Type III Secretion System-Dependent Translocation of Ectopically Expressed Yop Effectors into Macrophages by Intracellular

Yersinia pseudotuberculosis

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Yersinia pseudotuberculosis is a Gram-negative bacterial pathogen. Virulence in Y. pseudotuberculosis requires the plasmid-encoded Ysc type III secretion system (T3SS), which functions to translocate a set of effectors called Yops into infected host cells. The effectors function to antagonize phagocytosis (e.g., YopH) or to induce apoptosis (YopJ) in macrophages infected with Y. pseudotuberculosis. Additionally, when antiphagocytosis is incomplete and Y. pseudotuberculosis is internalized by macrophages, the bacterium can survive in phagosomes. Previous studies have shown that delivery of effectors into host cells occurs efficiently when Yersinia is extracellular. However, it is not clear whether the T3SS can be utilized by intracellular Y. pseudotuberculosis to translocate Yops. This possibility was investigated here using Y. pseudotuberculosis strains that express YopJ or YopH under the control of an inducible promoter. Bone marrow-derived murine macrophages were infected with these strains under conditions that prevented the survival of extracellular bacteria. Effector translocation was detected by measuring apoptosis or the activities of Yop-β-lactamase fusion proteins. Results showed that macrophages underwent apoptosis when YopJ expression was induced prior to phagocytosis, confirming that delivery of this effector prior to or during uptake is sufficient to cause cell death. However, macrophages also underwent apoptosis when YopJ was ectopically expressed after phagocytosis; furthermore, expression of the translocator YopB from intracellular bacteria also resulted in increased cell death. Analysis by microscopy showed that translocation of ectopically expressed YopH- or YopJ-β-lactamase fusions could be correlated with the presence of viable Y. pseudotuberculosis in macrophages. Collectively, our results suggest that the Ysc T3SS of Y. pseudotuberculosis can function within macrophage phagosomes to translocate Yops into the host cytosol.

The three species of Yersinia that are pathogenic for humans are the genetically related Yersinia pseudotuberculosis and Y. pestis and the more distantly related Y. enterocolitica (1, 50). Y. pestis is the causative agent of plague. Similar to Y. enterocolitica, Y. pseudotuberculosis is an enteropathogen that commonly causes terminal ileitis and mesenteric adenitis in humans. In rodents such as guinea pigs and laboratory strains of mice, Y. pseudotuberculosis disseminates from the intestinal tract to organs such as spleen and liver and causes systemic plague-like disease. The virulence of these pathogenic Yersinia species depends on a plasmid-encoded type III secretion system (T3SS) (11). T3SSs are also found in a number of other Gram-negative pathogens, including both intracellular and extracellular pathogens, where they play key roles in promoting bacterial pathogenesis (16, 19).

The T3SS of Yersinia is comprised of a Ysc injectisome, a tip complex formed by the LcrV protein, and translocators YopB and YopD, which are implicated in the formation of a translocon in the host cell plasma membrane (16, 28, 47). Yersinia utilizes the T3SS to deliver a set of seven effector proteins called Yops into eukaryotic cells upon bacterium-host cell interaction (8, 11). The seven Yop effectors are YopE, YopH, YopT, YopM, YopK, YopJ, and YpkA; the last two are also referred to as YopP and YopO in Y. enterocolitica (11). After translocation into the cytosol of eukaryotic cells, these Yops modulate eukaryotic signaling pathways to counteract innate and adaptive immune responses to the pathogen (11, 47).

YopE, YopH, YopT, and YpkA function together to disturb the actin cytoskeleton, resulting in inhibition of phagocytosis. YopH is a tyrosine phosphatase that dephosphorylates multiple focal adhesion proteins (4, 7, 18, 35). YopE, YopT, and YpkA disrupt either the activity or the regulation of the Rho family of small GTPases (47, 49). YopJ has acetyltransferase activity (25, 29). YopJ acetylates Ser and Thr residues in mitogen-activated protein (MAP) kinase kinases (MEKs) and the inhibitor kinase β (IKKβ) to inactivate these key signaling molecules, resulting in the suppression of multiple MAP kinase and nuclear factor κB (NF-κB) pathways (25, 29). YopJ inhibits the production of tumor necrosis factor alpha (TNF-α) in macrophages infected with Yersinia (32). In addition, activation of MAP kinases and NF-κB is important to counteract a Toll-like receptor 4 (TLR4)-dependent apoptotic pathway of macrophages induced during infection with Gram-negative pathogens. Therefore, YopJ promotes apoptosis of macrophages during infection (21, 26, 53, 55).

Y. pseudotuberculosis is considered to be primarily an extracellular pathogen in vivo. For example, in mice experimentally...
infected with *Y. pseudotuberculosis*, the majority of bacteria were found to exist extracellularly in tissues, including the liver (45). However, *Y. pseudotuberculosis* can survive in macrophages in vitro (17, 36, 54), and the ability of *Y. pseudotuberculosis* to survive in macrophages is important for virulence (17). Furthermore, a recent study that employed an ex vivo culture system to study *Y. pseudotuberculosis* to survive in macrophages is important for virulence (17).

