

Cloning Vectors and Fluorescent Proteins Can Significantly Inhibit *Salmonella enterica* Virulence in Both Epithelial Cells and Macrophages: Implications for Bacterial Pathogenesis Studies

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Plasmid vectors and fluorescent protein reporter systems are commonly used in the study of bacterial pathogenesis. Here we show that they can impair the ability of *Salmonella enterica* serovar Typhimurium to productively infect either cultured mammalian cells or mice. This has significant implications for studies that rely on these systems.

The facultative intracellular pathogen *Salmonella enterica* causes gastroenteritis and systemic infections. Pathogenesis is dependent on the pathogen's ability to survive and/or replicate within host cells and is mediated by two type III secretion systems, encoded on *Salmonella* pathogenicity islands 1 and 2 (SPI1 and -2, respectively), which translocate bacterial effector proteins into host cells (11). Characterization of the function and regulation of these virulence factors is essential to our understanding of *Salmonella* pathogenesis and has been the subject of intensive study. Many of these studies rely on the use of plasmid vectors for complementation analysis of protein function or for monitoring gene expression. In particular, plasmid-borne genes encoding green fluorescent protein (GFP) or related molecules have been used as reporters for gene expression (6, 9, 21, 27, 28) or to localize bacteria inside host cells (7, 13, 17, 18, 32). However, there is an inherent fitness cost associated with maintaining plasmids or high levels of fluorescent proteins, and even plasmids that do not appear to have a metabolic cost under normal laboratory growth conditions may significantly reduce the ability of a bacterial pathogen to adapt to the stress of intracellular life (1, 4).

Salmonella can be internalized into host cells by several different mechanisms (Table 1). Active invasion of nonphagocytic and phagocytic cells occurs via a "trigger"-type process that involves extensive actin rearrangements and plasma membrane ruffles and is mediated by the SPI1-encoded type III secretion system (12). Non-SPI1-induced *Salmonella* is unable to invade nonphagocytic cells but is internalized, albeit relatively inefficiently, into phagocytic cells (26). Opsonization of *Salmonella* with complement or specific antibodies considerably enhances the efficiency of this phagocytic uptake (Table 1).

We hypothesized that the presence of plasmid vectors or the

production of fluorescent proteins could affect the ability of *Salmonella* to establish an intracellular niche and that this might depend on the mechanism of entry. To investigate this possibility, we examined the effect of several plasmids on the ability of *Salmonella* to establish successful interactions with host cells. Five plasmids were selected for comparison using three selection criteria: (i) previous use in complementation studies of *Salmonella*, (ii) low to medium copy number, and (iii) the presence of different selectable markers (Table 2). The plasmids were electroporated into *S. enterica* serovar Typhimurium SL1344 (16) and maintained by the presence of antibiotics. These plasmids had no detectable effect on the growth of serovar Typhimurium in Luria-Bertani-Miller (LB-Miller) broth or on LB plates (data not shown). We compared the effects of these vectors on invasion and intracellular survival/replication in HeLa and RAW 264.7 cells, which have been widely used to study *Salmonella*-host cell interactions. The nonphagocytic epithelial-cell-like HeLa cells (ATCC CCL2) are efficiently invaded by SPI1-induced serovar Typhimurium (25). HeLa cells grown in 24-well plates (5×10^4 cells/well) were infected with a high multiplicity of infection (MOI) (≈ 50 to 100 CFU/cell) for a short time (10 min), after which extracellular bacteria were removed by washing the cells in Hanks balanced salt solution. After a short chase (10 min) in growth media at 37°C, the remaining extracellular bacteria were killed by the addition of gentamicin sulfate (100 μ g/ml for 1 h and then reduced to 10 μ g/ml). Intracellular bacteria were enumerated by solubilizing the cells in lysis buffer (1.0% Triton X-100, 0.1% sodium dodecyl sulfate in phosphate-buffered saline [PBS]) and plating on LB agar plates. Only one plasmid, pACYC184, significantly decreased the invasion efficiency of serovar Typhimurium under these conditions (Fig. 1). This plasmid also reproducibly reduced intracellular replication in HeLa cells, although without statistical significance (Fig. 1B).

