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Cumulative effect of prophage burden on Shiga toxin production in *Escherichia coli*

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Shigatoxigenic *Escherichia coli* (STEC) such as *E. coli* O157 are significant human pathogens, capable of producing severe, systemic disease outcomes. The more serious symptoms associated with STEC infection are primarily the result of Shiga toxin (Stx) production, directed by converting Stx bacteriophages. During phage-mediated replication and host cell lysis, the toxins are released en masse from the bacterial cells, and the severity of disease is linked inexorably to toxin load. It is common for a single bacterial host to harbour more than one heterogeneous Stx prophage, and it has also been recently proven that multiple isogenic prophage copies can exist in a single cell, contrary to the lambda immunity model. It is possible that in these multiple lysogens there is an increased potential for production of Stx. This study investigated the expression profiles of single and double isogenic lysogens of Stx phage $\phi 24_B$ using quantitative PCR to examine transcription levels, and a reporter gene construct as a proxy for the translation levels of *stx* transcripts. Toxin gene expression in double lysogens was in excess of the single lysogen counterpart, both in the prophage state and after induction of the lytic life cycle. In addition, double lysogens were found to be more sensitive to an increased induction stimulus than single lysogens, suggesting that maintenance of a stable prophage is less likely when multiple phage genome copies are present. Overall, these data demonstrate that the phenomenon of multiple lysogeny in STEC has the potential to impact upon disease pathology through increased toxin load.

INTRODUCTION

Shigatoxigenic *Escherichia coli* (STEC) are important food-borne pathogens responsible for a range of disease manifestations, varying from mild diarrhoea to fatal renal and central nervous system complications (Allison, 2007; Gyles, 2007). The more severe disease outcomes are caused by a subset of STEC termed enterohaemorrhagic *E. coli* (EHEC), which includes the O157 : H7 serogroup (O'Loughlin & Robins-Browne, 2001; Riley *et al.*, 1983). EHEC possess an array of virulence factors that enhance survival and invasion in the gut, but the principal factor associated with the most severe sequelae, haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP), is the production of Shiga toxin (Stx) (Kaper *et al.*, 2004; Spears *et al.*, 2006). It has recently been proposed that Stx overexpression in *E. coli* O157 : H7 strains is associated with severe human disease (Neupane *et al.*, 2011). Stx is encoded and disseminated by converting lambdoid bacteriophages, the Stx phages, which in addition to enhancing the pathogenic profile of virulent *E. coli* strains, can transform gut commensal members of the *Enterobacteriaceae* into toxin producers, with additional implications for disease progression (Allison, 2007; Gamage *et al.*, 2006; Herold *et al.*, 2004; O'Brien *et al.*, 1984).

Stx is an AB_5 holotoxin, structurally similar to cholera toxin, with broad systemic disease implications (O'Loughlin & Robins-Browne, 2001). The direct impact of Stx has been established, and its presence undoubtedly increases severe disease outcomes (Ashkenazi *et al.*, 1990). Bacteriophage-encoded Stxs are usually co-transcribed with the phage late genes, which include many of the components required for assembly, packaging and release of infective phage particles from the host cell (Brüssow *et al.*, 2004; Herold *et al.*, 2005; Neely & Friedman, 1998; Wagner *et al.*, 2001). A secondary mechanism for toxin production has been described, where expression from an additional promoter (pStx), located between pR′ and the toxin genes, can be initiated under conditions of iron depletion (Sung *et al.*, 1990). However, significant production and release of the toxin is still dependent upon

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Abbreviations: EHEC, enterohaemorrhagic *E. coli*; HUS, haemolytic uraemic syndrome; STEC, Shigatoxigenic *E. coli*; Stx, Shiga toxin.

pR′ and the phage-mediated lytic cascade (Wagner *et al.*, 2001, 2002). In a lysogen culture, Stx can be found at detectable levels in the medium, but this is primarily due to the relatively high level of background spontaneous lytic pathway induction (Shimizu *et al.*, 2009; Wagner *et al.*, 2002). Secretion of Stx by uninduced lysogen cells undoubtedly occurs (Shimizu *et al.*, 2007; Weinstein *et al.*, 1989), but is limited and therefore of much less importance.

