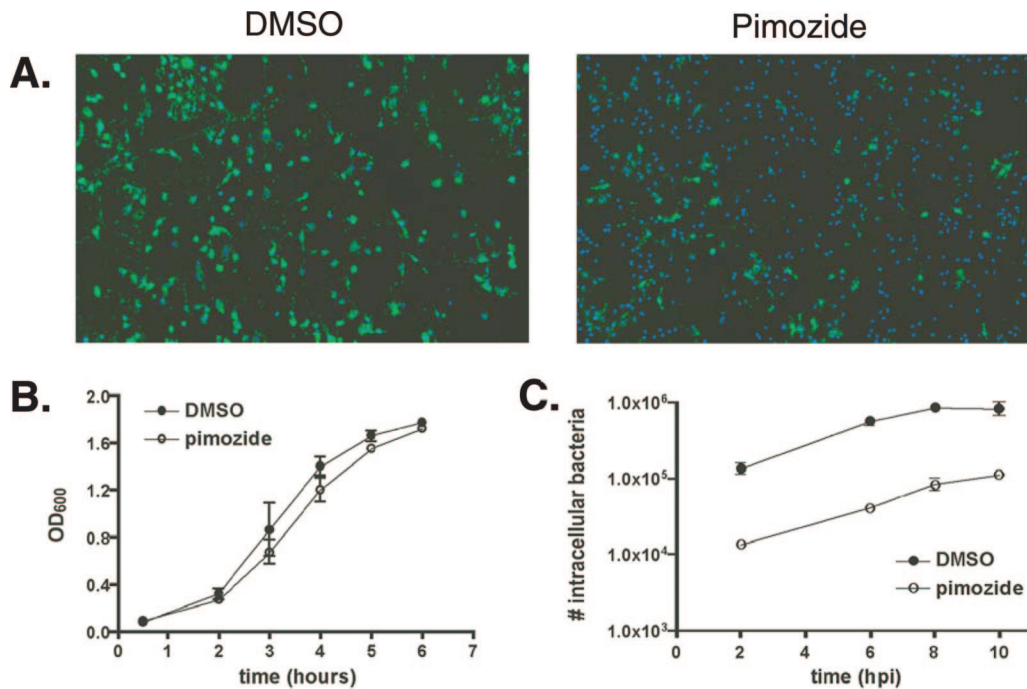
FIG. 1. Pimozide decreases intracellular infection by *L. monocytogenes*. (A) BMM were treated with 18.1 μM pimozide or an equal volume of DMSO and infected with *L. monocytogenes*-GFP (green) as described in Materials and Methods. Fifteen hours postinfection, BMM were fixed and stained with Hoechst dye (blue), and images were acquired by automated microscopy. Fluorescent images are representative of independent infections performed three times in duplicate. Images are similarly scaled and are shown at ×10 magnification. (B) Cultures of wild-type *L. monocytogenes* grown for 14 to 16 h were diluted 1:20 in BHI broth and grown in the presence of 18.1 μM pimozide or DMSO for 6 h at 37°C with shaking. Culture aliquots were taken at the indicated times, and the OD_{600} was determined. The means ± standard deviations of three independent experiments are shown. (C) Monolayers of BMM were infected with wild-type *L. monocytogenes* at an MOI of 1:2 in the presence of 18.1 μM pimozide or DMSO, and intracellular growth was determined as described in Materials and Methods. At the indicated hours postinfection (hpi) the number of intracellular bacteria was determined. Data represent the means ± standard deviations of one of three independent experiments performed in triplicate with similar results.

that treatment with pimozide decreases the ability of *L. monocytogenes* to be internalized by BMM. To quantitate bacterial uptake by BMM, we conducted gentamicin protection assays. BMM treated with pimozide internalized less than 1% of *L. monocytogenes* compared to that by DMSO-treated cells, indicating a significant defect in bacterial uptake (Fig. 2A). This effect was dose dependent, as decreasing concentrations of

pimozide resulted in a less pronounced inhibition of *L. monocytogenes* uptake (Fig. 2B).

