

Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system

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Summary

Shiga toxin (Stx) genes in Stx producing *Escherichia coli* (STEC) are encoded in prophages of the λ family, such as H-19B. The subpopulation of STEC lysogens with induced prophages has been postulated to contribute significantly to Stx production and release. To study induced STEC, we developed a selectable *in vivo* expression technology, SIVET, a reporter system adapted from the RIVET system. The SIVET lysogen has a defective H-19B prophage encoding the TnpR resolvase gene downstream of the phage P_R promoter and a *cat* gene with an inserted *tet* gene flanked by targets for the TnpR resolvase. Expression of resolvase results in excision of *tet*, restoring a functional *cat* gene; induced lysogens survive and are chloramphenicol resistant. Using SIVET we show that: (i) approximately 0.005% of the H-19B lysogens are spontaneously induced per generation during growth in LB. (ii) Variations in cellular physiology (e.g. RecA protein) rather than in levels of expressed repressor explain why members of a lysogen population are spontaneously induced. (iii) A greater fraction of lysogens with *stx* encoding prophages are induced compared to lysogens with non-Stx encoding prophages, suggesting increased sensitivity to inducing signal(s) has been selected in Stx encoding prophages. (iv) Only a small fraction of the lysogens in a culture spontaneously induce and when the lysogen carries two lambdoid prophages with different repressor/operators, 933W and H-19B, usually both prophages in the same cell are induced.

Introduction

Bacterial virulence factors are often encoded within the genomes of prophages (Barksdale and Arden, 1974;

Boyd and Brussow, 2002; Wagner *et al.*, 2002). Induction of toxin encoding prophages has been shown to markedly increase toxin production by a number of bacterial pathogens, including *Corynebacterium diphtheria*, *Vibrio cholerae*, *Streptococcus pyogenes*, and Shiga toxin producing *Escherichia coli* (STEC) (Wagner and Waldor, 2002), suggesting that prophage induction can play a direct and significant role in modulating the expression of phage-encoded virulence factors.

In nearly all strains of STEC isolated to date, the genes encoding the two subunits of Shiga toxin (Stx) are located in the genomes of resident prophages belonging to the lambdoid family of bacteriophages (Newland and Neill, 1988; Datz *et al.*, 1996; Unkmeir and Schmidt, 2000). Although STEC strains can express a number of factors that contribute to virulence, their ability to express Stx has been implicated in the most severe systemic manifestations associated with STEC infections (Sjogren *et al.*, 1994; Heyderman *et al.*, 2001). These systemic sequelae, which include haemorrhagic colitis, haemolytic uremic syndrome, and neurological disorders, can progress to be life-threatening, particularly in young children and the elderly (Karch *et al.*, 1999).

The lambdoid phage H-19B, obtained from the clinically isolated serogroup O26 *E. coli* H19, encodes Stx1, one of the two major variants of Stx (Smith and Linggood, 1971; O'Brien *et al.*, 1984; Calderwood *et al.*, 1987; De Grandis *et al.*, 1987). The repressed prophage is integrated in the bacterial chromosome and is replicated as part of the host genome. Our understanding of the nature of lysogeny by lambdoid phages is based primarily on the detailed information gained from studies with λ (Hendrix *et al.*, 1983). The prophage state can be maintained as long as CI repressor levels are sufficient (Ptashne, 1992). CI is inactivated by an auto-catalytic cleavage reaction that at high pH *in vitro* can occur efficiently in the absence of other proteins, but that at physiological pH is stimulated by activated RecA (Roberts and Devoret, 1983; Little, 1984; Kim and Little, 1993). RecA activation is mediated by single-stranded DNA as part of the bacterial SOS response following DNA damage (Little and Mount, 1982; Friedberg *et al.*, 1995). Hence, exposure of lysogens to agents that cause DNA damage, such as mitomycin C, results in a sharp decrease in the cellular concentration of functional CI repressor (Shinagawa and Ito, 1973). If CI levels are sufficiently reduced,

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phage lytic growth ensues. This transition from the repressed lysogenic state to the expression of phage lytic functions is known as induction. Lytic growth is characterized by autonomous phage genome replication, production of progeny viral particles, and phage mediated host cell lysis (Hendrix *et al.*, 1983). Thus both host-cell death and release of free phage particles can serve as indicators of prophage induction.

Lysogens with Stx encoding prophages in culture produce free phage in the absence of an inducing agent, suggesting that some prophages undergo spontaneous induction *in vitro*. The fraction of H-19B lysogens that spontaneously liberate phage appears to be relatively low, as the amount of phage obtained is orders of magnitude less than the phage titre produced when the same number of lysogens is treated with an inducing agent. These findings indicate that even grown in the absence of known inducing stimuli, the lysogens are comprised of two populations, one in which the prophage is repressed and a significantly smaller one in which the prophage has been induced. The release of phage from a lysogen in the absence of a known inducing agent has been called 'spontaneous phage release' (Lwoff, 1953). We will refer to that event as well as to prophage induction that results in host cell lethality without proof of viable phage production as 'spontaneous induction'. Moreover, we will refer to any loss of prophage repression that, in the absence of a known inducing agent, leads to expression of a phage encoded product, even if it does not result in phage production or host lethality, as 'spontaneous derepression'.

