GFP plasmid-induced defects in *Salmonella* invasion depend on plasmid architecture, not protein expression

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We have investigated the impact of plasmids and GFP expression on invasion of cultured epithelial cells by *Salmonella enterica* Typhimurium strain SL1344. The invasiveness of SL1344 carrying plasmids derived from pBR322, encoding promoterless GFP or constitutively expressed *rpsM*-GFP, was compared under optimal growth conditions with that of SL1344(pBR322), unmodified SL1344 and a strain with chromosome-integrated *rpsM*-GFP. The strain carrying pBR322 exhibited normal invasion, but the presence of modified plasmids impaired invasiveness, and impairment was exacerbated by plasmid-encoded chloramphenicol resistance (Cm^R). Using a different antibiotic resistance marker, kanamycin (Km^R), did not impair invasiveness. Despite the effect of plasmid-encoded Cm^R, the strain containing chromosomally encoded GFP, also carrying a Cm^R gene, was as invasive as the wild-type. To investigate the mechanism by which plasmid carriage decreases invasion, we monitored SPI-1 gene expression using *prgH* promoter activity as an index of SPI-1 activity. An SL1344 strain with a chromosome-integrated *prgH*::*gfp* reporter construct exhibited lower GFP expression during exponential phase when carrying plasmids incorporating Cm^R or *gfp*, mirroring invasion data. These data provide evidence that suppression of SPI-1 gene expression is a major factor in the loss of invasiveness associated with plasmid carriage. Our findings also indicate that some plasmids, especially those carrying Cm^R, should be used with caution, as virulence traits and gene expression may be affected by their presence. Integration of reporter proteins into the bacterial chromosome, however, appears to circumvent the adverse effects observed with plasmids.

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INTRODUCTION

Salmonella enterica is an important pathogen responsible for one of the most common food-borne diseases in humans. There are a reported 1.3 billion cases of salmonellosis worldwide per year with 3 million fatalities (Hansen-Wester & Hensel, 2001). Thus salmonellosis represents a serious public health and economic burden (Schlumberger & Hardt, 2006).

There is still much to learn about the virulence of *Salmonella* and how it is affected by environment. These pathogens have two specialized type 3 secretion systems that translocate bacterial effector proteins directly into the host cell to manipulate cellular functions for their own benefit. The type 3 secretion systems are encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). SPI-1 is required for invasion and SPI-2 promotes

intracellular survival by modulating the *Salmonella*-containing vacuole and preventing the oxidative killing responses of the host cell (Schlumberger & Hardt, 2006). SPI-1-mediated invasion results in actin rearrangements, allowing cellular engulfment of the bacterium, as a result of activity of several translocated effectors, including SopE, SopE2, SopB, SptP, SipA and SipC (Drecktrah *et al.*, 2006; Raffatellu *et al.*, 2005). These proteins are transported into the target cell via the needle complex, a supramolecular complex composed of SPI-1-encoded proteins, including PrgH, PrgK and InvG, each of which is essential for needle complex formation, effector translocation and SPI-1 stimulated invasion (Kubori *et al.*, 1998).

Infectious disease is a continual burden in both developed and developing countries, hence there is still pressure to increase the depth of understanding of pathogens, including their cellular signalling and host–pathogen interactions. Many molecular techniques have been

Abbreviation: SPI-1, SPI-2, *Salmonella* pathogenicity islands 1 and 2.

deployed to aid visualization of bacteria and monitor gene expression within cells. One of the most important of these is GFP from *Aequoria victoria* that fluoresces without any exogenous cofactors or substrates (Chalfie *et al.*, 1994). The *gfp* gene, most commonly inserted into bacteria on plasmids or alternatively integrated into the chromosome, is used in many studies and has a wide range of applications. Reporter GFP constructs have now been successfully produced, allowing the investigation of bacterial gene expression within particular environments or under specific conditions (Bongaerts *et al.*, 2002; Lee & Keasling, 2006). GFP expression can be studied microscopically, allowing visualization of gene expression within individual bacterial cells, or by the use of flow cytometry to determine the GFP expression level within a population.