To determine if the inhibitory effect of pimozide on bacterial uptake by BMM was specific for *L. monocytogenes* or represents a general effect on the phagocytosis of bacteria by macrophages, gentamicin protection assays were performed using additional bacterial species. We found that pimozide treatment
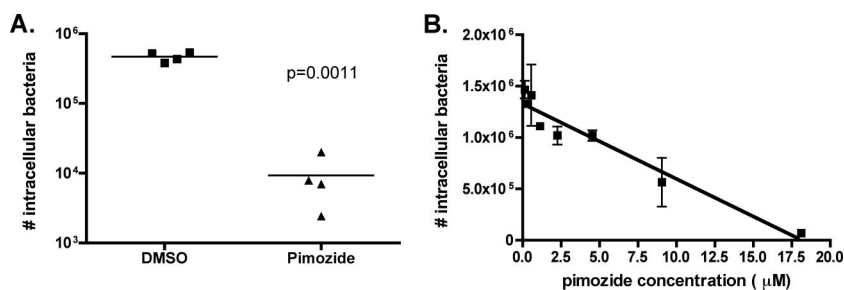

FIG. 2. Pimozide treatment decreases the internalization of *L. monocytogenes* by BMM in a dose-dependent manner. (A) BMM were treated for 2 h with 18.1 μM pimozide or DMSO prior to infection with wild-type *L. monocytogenes* (MOI, 10:1). At 30 min postinfection, BMM were washed with PBS, and medium containing 50 μg/ml gentamicin and 18.1 μM pimozide or DMSO was added to kill extracellular bacteria. At 90 min postinfection, intracellular bacteria were quantified as described in Materials and Methods. Each data point represents the mean of one individual experiment performed in triplicate. The horizontal bars represent the means of all data points shown. The *P* value is indicated. (B) BMM were pretreated with twofold dilutions of pimozide for 2 h prior to infection with wild-type *L. monocytogenes*, and the determination of internalized bacteria was performed as described for panel A. The means and standard errors of the means of three individual experiments performed in triplicate are shown. A line of linear regression is also shown.
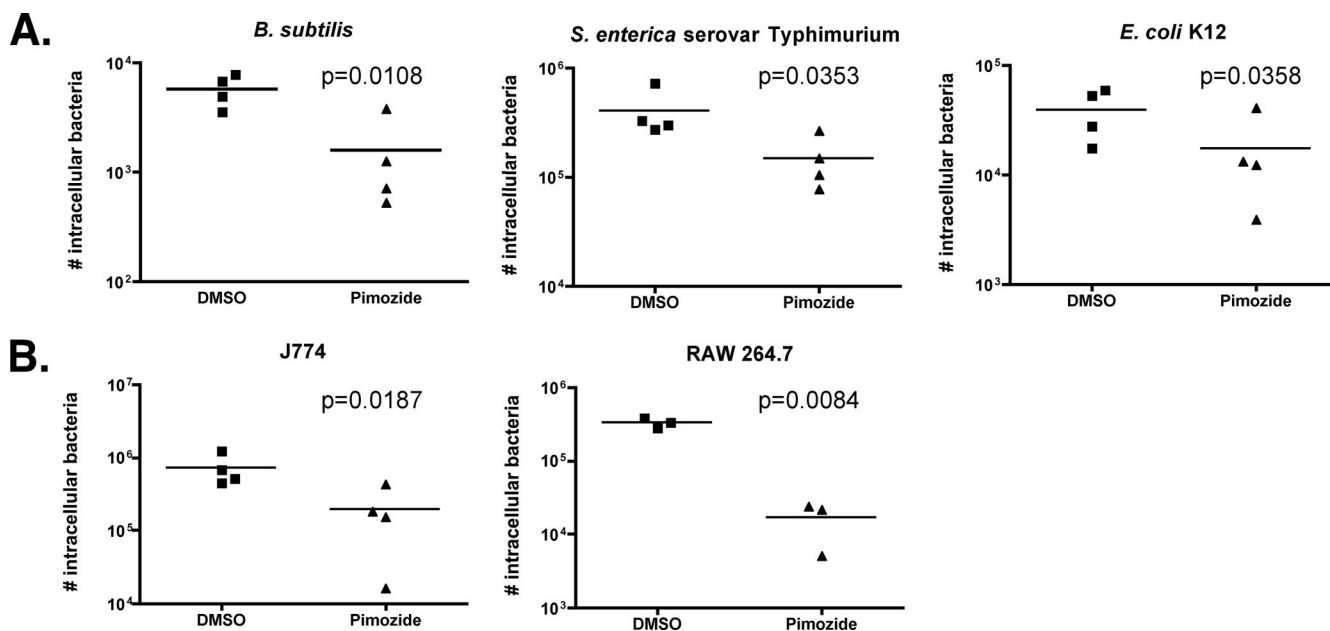
A.