Studies on the regulation of gene expression from the H-19B genome led to the suggestion that the subpopulation of induced STEC lysogens plays a major role in the production and release of Stx1 (Neely and Friedman, 1998a; Wagner *et al.*, 2002). Beyond the early classic experiments using the micromanipulator to look at the fate of a single bacterium (Lwoff, 1953), little, besides the fact that there is a small yield of phage particles, is known about the fraction of the lysogen population that is spontaneously induced. Even less is known about the fraction of lysogens that is induced during STEC infection of a mammalian host. Furthermore, the factors that cause a lysogen to be induced either spontaneously *in vitro* or *in vivo*, in the animal, are poorly understood. Studying the induced lysogen population presents a significant challenge, requiring a method to isolate and study bacteria that are normally destroyed by the phage lytic functions and likely comprise a small fraction of the total population of lysogens. To this end we have constructed a reporter lysogen that survives induction and, further, upon derepression of transcription from the early phage promoter P_R , undergoes a heritable and selectable genotypic change, thus allowing for the easy identification of the progeny of induced lysogens even when they represent a

small proportion of the total lysogen population. This approach, combining adaptations of the recombinase-based *in vivo* expression technology (RIVET) described by Camilli *et al.* (1994) and the λ prophage developed for reporting DNA damage by Toman *et al.* (1985), provides a method for directly isolating and quantifying cells in which the prophage has been induced. In addition, the selectable RIVET system, called SIVET, extends the utility of the RIVET system by facilitating the positive selection for bacteria in which expression from a promoter of choice has been turned on. The SIVET approach was used to measure the fraction of H-19B lysogens that is induced under standard culture conditions, compare the inducibility of H-19B and a number of other lambdoid prophages, and examine the factors within the bacterial cell that cause a lysogen to undergo spontaneous induction.

Results

Constructing a reporter H-19B lysogen that undergoes a heritable, phenotypic change upon prophage derepression

The recombinase based *in vivo* expression technology (RIVET) (Camilli *et al.*, 1994; Camilli and Mekalanos, 1995) was developed as a method for identifying the progeny of bacterial cells in which a promoter of interest has been activated. The main limitation of this approach is that the progeny of cells in which expression had been activated are isolated based on their sensitivity to tetracycline; isolating cells by screening or even by selection with fusaric acid (Bochner *et al.*, 1980) becomes impractical when the targeted cells represent an extremely small proportion of the total population. To circumvent this limitation, we developed a reporter system adapted from RIVET, known as selectable *in vivo* expression technology (SIVET), in which activation of transcription from a promoter of interest results in the acquisition of a selectable and heritable gain of chloramphenicol resistance (Fig. 1). The SIVET system is comprised of two components. The first is the gene encoding the $\gamma\delta$ TnpR resolvase (Reed *et al.*, 1982; Grindley, 1983; Heffron, 1983) inserted downstream of a promoter of interest. The second is a chloramphenicol transacetylation gene (*cat*) disrupted by an inserted tetracycline resistance gene (*tet*) flanked by altered resolvase target sequences (*resC*). Upon expression of TnpR, the *tet* gene and one *resC* site are excised, leaving one *resC* site inserted in *cat*. Excision results in a gene encoding a functional Cat protein, rendering the cell and its progeny resistant to chloramphenicol. Modification of *res* sites to *resC* was necessary because the *res* sequence remaining in the *cat* gene following excision would introduce a TAG stop codon in the *cat* reading frame. To eliminate this stop codon, the guanine of the

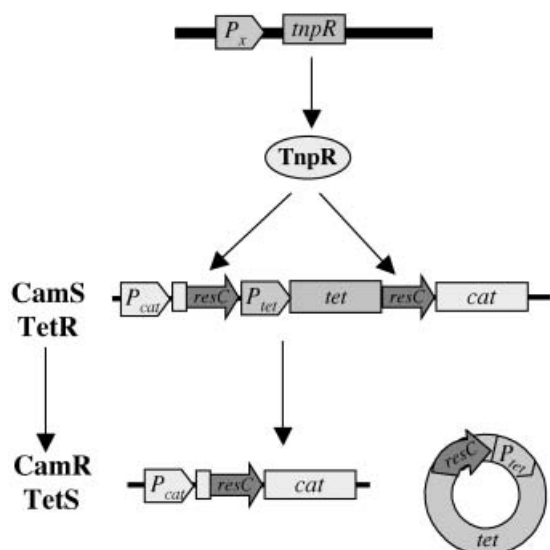


Fig. 1. General scheme of the selectable *in vivo* expression technology (SIVET). Expression of TnpR following activation of transcription from a promoter of interest results in excision and circularization of the *tet* gene and one modified resolvase excision site (*resC*). The excised DNA does not replicate and thus is lost by segregation. Excision of *tet* creates a functional *resC-cat* ORF. TnpR does not catalyse the reinsertion of *tet*, which is lost through segregation. Thus cells in which TnpR has been expressed undergo a genetic conversion which renders them and their progeny resistant to chloramphenicol and sensitive to tetracycline. Not drawn to scale.

TAG codon was changed to a cytosine in both *res* sites flanking the *tet* gene, producing *resC* sites.

To apply the SIVET scheme to identification and isolation of derepressed lysogens, we constructed a reporter H-19B prophage in which TnpR expression is dependent on transcription from the early phage promoter P_R . The *tnpR* gene with its ribosomal binding site (RBS) was inserted into the H-19B prophage downstream of P_R (Fig. 2). As first shown in λ and much later in H-19B, transcription from P_R is regulated by *Ci* repressor, which inhibits transcription by binding at both the P_R associated O_R and P_L associated O_L operators (Gussin *et al.*, 1983; Ptashne, 1992; Shi and Friedman, 2001). To ensure that

induction of the TnpR expressing prophage closely approximates the normal process of prophage induction, *tnpR* was inserted into the H-19B genome downstream of the *cro* gene, a location that would prevent the inserted gene from interfering with expression of phage functions and/or activity of sites involved in repression and derepression of the prophage. Although repressor binding at O_L does not directly influence transcription initiating at P_R , the design of the reporter prophage included an intact O_L . This design feature was based on findings that, in the wild-type λ prophage, repressor bound to O_R is further stabilized by its interaction with repressor bound at O_L (Revet *et al.*, 1999; Dodd *et al.*, 2001).