In macrophage infection assays carried out in vitro, the T3SS is only moderately effective at reducing phagocytosis, since ~5% of viable bacteria are located intracellularly at any given time in vivo, these intracellular bacteria could play an important role in pathogenesis and/or host responses.

In macrophage infection assays carried out in vitro, the T3SS is only moderately effective at reducing phagocytosis, since ~5% of *Y. pseudotuberculosis* bacteria that come into contact with macrophages are internalized (39, 40, 54). It is possible to study the function of the T3SS in the intracellular population of *Y. pseudotuberculosis* by the use of gentamicin to eliminate viable bacteria outside macrophages. Using this approach in recent studies, we showed that the presence of a functional T3SS and translocation of YopJ within phagosomes were important for *Y. pseudotuberculosis* to kill the host cell. This possibility was examined here using *Y. pseudotuberculosis* strains in which expression of Yop effectors or a translocator could be turned on before or after phagocytosis by macrophages.

### Table 1. Bacterial strains and plasmid used

<table>
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<tr>
<th>Strain or plasmid</th>
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<tr>
<td><strong>Yersinia strains</strong></td>
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<td>IP2666 pYY ΔyopJ::867, alternative name is IP26</td>
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<td>ysc</td>
<td>IP2666 pYY ysc::Tn5; alternative name is IP71; contains same pYV ysc::Tn5 as in previously published strain YP71</td>
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<td><strong>Plasmids</strong></td>
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<td>Contains <em>lacP</em> gene and <em>tac</em> promoter, Cmr</td>
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<td>pMMB67HE encoding YopHM45 under the control of the <em>tac</em> promoter, Ampr</td>
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<td>pYopJ</td>
<td>pMMB67HE encoding YopJM45 under the control of the <em>tac</em> promoter, Ampr; alternative name is lpL17</td>
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<td>pMMB67HE encoding YopB, Ampr</td>
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<td>This study</td>
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<tr>
<td>pYopJ*&lt;sup&gt;IC172A&lt;/sup&gt;-bla</td>
<td>pMMB207 encoding a YopJ&lt;sup&gt;IC172A&lt;/sup&gt;–TME-1 β-lactamase fusion protein under the control of the <em>tac</em> promoter, Cmr</td>
<td>This study</td>
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<td>pmCherry</td>
<td>pMMB67EH encoding mCherry under the control of the <em>tac</em> promoter, Ampr</td>
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### MATERIALS AND METHODS

**Bacterial strains.** The *Y. pseudotuberculosis* strains and plasmids used in this study are listed in Table 1. The strain ΔyopB (Table 1) was created by inactivating *yopB* in strain IP2666 as described previously (42). To construct pYopB2, the plasmid pYop containing containing *yopB* in the backbone of pMMB67HE that has been described before (42) was digested with restriction enzymes HindIII and NdeI to derive plasmid in vitro. Religation after treatment with T4 DNA polymerase resulted in pYopB2. To construct plasmids expressing Yop–TEM-1 β-lactamase (bla) translational fusions under the control of the *tac* promoter, DNA fragments containing coding sequences for YopH or YopJ<sup>IC172A</sup> with C-terminal M45 epo tag were amplified by PCR with primer 67NF (5′-CAAAA CAGCC AAGCT TACCA CTGTC TCAAA AGGAG G-3′) and primer M45R (5′-CAAAA CAGCC AAGCT TACCA CTGTC TCAAA AGGAG G-3′) from plasmid pYopH (Table 1) or pYopJ<sup>IC172A</sup> (Table 1), respectively. The coding region of TEM-1 β-lactamase was amplified by PCR using primer BlaF (5′-TTTGA GACAG AGACG CACCC AGAAAG CGGTG GTG-3′) and primer BlaR (5′-CCAAA CAGCG AACCT TACCA ATGCT TAAAC TGTA GA-3′) from plasmid pYopJ (Table 1). The resulting DNA fragments were ligated to create a Yop–TEM-1 β-lactamase (bla) translational fusion under the control of the *tac* promoter, DNA fragments containing coding sequences for YopH or YopJ<sup>IC172A</sup> were inserted into the plasmid pMMB207 (Table 1) between EcoRI and HindIII sites using an In-Fusion system (Clontech). The resulting plasmids were confirmed by restriction digestion analysis, and fusion protein expression was verified by immunoblotting analysis (see below). The plasmid pmCherry (Table 1) was constructed using the same strategy used to construct p207mCherry (37). Plasmids were introduced into *Y. pseudotuberculosis* strains through conjugation with *Escherichia coli* as described previously (36). Ampicillin (Amp, 100 µg/ml) and/or chloramphenicol (Cm, 25 µg/ml) was included in Luria-Bertani broth (LB) to maintain selection for pMMB67EH (Ampr)- or pMMB207 (Cmr)-derived plasmid in *E. coli* or *Y. pseudotuberculosis*.

**Macrophage cultures and infection conditions.** Bone marrow-derived macrophages (BMDMs) were obtained from female C57BL/6 mice (Jackson Laboratory) as described previously (32). Twenty-four hours before infection, the cells were seeded at a density of 1.5 × 10<sup>5</sup> cells/well in 24-well tissue culture plates in 1 ml Dulbecco modified Eagle medium containing 15% L-cell conditioned medium, 10% heat-inactivated fetal bovine serum (FBS; Gibco), 1 mM pyruvate, and 2 mM glutamate.