Despite widespread use, there are reports describing a fitness cost associated with maintaining plasmids or high levels of GFP expression (Abromaitis *et al.*, 2005; Knodler *et al.*, 2005; Rang *et al.*, 2003; Coulson *et al.*, 1994). In *Salmonella* studies, it has been shown that some plasmids can reduce invasion and/or intracellular survival in both *in vivo* and *in vitro* models (Abromaitis *et al.*, 2005; Coulson *et al.*, 1994; Knodler *et al.*, 2005). Coulson *et al.* (1994) showed that the cloning vector pUC19, derived from the commonly used pBR322, can reduce infection of mice spleens 200-fold compared to wild-type strains. Knodler *et al.* (2005) demonstrated that the pBR322 vector significantly reduces phagocytic uptake in mice, and reduces bacterial replication in RAW 264.7 cells. When plasmid-encoded GFP is expressed, invasion of HeLa and RAW 264.7 cells was also reduced under normal exponential-phase growth, an effect not seen with pBR322 (Knodler *et al.*, 2005). Previous studies have attempted to address the disadvantages associated with plasmid-encoded GFP expression, including plasmid instability, by integrating *gfp* genes with a promoter into the bacterial chromosome (Hautefort *et al.*, 2003). While use of traditional *gfp* genes, namely *GFPmut1* and 2, in single-copy transcriptional fusions produces GFP fluorescence bright enough to report only high levels of promoter activity, Hautefort *et al.* (2003) overcame this problem by the use of the GFP⁺ variant (Scholz *et al.*, 2000). GFP⁺ contains mutations resulting in improved protein folding and enhanced brightness, and with this variant a single-copy *gfp* gene fusion can be stably and reliably expressed within the chromosome, and readily detected even with low promoter activity (Hautefort *et al.*, 2003).

In this study we compared the effect of carrying pBR322 and derivative plasmids containing promoterless *gfp* or the constitutively expressed *rpsM::gfp*, and chromosome-integrated *rpsM::gfp* on *Salmonella enterica* serovar Typhimurium SL1344 under optimal growth conditions. To date, the mechanism by which GFP expression or plasmid presence reduces invasion has not been explored. To address this, we investigated whether plasmid carriage causes a reduction in SPI-1 gene expression using *prgH* promoter activity as a marker for this. Our findings

indicate that some studies using plasmid-encoded proteins should be treated with caution, as both invasion and gene expression were affected by their presence. Integration of reporter proteins into the bacterial chromosome, however, appears to circumvent the adverse effects observed with plasmids and may be the most reliable way to use this genetic technique.

METHODS

Bacterial strains. Details of *Salmonella* strains are given in Table 1. The GFP variant used in these strains was GFP⁺ (Scholz *et al.*, 2000); this contains the GFP_{uv} mutations F99S, M153T and V163A (Cramer *et al.*, 1996) as well as the enhanced GFP mutations F64L and S65T (Cormack *et al.*, 1996). These mutations lead to improved folding of the protein and enhanced brightness.

All bacterial strains were grown routinely in Luria–Bertani broth (LB) and on LB agar plates, with 30 µg chloramphenicol ml⁻¹ or 100 µg carbenicillin ml⁻¹ as appropriate.