B.

FIG. 3. Pimozide decreases the internalization of bacteria by professional phagocytic cells. (A) BMM were treated for 2 h with 18.1 μM pimozide or DMSO prior to the addition of bacteria. Bacterial species and MOIs used to infect BMM were the following: *B. subtilis*, 1.5:1; *S. enterica* serovar Typhimurium, 20:1; and *E. coli* K12, 20:1. At 30 min postinfection, BMM were washed with PBS, and medium containing 50 μg/ml gentamicin and 18.1 μM pimozide or DMSO was added to kill extracellular bacteria. At 90 min postinfection, intracellular bacteria were quantified as described in Materials and Methods. (B) Murine macrophage and macrophage-like RAW 264.7 and J774 cells, respectively, were treated for 2 h with 18.1 μM pimozide or DMSO prior to infection with wild-type *L. monocytogenes* (MOI = 10:1), and the quantification of internalized bacteria was performed as described for Fig. 2A. Data points in each panel represent the means of one individual experiment performed in triplicate. The horizontal bars represent the means of all data points shown. *P* values are indicated.

inhibited the uptake of *Bacillus subtilis*, a nonpathogenic gram-positive bacterium, by ~73% (Fig. 3A). In addition, we determined the ability of pimozide treatment to inhibit the internalization of the invasive gram-negative pathogen *Salmonella enterica* serovar Typhimurium. Bacterial uptake was decreased by ~64% (Fig. 3A), and similarly, the pimozide treatment of BMM inhibited the phagocytosis of a nonpathogenic strain of *Escherichia coli* K12 to a lesser extent (~60%) (Fig. 3A).

Furthermore, we determined if pimozide would inhibit the internalization of *L. monocytogenes* by additional professional phagocytic cell lines. Gentamicin protection assays indicated that pimozide treatment significantly decreased the uptake of *L. monocytogenes* by murine J774 and RAW 264.7 cells by ~75 and ~95%, respectively (Fig. 3B). Collectively, these data indicate that pimozide treatment decreases the ability of professional phagocytic cells to efficiently internalize both gram-positive and gram-negative bacteria.

Pimozide decreases vacuole escape by *L. monocytogenes*. While bacterial uptake by professional phagocytic host cells was decreased in the presence of pimozide, it is also possible that the decreased number of intracellular bacteria observed during the infection of BMM (Fig. 1C) was due partially to a decrease in the efficiency of vacuole escape subsequent to bacterial entry. To determine if pimozide treatment affected the vacuole escape of *L. monocytogenes*, BMM were infected with *L. monocytogenes*-GFP and vacuolar escape was assessed by phalloidin staining to determine if intracellular bacteria were present in the cytosol associated with polymerized F actin (15, 19). Cytosolic bacteria were quantified, and it was ob-

served that in the presence of pimozide, vacuolar escape was reduced by 26.3% (*P* = 0.0004) compared to that of the DMSO control.

It has been shown that the *L. monocytogenes* pore-forming cytolysin LLO is required for vacuole escape in BMM (33). To determine if pimozide treatment inhibits LLO activity, the hemolytic activity of LLO produced by *L. monocytogenes* grown in broth culture was determined. In addition to no difference in bacterial growth rate (Fig. 1B), no significant difference in hemolytic activity in culture supernatants was detected following growth in the presence of pimozide (227 ± 66 HU) compared to that of the DMSO control (204 ± 60 HU). Taken together, these results indicated that while pimozide treatment resulted in a decrease in vacuole escape following the phagocytosis of bacteria by BMM, this decrease was not due to the inhibition of LLO activity prior to bacterial uptake.