All known Stx phages are deemed lambdoid because their general genetic organization is that described for bacteriophage lambda (Ptashne, 2004). These similarities include the expression of an orthologous global repressor protein, CI, that is expected to effectively shut off all gene expression from the integrated prophage and provide immunity to superinfection from incoming homoimmune phages. The assumption that Stx phages conform to this immunity model was confounded when multiple lysogens of a differentially labelled, isogenic lambdoid Stx2-encoding phage ($\phi 24_B$) were created (Allison *et al.*, 2003). Furthermore, it was proved that at least three isogenic prophages could be harboured simultaneously within a single host at distinct integration sites (Fogg *et al.*, 2007), an eventuality not reproducible with bacteriophage lambda (Fogg *et al.*, 2010). Significantly, the rate at which these multiple infections occur is extremely high, increasing in frequency with each successive superinfection (Fogg *et al.*, 2007, 2011). Subsequently, several additional Stx phages have also been shown to circumvent this superinfection immunity system (Serra-Moreno *et al.*, 2008). Clearly this phenomenon is not an isolated event, and it has implications not only for the evolution of lysogen virulence and Stx phage diversity through intracellular recombination events in a multiply infected cell (Botstein, 1980; Brüssow *et al.*, 2004), but also for enhanced pathogenicity due to the presence of multiple copies of the *stx* gene in a single cell. However, a direct relationship between gene dosage and Stx toxin expression and production has yet to be established, and there is even some evidence that a multiple prophage burden actually inhibits Stx expression (Serra-Moreno *et al.*, 2008). The question that underpins this study is whether the level of Stx prophage burden actually alters the levels of Stx expression. There are two distinct possibilities. The first is that increased gene copy leads to increased protein production, and there are several reports in the literature that point towards this being the case for Stx production (Hayashi *et al.*, 2001; Muniesa *et al.*, 2003; Perna *et al.*, 2001; Wagner *et al.*, 1999). The second possibility is that gene expression levels of phage late genes are so great that the resources within an infected cell are not sufficient and are simply overwhelmed upon prophage induction. Thus, the protein made under these circumstances utilizes all the lysogen's resources and is not necessarily related to transcription levels or, possibly, the lysogen does not support the directed levels of transcription driven by multiple gene copies.

During lysogeny, substantial transcription from the pR′ promoter is inhibited by the presence of downstream termination sites (Fig. 1a). However, during the phage lytic replication cycle or upon prophage induction, an anti-terminator (*Q*) is produced that modifies the transcription complex initiated at pR′, enabling RNA polymerase to traverse the termination sites (Mühldorfer *et al.*, 1996; Plunkett *et al.*, 1999; Tyler *et al.*, 2004). In this study, we used a plasmid-based luminescence construct designed to exploit this transcriptional regulatory system (Fogg *et al.*, 2011). Quantitative PCR was used to measure toxin gene expression (transcription), and luminescence to determine protein production (translation), in isogenic single and double lysogen cultures of the Stx2-encoding phage $\Phi 24_B$ in the presence and absence of the SOS response-inducing agent norfloxacin. Transcription and translation levels were

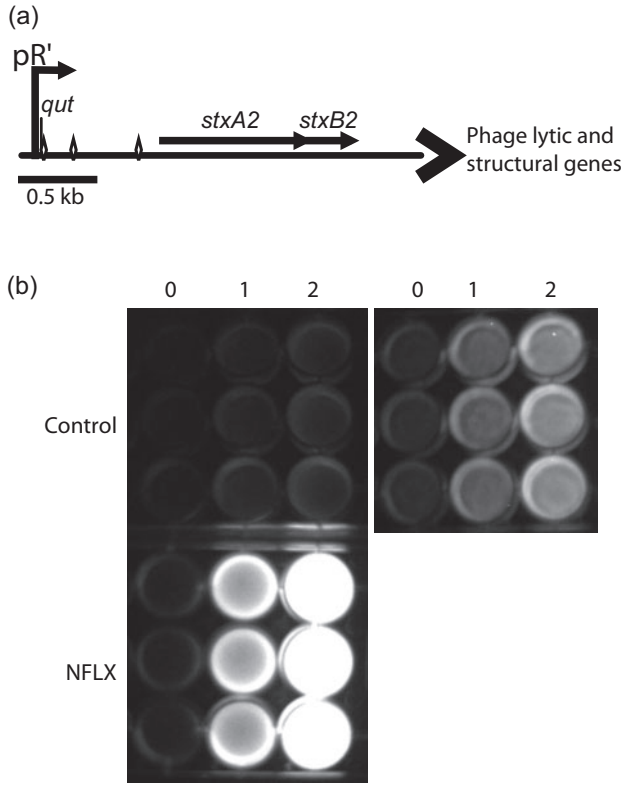

Fig. 1. Luminescence reporter gene construct. (a) Schematic of the location and transcription regulatory elements of the $\phi 24_B$ *stx2* genes. The pR′ promoter, which drives *stx* expression *in vivo*, is marked on the map immediately followed by three white diamonds representing rho-independent termination sites. These termination sites prevent significant expression of downstream genes in the absence of the Q anti-terminator protein, which acts at the marked Q utilization site (*qut*). The location and direction of the *stxA2* and *B2* subunit genes are indicated by annotated arrows. (b) A long-exposure luminescence image of the negative control (0), single lysogen (1) and double lysogen (2) cultures, either induced by addition of norfloxacin (NFLX; lower three rows) or without induction (upper three rows, Control). A longer exposure for the uninduced controls was included to allow differences in luminescence to be discerned (upper three rows, right panel, Control).

specifically uncoupled from each other in order to better explain some of the conflicting literature that has examined Stx expression levels (Neupane *et al.*, 2011; Serra-Moreno *et al.*, 2008; Wagner *et al.*, 1999).