Plasmid construction. The promoterless pZEP07 plasmid contains a Cm^R gene flanked by two *EcoRV* sites (Hautefort *et al.*, 2003). Thus the Cm^R gene was removed to produce pZEP07.1 via digestion with *EcoRV*. To produce pBR322.1, the primers Chloro_F (5'-GCATGAA-TTCCGTCATTCTGCCATTCATCC-3') and Chloro_R (5'-GCA-TGAATTCGGGCTAGCCGGCCCGACGC-3') were used to amplify the Cm^R gene with pZEP07 as a template. A Km^R gene was added to pBR322 to produce pBR322.2. The kanamycin cassette was amplified from pKD4 using Kan_F (5'-CATCAGCTGAATTCCACCAAGCGAA-CCGGAATTGCCAGC-3') and Kan_R (5'-GTCGCCATGAATTCA-CGCTCAGAAGAAGCTCGTCAAGAAGG-3'). These PCR cassettes were cloned into the *EcoRI* site of pBR322. pZEP07.1, pBR322.1 and pBR322.2 were isolated from DH5α cells and electroporated into SL1344.

Restriction digests, DNA ligations, bacterial electroporations, transformations and PCR amplifications were completed using standard conditions and according to standard protocols.

Curing strains of plasmids. Overnight cultures of pZEP16 and pZEP07 strains were prepared with no antibiotic, plated onto LB agar with no antibiotic and grown for 16 h at 37 °C. Individual colonies were selected and streaked simultaneously onto two LB agar plates, one of which contained no antibiotic and one which contained 30 µg chloramphenicol ml⁻¹. Only isolates able to grow on LB with no antibiotic were selected.

Cell culture and *Salmonella* infection. Madin–Darby canine kidney (MDCK) strain I cells were grown in Eagle's minimal essential medium supplemented with 2 mM L-glutamine, 10% fetal calf serum, 1% non-essential amino acids and 100 µg kanamycin ml⁻¹ at 37 °C in a humidified atmosphere of 5% CO₂. Cells at a concentration of 6.6 × 10⁴ were seeded onto 13 mm glass coverslips and grown for 2–3 days. For infection, the medium was replaced with 1 ml Krebs buffer (mM: NaCl, 137; KCl, 5.4; MgSO₄, 1; NaH₂PO₄, 0.3; CaCl₂, 2.4; glucose, 10; Tris/HCl, 10; adjusted to pH 7.4 at 37 °C with HCl), and 50 µl *Salmonella* culture was added and allowed to infect the cells at 37 °C for 15 min.

Infection using exponential-phase *Salmonella* cells. Overnight *Salmonella* cultures were prepared by inoculating colonies into 10 ml LB medium (with 30 µg chloramphenicol ml⁻¹ for plasmid- and GFP-expressing strains) and incubating for 16 h at 37 °C. Dilutions (1:100) were prepared in fresh LB medium and incubated at 37 °C

Table 1. Strains and plasmids used in this study

Strain/plasmid	Selectable marker	GFP promoter	Reference
Strains			
SL1344 WT	–	No GFP	Hoiseth & Stocker (1981)
SL1344 <i>rpsM::gfp</i>	Cm ^R	<i>rpsM</i> (constitutive)	Hautefort <i>et al.</i> (2003)
SL1344 <i>prgH::gfp</i>	Cm ^R	<i>prgH</i> (SPI-1 reporter)	Hautefort <i>et al.</i> (2003)
Plasmids			
pZEP07	Cm ^R Cb ^R	Promoterless	Hautefort <i>et al.</i> (2003)
pZEP16	Cm ^R	<i>rpsM</i> (constitutive)	Hautefort <i>et al.</i> (2003)
pBR322	Cb ^R Tc ^R	No GFP	This study
pZEP07.1	Cb ^R	Promoterless	This study
pBR322.1	Cm ^R Cb ^R Tc ^R	No GFP	This study
pBR322.2	Km ^R Cb ^R Tc ^R	No GFP	This study

with shaking at 100 r.p.m. for 3.5 h in order to reach exponential-phase growth. MDCK cells were infected at a mean m.o.i. of 50.

Differential staining of adhered and invaded *Salmonella*.