Following induction, lambdaoid prophages express a number of functions that lead to the death of the bacterial host cell (Court and Oppenheim, 1983). Therefore, the *tnpR* encoding prophage was modified to allow bacterial survival following induction. To this end we adopted a strategy based on the findings that host cell death following induction of a λ lysogen could be prevented by the inactivation of two phage genes (Eisen *et al.*, 1968): *N*, encoding a transcription elongation protein, and either *O* or *P*, encoding proteins required for initiation of phage DNA replication. We replaced the H-19B *N* gene with the kanamycin resistance gene (*kan*) and the adjacent *O* and *P* genes, with the ampicillin resistance gene (*amp*) yielding the H-19B $\Delta N::kan \Delta OP::amp$ prophage (Fig. 2). Following exposure to mitomycin C, the number of surviving H-19B $\Delta N::kan \Delta OP::amp$ lysogens was over four logs greater compared to the number of surviving wild-type H-19B lysogens and similar to the number of surviving non-lysogenic MC1000 cells. The *tnpR* gene was recombined into the $\Delta N::kan \Delta OP::amp$ downstream of P_R . This prophage, H-19B SIVET, was introduced into a derivative of strain MC1000 in which the *galK* gene had been replaced by the *resC-tet-resC* disrupted *cat* gene.

The following summarizes how the components of SIVET function to identify lysogens in which derepression of the H-19B prophage has taken place (Figs 1 and 2). Repressor inactivation leads to transcription from the P_R promoter resulting in expression of TnpR. TnpR excises

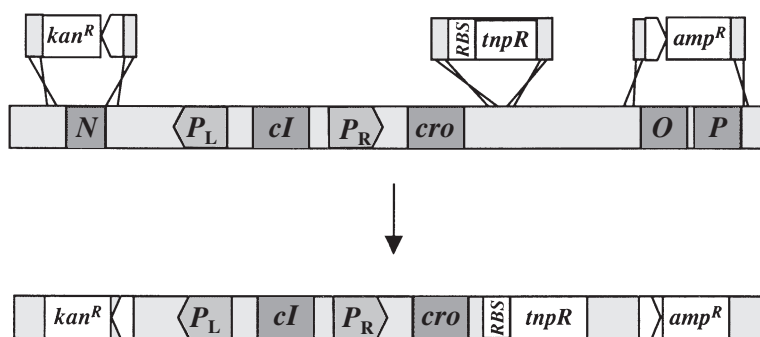


Fig. 2. Regulatory region of H-19B showing relevant genes and sites of allele replacement. Host killing by induced prophage was eliminated by replacing the *N* gene with a kanamycin resistance gene (*kan*) and the *O* and *P* genes with an ampicillin resistance gene (*amp*). The *tnpR* resolvase gene with its RBS was introduced into the H-19B prophage downstream of the *cro* gene in the P_R operon. Not drawn to scale.

the *resC-tet-resC* disrupting the *cat* gene in the chromosome, changing the phenotype of the lysogen from Tet^R to Cam^R. Thus, the SIVET scheme renders the progeny of cells that have expressed TnpR easily identifiable by selection for Cam^R. Because the SIVET prophage encodes resistance to kanamycin and ampicillin and cannot excise following induction, all SIVET lysogens, induced and non-induced, are resistant to those antibiotics. Thus the fraction of induced lysogens can be determined by dividing the number of cells resistant to chloramphenicol by the number of cells resistant to kanamycin and/or ampicillin.

Comparing the acquisition of Cam^R in the SIVET lysogen to wild-type prophage induction

The SIVET lysogen was used to measure *resC-tet-resC* excision under standard culture conditions. To minimize the background of lysogens in which *resC-tet-resC* had been excised, the SIVET lysogens were grown in LB supplemented with tetracycline before being diluted in LB and grown to stationary phase. Following growth to stationary phase in LB, approximately 0.11% of SIVET lysogens became Cam^R (Fig. 3). We note that as acquisition of Cam^R by the reporter lysogens is heritable and irreversible, the Cam^R SIVET lysogens isolated represent the progeny of all cells that are induced throughout growth of the culture. The SIVET lysogens underwent approxi-

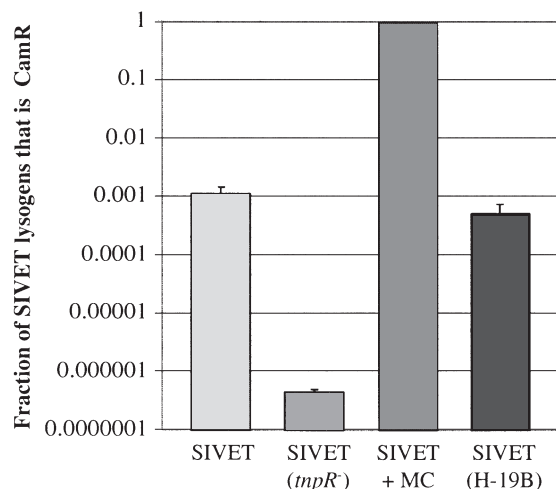


Fig. 3. Effect of TnpR expression from the H-19B SIVET prophage on the frequency of obtaining Cam^R lysogens. Cultures were grown to stationary phase in LB broth, some following treatment with mitomycin C (MC), and titres of induced and uninduced lysogens were determined using appropriate selection. See *Experimental procedures* for details. SIVET(*tnpR*) does not encode the *tnpR* gene; SIVET(H-19B) also carries a wild-type H-19B prophage. Fraction that are Cam^R is calculated as the number of colonies resistant to Cam, Amp, and Kan/the number of colonies resistant to Amp and Kan. Values shown correspond to the mean of at least three independent experiments with error bars indicating the standard error.

mately six rounds of cell division in the absence of antibiotics before reaching stationary phase. Assuming a similar fraction of the total population was spontaneously induced in each generation, the fraction of lysogens spontaneously induced per generation is approximately one-seventh of the fraction of lysogens that are Cam^R following growth to stationary phase, i.e. 0.016% or 1 in 6400 lysogens acquired Cam^R every generation. Acquisition of Cam^R is dependent on expression of TnpR, decreasing by three orders of magnitude in a SIVET strain lacking *tnpR*. Exposure of the SIVET lysogen to mitomycin C resulted in 95% of SIVET lysogens becoming Cam^R, suggesting that, like the wild-type H-19B lysogen, derepression leading to transcription from P_R promoter in the SIVET lysogen was stimulated by activation of the host SOS response. Additional proof for this assertion will be provided below.