METHODS

Luminescence reporter strains. The pStxCDF luminescence construct (Fig. 1a) was created as described previously (Fogg *et al.*, 2011), except that pCDF-Duet (Novagen) was used in place of pACYC. pStxCDF was transformed into competent MC1061 $\Phi24_B$::Kan lysogen cells (Allison *et al.*, 2003) and naïve *E. coli* MC1061 (James *et al.*, 2001) control cells. Single $\Phi24_B$ lysogens are preferentially integrated at a single location in *E. coli* MC1061, 5′ of *intS*, and the strain used here has been shown by Southern hybridization to harbour only one prophage (Fogg *et al.*, 2007). Transformants were selected on agar plates containing kanamycin (50 μg ml^{-1}) and spectinomycin (100 μg ml^{-1}). Double lysogens were created by infecting the above-mentioned single lysogen carrying pStxCDF (OD_{600} 0.5) with the isogenic bacteriophage $\phi24_B$::Cat at an m.o.i. of 0.1, as previously described (Allison *et al.*, 2003).

Induction protocol. Lux assays were carried out as previously described (Fogg *et al.*, 2011). Briefly, total protein was extracted from 1 ml samples by the method detailed in Görg *et al.* (2006) and was quantified using the Pierce bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). For experiments including a recovery period following norfloxacin treatment, each culture was diluted 1 : 10 after an initial exposure to norfloxacin for 1 h. Specific luminescence was calculated by dividing the luminescence readings by the corresponding total protein quantities, or OD_{600} values, for each sample. In addition, images were taken of single and double lysogen cultures plus a naïve negative control under inducing and non-inducing conditions (Fig. 1b). The cultures were treated as described above, with no recovery period. Three hours after addition of norfloxacin to the induced cultures, 1 ml samples were taken from each culture/condition and transferred to flat-bottomed, 12-well tissue culture plates. Bioluminescence was recorded following a 5 min exposure time using an ORCA II-BT-1024 16-bit camera cooled to −80 °C (Hamamatsu Photonics).

Effect of norfloxacin concentration. The above protocol for induction was modified to include final norfloxacin concentrations of 1, 2 and 3 μg ml^{-1}. Luminescence and OD_{600} measurements for both single and double lysogen cultures were made as described above. Specific luminescence was obtained by division of luminescence values by the OD_{600}, and these specific values were normalized with respect to the optimal induction concentration of 1 μg ml^{-1} (Matsushiro *et al.*, 1999; Piddock & Wise, 1987).

Quantitative PCR. Control and induced cultures were harvested 3 h following the 1 h norfloxacin treatment, in experiments with and without a recovery period. A recovery period is defined as the dilution of norfloxacin-treated cells (1 : 10) in fresh medium after 1 h to allow the host to recover from the norfloxacin induction treatment (1 μg ml^{-1} is greater than the MIC for *E. coli*), maximizing phage production. All induced cultures were treated with 1 μg norfloxacin ml^{-1}, except for the dose–response experiment, in which 1 and 2 μg ml^{-1} were used, as indicated. Preparation of samples and quantitative PCR were carried out as described previously (Fogg *et al.*, 2011). Transcripts (*stx* and *Q*) were amplified with primers *stx* qPCR F/R (5′-ATATCTGCGCCGGGTCTGG-3′ and 5′-CCAGTAACAGGCAC-AGTACC-3′) and *Q* qPCR F/R (Fogg *et al.*, 2010), respectively, and an endogenous 16S rRNA reference gene was amplified using *16S* qPCR F/R primers (Fogg *et al.*, 2010). Expression from test strains was quantified by the $2^{-\Delta\Delta C_t}$ relative abundance method, normalized to 16S rRNA gene expression and relative to the uninduced single lysogen control (Livak & Schmittgen, 2001).

RESULTS

Toxin expression levels from uninduced single and double lysogen cultures

A plasmid-based luminescence construct (Fogg *et al.*, 2011) was used to compare expression levels in single and double $\Phi24_B$ lysogen cultures of the *E. coli* K-12 strain MC1061. In the absence of any stimulus (i.e. norfloxacin) to induce the phage lytic cycle, low-level luminescence was clearly visible from both single and double lysogen cultures using long-exposure photography (Fig. 1b). Quantification of the luminescence revealed an approximate twofold increase in specific luminescence for the double lysogen compared with the single lysogen (Fig. 2). Relative quantitative PCR was carried out to determine whether these luminescence data (protein produced) correlated with transcript levels (gene expression levels). Abundance of both *stx* and *Q* mRNA was three- to fourfold higher in the uninduced double lysogen cultures than in the single lysogen control, indicating that transcriptional increases are not precisely reflected in translational output. However, the general trend of a moderate increase in toxin gene expression in double lysogens relative to single lysogens was apparent in the data for both transcript and protein levels. This level of disconnection was not unexpected, as there are many factors, such as RNA degradation rates and ribosome availability, that moderate the efficiency with which mRNA is translated to protein.