Cell-to-cell spread is decreased by pimozide treatment. Our results indicate that pimozide treatment negatively affects multiple stages of *L. monocytogenes* infection in BMM, including decreasing bacterial uptake and vacuole escape (Fig. 1 to 3). During intracellular infection, cell-to-cell spread also is an important component of *L. monocytogenes* pathogenesis. Therefore, we used plaque formation in L2 fibroblasts to determine the effect of pimozide on the cell-to-cell spread of bacteria. To ensure that pimozide did not affect the intracellular replication rate of *L. monocytogenes* within L2 fibroblasts, we analyzed intracellular growth during an 8-h infection period. As previously observed for BMM (Fig. 1C), pimozide treatment of L2 cells resulted in decreased numbers of intra-
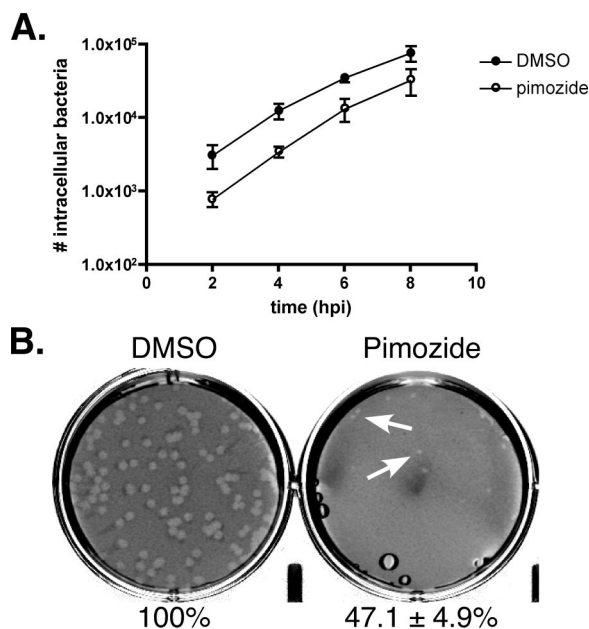
A.

B.

FIG. 4. Cell-to-cell spread of *L. monocytogenes* is decreased in the presence of pimozide. (A) Intracellular growth of *L. monocytogenes* in murine L2 fibroblasts. Monolayers of L2 fibroblasts were infected with wild-type *L. monocytogenes* in the presence of 18.1 μM pimozide or DMSO at an MOI of 50:1 as described in Materials and Methods. At the indicated times postinfection, the number of intracellular bacteria was determined. Data represent results of one of three independent experiments performed in triplicate with similar results. (B) Plaque formation in L2 fibroblasts. Wild-type *L. monocytogenes* was used to infect monolayers of L2 fibroblasts in the presence of 18.1 μM pimozide or DMSO as described in Materials and Methods. At 1 h postinfection, infected monolayers were washed with PBS, and a medium-agarose overlay containing 30 μg/ml gentamicin and 18.1 μM pimozide or an equivalent volume of DMSO was added. Intracellular growth and the cell-to-cell spread of bacteria were visualized after 96 h by the formation of clearing zones (plaques) within the L2 monolayers. The diameters of 10 plaques/sample were determined. Values are expressed as the percent diameter of plaques relative to those of the DMSO sample and represent the means ± standard deviations of three independent experiments. Arrows indicate small plaques present within the L2 monolayer.

cellular bacteria compared to those of DMSO-treated cells, yet the rate of intracellular replication in the presence or absence of pimozide was similar (Fig. 4A). We next performed plaquing analysis over a 96-h infection period to examine cell-to-cell spread. As expected, L2 cells infected in the presence of pimozide displayed a deficiency in bacterial entry, as demonstrated by a decrease in the number of plaques formed. Furthermore, pimozide treatment resulted in an average plaque size of 47.1% ± 4.9% of that formed in DMSO-treated L2 cells (Fig. 4B). These results are consistent with a cell-to-cell spread defect, potentially due to inefficient actin-based motility and/or decreased escape from secondary vacuoles following spread to neighboring cells.