Studies with RIVET have shown that extremely low levels of TnpR expression can result in excision of *res-tet-res* rendering the assay so sensitive as to interfere with its effectiveness at identifying active promoters (Lee *et al.*, 1999). This prompted concern that very low levels of transcription from P_R , although not sufficient to result in cell death through lytic development of a wild-type prophage, might result in sufficient expression of TnpR to excise *resC-tet-resC*. To determine how closely the level of derepression needed to excise *resC-tet-resC* in the SIVET lysogen corresponds to that required for induction of wild-type H-19B, a SIVET lysogen carrying a wild-type H-19B prophage, SIVET(H19B), was constructed and used to determine the number of lysogens in which prophage derepression leads to excision of *resC-tet-resC* but not killing by the wild-type prophage. The reasoning behind this strategy anticipates two possible outcomes. If the level of derepression that is sufficient to excise *resC-tet-resC* from the SIVET(H19B) lysogen is the same as the level required to activate the wild-type prophage, in nearly every bacterium in which derepression results in excision of *resC-tet-resC*, the wild-type prophage would be induced, resulting in death of the lysogen. Thus, the number of Cam^R colonies would be very low. Alternatively, if the level of derepression causing excision of *resC-tet-resC* in the SIVET(H19B) is significantly lower than that required to activate the wild-type prophage, in many bacteria in which the *resC-tet-resC* is excised, the wild-type prophage would not be induced. Thus the lysogen would survive and there would be nearly as many Cam^R colonies as found with the SIVET lysogen control.

The fraction of SIVET(H19B) lysogens that were Cam^R was only slightly lower than the fraction of SIVET lysogens that were Cam^R (Fig. 3) suggesting that, in a significant proportion of SIVET lysogens that lose *resC-tet-resC*, the wild-type H-19B prophage does not express

sufficient levels of phage functions to kill the host cell. Thus the low amounts of TnpR expression sufficient to cause *resC-tet-resC* excision make the SIVET lysogen, as constructed, too sensitive to provide an accurate measurement of the absolute number of wild-type H-19B lysogens that have undergone spontaneous induction. Furthermore, this result demonstrates that, in a subset of lysogens, derepression is sufficient to allow some transcription from P_R but not sufficient to result in levels of phage lytic function expression necessary to threaten cell viability.

We refer to the general approach of using a SIVET lysogen with a second intact prophage to compare the inducibility of the SIVET prophage with the inducibility of the intact prophage as the SIVET induction index test (IIT). The inducibility index refers to the ratio of the CamR fraction of SIVET lysogens with the intact second prophage to the CamR fraction of SIVET lysogens. The lower the ratio, the more similar is the inducibility of the intact prophage to the inducibility of the SIVET prophage. When the ratio is extremely low, it is not possible to discern whether the two phages induce similarly or the intact phage induces more readily.

Optimizing SIVET sensitivity

We adopted the strategy used to decrease the sensitivity of the RIVET procedure by Lee *et al.* (1999) to modify the SIVET lysogen so the fraction that are CamR closely reflects the fraction of spontaneously induced lysogens with a wild-type H-19B prophage. The ribosome bindings site (RBS) of *tnpR* was altered to increase the amount of *tnpR* transcription needed to attain levels of TnpR suffi-

cient for excision of *resC-tet-resC*. Two derivatives of the SIVET lysogen were constructed, each carrying one of the mutations in the RBS of *tnpR* reported by Lee *et al.* (1999), *tnpR168* and *tnpR184*, yielding strains SIVET¹⁶⁸ and SIVET¹⁸⁴. When cultures of the lysogens were grown to stationary phase in LB, 0.03% and 0.01% of SIVET¹⁶⁸ and SIVET¹⁸⁴ lysogens, respectively, acquired CamR (Fig. 4). This corresponds to approximately 0.0043% and 0.0014% of SIVET¹⁶⁸ and SIVET¹⁸⁴ cells, respectively, acquiring CamR per generation. When the two derivative strains were exposed to mitomycin C, 96% of SIVET¹⁶⁸ cells and only 8% of SIVET¹⁸⁴ cells became CamR (Fig. 4).

We next employed SIVET IIT to determine how closely *resC-tet-resC* excision in SIVET¹⁶⁸ and SIVET¹⁸⁴ corresponds to wild-type H-19B prophage induction. For both RBS-mutant derivatives carrying the wild-type H-19B prophage, very few CamR lysogens were isolated compared to the strains carrying only the SIVET prophage (Fig. 4). These results suggest that, unlike the SIVET^{WT} (H19B) strain, in nearly every SIVET¹⁶⁸(H19B) and SIVET¹⁸⁴(H19B) lysogen in which *resC-tet-resC* was excised, the wild-type prophage was induced. Thus, the fraction of SIVET¹⁶⁸ and SIVET¹⁸⁴ lysogens that become CamR is equal to or less than the fraction of lysogens that are spontaneously induced as measured by lethality to the host bacterium. Because SIVET¹⁶⁸ was found to spontaneously yield a higher fraction of CamR lysogens than did SIVET¹⁸⁴ yet was shown by the SIVET IIT assay not to be overly sensitive to low levels of transcription from P_R , SIVET¹⁶⁸ is the derivative in which acquisition of CamR most closely reflects spontaneous induction of the wild-type H-19B lysogen.