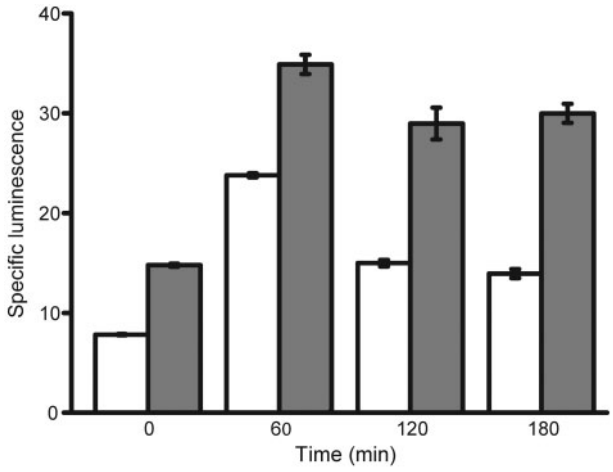

Fig. 2. Luminescence and promoter activity in uninduced lysogens. Luminescence emission from the pR′–*lux* fusion harboured in uninduced single (white bars) and double lysogen (grey bars) cultures. Specific luminescence was calculated per microgram of total protein in 1 ml of each sample.

Toxin expression levels from single and double lysogen cultures subjected to the inducing agent norfloxacin

Addition of the antibiotic norfloxacin induces lambdoid bacteriophages into the lytic life cycle (Matsushiro *et al.*, 1999; Walterspiel *et al.*, 1992) by activation of the RecA-mediated SOS response (Heddle *et al.*, 2000). RecA is then responsible for directing phage derepression via rapid autocleavage of the lambdoid repressor protein CI (Craig & Roberts, 1980). Under inducing conditions (1 μg norfloxacin ml^{-1}), there was the expected large increase in the levels of luminescence produced by both the single and double lysogens (Fig. 1b). Quantification of luminescence with respect to incubation time demonstrated that bacteriophage induction leading to expression from pR′ exhibited a lag period of 60–90 min following addition of norfloxacin, followed by a dramatic increase in luminescence at 120 min (Fig. 3). At this point, double lysogen pR′-driven expression achieved a 2.49-fold (SD 0.17-fold) increase in expression over the single lysogen, and by 180 min this difference had closed slightly to a 2.37-fold (SD 0.10-fold) increase in luminescence. Transcript level quantification of abundance revealed that there was a marked 2.11-fold increase in the number of *stx* transcripts in double lysogens compared with single lysogens (Fig. 4a). *Q* transcript levels were also in accordance with the trend observed for *stx*, i.e. a doubling, as would be predicted for a regulator of the pR′ promoter (Fig. 4b). Furthermore, levels of both *Q* and *Stx* were broadly in agreement with the trends observed in the luminescence data (Fig. 3), a measure of translation of the pR′ transcripts.

Dilution (1 : 10) in fresh medium after 1 h is routinely used *in vitro* to facilitate recovery from the norfloxacin induction treatment. When such a recovery step was included, both the single and double lysogen cultures exhibited higher specific luminescence values, although the difference between single and double lysogen luminescence was considerably reduced, with double lysogen luminescence recorded at 1.11-fold (SD 0.01-fold) of the single lysogen levels at 120 min and 1.24-fold (SD 0.02-fold) at 180 min (Fig. 3). However, the quantitative PCR data detected a much more substantial 2.56-fold increase in *stx* transcripts in the double lysogen compared with the single lysogen (Fig. 4a). This increase was even greater than the 2.11-fold increase observed in the absence of a recovery step. The failure of these increased *stx* transcript levels in the double lysogen to directly result in an increase in translated product (Fig. 3) could be due to translational ceiling effects restricting the number of transcripts that can be translated to functional protein. The abundance of *Q* gene mRNA copies in the double lysogen did not increase relative to the single lysogen, but actually decreased slightly (Fig. 4b). These effects may indicate that a state of maximal *Q* expression had been reached, and that the increased *stx* expression in the cells allowed to recover from the induction was due to increased metabolic potential and activity in the presence of fresh growth medium. This assumption is supported by the luminescence levels of the single lysogen with respect to the double lysogen with and without recovery at 120 and 180 min (Fig. 3).