Pimozide inhibition of *L. monocytogenes* entry into BMM is independent of extracellular calcium. Previous studies have shown that calcium-dependent host cell pathways indirectly control the success of *L. monocytogenes* infection within the macrophage-like cell line J774 (42), and that calcium influx is necessary for the entry of *L. monocytogenes* into nonprofes-
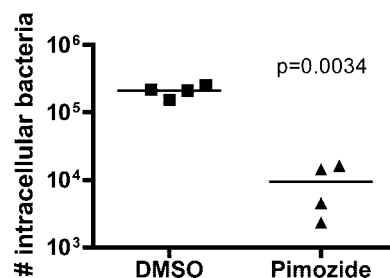

FIG. 5. Pimozide inhibition of *L. monocytogenes* entry into BMM is independent of extracellular calcium. BMM were pretreated for 30 min with 18.1 μM pimozide or DMSO in calcium-free medium. Monolayers were infected with wild-type *L. monocytogenes* at an MOI of 10:1. At 15 min postinfection, BMM were washed with PBS and calcium-free medium containing 50 μg/ml gentamicin with 18.1 μM pimozide or DMSO was added. At 45 min postinfection, the number of intracellular bacteria was determined. Each data point represents the mean of one individual experiment performed in triplicate. The horizontal bars represent the means of all data points shown. The P value is indicated.

sional phagocytic Hep-2 epithelial cells (9). Pharmacological inhibitors that affected calcium signaling altered the kinetics of bacterial uptake and vacuole escape in J774 cells (42). We hypothesized that the inhibitory effects of pimozide on *L. monocytogenes* infection may result from its calcium channel antagonist properties. To determine if the inhibitory effect of pimozide on bacterial uptake is dependent on extracellular calcium, we performed gentamicin protection assays in calcium-free medium. BMM infected with *L. monocytogenes* treated with pimozide in calcium-free medium displayed a significant (~95%) decrease in bacterial entry (Fig. 5). Thus, pimozide remained inhibitory to bacterial uptake in calcium-free medium. This result indicated that the mechanism of the pimozide inhibition of bacterial uptake in BMM was not dependent on the presence of extracellular calcium.

DISCUSSION

Traditional genetic-based approaches have led to the identification of both bacterial and host cell-specific factors that are required for intracellular pathogenesis. However, numerous questions remain regarding the mechanisms of host-pathogen interactions leading to infection and the development of disease. Small-molecule screening has proven a powerful approach for gaining insights into many diverse biological systems (23, 38). In the case of host-pathogen infection models, introduced compounds may affect both pathogen and host cell functions, resulting in alterations of infection that may not have been anticipated otherwise. The small-molecule screening approach developed in this report utilized primary host cells and the subsequent visual analysis of phenotypes to identify 21 compounds that affected *L. monocytogenes* infection. One particular compound, pimozide, was further studied to determine the specific aspects of *L. monocytogenes* infection that were affected by drug treatment. We chose to focus on pimozide, as it is an FDA-approved drug, and although pimozide is used clinically as a dopamine antagonist to treat Tourette's syndrome and schizophrenia (40), further develop-

ment may prove useful for application as an antibacterial therapeutic.

Pimozide was shown to have multiple inhibitory effects on *L. monocytogenes* infection. The most pronounced effect was on the bacterial uptake and invasion of professional and nonprofessional phagocytic host cells. The inhibitory effect of pimozide treatment on phagocytosis of *L. monocytogenes* by macrophages (Fig. 2A and 3B) may indicate a general defect in the recognition of bacterial surface components required for efficient uptake. Furthermore, pimozide treatment differentially affected the ability of macrophages to phagocytose other noninvasive bacterial species (Fig. 3A). While the bacterial species examined in this report were not exhaustive, it appears that the pimozide inhibition of macrophage phagocytosis may be more pronounced for the uptake of gram-positive bacteria than for that of gram-negative organisms (Fig. 2A and 3A). Furthermore, the pimozide inhibition of *L. monocytogenes* invasion into L2 fibroblasts (Fig. 4) indicates a general effect of pimozide on host cell actin dynamics, resulting in decreased phagocytosis or bacterium-induced invasion of host cells. Nonetheless, pimozide treatment did not have apparent negative effects on host cell fitness, as evidenced by the CellTiterGlo viability assay and the ability of intracellular bacteria to replicate at similar rates following access to the cytosol (Fig. 1C and 4A).