Similar results were obtained when phagocytic macrophage-like cells were infected with SPI1-induced serovar Typhi-

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TABLE 1. Methods of internalization used in this study

Method of internalization	Growth phase of <i>Salmonella</i>	Cell type	Efficiency of internalization ^a	Reference
SPI1-mediated invasion	Late log	Phagocytic or nonphagocytic (HeLa or RAW 264.7)	+++	25
Phagocytosis of opsonized bacteria	Stationary	Phagocytic (RAW 264.7)	++	2, 5
Phagocytosis of nonopsonized bacteria	Stationary	Phagocytic (RAW 264.7)	+	2, 10

^a +, low; ++, intermediate; +++, high.

murium. Invasion was carried out as described above, except that RAW 264.7 cells (ATCC TIB-71) were seeded in six-well tissue culture plates (1×10^6 cells/well), the MOI was ≈ 5 to 10 CFU/cell, and monolayers were lysed for bacterial enumeration at 1 h postinfection (p.i.) and 15 h p.i. The pACYC184-associated invasion defect was somewhat enhanced in RAW 264.7 cells compared to HeLa cells (Fig. 1A). Replication was also considerably reduced by the presence of pACYC184 to approximately 30% of that seen for the wild-type infections. We also observed decreased replication in RAW 264.7 cells of bacteria containing two other plasmids, pBR322 and pWSK29 (Fig. 1B), although these plasmids had no detectable effect on bacterial fitness in HeLa cells.

We next investigated whether these plasmids could affect the phagocytic uptake and/or subsequent intracellular replication of noninvasive bacteria (i.e., not SPI1 induced). *Salmonella* was inoculated into 10-ml LB-Miller broth with appropriate antibiotics for 16 to 18 h (stationary phase) and then opsonized by incubation in 14% normal human serum for 30 min or left untreated. Internalization was initiated (MOI of ~ 10 to 20 CFU/cell) by centrifugation at $1,000 \times g$ for 10 min at 25°C. After incubation at 37°C for 15 min, the remaining extracellular bacteria were killed by the addition of gentamicin sulfate. In contrast to what occurred with SPI1-mediated invasion, no plasmid had a detrimental effect on the ability of complement-opsonized bacteria to enter cells (Fig. 2A). However, entry of nonopsonized bacteria was compromised by pBR322 and pWSK29. As for SPI1-induced bacteria, pACYC184 reduced intracellular replication, although this was statistically significant only for nonopsonized bacteria (Fig. 2B).

GFP and its variants have been used as reporters of intracellular bacterial gene expression with some success (27–30), although a recent study proposed that GFP is costly for gastrointestinal bacteria and could affect the ability of *Salmonella*

to interact with host cells (22). Other fluorescent reporter proteins are likely to cause similar problems (24). We compared the effects of GFP (pFPV25.1) and DsRed (pRFP) on the ability of *Salmonella* to invade, and survive within, host cells. These plasmids comprise the same vector backbone, pFPV25, which has a promoterless *gfp* gene and was developed for gene expression analysis with *Salmonella* (27) (Table 2). In pFPV25.1, the promoter region of *rpsM* is added upstream of *gfp*, resulting in the constitutive synthesis of GFP. In pRFP, the DsRed gene simply replaces the *gfp* gene (Table 2). To construct pRFP, primers RFP-SD-For-XbaI (5'-GATTCTAGA TTTAAGAAGGAGATATACATATGAGGTCTTCCAAGA ATG-3') and RFP-Rev-SphI (5'-ACATGCATGCCTAAAGG AACAGATGGTGG-3') were used to amplify the DsRed gene of vector pDsRed (Clontech) with the Expand High Fidelity system (Roche). The forward primer contained a ribosome-binding site identical to that of pFPV25.1 (28). The product was digested by XbaI and SphI (restriction sites are underlined) and cloned in XbaI/SphI-digested plasmid pFPV25.1, thereby replacing the *gfp-mut3* gene. The construct was confirmed by DNA sequencing. Neither pFPV25.1 nor pRFP had any apparent effect on bacterial growth in LB-Miller broth (not shown).