Response of lysogens to increasing concentrations of norfloxacin

Multiple copies of the phage genome integrated into a single host result in additional copies of the phage regulatory genes, whose differential expression and activity *in trans* could alter the phage response to norfloxacin induction, either enhancing or depressing expression of phage late genes, accordingly. To address this, single and double lysogen cultures were exposed to different levels of norfloxacin (0–3 μg ml^{-1}) with no induction recovery period. Under all of these conditions, cell density in the double lysogen culture, as monitored by OD_{600} readings, was considerably lower than that of the single lysogen

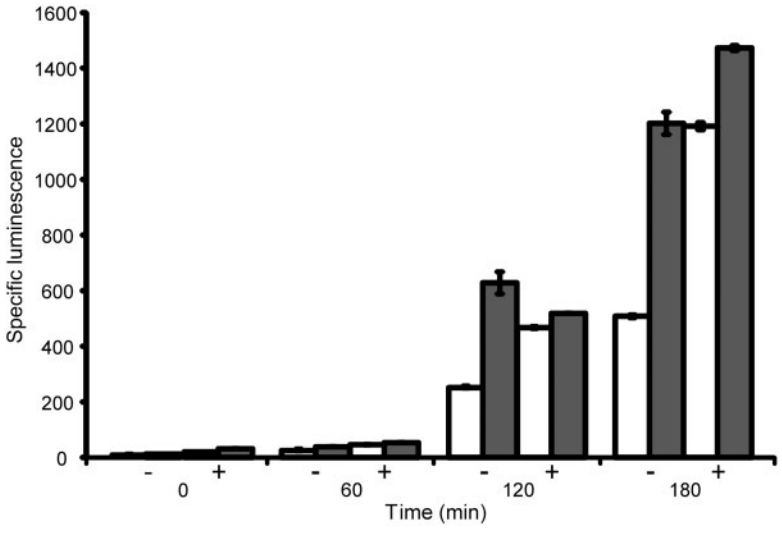

Fig. 3. Luminescence and promoter activity in induced lysogens. Luminescence emission from the pR′–*lux* fusion harboured in norfloxacin-induced single (white bars) and double lysogen (grey bars) cultures with no dilution and recovery period (–) and following a 1 : 10 dilution and recovery step after 1 h (+). Specific luminescence was calculated per microgram of total protein in 1 ml of each sample.

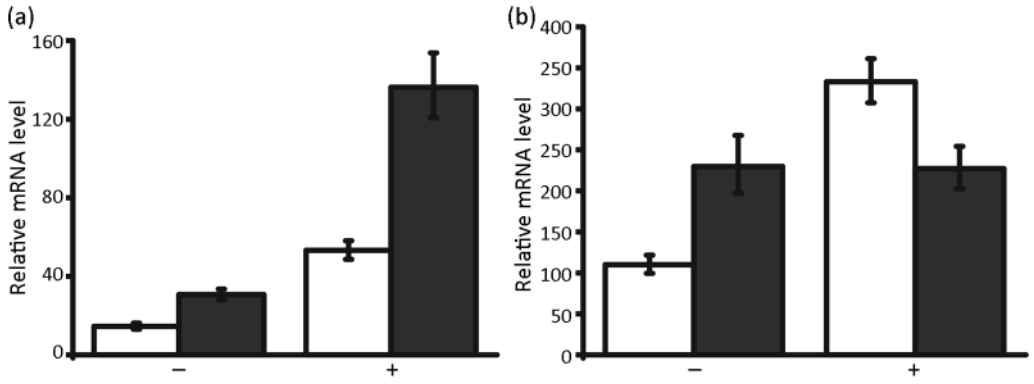

Fig. 4. *Q* and *stx* transcription in induced lysogens. Expression of *stx* (a) and *Q* (b) transcripts from induced single lysogen (white bars) and an induced double lysogen (grey bars) cultures, with no dilution and induction recovery period (–) and with dilution after 1 h and induction recovery (+). Samples were induced with 1 μg norfloxacin ml^{-1} and harvested at 3 h post-induction. All quantifications are relative to the uninduced single lysogen control.

counterpart, although the lysogens (single or double) possessed nearly identical growth patterns in the absence of norfloxacin (Fig. 5).

pR′–*lux* fusion luminescence data for single and double lysogen cultures induced in the same way produced decreased specific luminescence with higher concentrations of norfloxacin (Fig. 6a), although the effects were greater for the double lysogen. In order to examine what was occurring in these cultures on a single cell basis, specific luminescence was determined using OD_{600} readings as an indicator of cell number, enabling the comparison of the effects of 2 or 3 μg norfloxacin ml^{-1} compared with 1 μg norfloxacin ml^{-1} in both the single and double lysogens (Fig. 6b). The higher induction doses initially produced higher specific luminescence values relative to the 1 μg ml^{-1} control (Fig. 6b), peaking after 90 min for the single lysogen (124 % at 2 μg ml^{-1} and 119 % at 3 μg ml^{-1}) and after 60 min for the double lysogen (114 % at 2 μg ml^{-1} and 118 % at 3 μg ml^{-1}). Thereafter, the single lysogen appeared more tolerant to the increased norfloxacin concentrations, with the 2 μg ml^{-1} condition only decreasing to parity with the control after 150 min and remaining equivalent until 240 min (Fig. 6). Addition of 3 μg norfloxacin ml^{-1} resulted in a similar magnitude of peak luminescence, but expression dropped below the control earlier, at 120 min, falling to 72 % by 300 min.