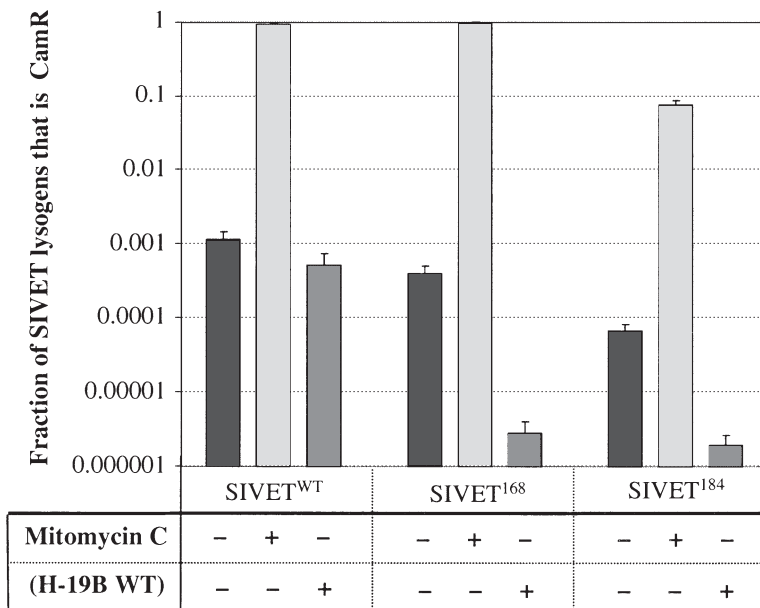


Fig. 4. Fraction of SIVET lysogens that is CamR in cultures of SIVET lysogens altered in their ability to express TnpR. Strains listed as SIVET¹⁶⁸ (K10449) and SIVET¹⁸⁴ (K10450) have nucleotide changes at different positions in the *tnpR*-associated RBS. Cultures were grown to stationary phase in LB broth, some following treatment with mitomycin C and titres of induced and uninduced lysogens were determined using appropriate selection. See *Experimental procedures* for details. Fraction that are CamR is calculated as described in Fig. 3. Values shown correspond to the mean of at least three independent experiments with error bars indicating the standard error.

Assessing the role of *Cl* auto-cleavage in H-19B spontaneous induction

To confirm that spontaneous H-19B induction required RecA mediated *Cl* autocleavage, we introduced a mutation into the *cl* gene of the SIVET prophage predicted to abolish auto-cleavage of the *Cl* protein (J.W. Little, pers. comm.). This prediction was based on work with other cleavable repressors, in which mutations changing either a conserved serine or lysine residue were shown to abrogate auto-catalytic activity (Lin and Little, 1988). Based on its location a likely candidate for the conserved serine codon in the *cl* gene of the prophage in SIVET¹⁶⁸ was replaced with an alanine codon, creating the *clind* mutant allele. When the H-19B SIVET¹⁶⁸ prophage encodes the *clind* allele, the fraction of lysogens that spontaneously acquire CamR is decreased by three logs compared to SIVET¹⁶⁸ (Fig. 5). This approaches the number of CamR lysogens observed in cultures of the SIVET derivative missing *tnpR*. These findings confirm that spontaneous induction of the SIVET lysogen, as expected, is due to host mediated *Cl* degradation. A culture of SIVET^{WT} lysogens with a *clind* SIVET prophage also spontaneously yielded extremely small numbers of CamR colonies similar to the numbers yielded by the SIVET lysogen missing a functional *tnpR* gene (Fig. 5). Hence, even low levels

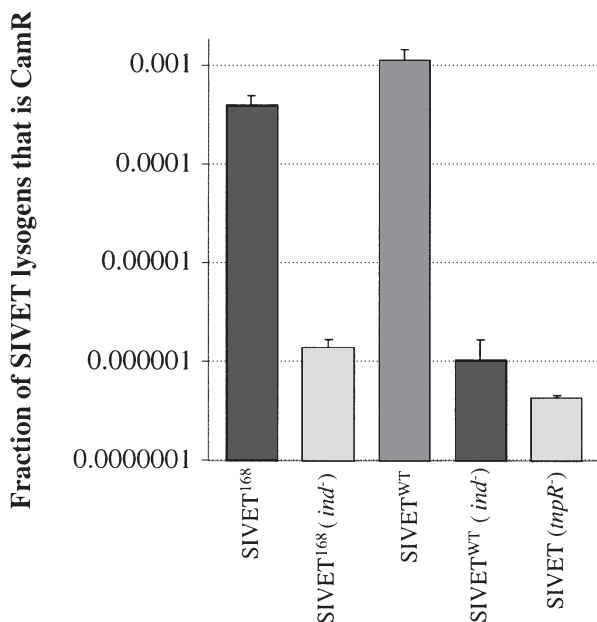


Fig. 5. Role of autocleavage in spontaneous induction of SIVET lysogens. Cultures were grown to stationary phase in LB broth and titers determined using appropriate selection. See *Experimental procedures* for details. Changes in the SIVET lysogens are indicated in parenthesis: *ind*⁻, a mutation in the *cl* gene resulting in a repressor that does not autocleave; *tnpR*⁻, no resolvase gene. Fraction of lysogens that is CamR was determined as described for Fig. 3. Values shown correspond to the mean of at least three independent experiments with error bars indicating the standard error.

of transcription from *P_R* that fail to promote prophage induction require repressor inactivation.

Determining the fraction of lysogens carrying the *Stx2* encoding phage 933 W that undergo spontaneous induction

We next applied the SIVET system to identify and quantify the fraction of lysogens carrying the *Stx* encoding lambdoid phage 933 W that are induced. 933 W carries the genes encoding *Stx2*, the other major variant of *Stx* (O'Brien *et al.*, 1984; Plunkett *et al.*, 1999). Whereas 933 W and H-19B share similar genomic organizations as well as some genes (Neely and Friedman, 1998a,b; Plunkett *et al.*, 1999), they have different immunities, meaning that each phage expresses a distinct *Cl* that cannot mediate repression of the other (O'Brien *et al.*, 1984). A SIVET¹⁶⁸ lysogen in which the SIVET prophage has the immunity of 933 W rather than the immunity of H-19B was constructed and named 933 W SIVET¹⁶⁸. We retain the nomenclature of SIVET without indication of the immunity for all constructs with SIVET prophages having the immunity of H-19B. Approximately 0.10% of the 933 W SIVET¹⁶⁸ lysogens acquired Cam resistance spontaneously when grown to stationary phase in LB. Correcting for the accumulation of CamR lysogens from previous rounds of cell division, this corresponds to approximately 0.014% or 1 in 7000 per cell generation.