Currently, the mechanism of the pimozide inhibition of bacterial entry and vacuole escape is unclear. Prior reports have shown that pimozide can function as a calcium channel antagonist (11, 14). Calcium is involved in many host cell processes, such as phagocytosis and the modulation of immune function. Previous studies have identified multiple calcium fluxes within host cells following infection by *L. monocytogenes*, and the bacterial LLO, PI-PLC, and PC-PLC proteins have been implicated in mediating calcium fluxes that can modulate bacterial entry and vacuole escape (42). While the mechanism of the pimozide inhibition of bacterial entry and vacuole escape is unclear, our studies indicated that the pimozide inhibition of bacterial uptake by BMM was independent of extracellular calcium. In addition, the infection of BMM in the presence of pimozide did not inhibit an early calcium flux previously observed during infection of J774 cells (42 and data not shown). Therefore, it is unclear how pimozide mediates its inhibitory effects on *L. monocytogenes* infection and the relationship to pimozide's calcium channel antagonist properties. One possibility is that pimozide affects macrophages directly, altering the ability of macrophages to phagocytose and kill intracellular bacteria. Alternatively, although pimozide did not affect the replication rate of *L. monocytogenes* in broth culture or intracellular bacteria that had gained access to the cytosol, pimozide may decrease bacterial infectivity by affecting the expression of bacterial determinants required for efficient invasion and vacuole escape. It should be noted that pimozide has been shown to decrease the phagocytosis of opsonized sRBCs by 30% in murine peritoneal macrophages (24), which may support a role for pimozide in directly affecting a host cell function. Pimozide also may interfere with host cell membrane fluidity, decreasing the ability of host cells to take up bacteria and the ability of bacteria to efficiently escape the phagocytic vacuole. Additional transcriptome and proteomic analyses of pimozide-treated cells will provide insights into potential alterations in gene expression and protein profiles that may

indicate the host cellular pathways that are affected. The ability of pimozide to decrease bacterial uptake by professional phagocytic cells during the infection of the host actually may exacerbate certain bacterial infections; however, the multiple inhibitory effects of pimozide on invasion, vacuole escape, and cell-to-cell spread of *L. monocytogenes*, coupled with a lack of toxicity to host cells and prior FDA approval, warrants additional study of the effects of pimozide on the in vivo infection of intracellular pathogens. Interestingly, a preliminary analysis of other antipsychotic drugs revealed inhibitory effects on *L. monocytogenes* infection (data not shown).

Our screening approach has yielded insights into the effects of several known biologically active small molecules on infection by *L. monocytogenes*, a model intracellular bacterial pathogen. This approach may be adaptable to other pathogenic species that are less tractable to study by traditional genetic methods. Ultimately, the use of classical genetic-based techniques in combination with small-molecule screening methods will provide an ideal approach for making significant advances in our understanding of the mechanisms governing host-pathogen interactions. Furthermore, as antibiotic resistance increases among several pathogenic species, a thorough understanding of the mechanisms of pathogenesis will aid in the development of alternative treatments for diseases. Compounds, such as pimozide, that have been previously approved for alternative clinical uses may be ideal targets for the further development of nontraditional therapeutics.