In contrast, specific luminescence for the double lysogen induced with 2 and 3 μg norfloxacin ml^{-1} decreased sharply compared with the 1 μg ml^{-1} control after 120 and 90 min, respectively (Fig. 6b). The former fell to approximately 80 %

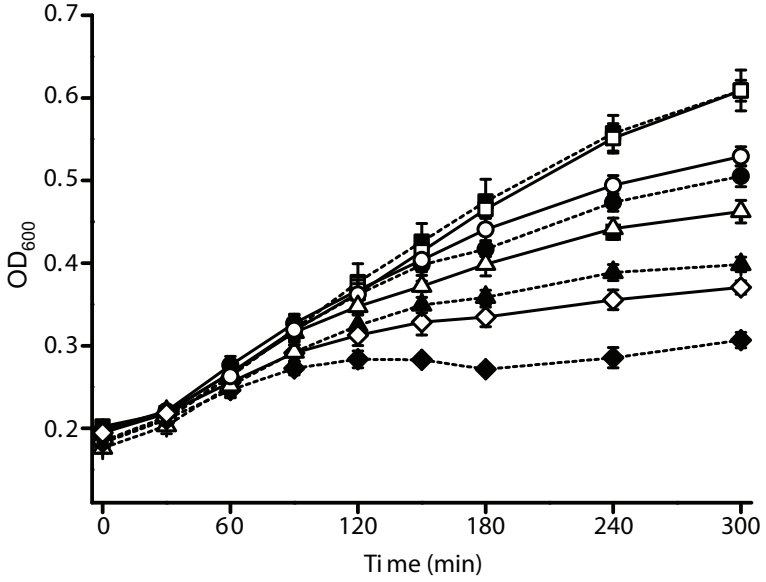

Fig. 5. Effect of norfloxacin concentration on lysogen growth. OD_{600} growth curves of single (open symbols, solid lines) and double lysogen (closed symbols, dashed lines) cultures induced by the addition of norfloxacin. The timescale begins immediately after norfloxacin induction (t=0). Final norfloxacin concentrations tested were 1, 2, 3 μg ml^{-1} (circles, triangles, diamonds, respectively) and a 0 μg ml^{-1} control (squares).

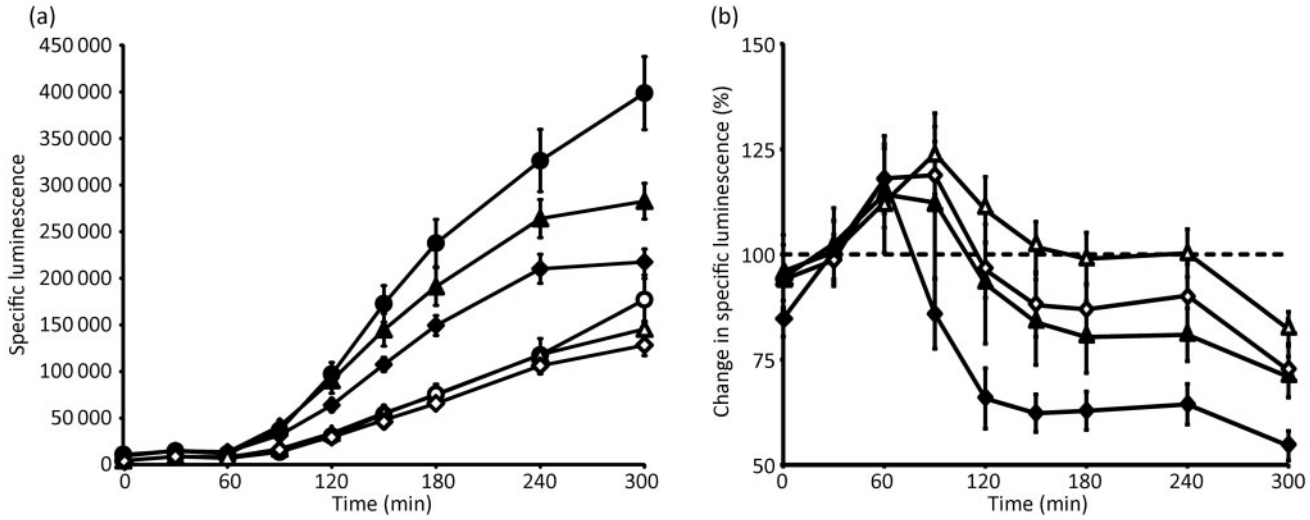

Fig. 6. Effect of increased norfloxacin concentrations on lysogen luminescence. (a) Luminescence produced from the pR′ fusion construct was determined in single lysogen (open symbols) and double lysogen (closed symbols) cultures induced with 1, 2 and 3 μg norfloxacin ml^{-1} (circles, triangles and diamonds, respectively). The timescale begins immediately after norfloxacin induction (t=0). (b) The difference in specific luminescence detected in cultures treated with 2 and 3 μg norfloxacin ml^{-1} (triangles and diamonds, respectively) is presented as a percentage of the 1 μg norfloxacin ml^{-1} concentration (dashed horizontal line). Single lysogen (open symbols) and double lysogen (closed symbols) values are relative to the specific luminescence of the respective cultures treated with 1 μg norfloxacin ml^{-1}. Specific luminescence was calculated by division of the raw luminescence counts by the OD_{600}.