For infection, overnight cultures in LB were seeded at a density of 1.5 × 10<sup>5</sup> cells/well in 24-well tissue culture plates in 1 ml Dulbecco modified Eagle medium containing 15% L-cell conditioned medium, 10% heat-inactivated fetal bovine serum (FBS; Gibco), 1 mM pyruvate, and 2 mM glutamate.

For infection, overnight Y. pseudotuberculosis cultures grown at 26°C in LB
with appropriate antibiotics were diluted to an optical density at 600 nm of 0.1 in LB supplemented with 2.5 mM calcium chloride. The cultures were shaken at 37°C for 2 h. Subsequently, bacteria were washed once, resuspended in pre-warmed Hanks balanced salt solution (HBSS), and diluted into fresh cell culture medium as described above to infect macrophages at a multiplicity of infection (MOI) of 10, unless otherwise indicated. After centrifugation for 5 min at 200 × g to bring the bacteria into contact with the macrophages, an incubation was performed at 37°C for 15 min; therefore, a total of 20 min was allowed for the cells to take up the bacteria. Next, the cells were incubated in cell culture medium containing gentamicin (Gm; 8 μg/ml) for 1 h to kill extracellular bacteria. Then, the wells were changed into medium containing Gm (4.5 μg/ml) until the indicated times. When indicated, isopropyl-β-D-thiogalactopyranoside (IPTG; to 0.1 mM) was included in the LB or cell culture medium to induce expression of genes under the control of tac promoters. Congo red-magnesium oxalate plating was done according to an established method (38) to determine the presence of the virulence plasmid in colonies of Y. pseudotuberculosis recovered from lysates of infected macrophages.

Propidium iodide (PI) and annexin V-FLUOS staining. BMDMs (1.5 × 10⁵ cells/well) in the 24-well plates were infected for 6 h. The cells were washed with phosphate-buffered saline and processed to stain by use of an annexin V-FLUOS staining kit (Roche) according to the manufacturer’s instructions. The live cells were examined by epifluorescence microscopy using a Zeiss Axioplan2 microscope with a ×40 objective, phase-contrast, green fluorescence, and red fluorescence. Centrioles were captured, sequentially pseudocolored using a black-and-white Spot camera (Diagnostic Instruments, Inc.), and assembled in Adobe Photoshop software.

Immunofluorescence microscopy to detect intracellular β-lactamase activity. Four hours after infection of BMDMs seeded in wells of a 96-well plate, CCF2-acetoxyethyl (CCF2-AM) substrate (Invitrogen) was prepared according to the manufacturer’s instructions and loaded directly into the medium as a 6× solution. BMDMs were incubated for 1 h at room temperature prior to observation with a Zeiss Axiovert 100 microscope using a ×20 or ×32 lens. To detect the cleavage of CCF2, a blue filter set with a D470/40 filter for excitation and an OG515 filter for emission (Chroma Technology Corp.) was used. Sequenced images of phase, green/blue, and red (mCherry), where applicable, were captured with a true-color Spot camera (Diagnostic Instruments, Inc.) and assembled in Adobe Photoshop software. BMDMs containing red bacteria were counted in pictures taken from one random field per experiment per infection strain and scored for green or blue fluorescence or the absence of fluorescence. On average, 50 cells were counted in each field. Alternatively, BMDMs with blue fluorescence from cells infected with either Δyop/pYopH-bla or Δyop/pYopJ(E772A)-bla were counted for the presence or absence of red bacteria, and totals of 163 and 20 such cells were counted, respectively.

Western blot analysis. To detect expression of YopJ in Y. pseudotuberculosis, bacterial lysates were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblotting with antibodies against YopJ (clone C2 and D3) and Dnak (clone 8E2/2; Stressgen) as described before (54). The secondary antibody used was IRDye800-conjugated anti-mouse IgG (Rockland). The membrane was then scanned with an Odyssey VI scanner (LI-COR Biosciences).

To detect secretion of Yop–TEM-1 fusion proteins or native Yop proteins, Y. pseudotuberculosis cultures were grown under low-Ca²⁺ conditions as described previously (52). Briefly, overnight cultures grown at 28°C in LB were diluted to an optical density at 600 nm of 0.1 into LB supplemented with 20 mM magnesium chloride and 20 mM sodium oxalate. IPTG was included at 0.1 mM when indicated. Then, after shaking at 28°C for 1 h and 37°C for 4 h, Yops secreted into the growth media were precipitated, resolved using 10% SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blot analysis using total bacterial lysates and a mixture of monoclonal antibodies specific for YopJ. YopJ protein was detected using a monoclonal antibody to β-lactamase (QED Bioscience Inc.).

LDH release. The lactate dehydrogenase (LDH) content in the supernatant collected from tissue culture plate wells containing uninfected or infected BMDMs was measured in triplicate with the CytoTox 96 nonradioactive cytotoxicity assay (Promega) following the manufacturer’s instructions. Total LDH was determined from macrophages from separate uninfected wells that had been lysed by a freeze-thaw cycle. The percentage of LDH released was calculated with the formula 100 × (LDH_released/LDH_total).