HeLa or RAW 264.7 cells were infected with SPI1-induced bacteria bearing either pFPV25 or one of the fluorescent-protein-producing pFPV25 derivatives, pFPV25.1 or pRFP. We found that the presence of either GFP or red fluorescent protein (RFP) significantly decreased bacterial invasion in both cell lines (Fig. 3). Furthermore, RFP- but not GFP-producing bacteria displayed a severe replication defect in HeLa cells (Fig. 3B). In contrast, fluorescent protein production did not appear to affect entry or replication when non-SPI1-induced bacteria were internalized into RAW 264.7 cells via phagocytosis (data not shown).

Our results suggested that the presence of certain cloning vectors or the production of fluorescent proteins can significantly impair the ability of serovar Typhimurium to invade and survive/replicate within host cells under certain conditions. To investigate whether such defects are also observed in vivo, we carried out competitive index studies with mice (19, 20). Since our in vitro experiments indicated that the pACYC184 vector and GFP and RFP production had the greatest influence on host cell interactions, only bacteria carrying pACYC184, pACYC177, pFPV25, pFPV25.1, or pRFP were tested against wild-type SL1344. Animal protocols were in direct accordance with guidelines drafted by the University of British Columbia's Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. Bacteria were grown to the stationary phase by shaking them overnight at 37°C in 10 ml of LB broth

TABLE 2. Plasmids used in this study

Plasmid	Relevant characteristics	Fluorescent-protein production ^a	Reference
pACYC177	Kan ^r Amp ^r p15A <i>ori</i>	NA	8
pACYC184	Cm ^r Tc ^r p15A <i>ori</i>	NA	8
pBAD30	Amp ^r M13 p15A <i>ori</i>	NA	15
pBR322	Amp ^r Tc ^r pMB1 <i>ori</i>	NA	3
pWSK29	Amp ^r pSC101 <i>ori lacZ</i> α	NA	31
pFPV25	Amp ^r ColE1 <i>ori</i> ; promoterless <i>gfp</i>	None	27
pFPV25.1	pFPV25 ϕ rpsM'- <i>gfpmut3</i>	GFP	27
pRFP	pFPV25 ϕ rpsM'- <i>rfp</i>	RFP	This study

^a NA, not applicable.