of the control and the latter to as low as 62 % of the control by 180 min, before eventually reaching 71 % and 55 %, respectively. Double lysogen specific luminescence did, however, remain higher than that of the single lysogen cultures under all norfloxacin induction concentrations (Fig. 6a). Quantitative PCR measurement of *stx* transcripts revealed a 2.4-fold higher transcription level for the single lysogen induced with 2 μg ml^{-1} over 1 μg ml^{-1}, whilst for the double lysogen there was a twofold increase (Fig. 7). These luminescence and quantitative PCR data in conjunction with the growth curves clearly indicate a greater sensitivity of the double lysogen to norfloxacin induction, possibly due to decreased lysogen stability.

DISCUSSION

Stx expression is an important virulence factor for STEC, and its importance to this group of pathogenic organisms was recently expanded through the demonstration that Stx can provide defence against grazing by the eukaryotic predator *Tetrahymena thermophila*, and enhance the survival of *E. coli* O157 after engulfment by human macrophages (King *et al.*, 1999; Lainhart *et al.*, 2009; Poirier *et al.*, 2008). Under conditions mimicking one of the potential stresses faced by bacteria in the gut (3 mM H_2O_2), high levels of phage production and *stx* expression have been reported, comparable to antibiotic induction with norfloxacin and mitomycin C (Loś *et al.*, 2009, 2010). Consequently, this is evidence that Stx could play an important role in survival of the EHEC or STEC pathogen within the host as well as in the environment.

Stx-encoding phages have been shown to be able to multiply infect, and form stable lysogens within, a single

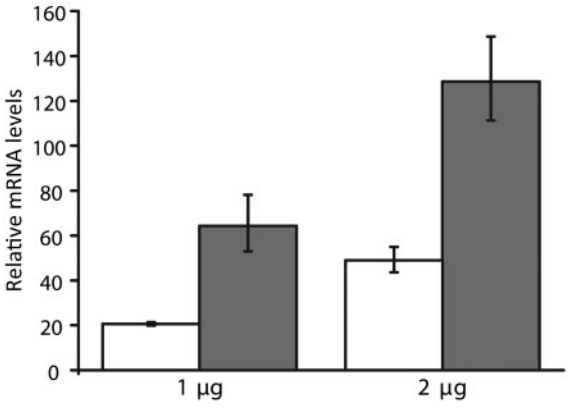

Fig. 7. *Q* and *stx* transcription induced by increased norfloxacin concentrations. Quantitative PCR was used to monitor *stx* transcript abundance in response to norfloxacin induction conditions at final concentrations of 1 μg ml^{-1} (1 μg) and 2 μg ml^{-1} (2 μg). All quantifications are relative to the uninduced single lysogen control expression. White bars represent induced single lysogen cultures and grey bars represent induced double lysogen cultures. Samples were harvested at 3 h post-induction.

host cell (Allison *et al.*, 2003; Fogg *et al.*, 2007; Serra-Moreno *et al.*, 2008). Theoretically, this ability has clear implications for the severity of Stx-mediated disease symptoms, commensurate with an increased number of toxin gene copies and increased toxin load, but previously this had not been directly and unequivocally demonstrated. Different *stx* genes, associated with different prophages or prophage remnants, are regularly found in combination within a single host (Allison *et al.*, 2003; Bielaszewska *et al.*, 2007; Eklund *et al.*, 2002; Muniesa *et al.*, 2003; Zheng *et al.*, 2008), and virulence profiles comprising two *stx2* genes are frequently associated with HUS and bloody diarrhoea (Banatvala *et al.*, 1996; Eklund *et al.*, 2002; Elliott *et al.*, 2001; Tozzi *et al.*, 2003; Woodward *et al.*, 2002). Furthermore, the presence of two *stx* operons can lead to increased *in vitro* toxin expression (Bielaszewska *et al.*, 2006; Cornick *et al.*, 2002; Eklund *et al.*, 2002; Muniesa *et al.*, 2003), though this observation is not without exceptions (Serra-Moreno *et al.*, 2008), nor does it necessarily equate to more severe clinical disease (Bielaszewska *et al.*, 2006; Cornick *et al.*, 2002). This disparity may be due to variation between *in vitro* and *in vivo* expression, resulting from different sensitivities to inducing agents or overall fitness states of the lysogens. In addition, previous studies have often focussed on 'hetero-lysogenic' *E. coli* strains, in which distinct differences between *stx* regulatory mechanisms were neither explored nor considered. Here, the question of whether *in vitro* toxin expression is significantly higher in isogenic double lysogens was specifically addressed. The results indicate that, even in the absence of induction of any kind, there is significant upregulation of Stx expression in an isogenic double lysogen, compared with a single lysogen. Therefore, colonization of the gut with toxigenic *E. coli*, and perhaps converted members of the normal intestinal microflora (Gamage *et al.*, 2006), harbouring multiple isogenic prophages, could produce an increased toxin load for the host. Under these conditions, Stx would be able to accumulate within the cell until phage-directed bacterial cell lysis enabled its release. Some of the well-characterized Stx2 phages, in particular, undergo spontaneous induction at a relatively high frequency, resulting in the presence of Stx2 in the extracellular fraction in the absence of any applied induction (Shimizu *et al.*, 2007, 2009; Strockbine *et al.*, 1986; Weinstein *et al.*, 1989). Spontaneous lytic and non-lytic toxin release coupled with increased toxin production in multiple lysogens could be an important combination for the onset of initial, localized disease symptoms such as inflammation and bloody diarrhoea.