Statistical analysis. Statistical analysis was performed with Prism (version 4.0; Graphpad) software. The tests used are as indicated in the figure legends or main text. A P value of less than 0.05 was considered significant.

RESULTS

Ectopic expression of YopJ induces apoptosis in macrophages containing Y. pseudotuberculosis. To test the possibility that YopJ synthesized by Y. pseudotuberculosis in phagosomes could trigger macrophage apoptosis, the strain Δyop/pYopJ (Table 1) was constructed. This strain contains a deletion of yopJ on the virulence plasmid pYV and carries a low-copy-number expression vector (pMMB67EH, Amp r) in which the yopJ open reading frame appended by an M45 epitope tag is placed under the control of the tac promoter, allowing regulation by IPTG. A control strain carrying the empty vector (Δyop/pVector; Table 1) was also constructed. Since YopJ does not interfere with phagocytosis or intracellular replication of Y. pseudotuberculosis (54), these processes will not be affected by the presence or absence of YopJ in infected macrophages. To demonstrate regulated expression of YopJ by IPTG in Δyop/pYopJ, immunoblotting analysis was carried out using total bacterial lysates and a mixture of monoclonal antibodies specific for YopJ. YopJ protein was detected when Δyop/pYopJ was grown in LB containing 2.5 mM CaCl₂ at 37°C for 2 h in the presence of IPTG but not in the absence of IPTG (Fig. 1; compare lane 2 and lane 3). When IPTG was removed and the bacteria were incubated in tissue culture medium for 20 min, the steady-state level of YopJ protein decreased (Fig. 1, lane 4). In comparison, the parental wild-type strain IP2666 also expressed undetectable YopJ when grown in LB containing 2.5 mM CaCl₂ at 37°C (Fig. 1, lane 5) but demonstrated robust YopJ expression when grown in LB.
containing a low Ca\textsuperscript{2+} concentration (Fig. 1, lane 6), which was considered to mimic the growth condition when the bacterium is in contact with a eukaryotic cell.

BMDMs in duplicate wells were left uninfected or infected for 20 min with \(\Delta\text{yopJ}/\text{pVector}\) or \(\Delta\text{yopJ}/\text{pVector}\) grown in LB containing 2.5 mM CaCl\textsubscript{2} at 37°C in the absence of IPTG. After the BMDMs had been treated with 8 \(\mu\text{g/ml}\) Gm for 1 h, IPTG was added to half of the wells for an additional 5 h. Apoptosis was measured by PI and annexin V staining assay (see Materials and Methods). Annexin V staining (apoptosis) or annexin V and PI staining (late apoptosis or necrosis) above the background level was observed only among the BMDMs infected with \(\Delta\text{yopJ}/\text{pYopJ}\) that were treated with IPTG and not among the cells similarly infected but not treated with IPTG (compare Fig. 2E and F) or among the BMDMs left uninfected (Fig. 2A and B) or infected with \(\Delta\text{yopJ}/\text{pVector}\) (Fig. 2C and D). To quantify cell death under these conditions, LDH release assays were performed at 21 h postinfection. The amount of LDH released from the BMDMs infected with \(\Delta\text{yopJ}/\text{pYopJ}\) and treated with IPTG (32%; lane 3 in Fig. 3) was significantly higher (\(P < 0.01\)) than the amount released from macrophages infected with the same strain yet not treated with IPTG (7%; lane 2 in Fig. 3). Together, these results suggested that YopJ synthesized by intracellular \(Y.\ pseudotuberculosis\) was able to promote macrophage apoptosis.

A YopJ removal infection experiment was performed to determine if continuous YopJ expression by intracellular \(Y.\ pseudotuberculosis\) was required for macrophage apoptosis. Strain \(\Delta\text{yopJ}/\text{pYopJ}\) was grown in the presence of IPTG and used to infect BMDMs. Then, IPTG was removed from the cultures at the time of infection or 20 min after the initial contact between the BMDMs and the bacteria or was maintained throughout. Cell death was quantified by measuring LDH release at 21 h postinfection. When IPTG was removed at the time of infection, the level of LDH released (23%; lane 4 of Fig. 3) was significantly higher (\(P < 0.05\)) than the level of LDH released from BMDMs infected with \(\Delta\text{yopJ}/\text{pYopJ}\) grown in the absence of IPTG (7%; lane 2 of Fig. 3). This result indicated that continuous YopJ synthesis from intracellular bacteria was not required to induce macrophage apoptosis. However, and interestingly, if IPTG was removed 20 min after the initial contact or maintained throughout the infection, even higher levels of LDH were released (38% and 50%, respectively; lanes 5 and 6 of Fig. 3), and in the latter case, this difference was significant (\(P < 0.01\)) (Fig. 2). Therefore, although continuous synthesis of YopJ by intracellular \(Y.\ pseudotuberculosis\) was not required for macrophage apoptosis, YopJ synthesized by intracellular bacteria contributed to higher levels of cell death.

