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Yersinia enterocolitica Inhibits *Salmonella enterica* Serovar Typhimurium and *Listeria monocytogenes* Cellular Uptake

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Yersinia enterocolitica **biovar 1B employs two type three secretion systems (T3SS), Ysa and Ysc, which inject effector proteins into macrophages to prevent phagocytosis. Conversely, *Salmonella enterica* serovar Typhimurium uses a T3SS encoded by *Salmonella* pathogenicity island 1 (SPI1) to actively invade cells that are normally nonphagocytic and a second T3SS encoded by SPI2 to survive within macrophages. Given the distinctly different outcomes that occur with regard to host cell uptake of *S.* Typhimurium and *Y. enterocolitica*, we investigated how each pathogen influences the internalization outcome of the other. *Y. enterocolitica* reduces *S.* Typhimurium invasion of HeLa and Caco-2 cells to a level similar to that observed using an *S.* Typhimurium SPI1 mutant alone. However, *Y. enterocolitica* had no effect on *S.* Typhimurium uptake by J774.1 or RAW264.7 macrophage-like cells. *Y. enterocolitica* was also able to inhibit the invasion of epithelial and macrophage-like cells by *Listeria monocytogenes*. *Y. enterocolitica* mutants lacking either the Ysa or Ysc T3SS were partially defective, while double mutants were completely defective, in blocking *S.* Typhimurium uptake by epithelial cells. *S.* Typhimurium encodes a LuxR homolog, SdiA, which detects *N*-acylhomoserine lactones (AHLs) produced by *Y. enterocolitica* and upregulates the expression of an invasin (Rck) and a putative T3SS effector (SrgE). Two different methods of constitutively activating the *S.* Typhimurium SdiA regulon failed to reverse the uptake blockade imposed by *Y. enterocolitica*.**

Salmonella enterica serovar Typhimurium is one of the leading causes of severe gastroenteritis in humans (1–4). It also causes a systemic typhoid-like disease in susceptible mice (*Slc11A1*$^{-/-}$, previously known as *Nramp1*$^{-/-}$) (5, 6). As a facultative intracellular pathogen, *S.* Typhimurium can invade cells that are normally nonphagocytic (7), such as intestinal epithelial cells, and is also capable of surviving in phagocytic cells during systemic infection (8). The ability of *S.* Typhimurium to cause disease is largely due to two type three secretion systems (T3SS) which mediate translocation of more than 40 effector proteins from the bacterial cytoplasm into the host cell cytosol (9–13). T3SS1 is encoded within SPI1 (*Salmonella* pathogenicity island 1); it confers the ability to invade nonphagocytic cells and plays a role in the biogenesis of the *Salmonella*-containing vacuole (SCV) (7, 14, 15). T3SS2 is encoded within SPI2 and is required for transit of *S.* Typhimurium from the apical to the basolateral side of epithelial cells, for survival within macrophages, and for systemic infection (11, 16).

Internalization of *S.* Typhimurium into host cells can occur by the trigger mechanism or the zipper mechanism (17). The trigger mechanism is induced by T3SS1 effectors that activate Rho guanosine triphosphatase proteins (Rho GTPases), leading to host cell cytoskeleton rearrangements and bacterial engulfment (18–21). Phagocytic cells can zipper around bacteria opsonized with antibody or complement, while *Salmonella* can induce the zipper mechanism in nonphagocytic cells using the outer membrane protein Rck (22). Similar to the T3SS1 effectors, Rck activates the Rho GTPases Rac1 and Cdc42 to promote *Salmonella* uptake (22–24).

Yersinia enterocolitica bv. 1B is also a food-borne pathogen and, like *S.* Typhimurium, translocates numerous T3SS effector proteins, called Yops, into the host cell cytosol using both the Ysc T3SS encoded on the virulence plasmid, pYV, and the Ysa T3SS encoded on the chromosome (25–29). However, in contrast to *S.* Typhimurium effectors, the *Y. enterocolitica* effectors prevent phagocytosis to promote extracellular replication within lymphoid tissues (25, 30–36). Inhibition of phagocytosis results from two sequential events. First, outer membrane proteins such as invasin, YadA, and Ail mediate *Y. enterocolitica* attachment to host cells through interaction with β1-integrin, collagen, fibronectin, laminin, and heparan sulfate proteoglycan (37–40). Second, adhesion is rapidly followed by translocation of T3SS effectors YopE, YopH, YopO/YpkA, and YopT. Each of these effectors specifically hinders *Y. enterocolitica* phagocytosis by counteracting signaling cascades controlled by Rho family GTPases (31–33, 41).

Previously, we have described a mouse model system for the study of coinfections using *S.* Typhimurium and *Y. enterocolitica*. This coinfection model has already provided insight on how *S.* Typhimurium detects and responds to the *N*-acylhomoserine lactones (AHLs) produced by *Y. enterocolitica* (42). This interspecies detection event requires the *yenI* gene of *Y. enterocolitica*, which encodes an AHL synthase, and the *sdiA* gene of *S.* Typhimurium, which encodes a LuxR-type AHL receptor. In response to AHLs, SdiA increases the expression of the *rck* operon and a gene named *srgE* that encodes a putative T3SS effector of unknown function (43–45). Given the distinctly different outcomes that occur with regard to host cell uptake of *S.* Typhimurium and *Y. enterocolitica*, and that *S.* Typhimurium expresses an alternate invasin and a

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
Salmonella Typhimurium		
14028	Wild-type *Salmonella enterica* serovar Typhimurium	ATCC
BA612	14028 *sdiA1*::mTn*3*	49
BA3100	14028 *invA*::Cam	This study
JLD1201	14028 λP_R-*yenI*-FRT-kan-FRT	42
MJW1836	14028 *ssaK*::kan *invA*::Cam	Micah Worley
Yersinia enterocolitica		
JB580v	Biovar 1 B, serotype O:8 (R^-M^+)	71
GY4415	JB580v *ysaT*::mTnMod-R-Kan	72
GY4481	JB580v *ysaT*::mTnMod-R-Kan, pYV8081$^-$ (lacks *ysc* T3SS on the virulence plasmid and *ysa* T3SS on the chromsome)	72
GY5456	JB580v *yenR*::kan, pYV8081$^-$	This study
GY5465	JB580v + pML10::*gfp* (Tet^r)	This study
NCTC10460	biovar 3, serotype O:1	73
Listeria monocytogenes		
DP10403S	Wild-type *Listeria monocytogenes*	74
Plasmids		
pBAD33	pACYC vector for arabinose-conditional expression (Cam^R and Amp^r)	75
pJVR2	pBAD33 carrying *sdiA* (Amp^r and Cam^r)	49
pNF8	pAT18::*gfp* (Tet^r Erm^r)	47