Stimulation of the prophage lytic replication cycle with the antibiotic norfloxacin elicited a rapid increase in toxin expression levels in both single and double lysogen cultures after approximately 90 min. Whilst quantitative PCR generally supported the luminescence data, the magnitude of expression was far greater at the transcript level, suggesting that there may be a translational bottleneck that inhibits full realization of potential toxin production from an increased *stx* gene transcript copy number (Díaz *et al.*, 2011; Niebauer *et al.*, 2004). The abundance and availability of tRNAs, amino acids and ribosomes have been suggested as limiting factors in protein production (Brockmann *et al.*, 2007). Moreover, convergence of the induced single and double lysogen luminescence levels observed in the presence of a recovery step further supports the idea of a translational ceiling, as the double lysogen luminescence is unable to increase appreciably over that of the unrecovered culture, whereas single lysogen expression rises to approach double lysogen levels. This suggests that there is a limit to the amount of expression that host cells are able to support at the protein level, which can dampen Stx translation efficiencies to limit the increase that can be achieved by multiple gene copies.

Optical density measurement-derived growth curves for the single and double lysogen cultures revealed a greater susceptibility of the double lysogen to increasing levels of norfloxacin antibiotic. Control growth curves, in the absence of norfloxacin induction, were almost identical for single and double lysogen cultures, whilst at the highest induction level tested (3 μg ml^{-1}), the difference in OD_{600} readings was as much as 20 %. This observation, taken in conjunction with the increased expression from the pR′–*lux* fusion at 1 μg norfloxacin ml^{-1}, would seem to indicate that double lysogens are more sensitive to phage induction, thus leading to host cell lysis. Increased sensitivity to induction *in vivo*, and its associated increased toxin expression, could lead to more serious clinical symptoms (Neupane *et al.*, 2011). However, at the higher concentrations of norfloxacin induction there was a brief peak of expression for both lysogens after 60 min, before the sensitivity to induction actually led to reduced levels of luminescence as compared with the 1 μg ml^{-1} control dose. This dampening effect was far more pronounced for the double lysogen, where 3 μg norfloxacin ml^{-1} almost halved the observed luminescence, whilst the single lysogen remained relatively unaffected.

Nonetheless, even at the highest induction concentration, double lysogen cultures luminesced at higher levels than their single lysogen counterparts. Again, quantitative PCR data indicated that this effect was not regulated at the transcriptional level, as transcript amounts increased in concordance with the concentration of inducing agent. Therefore, the reduced luminescence observed at higher antibiotic concentrations is most likely to be due to decreased translational capacity brought about by a greater drain on cellular resources (two prophages add an additional 117 kb, representing an increase of 2.6 % in the K12 genome of highly expressed genes upon induction). It is important to note that a limit of any reporter gene system is that translation of the reporter gene is expected to mimic that of the endogenous product, in this case Stx, so luminescence is only an approximation of what may happen and not an absolute. However, these data may also go some way to explain why clinical outcome would not necessarily correlate with increased toxin expression

under laboratory conditions, as exposure to the inducing agent may vary in different environments and conditions.

To summarize, the presence of two isogenic prophages within a single host leads to an increase in Stx expression (transcription) under both non-inducing and inducing conditions. However, there appears to be a translational limit to the upregulation achievable, which becomes more pronounced when the cell is subjected to greater inductive stress. As a result, double lysogens, though seemingly more sensitive to induction with norfloxacin because they respond in a shorter time and their growth rates are more depressed, produce the toxin at levels that are inversely proportional to antibiotic concentrations. These observations suggest that, for double isogenic lysogens, the resultant toxin load may be higher or lower than in a respective single lysogen, and this outcome is dependent upon environmental factors. *In vitro*, the quantity of toxin produced by STEC strains harbouring more than one Stx prophage may vary depending on the experimental parameters. Relative *in vivo* toxin production by single and double lysogens is difficult to predict and may therefore depend on the host immune response as well as the health, composition and complexity of the intestinal microbiota.

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