Apoptosis induced by ectopic expression of YopJ in \(Y.\ pseudotuberculosis\) in macrophages requires a functional T3SS. The above-described results suggested that YopJ could be translocated across the phagosomal membrane by \(Y.\ pseudotuberculosis\) in macrophages. To investigate whether the process required the Ysc T3SS encoded on the virulence plasmid, the pYopJ vector was introduced into the \(Y.\ pseudotuberculosis\) ysc mutant (Table 1), which does not assemble an injectisome, or the \(\Delta\text{yopB}\) mutant (Table 1), which is defective for translocation. The resulting strains were compared to strain \(\Delta\text{yopJ}/\text{pYopJ}\) for the ability to induce death of BMDMs following ectopic expression of YopJ after the extracellular bacteria were killed with Gm.
for 1 h. Cell death was assessed by measuring LDH release after 24 h of infection. As shown in Fig. 4A, ΔyopB/pYopJ and ysc/ pYopJ were defective in inducing BMDM apoptosis. To determine if the catalytic activity of YopJ was required for cell death, a vector encoding yopJ(C172A) (pYopJ*) (Table 1) was introduced into ΔyopJ. Following infection with ΔyopJ/pYopJ*, ectopic expression of YopJ* from intracellular bacteria failed to increase apoptosis in BMDMs (Fig. 4A). These results indicated that a functional Ysc T3SS and acetyltransferase activity were required for macrophage apoptosis to occur following ectopic expression of YopJ in intracellular Y. pseudotuberculosis.

Next we tested whether endogenously expressed YopJ was sufficient to mediate macrophage death when injected from the intracellular location. BMDMs were left uninfected (UI) or infected with the indicated Y. pseudotuberculosis strains carrying pYopJ, pYopJ*C172A, or pYopB2. (A) YopJ expression from intracellular bacteria was either not induced (white bars) or induced with IPTG (checked bars) after 1 h treatment with 8 μg/ml Gm. (B) YopB expression from intracellular ΔyopB/ pYopB2 bacteria was not induced (no IPTG) or induced with IPTG (IPTG) after extracellular bacteria were killed with 8 μg/ml Gm for 1 h. The percentage of LDH released into the medium was determined at 24 h postinfection. Data shown are means and SEMs from three independent experiments. P values were determined by two-way analysis of variance, followed by Bonferroni’s multiple-comparison test (A) or the Mann-Whitney test (B). *P < 0.05 for comparison of the values between IP2666 and either IP2666Gm or ΔyopJ of the same MOI; **P < 0.05 for comparison of the values between IP2666Gm and ΔyopJ.

located from intracellular bacteria was sufficient to induce death of the infected BMDMs.

Exposure of bacteria to Gm before uptake is sufficient to prevent death of the BMDMs infected with parental wild-type Y. pseudotuberculosis. The above-described results indicated that when YopJ was ectopically expressed by IPTG induction from intracellular bacteria, the endogenously expressed T3SS was sufficient to mediate its translocation; on the other hand, when YopB was ectopically expressed through IPTG induction from intracellular bacteria, the endogenously expressed YopJ was also translocated. However, the above conclusions assume that any intracellular bacteria that may escape the macrophage would be unable to reinfet or mediate YopJ translocation due to the bactericidal action of Gm. To test this, the parental wild-type Y. pseudotuberculosis IP2666 strain was used to infect BMDMs at an MOI of 10 in medium already containing Gm at 8 μg/ml. For controls, death of the BMDMs infected with either wild-type strain IP2666 or the ΔyopJ mutant was determined under our standard intracellular infection conditions (MOI of 10 with addition of Gm at 8 μg/ml after 20 min of infection for 1 h and then incubation with Gm at 4.5 μg/ml for another 23 h). As expected from published results obtained with serogroup I Y. pseudotuberculosis strain 32777 (54), the IP2666 strain used in this study also caused cell death under the standard intracellular infection condition (Fig. 5, IP2666). Release of LDH was significantly lower in BMDMs infected with the ΔyopJ strain (Fig. 5, ΔyopJ). When IP2666 was used to infect BMDMs in medium already containing Gm (Fig. 5, IP2666 Gm), the amount of LDH released was not significantly different from that released from macrophages left uninfected or infected with ΔyopJ. Similar results were obtained when the MOI was increased to 20 (Fig. 5). With a higher MOI of either 40 or 100, infection with IP2666 in the presence of Gm did
result in significant LDH release compared to the negative control (ΔyopJ) (Fig. 5). Importantly, the level of LDH released when Gm was always present was significantly lower than that under the standard intracellular infection condition regardless of the MOI (Fig. 5). Given the fact that an MOI of 10 was our standard intracellular infection condition, this result indicated that even though escape of the intracellular bacteria and reinfection could occur, they are far from sufficient to explain the level of BMDM death observed.