putative T3SS effector in the presence of *Y. enterocolitica* AHLs, we investigated how each pathogen influences the internalization outcome of the other. We determined that *Y. enterocolitica* is dominant in that it inhibits the uptake of *S.* Typhimurium into some cell types, while *S.* Typhimurium does not influence the uptake of *Y. enterocolitica*. The ability of *Y. enterocolitica* to inhibit *S.* Typhimurium uptake required both *Y. enterocolitica* T3SS. The expression of *rck* and *srgE* by *S.* Typhimurium could not bypass the inhibition of uptake imposed by *Y. enterocolitica*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium strain ATCC 14028, *Yersinia enterocolitica* bv. 1B strain JB580v, and *Listeria monocytogenes* strain DP10403S and their isogenic derivatives are listed in Table 1. *Salmonella* strains were grown standing in Luria-Bertani (LB) broth (EMD Chemicals, Germany) at 37°C overnight. *Yersinia* strains were grown overnight with shaking in LB broth at 26°C. *L. monocytogenes* was grown in brain heart infusion (BHI) broth (Becton, Dickinson Company, Sparks, MD) overnight with shaking at 37°C. The following morning, the overnight cultures were subcultured 1:100 and grown to an optical density at 600 nm (OD_{600}) of 0.7 to 0.8. The antibiotics kanamycin, chloramphenicol, tetracycline, and erythromycin were added to the media at concentrations of 100, 50, 20, and 10 μg/ml, respectively, as necessary (Sigma-Aldrich, St. Louis, MO). Expression of *sdiA* from plasmid pJVR2 was induced by growth in LB broth containing 0.2% arabinose (Sigma-Aldrich).

Cell culture. HeLa, RAW264.7, and J774.1 cell lines were obtained from the ATCC and cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Rockville, MD) containing 10% fetal bovine serum (FBS) (Biowest, Miami, FL), 1% nonessential amino acids, and 1 mM L-glutamine. The Caco-2 cell line was obtained from ATCC and maintained in minimal essential medium (MEM; Gibco) containing 20% FBS. All cells were incubated at 37°C in the presence of 5% CO_2.

Gentamicin protection assays. Gentamicin protection assays were performed as previously described (46). A total of 5×10^5 HeLa, RAW264.7, J774.1, or Caco-2 cells were seeded overnight in 24-well plates (Becton, Dickinson, Franklin Lakes, NJ) to allow them to adhere. Cultures of *S.* Typhimurium, *L. monocytogenes*, and/or *Y. enterocolitica* were added to adherent cells at a multiplicity of infection (MOI) of 10. To synchronize the infection, cells were centrifuged for 5 min at 1,000 rpm at room temperature and then incubated for 1 h at 37°C in the presence of 5% CO_2. The cells were then washed three times with tissue culture medium to remove nonadherent bacteria and incubated for another hour in tissue culture medium containing 100 μg/ml of gentamicin (Gibco, Rockville, MD) to kill extracellular bacteria. In experiments in which two bacterial strains were added sequentially, the first strain was centrifuged onto the tissue culture cells and incubated for 1 h as described above, the cells were washed three times with tissue culture medium, and then the second strain was added to the cells via centrifugation and incubated for 1 h, followed by washing and incubation in gentamicin as per the single-infection experiments. To enumerate intracellular bacteria, the infected cells were lysed with 1% Triton X-100 in phosphate-buffered saline (PBS; pH 7.0) for 5 min at room temperature and serial dilutions were plated onto LB agar plates for single infections, onto Difco xylose-leucine-desoxycholate (XLD) agar plates (Becton, Dickinson Company, Sparks, MD) for differentiation of *Salmonella* and *Yersinia* colonies, onto Difco *Yersinia* selective agar (cefsulodin-Irgasan-novobiocin [CIN]) plates to select for *Yersinia*, or onto BHI agar plates containing 10 μg/ml of erythromycin to select for *Listeria* (47).

Immunofluorescence staining. Immunofluorescence staining was used to distinguish intracellular *Salmonella* or *Listeria* from extracellular *Salmonella* or *Listeria* in single-infection and coinfection assays. *Yersinia* was detected using green fluorescent protein (GFP) or rabbit anti-YadA (generously provided by Jürgen Heesemann, Max von Pettenkofer-Institute of Hygiene and Medical Microbiology, Munich, Germany). Infected tissue culture cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Cells were labeled for 30 min with rabbit anti-*Salmonella* (O and H antigens) antibody (Novus Biologicals, Littleton, CO) at a 1/500 dilution or rabbit anti-*Listeria* antibody (GeneTex Inc., Irvine, CA) at a 1/800 dilution to stain extracellular bacteria or rabbit anti-YadA antibody at a 1/100 dilution. Rabbit anti-*Salmonella*, anti-*Listeria*, and anti-*Yersinia* primary antibodies were detected with a red fluorescence Alexa Fluor 555-conjugated anti-rabbit antibody (1/4,000 dilution; Invitrogen, Carlsbad, CA). Following the labeling of extracellular bacteria, infected cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature to label intracellular and extracellular bacteria with the same primary antibodies followed by secondary anti-rabbit antibody conjugated with Alexa Fluor 405 (blue fluorescence) or Alexa Fluor 488 (green fluorescence) (1/4,000 dilution) (Invitrogen). Fixed samples were examined using an Olympus FV1000 spectral laser-scanning confocal microscope. On average, 8 to 15 0.2-μm serial Z sections of each image were captured and stored for additional analyses, using Adobe Photoshop CS6 software. At least 100 cells infected were analyzed per well.

Statistical methods. For gentamicin protection and single-cell analysis assays, all experiments were performed in triplicate wells on three occasions. The statistical analysis was performed with Prism 5 software (GraphPad) with one-way analysis of variance (ANOVA) and Bonferroni analyses or two-tailed unpaired Student's *t* test. A *P* value of <0.05 was considered significant (*, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0005$).