Following infection, MDCK cells on coverslips were washed thoroughly in PBS to remove non-adhered bacteria, and fixed in 2 % paraformaldehyde (in PBS) at 4 °C for 45 min. The coverslips were then labelled to localize adhered and invaded *Salmonella* as described previously (Perrett & Jepson, 2007). Briefly, this involved incubation with goat anti-*Salmonella* antibody (CSA-1 antibody; Kirkegaard and Perry Laboratories) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat immunoglobulin (Sigma) to label extracellular bacteria. Following permeabilization in 0.1 % Triton X-100, all cell-associated *Salmonella* were labelled by repeating the incubation with goat anti-*Salmonella* antibodies followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-goat immunoglobulin (Sigma). Cells were then washed in PBS and mounted in Vectashield (Vector laboratories) containing DAPI. Counts of MDCK cells (enabled by DAPI labelling of nuclei), and of adhered and total bacteria were made using a Leica DM LB2 fluorescence microscope. The numbers of invaded bacteria per cell were calculated from these figures. Ten random fields were examined on each coverslip, comprising a total of at least 200 MDCK cells.

Comparison of *prgH* expression. Strains containing the chromosomal *prgH::gfp* construct, with and without various plasmids, were grown to exponential phase and examined microscopically with a haemocytometer and a Leica DM LB2 microscope using a ×63 phase-contrast objective. The number of green-fluorescent bacteria, hence those with detectable *prgH* promoter activity, were counted and compared to the total number of bacteria; this was used to calculate percentage *prgH* expression. Phase-contrast and fluorescence images were acquired on a Leica DMIRB inverted microscope with a ×100 objective using a Hamamatsu ORCA-ER CCD camera and Openlab 4 software (Improvision).

Measurement of bacterial growth. Overnight cultures were prepared via inoculation of a single colony into 2 ml LB which was incubated statically at 37 °C. Volumes (10 µl) of these cultures were diluted into 990 µl LB broth. The diluted cultures were grown in 200 µl volumes in a 100-well honeycomb plate at 37 °C for 48 h using a Bioscreen C automatic turbidometric analyser (Thermo Electron Corp.). Optical density was measured every hour after shaking.

Statistical analyses. All data are expressed as means ± SD. Significance of difference between mean values was assessed using two-tailed unpaired Student's *t* tests with significance set at $P \leq 0.05$.

RESULTS

Salmonella invasion is decreased by GFP-expressing and -non-expressing plasmids

To compare the impact of plasmid and/or GFP expression on *Salmonella* invasion we compiled a set of strains derived from wild-type *S. Typhimurium* SL1344 (Table 1) modified by the introduction of plasmids pBR322, pZEP07 (promoterless, non-expressing GFP) or pZEP16 (*rpsM::gfp*, constitutively expressing GFP), or by chromosomal insertion of an identical *rpsM::gfp* construct. The effect of carrying a plasmid and/or GFP expression was investigated in *Salmonella* cells that were grown to exponential phase in LB medium and allowed to infect MDCK cells for 15 min. Invasion was measured by differential immunolabelling of adhered and invaded bacteria. The chromosomal *rpsM::gfp* and pBR322-carrying strains both exhibited the same level of invasion as the wild-type (Fig. 1). However, the strains carrying plasmids *rpsM::gfp* and promoterless *gfp* both exhibited similarly attenuated invasion compared to wild-type and the chromosomal *rpsM::gfp* strain ($P < 0.01$). Thus, the plasmid-induced invasion defect is not due to GFP expression.

To ensure the observed difference in invasion was a result of the presence of the plasmid, the SL1344(pZEP16) and SL1344(pZEP07) strains were cured of their plasmids. The cured strains were grown to exponential phase and used to infect MDCK cells for 15 min; differential immunolabelling was used to determine invasion as above. The cured plasmid strains both exhibited invasion levels which were indistinguishable from that of the wild-type strain, and were significantly ($P < 0.01$) higher than the non-cured strains (data not shown).