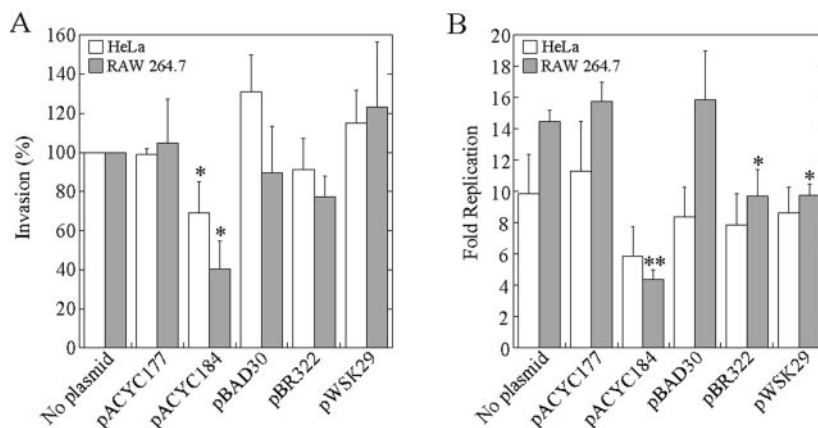


FIG. 1. Effect of plasmids on SPI1-mediated invasion and subsequent replication in epithelial-cell- and macrophage-like cell lines. HeLa cells (white bars) or RAW 264.7 cells (gray bars) were infected with SPI1-induced serovar Typhimurium (late log phase) for 10 min. Cells were lysed and plated on LB agar for enumeration of CFU (intracellular bacteria). (A) Invasion was measured at 1.5 h p.i. for HeLa cells and at 1 h p.i. for RAW 264.7 cells. Invasion of the plasmid-containing strains is shown relative to that of wild-type bacteria (set to 100%). (B) Replication of all strains is shown as the number of CFU at 6 h divided by the number of CFU at 1.5 h (HeLa cells) or the number of CFU at 15 h divided by the number of CFU at 1 h (RAW 264.7 cells). Data are the means \pm standard deviations from at least three separate experiments. Data points that are significantly decreased (analysis of variance and Dunnett's post hoc analysis) compared to those for the wild type are indicated (*, $P < 0.05$; **, $P < 0.01$).

containing the appropriate antibiotics. Plasmid-containing strains were diluted in PBS and mixed with equal numbers of CFU of wild-type SL1344 (no plasmids). Female BALB/c mice (6 to 8 weeks old; Jackson Laboratories) were inoculated with a total of 1×10^5 bacteria in 300 μ l by intraperitoneal injection. Mice were euthanized 48 h postinoculation by cervical dislocation, and the infected spleens were removed and homogenized in PBS. Bacteria were enumerated by serial dilutions onto LB agar containing streptomycin to enumerate total bacteria or onto selective media to enumerate plasmid-containing bacteria. The competitive index was calculated by dividing the ratio of the number of plasmid-carrying bacteria in the output to the number of total bacteria in the spleens (out-

put CFU) by the ratio of the number of plasmid-carrying CFU to the total number of CFU in the inoculum (input CFU). Experiments were repeated at least twice, with a total of 6 to 10 mice being used per group. Neither pACYC177 nor pACYC184 affected the virulence of serovar Typhimurium in the mouse model of infection, as measured by determining the competitive index (data not shown). In contrast, the mean competitive indices for pFPV25.1 and pRFP were 0.45 ($P = 0.0016$) and 0.25 ($P < 0.0001$), respectively, compared to 0.79 for the empty pFPV25 vector, indicating that production of the fluorescent proteins significantly reduced the ability of bacteria to compete against wild-type bacteria during a systemic infection. It remains possible that the pFPV25.1 and pRFP plasmids

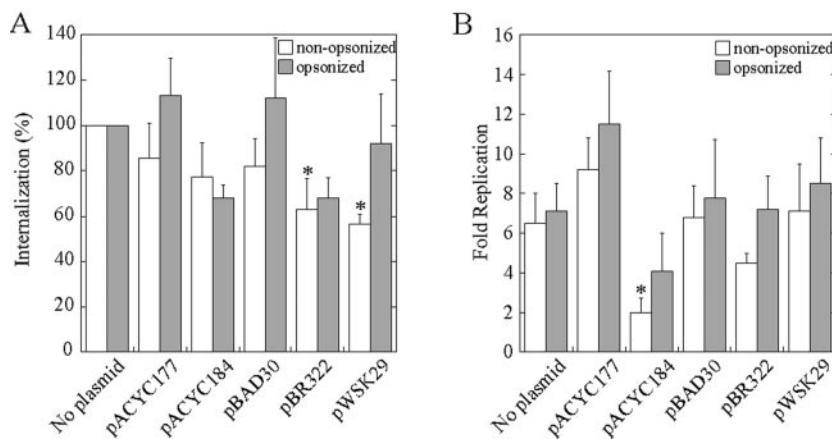


FIG. 2. Effect of plasmids on phagocytic uptake and subsequent replication of serovar Typhimurium in RAW 264.7 cells. Nonopsonized (white bars) or complement-opsonized (gray bars) stationary-phase bacteria were internalized for 10 min with centrifugation, followed by a further 15-min incubation at 37°C. Cells were lysed and plated on LB agar for enumeration of CFU (intracellular bacteria). (A) Bacterial entry was measured at 1 h p.i. Internalization of the plasmid-containing strains is shown relative to that of the wild type (100%). (B) Replication of all strains is shown as the number of CFU at 24 h divided by the number of CFU at 1 h for nonopsonized bacteria or the number of CFU at 20 h divided by the number of CFU at 1 h for opsonized bacteria. Data are the means \pm standard deviations from three separate experiments. Data points that are significantly different ($P < 0.05$) from those for the wild type are indicated (*).