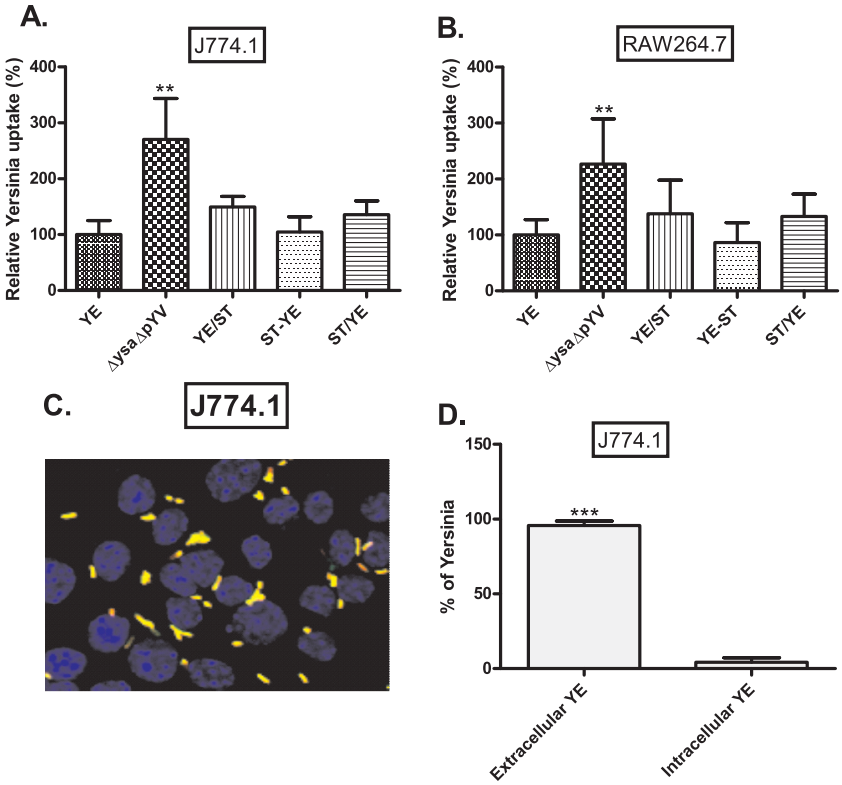

FIG 1 *Y. enterocolitica* prevents its own uptake into phagocytic cells. J774.1 (A, C, and D) and RAW264.7 (B) cells were infected with wild-type (WT) *Y. enterocolitica* (YE) (JB580v) or a ΔpYV *ysa* double mutant (GY4481) or simultaneously or sequentially coinfected with JB580v and *S.* Typhimurum (ST) WT (14028). The simultaneous infections are indicated by the two strain names separated by a dash. The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. The uptake of *Y. enterocolitica* in each scenario is plotted relative to the uptake of JB580v in a single infection. (C) Representative images of intracellular *Y. enterocolitica* (green) and extracellular *Y. enterocolitica* (yellow) and host cell nucleus (blue). (D) Data points are the percentages of intracellular and extracellular *Y. enterocolitica* per J774.1 infected cell. Results are expressed as the means ± SEMs of three independent experiments. **, $P < 0.005$; ***, $P < 0.0005$.

RESULTS

***Y. enterocolitica* inhibits its own uptake into phagocytic but not into nonphagocytic cells.** *Y. enterocolitica* is reported to use its T3SS to deliver effectors into phagocytic cells to resist internalization (31–33, 41). To confirm this, we performed gentamicin protection assays and observed that *Yersinia* lacking T3SS (a *ysa* ΔpYV double mutant) was internalized by macrophage-like cells (J774.1 and RAW264.7) more than the wild type (Fig. 1A and B). *Salmonella* did not affect *Yersinia* uptake. The localization of *Yersinia* on the outside J774.1 cells was confirmed using microscopy.

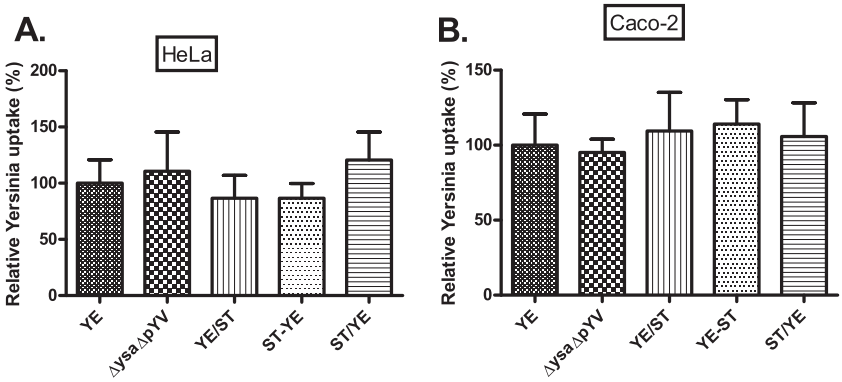

FIG 2 *Salmonella* does not inhibit *Y. enterocolitica* uptake into nonphagocytic cells. HeLa (A) and Caco-2 (B) cells were infected with WT *Y. enterocolitica* (YE) (JB580v) or a ΔpYV *ysa* double mutant (GY4481) or simultaneously or sequentially coinfected with JB580v and *S.* Typhimurum (ST) WT (14028). The simultaneous infections are indicated by the two strain names separated by a hyphen. The sequential infections are indicated by the strain that is added first, a slash, and then the strain that was added one hour later. The uptake of *Y. enterocolitica* in each scenario is plotted relative to the uptake of JB580v in a single infection. Results are expressed as the means ± SEMs of three independent experiments.