**Detection of ectopically expressed Yop-β-lactamase fusion proteins translocated from Y. pseudotuberculosis into macrophages.** The above-described results assessed translocation of YopJ from intracellular *Y. pseudotuberculosis* indirectly through measuring the death of infected BMDMs. To allow detection of Yop translocation at the single-cell level, plasmids were constructed to encode translational fusions between a Yop and the mature domain of TEM-1 β-lactamase (Yop-bla). Detection of cytosolic β-lactamase activity would indicate translocation of the respective effector (10). The activity of β-lactamase was detected with the substrate CCF2-AM. This reagent is cell permeant and is trapped inside cells after hydrolysis of its ester functionalities. Thus, the CCF2-AM-loaded cells will fluoresce green. Upon cleavage by β-lactamase, the product emits blue fluorescence due to the disruption of resonance energy transfer (56). Since the wild-type YopJ protein causes death of BMDMs, which could interfere with detection of fluorescence, the catalytically inactive YopJ<sup>C172A</sup> protein was fused to TEM-1 (pYopJ<sup>C172A-bla</sup>; Table 1). In addition, a plasmid encoding YopH-bla (pYopH-bla; Table 1) was constructed to examine whether Yops other than YopJ could be translocated from *Y. pseudotuberculosis* in macrophages. These plasmids were conjugated into ΔyopJ and ΔyopB. The resulting strains and the parent ΔyopJ strain were cultured under low-Ca<sup>2+</sup> conditions at 37°C to stimulate secretion of Yops by the T3SS, and proteins secreted into culture supernatants were analyzed by gel electrophoresis and immunoblotting (see Materials and Methods). Secretion of the Yop-bla fusion proteins into the growth medium was detected from both ΔyopJ and ΔyopB strains (Fig. 6). Next, BMDMs were infected with these strains grown in the absence of IPTG. As before, IPTG was added after 1 h treatment with Gm to induce expression of the Yop-bla fusion proteins from intracellular *Y. pseudotuberculosis*. Three hours later, the infected BMDMs were loaded with CCF2-AM. After an additional hour, the infected BMDMs were analyzed by two-color fluorescence microscopy. As shown in Fig. 7, blue fluorescent BMDMs indicating the presence of β-lactamase activity were detected after infection with the translocation-competent ΔyopJ derivatives but not with the translocation-defective ΔyopB strains. Furthermore, consistent with the possibility that YopH is translocated at higher levels, more BMDMs showed blue fluorescence when infected with strains producing YopH-bla than strains producing YopJ<sup>C172A-bla</sup> (compare the numbers of blue cells in Fig. 7A and B). This result provided evidence that *Y. pseudotuberculosis* is capable of translocating ectopically expressed YopJ-bla and YopH-bla fusion proteins from inside macrophages.

**Translocation of ectopically expressed Yop-β-lactamase fusion proteins can be correlated with the presence of Y. pseudotuberculosis in macrophages.** We next determined if detection of translocated Yop-bla fusion proteins in BMDMs could be correlated with the presence of viable intracellular *Y. pseudotuberculosis*. To facilitate the detection of intracellular bacteria, a low-copy-number plasmid encoding the monomeric red fluorescent protein mCherry under the control of the tac promoter was constructed from pMMB207 (pmCherry, Amp<sup>+</sup>; Table 1). The ΔyopJ and ΔyopB strains encoding the Yop-bla fusion proteins were transformed with pmCherry. The presence of different selectable markers on the pMMB67EH (Amp<sup>+</sup>- and pMMB207 (Cm<sup>+</sup>)-derived plasmids allowed maintenance of both vectors in *Y. pseudotuberculosis*. BMDMs were infected with these strains, and as described before, after 1 h of treatment with Gm to kill extracellular bacteria, IPTG was included with a lower concentration of Gm for an additional 4 h to induce de novo expression of both the Yop-bla fusion protein and mCherry. Then, substrate CCF2-AM was loaded on the BMDMs for 1 h before analysis by three-color fluorescence microscopy. As shown in Fig. 8, it was possible to identify BMDMs that contained intracellular bacteria (red), were loaded with CCF2-AM (green), and had received translocated

![FIG. 6. Secretion of Yop-bla fusion proteins from Y. pseudotuberculosis strains. Y. pseudotuberculosis ΔyopJ and ΔyopB strains carrying either pYopH-bla (YopH-bla), pYopJ<sup>C172A-bla</sup> (YopJ-bla), or no vector were grown in LB containing 20 mM MgCl<sub>2</sub>, 20 mM sodium oxalate, and IPTG for 4 h at 37°C. Secreted proteins were analyzed by SDS-PAGE and GelCode protein stain (A) or Western blotting (WB) for the M45 epitope (B) or β-lactamase (C). The positions of the protein size markers (in kilodaltons) are indicated on the left, and the locations of the secreted proteins are indicated on the right.](http://iai.asm.org/)
Yop-bla fusion protein (blue). While intracellular bacteria could be detected under all conditions, only BMDMs infected with Δ\textit{yopJ} or Δ\textit{yopB} strains carrying p\textit{YopH-bla} (\textit{YopH-bla}) or p\textit{YopJC172A-bla} (\textit{YopJ-bla}). After 1 h of treatment with 8 μg/ml Gm, IPTG was added and included during the last 3 h of infection. BMDMs were then incubated with CCF2-AM for 1 h. Green and blue images were captured by fluorescence microscopy. Arrowheads indicate some blue cells that contain translocated Yop-bla fusion proteins.