Growth in chloramphenicol-containing medium has no effect on invasion

The finding that both the modified plasmids pZEP07 and pZEP16, but not their parent plasmid pBR322, decreased

Fig. 1. Comparison of invasion of GFP-expressing and plasmid-carrying strains. *Salmonella* cultures were grown at 37 °C with shaking at 100 r.p.m. for 3.5 h. A volume (50 µl) of each culture was used to infect MDCK cells grown on coverslips resting in 1 ml Krebs buffer. Differential immunolabelling was used to determine invasion. Data are means $\pm$ SD of three independent experiments. * indicates significant difference from wild-type and chromosomal *rpsM::gfp* at $P < 0.01$.

SL1344 invasion led us to investigate why the modified plasmids reduce invasion. Since the overnight cultures of SL1344(pZEP07) and SL1344(pZEP16) were grown in LB containing chloramphenicol, and it is documented that chloramphenicol can have a negative effect upon bacterial physiology (Steel *et al.*, 2004), we investigated if the presence of this antibiotic in LB medium was the cause of the observed decrease in invasion. pZEP07 encodes two resistance genes, chloramphenicol and carbenicillin. Therefore, three overnight cultures were prepared in triplicate; an SL1344 culture containing no antibiotics, and two SL1344(pZEP07) cultures, one of which contained chloramphenicol and the other carbenicillin. The overnight cultures were subcultured, grown to exponential phase in LB medium and used to infect MDCK cells for 15 min. Both SL1344(pZEP07) cultures grown in either chloramphenicol or carbenicillin showed the same impaired invasion compared to the wild-type. Following growth in

carbenicillin, the level of invasion of SL1344(pZEP07) was 0.31 ± 0.05 bacteria per cell compared with 0.34 ± 0.04 following growth in chloramphenicol. This was significantly reduced ($P < 0.01$) compared to the wild-type invasion level of 1.16 ± 0.08 bacteria per cell. Thus medium containing chloramphenicol had no effect on invasion of the plasmid-bearing strain, confirming data with the chromosomal GFP strain which exhibited invasion levels the same as wild-type, despite being grown in medium containing chloramphenicol (Fig. 1).

Salmonella invasion is decreased by modified plasmids

Having discounted growth in chloramphenicol as a significant factor in the decreased invasion of SL1344 carrying the GFP plasmids (pZEP07 and pZEP16) compared with SL1344(pBR322), we next investigated if invasion was affected by the presence of multiple copies of the gene conferring Cm^R . The Cm^R gene was removed from pZEP07 to create pZEP07.1, and for the previously chloramphenicol-sensitive pBR322, the Cm^R gene was added to create pBR322.1. Invasion of bacterial strains carrying these plasmids was compared to a strain carrying pBR322.2 with a Km^R gene under optimal conditions by infecting MDCK cells for 15 min and differentially immunolabelling adhered and invaded bacteria. As before, SL1344(pZEP07) exhibited attenuated invasion ($P < 0.01$) compared to wild-type and the chromosomal *rpsM::gfp* strain (Fig. 2). SL1344(pZEP07.1), lacking Cm^R , was more invasive ($P < 0.05$) than SL1344(pZEP07); however, it did not reach wild-type levels of invasiveness (Fig. 2). Similarly, SL1344(pBR322.1), which contained Cm^R , was less invasive ($P < 0.05$) than SL1344(pBR322), but remained more invasive than SL1344(pZEP07). In contrast, SL1344(pBR322.2) containing Km^R had invasion levels comparable to wild-type ($P < 0.01$) (Fig. 2). All cultures exhibited indistinguishable growth characteristics when compared by Bioscreen (data not shown), indicating that there was no prominent metabolic effect of plasmid carriage, at least under the conditions used for invasion assays.