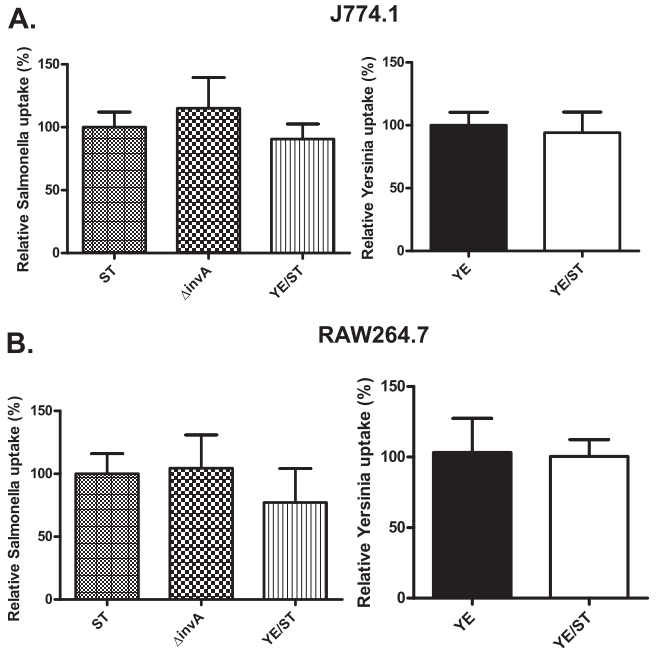

FIG 3 *Y. enterocolitica* does not inhibit *S.* Typhimurium uptake into phagocytic cells. J774.1 (A) and RAW264.7 (B) cells were infected with WT *Y. enterocolitica* (YE) (JB580v) or *S.* Typhimurium (ST) WT (14028) or an *invA* mutant of *S.* Typhimurium (BA3100) or sequentially coinfected with JB580v and 14028. The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. The first three columns show the uptake of *Salmonella* relative to the uptake of 14028 in a single infection. The last two columns show the uptake of *Yersinia* relative to the uptake of JB580v in a single infection. Results are expressed as the means ± SEMs of three independent experiments.

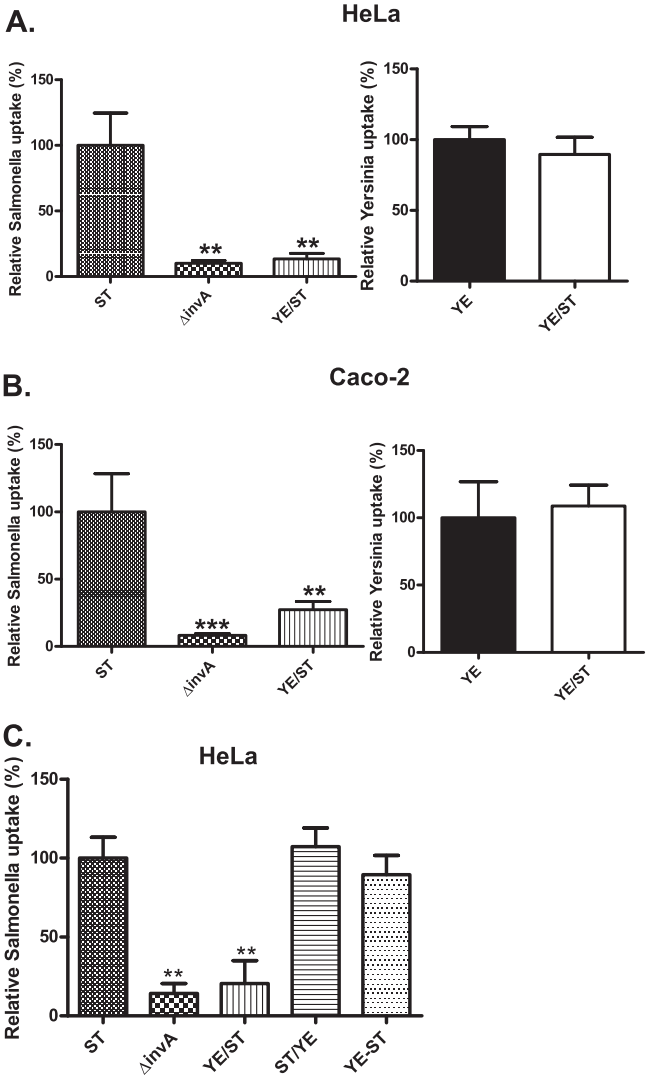

FIG 4 *Y. enterocolitica* inhibits *S.* Typhimurium uptake into nonphagocytic cells. HeLa (A and C) and Caco-2 (B) cells were infected with WT *Y. enterocolitica* (YE) (JB580v) or *S.* Typhimurium (ST) WT (14028) or an *invA* mutant of *S.* Typhimurium (BA3100) or simultaneously or sequentially coinfected with JB580v and 14028. The simultaneous infections are indicated by the two strain names separated by a dash. The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. In panels A and B, the first three columns show the uptake of *Salmonella* relative to the uptake of 14028 in a single infection, whereas the last two columns show the uptake of *Yersinia* relative to the uptake of JB580v in a single infection. In panel C, all columns show the uptake of *Salmonella* relative to the uptake of 14028 in a single infection. Results are expressed as the means ± SEMs of three independent experiments. **, $P < 0.005$; ***, $P < 0.0005$.

The microscopic analysis gave more striking results, with 96% of *Yersinia* organisms remaining extracellular (Fig. 1C and D). With epithelial cell lines, the uptake of a *Yersinia ysa* ΔpYV double mutant was similar to that of the wild type in Caco-2 cells and in HeLa cells, and the presence of *Salmonella* did not change this (Fig. 2).

***Y. enterocolitica* inhibits uptake of *S.* Typhimurium.** Next, we tested the hypothesis that the orchestrated mechanisms used by *Y. enterocolitica* to prevent its own uptake by macrophages can disrupt the uptake of other invasive pathogens, such as *S.* Typhimurium. Surprisingly, *Y. enterocolitica* did not significantly impair the uptake of *S.* Typhimurium into J774.1 or RAW264.7 macrophage-like cells (Fig. 3). However, when using the nonphagocytic cell lines, HeLa and Caco-2, *Y. enterocolitica* was able to significantly reduce the uptake of *S.* Typhimurium, by 86% and 73%, respectively (Fig. 4). Interestingly, the number of *S.* Typhimurium organisms recovered from HeLa cells after *Y. enterocolitica* preinfection was comparable to the number recovered when using a noninvasive *S.* Typhimurium *invA* mutant (lacking T3SS1) in single infection (Fig. 4). However, when *Y. enterocolitica* and *S.* Typhimurium were added to HeLa cells simultaneously or when *Yersinia* was added 1 h after *S.* Typhimurium, *S.* Typhimurium uptake was not impaired (Fig. 4C).