Comparing the inducibility of H-19B with that of other lambdoid phages

The SIVET IIT approach was used to compare the inducibility of the SIVET¹⁶⁸ H-19B prophage with the inducibility of lambdoid prophages encoding other immunity regions. The SIVET¹⁶⁸ strain was lysogenized with 933 W, λ , P21, or λ *imm434*, yielding strains SIVET¹⁶⁸(933 W), SIVET¹⁶⁸(λ), SIVET¹⁶⁸(P21), and SIVET¹⁶⁸(λ *imm434*). Following growth of these double lysogen strains to stationary phase, the fraction of SIVET¹⁶⁸(933 W) cells that was CamR was found to be nearly 30-fold less than that observed in the SIVET¹⁶⁸ strain (Fig. 6), indicating that in almost every cell in which the H-19B prophage was induced the 933 W prophage was also induced. The number of CamR SIVET¹⁶⁸(λ *imm434*) lysogens obtained was reduced only about threefold compared to SIVET¹⁶⁸, indicating that in a significant subset of cells in which SIVET¹⁶⁸ had been induced, the λ *imm434* prophage had not been induced (Fig. 6). This threefold decrease was substantially less than that seen in SIVET¹⁶⁸(933 W), suggesting that the fraction of lysogens with a λ *imm434* prophage undergoing spontaneous induction is significantly less than lysogens with either H-19B or 933 W prophages. The fractions of SIVET¹⁶⁸(λ) and

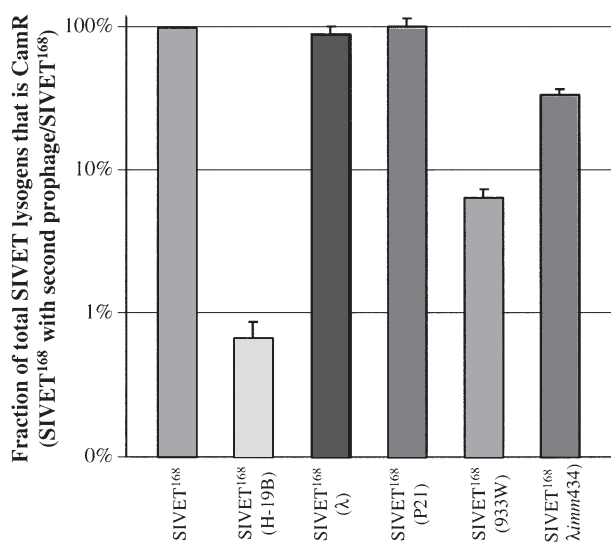


Fig. 6. Comparison of spontaneous induction frequencies of H-19B and lambdoid phages with different repressor-operator systems (immunity regions) using SIVET IIT. Cultures were grown to stationary phase in LB broth and titers determined using appropriate selection. See *Experimental procedures* for details. Indicated in parenthesis are the intact second prophages. The indicated values are normalized to the fraction of total SIVET¹⁶⁸ lysogens that are CamR which is set at 100% and represented by the left most bar. Raw data were calculated as the number of colonies resistant to Cam, Amp, and Kan/the number of colonies resistant to Amp and Kan. Values shown correspond to the mean of at least three independent experiments with error bars indicating the standard error.

SIVET¹⁶⁸(P21) that were CamR were similar to those observed in SIVET¹⁶⁸ (Fig. 6), indicating that, in the vast majority of cells in which *resC-tet-resC* is excised, neither λ nor P21 were induced. These findings suggest that spontaneous induction of H-19B either occurs in a larger fraction of cells than does either λ, P21, or 434 induction, or through a mechanism distinct from that causing induction of λ, P21 and 434.

To differentiate between these two possibilities, we repeated SIVET IIT analysis using λ*cl857ind*⁺ or λO_R323 as the intact prophages. λ*cl857ind*⁺, a recombinant derived from the original λ*cl857ind*⁻ phage (Sussman and Jacob, 1962), encodes a *cl* with a point mutation resulting in a relatively unstable repressor (S. Adhya, pers. comm.); λO_R323 has changes in the right operator (Little *et al.*, 1999). Based on the observation that lysogens with these λ prophages spontaneously produce more phage than do wild-type λ lysogens (S. Adhya, pers. comm.; Little *et al.*, 1999) we reasoned that these prophages undergo spontaneous induction more readily than wild-type λ. Thus, if spontaneous induction of H-19B and λ is mediated by the same host-specific factor, SIVET¹⁶⁸ lysogens carrying these mutant λ prophages should produce a lower proportion of CamR cells than SIVET¹⁶⁸. As shown in Fig. 7, the fraction of lysogens that were CamR was significantly

reduced in cultures of the SIVET¹⁶⁸(λ*cl857ind*⁺) and SIVET¹⁶⁸(λO_R323) strains. These findings suggest that in nearly every cell in which H-19B was induced, the λ*cl857ind*⁺ and λO_R323 prophages were induced as well. Therefore, we conclude that induction of H-19B is mediated by the same factor, presumably activated RecA, that causes induction of λ, but that spontaneous λ prophage induction requires a relatively higher level of this factor and thus occurs in a smaller proportion of cells than does spontaneous H-19B induction.