FIG. 7. Detection of translocated Yop-bla fusion protein expressed from intracellular \textit{Y. pseudotuberculosis}. BMDMs were infected with Δ\textit{yopJ} or Δ\textit{yopB} strains carrying p\textit{YopH-bla} (\textit{YopH-bla}) or p\textit{YopJC172A-bla} (\textit{YopJ-bla}). After 1 h of treatment with 8 μg/ml Gm, IPTG was added and included during the last 3 h of infection. BMDMs were then incubated with CCF2-AM for 1 h. Green and blue images were captured by fluorescence microscopy. Arrowheads indicate some blue cells that contain translocated Yop-bla fusion proteins.

FIG. 8. Detection of BMDMs containing translocated Yop-bla fusion proteins and intracellular \textit{Y. pseudotuberculosis}. BMDMs were infected with Δ\textit{yopJ} or Δ\textit{yopB} strains carrying pmCherry and p\textit{YopH-bla} (\textit{YopH-bla}) or p\textit{YopJC172A-bla} (\textit{YopJ-bla}), as indicated. IPTG induction and substrate loading were carried out as described in the legend to Fig. 7. Overlaid red, green, and blue images were captured sequentially by fluorescence microscopy. (A to D) Representative images from three independent experiments are shown. (E) The percentage of cells that carried mCherry-positive (red) bacteria that were also positive for translocation (blue, translocation) or negative for substrate loading (neither red nor blue, no substrate) or the percentage of cells in which translocation was not detectable (green, no translocation) was determined by scoring the cells in multiple images. (F) The percentage of blue cells that were positive (bacteria) or negative (not detectable) for red intracellular bacteria was determined by scoring the cells in multiple images. Results shown are the means and SEMs of three to four independent experiments. \(P\) values of less than 0.05 by the Mann-Whitney test are indicated.
cated that all colonies still contained the virulence plasmid (data not shown).

It was noticed that some BMDMs that fluoresced blue did not contain detectable bacteria. To quantify these cells, images of random fields from the above-described experiments were analyzed to identify BMDMs that fluoresced blue and to score those cells for the presence of intracellular bacteria. As summarized in Fig. 8F, in BMDMs infected with ΔyopJ/pYopH-bla, 61% of the blue cells contained bacteria. In contrast, in BMDMs infected with ΔyopJ/pYopJC172A-bla, 95% of the blue cells contained bacteria. Overall, the finding that the majority of blue BMDMs contain bacteria argues that the Yop-bla fusion proteins are being translocated from intracellular Y. pseudotuberculosis.

**DISCUSSION**

Here we provide the first evidence that intracellular Y. pseudotuberculosis is able to deliver Yops into the macrophage cell cytosol utilizing the Ysc T3SS encoded on the virulence plasmid. The key to our approach was the use of an inducible system that allowed the expression of a translocator or effector to be turned on after Y. pseudotuberculosis was internalized by macrophages. In addition, two sensitive enzymatic reaction-based methods were utilized to detect effector translocation. First, taking the advantage that translocated YopJ induces death of infected macrophages, YopJ expression was ectopically induced after extracellular bacteria were killed with Gm. As indicated before, YopJ does not interfere with phagocytosis or the survival of the intracellular Y. pseudotuberculosis; so cell death resulting from YopJ activity could be directly correlated to its translocation. As shown in Fig. 2 to 4, induction of YopJ expression in intracellular Y. pseudotuberculosis indeed resulted in macrophage death. Alternatively, when the expression of translocator YopB was induced ectopically from intracellular bacteria, increased death of the BMDMs was also observed (Fig. 4B). As cell death resulted from reciprocal usage of either native or ectopically expressed YopJ or YopB, the results are highly consistent with translocation events occurring from intracellular bacteria. Second, it was possible to detect the translocation of β-lactamase fused to either YopJC172A or YopH under the same infection conditions (Fig. 7 and 8), indicating that the Ysc T3SS of intracellular Y. pseudotuberculosis can mediate translocation of multiple ectopically expressed Yop effectors. More YopH-bla than YopJC172A-bla appeared to be translocated (Fig. 7 and Fig. 8E), possibly because YopH has a dedicated chaperone (SycH) that may facilitate its translocation (48).

Our results also indicated that translocation of ectopically expressed YopJ from intracellular Y. pseudotuberculosis required the Ysc injectisome and the YopB translocon protein (Fig. 4). A chromosomally encoded T3SS, similar to the SPI-2-encoded system of Salmonella enterica, is found in Y. pseudotuberculosis (9, 36) and Y. pestis (13, 34). A distinct T3SS is encoded on the chromosome of biovar 1B strains of Y. pestis, and this Ysa system has been shown to be important for translocation of YopP into macrophages (24, 51). Our results (Fig. 4A) rule out the possibility that the chromosomal T3SS in Y. pseudotuberculosis can function in the absence of the Ysc system to mediate bacterial translocation of ectopically expressed YopJ from phagosomes into the cytosol of macrophages.