To corroborate the results obtained using gentamicin protection assays, we performed microscopic analysis of HeLa and RAW264.7 cells coinfected with both pathogens. The average number of intracellular *S.* Typhimurium organisms in HeLa cells was reduced by 50% when *Y. enterocolitica* was added first. Consistent with the gentamicin protection assay results, this level of *S.* Typhimurium entry is similar to the entry level of an *S.* Typhimurium *invA* mutant alone (Fig. 5). Also consistent with the gentamicin protection assays, *Y. enterocolitica* did not prevent entry of *S.* Typhimurium into RAW264.7 cells but did prevent entry into HeLa cells (Fig. 5). Similar inhibition of *S.* Typhimurium uptake was observed using another *Y. enterocolitica* strain (NCTC10460 [data not shown]). Collectively, the gentamicin protection assays

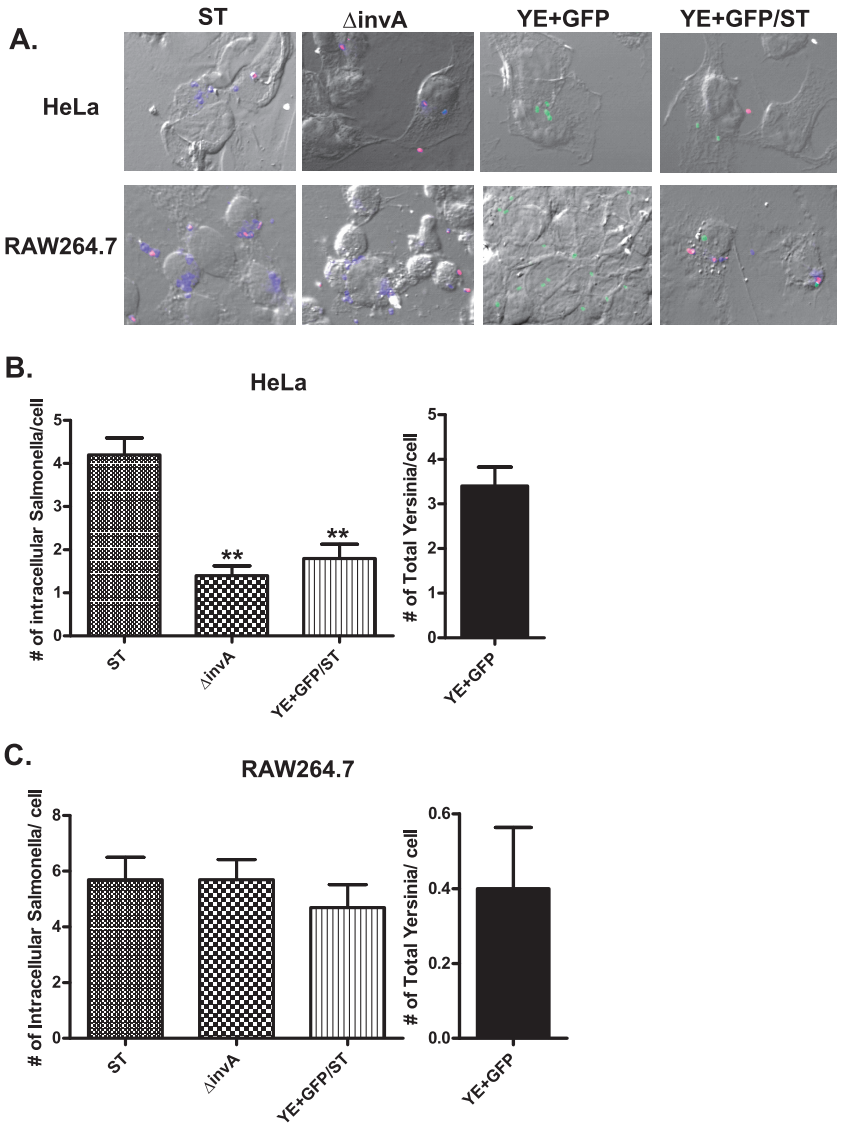

FIG 5 Single-cell analysis of *Y. enterocolitica* inhibition of *S.* Typhimurium uptake into phagocytic and nonphagocytic cells. HeLa and RAW264.7 cells were infected with the *Y. enterocolitica* (YE+GFP) WT carrying *gfp* (GY5465) or the *S.* Typhimurium (ST) WT (14028) or an *invA* mutant of *S.* Typhimurium (BA3100) or sequentially coinfected with GY5465 and 14028. The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. (A) Representative images of intracellular *S.* Typhimurium (blue), extracellular *S.* Typhimurium (pink), and total *Y. enterocolitica* (green). Data points are the numbers of intracellular *S.* Typhimurium or total *Y. enterocolitica* organisms per infected HeLa (B) and RAW264.7 (C) cell. Results are expressed as the means ± SEMs of three independent experiments. **, $P < 0.005$; ***, $P < 0.0005$.

and confocal microscopy analysis indicate that *Y. enterocolitica* strongly inhibits *S.* Typhimurium uptake into HeLa and Caco-2 cells but does not inhibit *S.* Typhimurium uptake into J774.1 and RAW264.7 cells.

Additive effect of *Y. enterocolitica* Ysa and Ysc T3SS in reducing *S.* Typhimurium uptake. Upon attachment to host cells, *Y. enterocolitica* utilizes T3SS to inject effector proteins into the host cell cytosol to prevent its own uptake (31–33, 41). To test whether *Y. enterocolitica* the Ysa and/or Ysc T3SS is required for blocking *S.* Typhimurium internalization, we infected HeLa cells with *Y. enterocolitica* strains lacking either one or both T3SS for 1 h prior to *S.* Typhimurium infection and examined the level of *S. Typhimurium* uptake relative to that of *S.* Typhimurium in a single infection. Both the *ysa* and ΔpYV *Y. enterocolitica* single mutants were able to partially block *S.* Typhimurium uptake, while the double mutant was unable to block *S.* Typhimurium uptake (Fig. 6). Uptake of *S.* Typhimurium was reduced by 57%, 41%, and 17% in coinfection with a *Y. enterocolitica* ΔpYV single mutant, *ysa* single mutant, and ΔpYV *ysa* double mutant, respectively (Fig. 6). This result demonstrates that the *Y. enterocolitica* Ysa and Ysc T3SS act in concert to inhibit *S.* Typhimurium entry into infected cells.