Fig. 2. The effect of plasmid modification on invasion. *Salmonella* cultures were grown overnight in LB medium with the appropriate antibiotic, subcultured and grown to exponential phase. A volume (50 µl) of each culture was used to infect MDCK cells grown on coverslips, and differential immunolabelling was used to determine invasion. Data are means $\pm$ SD of three independent experiments.

prgH expression is suppressed in plasmid-bearing strains

We hypothesized that the plasmid-induced reduction in invasion may be related to reduced SPI-1 expression. Expression of *prgH* has previously been used as a marker of SPI-1 activity (Hautefort *et al.*, 2003) and was visualized here using a bacterial strain with a chromosomally integrated *prgH::gfp* construct. Comparisons of *prgH* expression were made between this strain and ones derived from the same parental *prgH::gfp* strain containing pBR322 and the various modified plasmids. When exponential-phase cultures were examined microscopically, it was seen that strains carrying pBR322 and pBR322.2 showed the same level of *prgH::gfp* expression as the wild-type culture. However, the pZEP07-carrying strain showed a significant drop in *prgH::gfp* expression compared to the culture lacking the plasmid ($P<0.05$) (Figs 3 and 4). The strains containing pZEP07.1 (without Cm^R) and pBR322.1 (with Cm^R) also showed a drop in *prgH::gfp* expression compared to wild-type ($P<0.05$), but both had a significantly higher

expression level than the SL1344(pZEP07) strain ($P<0.05$) (Figs 3 and 4). The close correlation between the effects of plasmids on expression of the SPI-1 transcriptional reporter *prgH* and on invasiveness (Figs 2 and 4) provides compelling evidence that suppression of SPI-1 gene expression is a major contributory factor in reduced *Salmonella* invasion seen under these conditions.

DISCUSSION

It was observed that invasion of MDCK cells by exponential-phase *Salmonella* SL1344 was reduced when carrying plasmids constitutively expressing GFP (pZEP16) or containing, but not expressing the *gfp* gene (pZEP07). However, SL1344 carrying the chromosomal *rpsM::gfp* or unmodified pBR322 were as invasive as the wild-type. Previous reports on the effects of cloning vectors on bacterial virulence are equivocal; Coulson *et al.* (1994) and Abromaitis *et al.* (2005) found reduced virulence with derivatives of the popular cloning vector pBR322, while

Fig. 3. *prgH::gfp* expression in the *prgH::gfp*-expressing strain SL1344 with no plasmid and the equivalent strain with the indicated plasmids during optimal growth. Expression of *prgH::gfp* was reduced by plasmids derived from pBR322 and including either *gfp* or Cm^R , but not with the plasmid including Km^R (pBR322.2). Each panel includes phase-contrast (monochrome), GFP fluorescence and merged images (with GFP in green). Field of view: $90\times 68\ \mu\text{m}$.

Fig. 4. *PrgH* expression in exponential-phase bacteria. Overnight *Salmonella* cultures carrying chromosomal *prgH::gfp* with and without the indicated plasmids were grown in LB medium for 3.5 h. The proportion of bacteria expressing detectable GFP was counted microscopically. Data are means $\pm$ SD of four independent experiments.

Knodler *et al.* (2005) reported that the invasion of HeLa cells and macrophages was unaffected by the presence of pBR322, but reduction in replication within macrophages was observed. Since two pBR322-derived plasmids (pZEP07 and pZEP16), differing in their expression of GFP, both had a pronounced negative impact on invasiveness, supported by other studies using pBR322 derivatives (Abromaitis *et al.*, 2005; Coulson *et al.*, 1994), we conclude that any effect was due to the burden or nature of the extra DNA rather than GFP expression per se. It is known that chloramphenicol treatment can lead to bacterial alterations, including the formation of filamentous cells (Steel *et al.*, 2004); hence we considered the possibility that invasion defects were due to growth in chloramphenicol. Following growth in medium containing chloramphenicol or carbenicillin, the pZEP07 strain showed a similar decrease in invasion, verifying data provided by the chromosomal *gfp* strain, which exhibited invasion levels the same as wild-type despite chloramphenicol treatment. Therefore, it was deduced that the presence of chloramphenicol in the growth medium itself had no detrimental effect on invasion. Subsequently, the possibility of overexpression of Cm^R due to plasmid copy number was investigated. The results from the invasion assay, involving pZEP07.1 (without Cm^R), pBR322.1 (with Cm^R) and pBR322.2 (with Km^R), suggest that the carriage of Cm^R does indeed lower the level of invasion. However, the invasion level of pZEP07.1 does not reach wild-type levels, and similarly pBR322.1 invasion was higher than that of SL1344 carrying either pZEP07 or pZEP16. Therefore, although Cm^R has a significant negative effect on invasion, it may not be the sole cause of the invasion defects observed in this study. It is possible that the increased size of these plasmids when compared to the various derivatives of pBR322 creates a larger burden on the bacteria, leading to invasion reduction without affecting bacterial growth. In contrast, the single copy of Cm^R in the chromosomal *rpsM::gfp* had no detectable effect on invasion. This finding has important implications for many studies that use such plasmids either to label cells or to investigate the function of specific bacterial proteins using plasmid complementation. The increase in DNA content or