Discussion

The SIVET system

Selectable *in vivo* expression technology was developed to extend the utility of RIVET technology by allowing the isolation of a small fraction of a population of bacteria in which transcription from a promoter of interest had been activated. In the SIVET system, expression of TnpR leads to a genetic change resulting in cells that started as stably TetR and CamS becoming CamR and TetS. Because they are identified by selection rather than by screening, the induced bacteria can be isolated and quantified even when they represent an extremely small fraction of the total population. Furthermore, as acquisition of CamR is

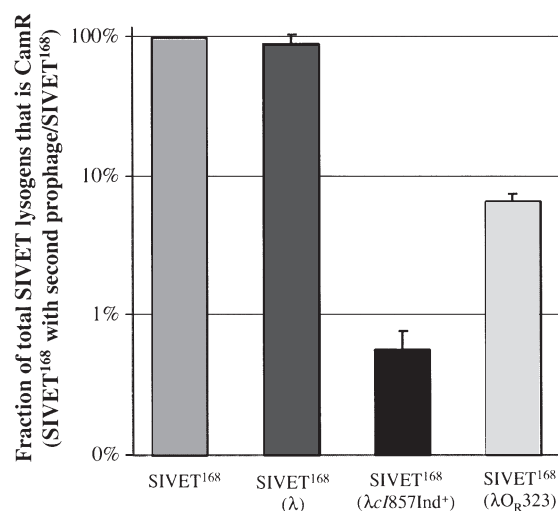


Fig. 7. Comparison of spontaneous induction frequencies of H-19B and lambdoid phages with repressor-operator systems (immunity regions) containing mutations affecting their inducibility using SIVET IIT. Cultures were grown to stationary phase in LB broth and titers determined using appropriate selection. See *Experimental procedures* for details. Listed in parenthesis are the intact second prophages; *cl857Ind*⁺ has a point mutation in λ *cl* and λO_R323 mutations in the right operator (see text for details). The indicated values are normalized to the fraction of total SIVET¹⁶⁸ lysogens that are CamR which is set at 100% and represented by the left most bar. Raw data were calculated as described in Fig. 5. Values shown correspond to the mean of at least three independent experiments with error bars indicating the standard error.

both irreversible and heritable, identifying induced cells using SIVET does not require that selection take place during growth under the environmental conditions being tested.

The SIVET approach was applied to the study of spontaneous prophage induction *in vivo*, with a long-term goal of assessing prophage induction during STEC infection. To reduce the sensitivity of the system, we altered the RBS of the *tnpR* gene as described by Lee *et al.* (1999). This ability to alter the sensitivity of the system allowed us to make a derivative of the H-19B SIVET lysogen, SIVET¹⁶⁸, in which acquisition of CamR closely matched induction of the intact H-19B prophage. Hence, the fraction of SIVET¹⁶⁸ lysogens that become CamR per cell generation is a close approximation of the fraction of lysogens carrying the intact H-19B prophage that undergoes spontaneous induction. We therefore will use induction throughout the rest of the discussion whether discussing either SIVET¹⁶⁸ or lysogens carrying intact prophages.

Fraction of lysogens spontaneously induced

Using the SIVET¹⁶⁸ lysogen, we showed that, under standard culture conditions, approximately 1 in 20 000 lysogens carrying the Stx-1 encoding phage H-19B and 1 in 7000 lysogens carrying the Stx-2 encoding phage 933 W are spontaneously induced per cell generation. Whereas the fraction of the H-19B lysogen population that is induced in the intestinal environment was not directly examined here, host derived factors such as pharyngeal cells and neutrophils have been shown to stimulate phage production *in vitro* by *S. pyogenes* and *E. coli* lysogens, respectively (Broudy *et al.*, 2001; Wagner *et al.*, 2001a), suggesting that the fraction of the H-19B and 933 W lysogen populations induced during infection may be greater than that observed in culture.

On the role of RecA

Previous studies with λ identified activated RecA as an important contributor to spontaneous induction. First, a lysogen carrying a λ *ind⁻* prophage produces very few phage spontaneously when compared to a lysogen with a λ *ind⁺* prophage (Roberts and Devoret, 1983). Second, a *recA⁻* lysogen carrying a λ *ind⁺* prophage shows a similar reduction in spontaneous phage production, indicating that the physiological state of the *recA⁻* lysogen results in an *Ind⁻* phenotype (Brooks and Clark, 1967; Hertman and Luria, 1967). Third, Fuchs *et al.* (1999) showed that there were unmeasurable levels of phage produced from a *recA⁻* derivative of a phage producing EHEC strain.

Our studies with the H-19B SIVET lysogens address the roles of CI expression and inactivation in the sponta-

neous induction of the H-19B prophage. First, the H-19B SIVET lysogens in which the SIVET prophage *cl* gene encodes the *ind⁻* mutation are essentially uninducible, showing a greater than two log decrease in the fraction of lysogens that becomes CamR. Hence, a host encoded product that stimulates repressor autocleavage, presumably activated RecA, is required for spontaneous induction of H-19B. Second, studies with derivatives of the H-19B SIVET lysogen carrying an additional prophage provide evidence that variation in levels of repressor expression is likely to play a minor role, if any, in spontaneous derepression leading to prophage induction. When the intact prophage in the SIVET IIT is either 933 W or λ O_R323, there is a ~95% decrease in the number of CamR colonies. Hence, in the vast majority of bacteria in which sufficient levels of spontaneous derepression occur, phages expressing different repressor proteins are both induced. Because spontaneous induction of H-19B and 933 W, as determined by SIVET analysis, is 5×10^{-5} and 1.4×10^{-4} , respectively, if induction of prophages in the same bacterium were independent events then the proportion of cells in which coinduction of H-19B and 933 W had occurred would be $\sim 7 \times 10^{-9}$. Therefore, if spontaneous induction of prophages with different repressors occurs independently of each other there should be no observable difference in the number of CamR colonies in the H-19B SIVET strain whether or not it carries a 933 W prophage. As this is obviously not the case, these results provide compelling evidence that stochastic fluctuations of repressor levels within a population of a specific lysogen are unlikely to play a significant role in spontaneous induction. In making this argument we assume that it is unlikely that there is a mechanism dedicated, in some unexplainable way, toward specifically reducing expression of a number of different phage repressor genes in lysogens undergoing spontaneous induction. Therefore, the simplest explanation is that spontaneous induction occurs solely on the basis of the level of activated RecA reaching the critical level for prophage induction.