Induction of the *S.* Typhimurium SdiA regulon cannot bypass the uptake inhibition mediated by *Y. enterocolitica*. *S.* Typhimurium is known to use the SdiA transcription factor to detect AHL signaling molecules produced by the *Y. enterocolitica* YenI protein (42, 43, 48). Detection of AHL leads to the upregulation of the *rck* operon and a putative type III secreted effector, SrgE (42,

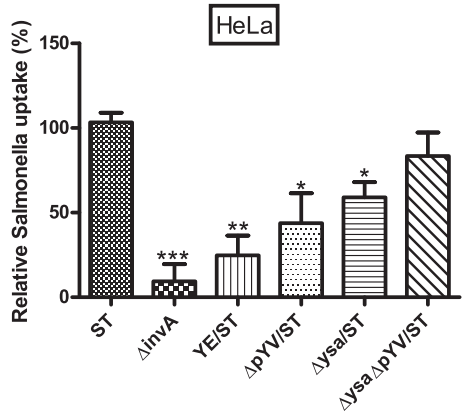

FIG 6 *Y. enterocolitica* inhibition of *S.* Typhimurium uptake requires both T3SS. HeLa cells were infected with the *S.* Typhimurium (ST) WT (14028) or *S.* Typhimurium *invA* mutant (BA3100) or sequentially infected first with the *Y. enterocolitica* (YE) WT (JB580v), ΔpYV mutant (GY5456), *ysa* mutant (GY4415), or ΔpYV *ysa* double mutant (GY4481) for 1 h, followed by the *S.* Typhimurium WT (14028). The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. The uptake of *S.* Typhimurium is plotted relative to the uptake of 14028 in a single infection. Results are expressed as the means ± SEMs of three independent experiments. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

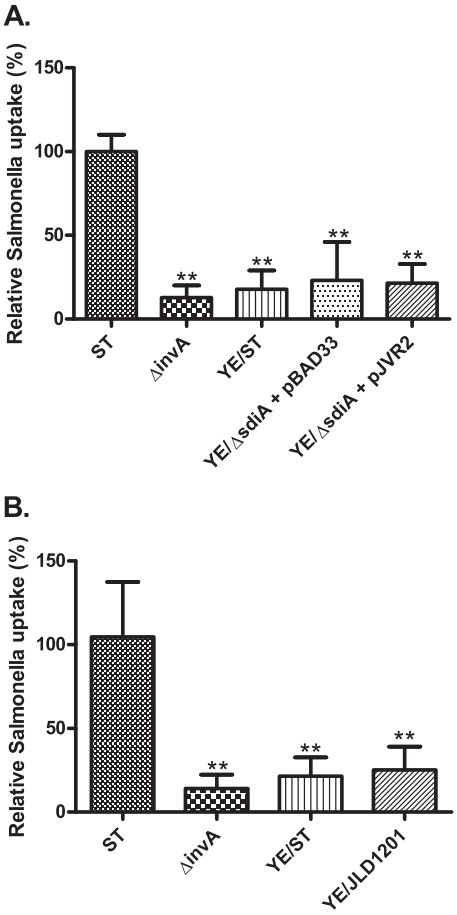

FIG 7 The SdiA regulon fails to reverse *Y. enterocolitica* inhibition of *S.* Typhimurium uptake. HeLa cells were infected with the *S.* Typhimurium (ST) WT (14028) or *S.* Typhimurium *invA* mutant (BA3100) or sequentially infected first with *Y. enterocolitica* WT for 1 h, followed by the *S.* Typhimurium WT or BA612+pBAD33 (overexpression vector control) or BA612+pJVR2 (overexpresses *sdiA*) (A) or *S.* Typhimurium *yenI*$^+$ (JLD1201) (B). The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. The uptake of *S.* Typhimurium is plotted relative to the uptake of 14028 in a single infection. Results are expressed as the means ± SEMs of three independent experiments. **, $P < 0.005$.

43, 48, 49). Rck is known to mediate *S.* Typhimurium internalization by a zipper-like mechanism through the activation of small GTPases Rac1 and Cdc42 (22–24). Therefore, we investigated whether the *S.* Typhimurium Rck-mediated zippering mechanism, possibly with the aid of the putative T3SS effector SrgE, can bypass the *Y. enterocolitica*-mediated uptake blockade. Since gentamicin protection assays include several washing steps that would remove AHLs, and SdiA activity is low in liquid medium even in the presence of AHLs (43), we used two different approaches of "locking ON" the SdiA regulon of *S.* Typhimurium during the invasion assays. The first method used overexpression of *sdiA*, which has been shown to lead to SdiA regulon expression even in the absence of AHL (48, 49). The uptake of the *Salmonella* strain carrying *sdiA* on a plasmid was inhibited to the same extent by *Y. enterocolitica* as wild-type *Salmonella* or a strain carrying the vector control (Fig. 7A). The second method used an *S.* Typhimurium strain, JLD1201, which expresses the *Y. enterocolitica yenI* gene from a constitutive promoter in the *Salmonella* chromosome (42). The uptake of wild-type *S.* Typhimurium and that of JLD1201 were inhibited to the same extent (Fig. 7B). Both of these results indicate that the induction of the *Salmonella* SdiA regulon cannot bypass the inhibition of uptake imposed by *Y. enterocolitica*.

***Y. enterocolitica*-mediated inhibition of uptake is not specific to *S.* Typhimurium.** It is well established that *L. monocytogenes* is able to invade nonphagocytic cells, such as epithelial cells, to cross multiple host barriers during pathogenesis (50–57). *L. monocytogenes* invades nonphagocytic cells via internalin A (InlA) and/or InlB surface proteins that interact with host cell E-cadherin and the Met receptors to trigger its zippering mechanism of entry. During this process, small GTPases Rac1 and Cdc42 are activated, leading to its internalization. To determine if the ability of *Y. enterocolitica* to inhibit cellular uptake was specific to *S.* Typhimurium, we tested the ability of *Y. enterocolitica* to inhibit *L. monocytogenes* uptake into epithelial and phagocytic cells. As described above, we performed a gentamicin protection assay and a microscopic analysis of cells sequentially coinfected with *Y. enterocolitica* and *L. monocytogenes*. The gentamicin protection assay showed that *Y. enterocolitica* significantly reduced *L. monocytogenes* uptake, by 40%, 24%, and 30%, compared to *L. monocytogenes* single infection in HeLa, RAW264.7, and J774.1 cells, respectively (Fig. 8). Consistent with the results from the gentamicin protection assay, microscopic analysis indicated that the average number of *L. monocytogenes* organisms per infected cell was reduced from 3 intracellular bacteria per HeLa cell in *L. monocytogenes* single infection to 1 bacterium per cell in *Y. enterocolitica*-coinfected cells (Fig. 9A and C). Similarly, the average number of intracellular *L. monocytogenes* organisms per infected RAW264.7 cell was decreased from 10 bacteria per cell to 5 bacteria per cell when *Y. enterocolitica* was added first (Fig. 9B and D). Collectively, these data indicate that *Y. enterocolitica* inhibits *L. monocytogenes* uptake by nonphagocytic and phagocytic cells.