REFERENCES

1. **Agaisse, H., L. S. Burrack, J. A. Philips, E. J. Rubin, N. Perrimon, and D. E. Higgins.** 2005. Genome-wide RNAi screen for host factors required for intracellular bacterial infection. Science **309:**1248–1251.
2. **Alberti-Segui, C., K. R. Goeden, and D. E. Higgins.** 2007. Differential function of *Listeria monocytogenes* listeriolysin O and phospholipase C in vacuolar dissolution following cell-to-cell spread. Cell Microbiol. **9:**179–195.
3. **Burrack, L. S., and D. E. Higgins.** 2007. Genomic approaches to understanding bacterial virulence. Curr. Opin. Microbiol. **10:**4–9.
4. **Caamano, J., J. Alexander, L. Craig, R. Bravo, and C. A. Hunter.** 1999. The NF-kappa B family member RelB is required for innate and adaptive immunity to *Toxoplasma gondii*. J. Immunol. **163:**4453–4461.
5. **Carey, K. L., N. J. Westwood, T. J. Mitchison, and G. E. Ward.** 2004. A small-molecule approach to studying invasive mechanisms of *Toxoplasma gondii*. Proc. Natl. Acad. Sci. USA **101:**7433–7438.
6. **Carpenter, A. E.** 2007. Image-based chemical screening. Nat. Chem. Biol. **3:**461–465.
7. **Dabiri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick.** 1990. *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA **87:**6068–6072.
8. **De La Fuente, R., N. D. Sonawane, D. Arumainayagam, and A. S. Verkman.** 2006. Small molecules with antimicrobial activity against *E. coli* and *P. aeruginosa* identified by high-throughput screening. Br. J. Pharmacol. **149:**551–559.
9. **Dramsi, S., and P. Cossart.** 2003. Listeriolysin O-mediated calcium influx potentiates entry of *Listeria monocytogenes* into the human Hep-2 epithelial cell line. Infect. Immun. **71:**3614–3618.
10. **Dussurget, O., J. Pizarro-Cerda, and P. Cossart.** 2004. Molecular determinants of *Listeria monocytogenes* virulence. Annu. Rev. Microbiol. **58:**587–610.

11. **Fulop, G., R. A. Phillips, A. K. Shapiro, J. A. Gomes, E. Shapiro, and J. W. Nordlie.** 1987. ECG changes during haloperidol and pimozide treatment of Tourette's disorder. Am. J. Psychiatry **144:**673–675.

12. **Gellin, B. G., and C. V. Broome.** 1989. Listeriosis. JAMA **261:**1313–1320.

13. **Goldfine, H., and C. Knob.** 1992. Purification and characterization of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C. Infect. Immun. **60:**4059–4067.

14. **Gould, R. J., K. M. Murphy, I. J. Reynolds, and S. H. Snyder.** 1983. Antischizophrenic drugs of the diphenylbutylpiperidine type act as calcium channel antagonists. Proc. Natl. Acad. Sci. USA **80:**5122–5125.

15. **Gründling, A., M. D. Gonzalez, and D. E. Higgins.** 2003. Requirement of the *Listeria monocytogenes* broad-range phospholipase PC-PLC during infection of human epithelial cells. J. Bacteriol. **185:**6295–6307.

16. **Hung, D. T., E. A. Shakhnovich, E. Pierson, and J. J. Mekalanos.** 2005. Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization. Science **310:**670–674.

17. **Ireton, K., B. Payrastre, and P. Cossart.** 1999. The *Listeria monocytogenes* protein InlB is an agonist of mammalian phosphoinositide 3-kinase. J. Biol. Chem. **274:**17025–17032.

18. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.

19. **Jones, S., and D. A. Portnoy.** 1994. Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. Infect. Immun. **62:**5608–5613.

20. **Jones, S., and D. A. Portnoy.** 1994. Intracellular growth of bacteria. Methods Enzymol. **236:**463–467.

21. **Jones, S., and D. A. Portnoy.** 1994. Small plaque mutants. Methods Enzymol. **236:**526–531.

22. **Junker, L. M., and J. Clardy.** 2007. High-throughput screens for small-molecule inhibitors of *Pseudomonas aeruginosa* biofilm development. Antimicrob. Agents Chemother. **51:**3582–3590.

23. **Kawasumi, M., and P. Nghiem.** 2007. Chemical genetics: elucidating biological systems with small-molecule compounds. J. Investig. Dermatol. **127:**1577–1584.

24. **Key, K. A., A. C. Oakley, and S. V. Pizzo.** 1984. Effects of pimozide and penfluridol on the binding and endocytosis of alpha 2-macroglobulin-CH₃NH₂ by mouse peritoneal macrophages. Biochem. Pharmacol. **33:**3712–3714.