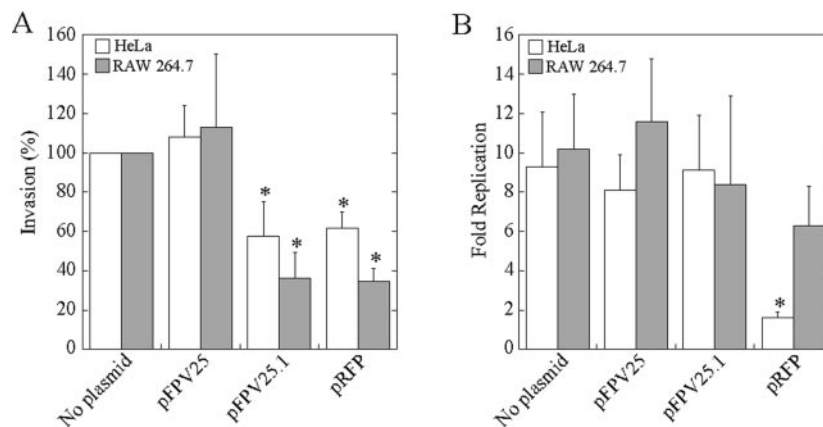


FIG. 3. Effect of fluorescent-protein production on SPII-mediated invasion and subsequent replication in epithelial-cell- and macrophage-like cell lines. HeLa cells (white bars) or RAW 264.7 cells (gray bars) were infected with SPII-induced serovar Typhimurium for 10 min as described in the legend for Fig. 1. Cells were lysed at the indicated times and plated on LB agar for enumeration of CFU (intracellular bacteria). (A) Invasion of the plasmid-containing strains is shown relative to that of the wild type at 1 h p.i. (100%). (B) Replication of all strains is shown as the number of CFU at 6 h divided by the number of CFU at 1.5 h (HeLa cells) or the number of CFU at 15 h divided by the number of CFU at 1 h (RAW 264.7 cells). Data are the means \pm standard deviations from three separate experiments. Data points that are significantly different ($P < 0.05$) from those for the wild type are indicated (*).

were lost during the course of infection due to the absence of antibiotic selection, which would also result in a lower confidence interval, although we consider this unlikely in these experiments (6, 23, 28).

In conclusion, our data demonstrate that the outcome of *Salmonella* infection can be impaired by the presence of plasmids or the production of fluorescent proteins, and the mechanism by which *Salmonella* is internalized into tissue culture cells is a major determining factor. Bacterial fate is more compromised under conditions where bacterial fitness is requisite, i.e., SPII-mediated (bacterium-driven) invasion versus host cell-driven phagocytosis. Three of the plasmids we tested, pACYC184, pACYC177, and pBAD30, have the same origin of replication, yet only pACYC184 significantly impaired the ability of serovar Typhimurium to interact with host cells. The most likely explanation for this is that these plasmids carry different antibiotic resistance markers (Table 2), and indeed the *tet* gene, present in pACYC184, has recently been shown to have deleterious effects on *Salmonella* survival in macrophages (1). In our experiments, the ability of *Salmonella* to colonize a murine host was reduced by fluorescent proteins but not significantly affected by the presence of either pACYC184 or pACYC177. The differences observed between in vitro and in vivo studies presumably reflect the different stresses experienced by bacteria in these infections and highlight the importance of including suitable controls when using plasmids in complementation studies (14). Furthermore, results from in vitro and in vivo experiments that rely exclusively on GFP- and RFP-expressing bacteria should be interpreted with some caution. While our conclusions serve as a cautionary note, these tools remain a powerful asset to the study of bacterium-host cell interactions.

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