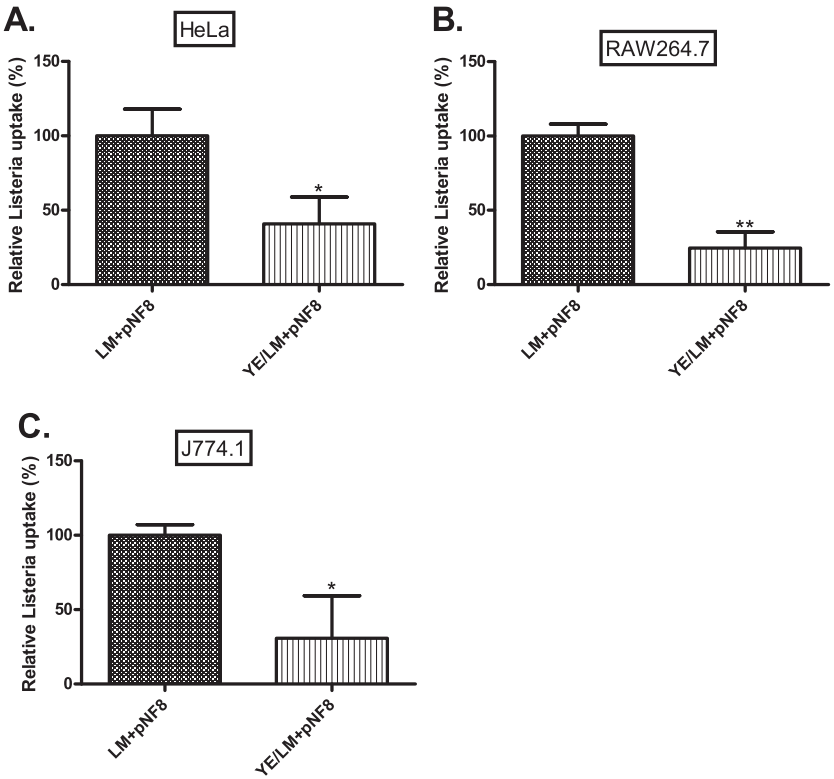

FIG 8 *Y. enterocolitica* is able to inhibit uptake of *L. monocytogenes* into phagocytic and nonphagocytic cells. HeLa (A), RAW264.7 (B), and J774.1 (C) cells were infected with *L. monocytogenes* (LM) WT (DP10403S+pNF8) or sequentially infected first with the *Y. enterocolitica* (YE) WT (JB580v) for 1 h, followed by *L. monocytogenes* WT (DP10403S+pNF8). The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. The erythromycin resistance cassette on plasmid pNF8 allows the differentiation of *L. monocytogenes* from *Y. enterocolitica* on BHI plates containing erythromycin. The uptake of *L. monocytogenes* is plotted relative to the uptake of DP10403S+pNF8 in a single infection. Results are expressed as the means ± SEMs of three independent experiments. *, $P < 0.05$; **, $P < 0.005$.

DISCUSSION

Infectious disease is generally described as the outcome of infection by a single species of pathogen. While this description is academically useful, it is too simplistic to describe the variety of disease outcomes naturally occurring in the case of pathogen coinfections. Food-borne pathogens offer an excellent venue for shedding light on alternative infectious outcomes that can emerge due to coinfection. *Y. enterocolitica*, *S.* Typhimurium, and *L. monocytogenes* are enteric pathogens that are known to colonize domesticated animals and are likely to participate in coinfections (58). In this report we demonstrated that *Y. enterocolitica* blocks the uptake of *S.* Typhimurium into epithelial cells in a T3SS-dependent manner but does not block *S.* Typhimurium uptake by macrophages. This was surprising, because *Y. enterocolitica* is known to inhibit its own entry into phagocytic cells but not into epithelial cells.

S. Typhimurium translocates effector proteins (SipA, SipC, SopE, SopE2, and SopB/SigD) into nonphagocytic cells via T3SS1 to induce a "triggering" mode of uptake, whereas *Y. enterocolitica* translocates effectors (YopE, YopH, YopO, and YopT) into phagocytic cells to inhibit its own uptake (17, 19, 25, 29). When *Y. enterocolitica* is added to host cells first, *S.* Typhimurium invasion drops to a level similar to that of an *S.* Typhimurium *invA* mutant, which lacks T3SS1 (Fig. 4 to 7). Thus, the *Y. enterocolitica* effectors appear to be dominant over the *S.* Typhimurium effectors. This suggests that the T3SS1-dependent uptake pathway is blocked and that other invasion pathways may remain active. Indeed, alternative uptake pathways have been published for *S.* Typhimurium (17, 23, 24, 59). One of these is mediated by the outer membrane protein Rck, which confers a "zippering" mode of entry. Both the trigger and zipper mechanisms of *S.* Typhimurium invasion converge at the activation of Rho GTPases Rac1 and Cdc42 (17–24, 60). However, in addition to Rac1 and Cdc42, the trigger mechanism uses another set of Rho GTPases (RhoA and RhoG) and Arf GTPases to induce actin polymerization (19, 61, 62), highlighting the morphological differences in membrane ruffling induced by the trigger and zipper mechanisms of *S.* Typhimurium invasion. *Y. enterocolitica* Yop proteins are known to specifically deactivate Rac1, Cdc42 and RhoA, while the role of Yops in counteracting Arf GTPases has not yet been reported (62). Induction of actin polymerization has been observed in other invasive pathogens, including *L. monocytogenes* (63), *Shigella flexneri* (64–66), and vaccinia virus (67, 68). Likewise, *L. monocytogenes* uses the InlA and/or InlB surface protein to perform a "zippering" type of uptake using the same pathway (50–52). Consistent with this, we observed that *Y. enterocolitica* inhibits both *S.* Typhimurium and *L. monocytogenes* uptake into HeLa (Fig. 3, 4, 5, 8, and 9) but additionally inhibits *L. monocytogenes* uptake into J774.1 cells and RAW264.7 cells (Fig. 4 and 8C). This suggests the existence of additional pathways that allow *S.* Typhimurium but not *L. monocytogenes* uptake into J774.1 and RAW264.7 cells in the presence of *Yersinia*. In support of this assumption, Rosselin et al. have dem-