25. **Kim, S. S., L. F. Peng, W. Lin, W. H. Choe, N. Sakamoto, N. Kato, M. Ikeda, S. L. Schreiber, and R. T. Chung.** 2007. A cell-based, high-throughput screen for small molecule regulators of hepatitis C virus replication. Gastroenterology **132:**311–320.

26. **Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon, and P. Cossart.** 1992. *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. Cell **68:**521–531.

27. **Lee, V. T., S. Pukatzki, H. Sato, E. Kikawada, A. A. Kazimirova, J. Huang, X. Li, J. P. Arm, D. W. Frank, and S. Lory.** 2007. Pseudolipasin A is a specific inhibitor for phospholipase A2 activity of *Pseudomonas aeruginosa* cytotoxin ExoU. Infect. Immun. **75:**1089–1098.

28. **Marquis, H., V. Doshi, and D. A. Portnoy.** 1995. The broad-range phospholipase C and a metalloprotease mediate listeriolysin O-independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. Infect. Immun. **63:**4531–4534.

29. **Nhieu, G. T., C. Clair, G. Grompone, and P. Sansonetti.** 2004. Calcium signalling during cell interactions with bacterial pathogens. Biol. Cell. **96:**93–101.

30. **Nhieu, G. T., J. Enninga, P. Sansonetti, and G. Grompone.** 2005. Tyrosine kinase signaling and type III effectors orchestrating *Shigella* invasion. Curr. Opin. Microbiol. **8:**16–20.

31. **Pizarro-Cerda, J., and P. Cossart.** 2006. Bacterial adhesion and entry into host cells. Cell **124:**715–727.

32. **Portnoy, D. A., V. Auerbuch, and I. J. Glomski.** 2002. The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. J. Cell Biol. **158:**409–414.

33. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167:**1459–1471.

34. **Ridley, A. J., M. A. Schwartz, K. Burridge, R. A. Firtel, M. H. Ginsberg, G. Borisy, J. T. Parsons, and A. R. Horwitz.** 2003. Cell migration: integrating signals from front to back. Science **302:**1704–1709.

35. **Schlech, W. F., III.** 2000. Foodborne listeriosis. Clin. Infect. Dis. **31:**770–775.

36. **Schreiber, K., W. Ckurshumova, J. Peek, and D. Desveaux.** 2008. A high-throughput chemical screen for resistance to *Pseudomonas syringae* in *Arabidopsis*. Plant J. **54:**522–531.

37. **Shen, Y., M. Naujokas, M. Park, and K. Ireton.** 2000. InlB-dependent internalization of *Listeria* is mediated by the Met receptor tyrosine kinase. Cell **103:**501–510.

38. **Spring, D. R.** 2005. Chemical genetics to chemical genomics: small molecules offer big insights. Chem. Soc. Rev. **34:**472–482.

39. **Swaminathan, B., and P. Gerner-Smidt.** 2007. The epidemiology of human listeriosis. Microbes Infect. **9:**1236–1243.

40. **Tueth, M. J., and J. A. Cheong.** 1993. Clinical uses of pimozide. South. Med. J. **86:**344–349.

41. **Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft.** 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14:**584–640.

42. **Wadsworth, S. J., and H. Goldfine.** 1999. *Listeria monocytogenes* phospholipase C-dependent calcium signaling modulates bacterial entry into J774 macrophage-like cells. Infect. Immun. **67:**1770–1778.

43. **Wadsworth, S. J., and H. Goldfine.** 2002. Mobilization of protein kinase C in macrophages induced by *Listeria monocytogenes* affects its internalization and escape from the phagosome. Infect. Immun. **70:**4650–4660.

44. **Zolli-Juran, M., J. D. Cechetto, R. Hartlen, D. M. Daigle, and E. D. Brown.** 2003. High throughput screening identifies novel inhibitors of *Escherichia coli* dihydrofolate reductase that are competitive with dihydrofolate. Bioorg. Med. Chem. Lett. **13:**2493–2496.