Despite the detection of translocation of Yops from intracellular bacteria, it is important to reiterate that translocation of effectors through T3SS does not benefit the intracellular bacteria through promoting bacterial survival or replication. Rather, T3SS decreases survival of intracellular bacteria. As shown before, at 5 h postinfection, intracellular bacteria have been cleared from about half of the cells that harbored intracellular bacteria initially (54). The same phenomenon was seen in this study when the presence of intracellular mCherry-expressing Y. pseudotuberculosis was correlated with the translocation of a Yop-bla fusion protein. When BMDMs that were positive for the YopJ-bla fusion protein were scored for the presence of mCherry-positive Y. pseudotuberculosis, there was an excellent correlation, as 95% of the blue fluorescent macrophages contained red bacteria (Fig. 8F). In the case of BMDMs infected with the strain expressing the YopH-bla fusion protein, a majority of the blue fluorescent macrophages contained red bacteria (61%), but 39% did not (Fig. 8F). There are several explanations for the origin of the 39% of BMDMs that contain translocated YopH-bla but not bacteria. We favor the idea that more YopH-bla than YopJ-bla was translocated from intracellular bacteria, and therefore, fewer viable intracellular bacteria were needed to reach the threshold of the Yop-bla fusion protein for detection. The single cell assay also revealed that among the BMDMs containing intracellular bacteria, translocation was undetectable in a considerable population (Fig. 8B, arrow, and E). For example, in 32% of the cells, translocation of YopH-bla was undetectable at 5 h postinfection, and the value for YopJ-bla was 54% (Fig. 8E). The lack of Yop-bla translocation suggested that these bacteria probably downregulated the expression of the Ysc T3SS. Alternatively, these bacteria may not have had the opportunity to express the Ysc T3SS before the initial contact with BMDMs. This phenomenon reflected the potential heterogeneity in the expression or functional profiles of the intracellular bacteria.

Given the detection of these translocation events, it is important to emphasize that Ysc T3SS-mediated translocation is likely a continuous process. Furthermore, it is well established that translocation of effectors by the Ysc T3SS does not require internalization of Yersinia into host cells. Two previous studies concluded that Yops are not translocated by intracellular Yersinia. Rosqvist et al. showed that the ability of Y. pestis to cause cytotoxicity (an outcome of YopE translocation) was inhibited when the bacteria were internalized into HeLa cells (40). In addition, as shown by immunoblotting, the intracellular bacteria were shown to express substantially smaller amounts of Yops than Y. pestis attached to the surface of HeLa cells (40). Similarly, Cowan et al., using immunoblotting, obtained evidence that intracellular Y. pestis is unable to translocate YopH into macrophages (12). Our results agree with these findings that the intracellular bacteria are not as efficient as their extracellular counterparts in injecting the Yops into host cell cytosol. However, by employing more sensitive detection methods, we were able to detect that the Ysc T3SS remains functional for some time within the macrophage phagosomes, such that ectopically expressed effectors can be translocated from this location.

Studies show that a pool of effectors is synthesized in the
bacteria prior to host cell contact and these effectors can be translocated within minutes of interaction with phagocytes (2, 6, 23). Our own results are consistent with this concept, in that the induction of YopJ in *Y. pseudotuberculosis* prior to infection of BMDMs is sufficient to cause apoptosis (Fig. 3). Our results also suggested the possibility that the T3SS remains functional for some time after internalization of the bacteria to mediate translocation of the native Yop effectors. Given the strong expression of the Yops after contact with a eukaryotic cell, it is conceivable that when the existing pool of Yops is large enough or the synthesis of the Yops is able to keep up with translocation, T3SS-mediated translocation of Yops should continue, despite the closure of the phagocytic cup. However, translocation of the effectors through T3SS may be regulated at discrete steps. For example, for *Salmonella enterica*, contact with a host cell triggered effector translocation within seconds, and translocation continued for several minutes, until the intrabacterial pool of effectors was depleted (43). For *Yersinia*, the kinetics of effector translocation has yet to be revealed in such detail. At least by microarray analysis, the situation of *Yersinia* seems to be different from that of *Salmonella*, in that *Y. pestis* inside macrophages expresses yop mRNA at detectable levels (14). Therefore, it is possible that the intracellular *Yersinia* may translocate some newly synthesized effectors.

So if intracellular *Yersinia* bacteria mediate translocation of effectors through T3SS at the cost of their very survival, why would they do this? The process is certainly detrimental to the individuals located intracellularly, but it benefits the whole population, especially the ones located extracellularly. It is possible that the role of intracellular bacteria is important especially initially during *in vivo* infection, when the ratio of bacteria to host cells is low. In addition, since the function of T3SS depends on contact with the host cell and phagocytes are continuously recruited to sites of bacterial presence during infection, increased levels of translocated effectors will help to thwart the host innate response, regardless of the source. Furthermore, the amount of translocated proteins from the intracellular population seems to be sparse; however, as the majority of the extracellular bacteria replicate in microcolonies that do not make direct contact with host cells (20), the small amount could become important.

Overall, the evidence presented here supports a model where translocation of the Yop effectors initiated strongly during phagocytosis; once the bacteria were internalized, translocation of Yops persisted for some bacteria, while others may replicate through restricting the expression of the T3SS. During *Yersinia* infection, the coexistence of both extracellular and intracellular bacteria can diverge the host immune responses, while the presence of different functional populations of intracellular bacteria can diverge such responses even further. This could be the basis of the daunting task that multiple branches of the host immune responses have to be activated for a successful vaccine against *Yersinia* (46).

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