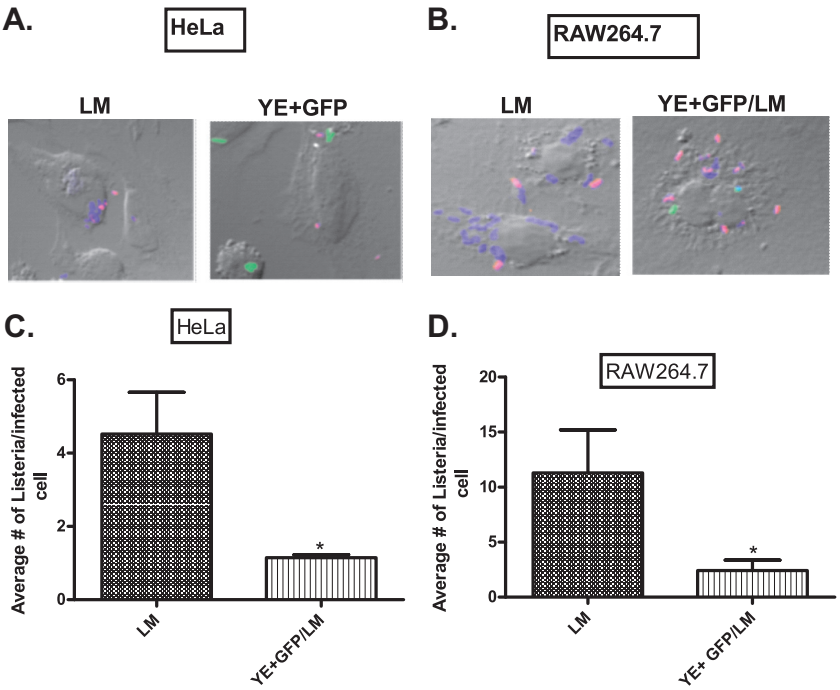

FIG 9 Single-cell analysis of *Y. enterocolitica* inhibition of *L. monocytogenes* uptake in nonphagocytic and phagocytic cells. HeLa (A) and RAW264.7 (B) cells were infected with the *L. monocytogenes* (LM) WT (DP10403S) or sequentially infected first with the *Y. enterocolitica* (YE) WT carrying *gfp* (GY5465) for 1 h, followed by the *L. monocytogenes* WT. The sequential infections are indicated by the strain names separated by a slash, in which case the first strain listed was added first and the second was added 1 h later. Representative images of intracellular WT *L. monocytogenes* (blue), extracellular WT *L. monocytogenes* (pink), and total *Y. enterocolitica* (green) in HeLa cells (C) and in RAW264.7 cells (D) are shown. Results are expressed as the means ± SEMs of three independent experiments. *, $P < 0.05$.

onstrated that in addition to PagN, Rck, and T3SS-1, other invasion factors are capable of promoting *Salmonella* zipper or trigger entry, perhaps involving distinct active Rho GTPases that *Y. enterocolitica* effectors are incapable of inactivating (17, 59). The ability of *Y. enterocolitica* to inhibit *S.* Typhimurium uptake is in agreement with a recent study conducted by Martins et al. that shows that incubation of HeLa cells overnight with the yeast *Saccharomyces boulardii* reduced *S.* Typhimurium uptake (69). In this study, Rac1 and Cdc42 were found to be downregulated by *S. boulardii*. Apparently, both *Y. enterocolitica* and *S. boulardii* deactivate the Rho GTPases, Rac1, and Cdc42 to prevent *S.* Typhimurium entry into coinfected cells. Similar bacterial interceptions of actin polymerization pathways have been reported for dual infections. Recently, it was demonstrated that enteropathogenic *Escherichia coli* (EPEC) inhibits *H. pylori*-induced activation of Rho GTPase Rac1 via Tir-intimin interaction (70). Our data clearly show that inhibition of *Salmonella* uptake in HeLa cells requires *Y. enterocolitica* Ysa T3SS at 37°C, but another recent study conducted by Walker et al. (26) has identified an invasion phenotype for Ysa using S2 insect cells at 26°C. Thus, our results suggest an additional role played by Ysa at 37°C (Fig. 6).

S. Typhimurium encodes a LuxR-type transcription factor, SdiA, which can detect the AHLs produced by *Y. enterocolitica* in mice (42). SdiA increases the expression of the *rck* operon and a putative T3SS effector of unknown function, SrgE. It is not known if *Y. enterocolitica* AHL production is relevant to *S.* Typhimurium in nature, although both are prevalent in swine, where they presumably encounter one another (58). We tested the hypothesis that the SdiA regulon (*rck* and *srgE*) is used to reverse the host cell uptake blockade imposed by *Y. enterocolitica*. However, two methods of maximizing the expression of the SdiA regulon failed to reverse the inhibition of uptake imposed by *Y. enterocolitica* (Fig. 7).

Bacterial pathogens and their individual components have long been considered to be fascinating tools with regard to studying host cell physiology. The use of two pathogens simultaneously takes this one step further. The use of defined mutations in each pathogen allows a molecular dissection of host cell pathways. In this particular instance, we discovered that the *Y. enterocolitica* effectors are dominant over the *S.* Typhimurium effectors with regard to cellular uptake. Additionally, the ability of *Y. enterocolitica* to prevent the uptake of *S.* Typhimurium was found to occur only in particular cell types and to require both T3SS of *Y. enterocolitica*. Surprisingly, the *Y. enterocolitica* effectors could prevent *S.* Typhimurium uptake into epithelial cells but not into J774.1 and RAW264.7cells, while *Y. enterocolitica* could prevent the uptake of *L. monocytogenes* into all cell types tested. The mechanism by which *Y. enterocolitica* blocks its own uptake and the uptake of *L. monocytogenes* into J774.1 and RAW264.7 cells without inhibiting the uptake of *S.* Typhimurium is not known.

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