inclusion of Cm^R in the plasmid could affect normal function in such a way that the importance of the protein of interest may be wrongly interpreted. On the other hand, we found that GFP expression itself has minimal, if any, effect on *Salmonella* invasiveness during optimal growth; the reduction in invasion being exclusively due to presence of the Cm^R and the *gfp* gene, regardless of GFP expression. The observation that chromosomally encoded GFP had no effect on invasion is of relevance to vaccine development where use of plasmids to express heterologous proteins raises issues over their safety and the need for antibiotic selection to retain plasmids. Our finding that stable integration of *gfp* into the chromosome led to efficient protein synthesis without appearing to affect invasiveness supports the use of similar strategies for generation of vaccine strains by chromosomal integration of heterologous proteins (McKelvie *et al.*, 2004).

GFP expression from a chromosomally inserted gene under control of the *prgH* promoter has previously been used as a reporter of expression of the SPI-1 invasion machinery (Hautefort *et al.*, 2003). It has been deduced that *prgH* expression is regulated in an all-or-nothing manner, meaning that individual cells within the population either fully express *prgH* or do not express it at all (Hautefort *et al.*, 2003; Temme *et al.*, 2008), enabling accurate determination of *prgH* status at the single-cell level by microscopical analysis. We have confirmed the utility of this reporter system and, furthermore, have shown that reduced *prgH* expression is closely correlated with invasiveness in plasmid-carrying strains. Thus, *prgH::gfp* expression was similar in wild-type SL1344, SL1344(pBR322) and SL1344(pBR322.2) strains, but reduced in strains carrying other modified plasmids, with repression being greater in SL1344 carrying pZEP07 than pZEP07.1 or pBR322.1. Thus *prgH::gfp* expression was closely correlated with invasiveness of all plasmid-bearing and wild-type strains. Taken together these data provide further validation of the use of chromosomal expression of a *prgH::gfp* fusion to monitor SPI-1 activity, since expression of GFP from this insertion correlated well with invasiveness in optimal conditions and when invasiveness was compromised by the presence of plasmid.

In summary, our data suggest that the presence of modified plasmids can place a significant burden on cells, hindering energy-expensive bacterial functions such as invasion; this effect is exacerbated by the presence of the Cm^R gene. Serious implications for studies using plasmid complementation are thus inferred; those carrying out such studies should be wary of the possibility of creating biased results. GFP is a valuable molecular biology tool and may be used with plasmid expression, although our data suggest that use of even low copy plasmids should be treated with caution because of possible adverse effects of the plasmid itself. Although we observed no additional effect of GFP expression itself under optimal growth conditions, the possibility remains that, as suggested by Knodler *et al.* (2005), GFP expression may provide an additional burden on bacteria that can modify their behaviour under some conditions. Although more labour intensive to produce, chromosomally encoded GFP or other proteins of interest appears to be the most reliable option, since we found no effect on invasion or stress responses, despite readily detectable GFP expression levels.

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