SIVET as a tool to measure relative inducibility of prophages

The SIVET lysogen was also used to directly compare levels of spontaneous induction of prophages that differ in their repressors and cognate operators (i.e. differ in their immunity regions). Called the SIVET-induction index test (IIT), the comparison is between the levels of induction of the defective SIVET prophage and a second fully functional prophage with a different immunity region. The prophages tested could be divided into three classes. In one class, composed of phages with the immunity regions of lambdoid phages λ and 21, spontaneous induction occurred in a significantly smaller proportion of

the lysogen population than did induction of H-19B. In the second class, composed of the Stx encoding phages 933 W, spontaneous induction occurred in a similar fraction of the lysogen population as did induction of H-19B. In the third class, composed of the phage with the immunity of 434, spontaneous induction occurred in a smaller fraction of lysogens than did induction of H-19B but in a somewhat higher fraction of lysogens than did induction of prophages with the immunity regions of λ or P21. This result is consistent with previous studies showing that phages with the immunity of 434 express less repressor and induce more readily than do phages with the immunity of λ (Pirrota and Ptashne, 1969; Levine *et al.*, 1979).

In this regard it is interesting to note a study by Bertani (1951) using a multiple lysogen where, unlike the multiple lysogens studied here where both phages were inducible by the activated SOS system, in the Bertani experiment one phage was inducible by the SOS system, while the other was not. Examination of phage spontaneously released from single bacterium of the latter type of multiple lysogen, shows that spontaneous induction of the two types of phages are independent events, a single bacterial lysogen even though carrying both prophage types spontaneously produces one or the other.

On the differences in spontaneous induction of H-19B and λ

The results with the mutant λ prophages allowed us to rule out one possible explanation for the lower spontaneous induction of the λ prophage compared to induction of H-19B, namely, that in addition to activated RecA another bacterial factor(s) contributes to the induction of H-19B but not λ . The observation that the λ c857ind⁺ and λ O_R323 prophage cause a significant reduction in CamR colonies in SIVET IIT shows that both these λ derivative prophages are efficiently induced in the same cells in which the H-19B prophage is induced. Because the repressor encoded by O_R323 and the operator sites encoded by c857ind⁺ are identical to those encoded by the wild-type λ , the difference in the induction of H-19B and λ measured by the SIVET IIT test can not reflect a difference in repressor response to a host factor and therefore must reflect differences in repressor action with the operator regions of the two phages. Thus, spontaneous induction of prophages with λ immunity results from a response to the same environmental signal(s) that acts to spontaneously induce the H-19B prophage.

Prophage inducibility and Stx production

One striking finding from these studies is that the two phages that spontaneously induce most readily, or by the

terminology of Little *et al.* (1999) have 'hair triggers', are those with *stx* genes, H-19B and 933 W. Those authors proposed that the stability of the repressor-operator system of λ and presumably those of other lambdoid phages have evolved so that prophage induction occurs only when the health of the host bacterium is significantly compromised. They argue that an increased sensitivity to inducing stimuli may decrease the relative fitness of the λ prophage by causing it to induce even under conditions that are favourable to the survival of the host bacterium and its prophage. Whereas this reasoning may be applicable to prophages not encoding Stx, it is unlikely to apply to H-19B and 933 W. If, as we have previously proposed (Neely and Friedman, 1998a; Wagner *et al.*, 2001b; Wagner *et al.*, 2002), induction of Stx-producing prophages plays a significant role in the expression and release of Stx, these Stx encoding prophages may be subject to selective pressures not operating on other prophages. The assumption underlying this argument is that both modes of a temperate phage existence have an impact on temperate phage evolution; if the phage in its prophage state contributes to the growth advantage of its bacterial host, the responsible trait(s) and the mechanism controlling its expression, in this case a higher inducibility, will be selected even though the selected trait may not provide a selective advantage to the phage during lytic growth. Assuming that the small fraction of the STEC lysogens that is spontaneously induced contributes in a significant way to Stx expression and/or release, then an increase in the induced fraction could significantly influence the level of Stx production and/or release. If Stx contributes to the fitness of the STEC strain to its environment, then it seems plausible that there could be selective pressure on the prophage to induce more readily, explaining why, of phages we examined, those that exhibit substantially greater spontaneous induction are the Stx encoding phages H-19B and 933 W. But how can selection at the level of the lysogen work if, by this argument, the very lysogens that are responsible for Stx production and/or release are those lysogens that are destroyed by the induced prophage? Although being high relative to lysogens with prophages not carrying *stx* genes, the fraction of spontaneously induced lysogens with prophages carrying *stx* genes is still low; most of the lysogens survive. Therefore, assuming that Stx provides a growth advantage to the STEC strain, we suggest that, based on what admittedly is a small sample, the selection is for the remaining population of lysogens of the clone from which the destroyed lysogens were derived. A few have been sacrificed to give a growth advantage to the many. The desired trait expressed by the dead lysogens is maintained in the population of survivors; the diarrhoea caused by Stx promotes the spread of the surviving descendants of the original clone.

Experimental procedures

Bacteria, phage, and plasmids

Bacterial strains, relevant genotypes, and sources are listed in Table 1. Phages λ , λ cI857ind⁺, λ imm434, and P21 (21GP) were obtained from the NIH collection. Phage λ O_R323 was isolated from strains JL5938 provided by J. Little. Phages H-19B and 933 W were isolated from strains C600(H-19B) and C600(933 W), respectively, which were provided by A. O'Brien.

To construct pJLTnpR, the *tnpR* gene with its associated RBS was PCR amplified from pIVET5 (see below) with primers 39 and 40 (*Supplementary material*, Table S1) and cloned into the expression vector pTrc99A (Amersham Biosciences) as described (Sambrook and Fritsch, 1989).

Sources of resistance markers, *tnpR* and *res* sites

The *tet* and *amp* genes were amplified from vector pBR322 (Sutcliffe, 1979). The *cat* and *kan* genes were amplified from plasmids pKan (Carolina Biological Supply Company) and pBR325 (Bolivar, 1978) respectively. The *cat-sacB* cassette was amplified from strain DY406. The *res* sites and *tnpR* gene were amplified from vectors pAC21 and pIVET5 (Camilli and Mekalanos, 1995) respectively.

Media

LB and top agar media were prepared as described (Miller and Friedman, 1980). Except where noted, chloramphenicol, kanamycin, ampicillin, tetracycline, and mitomycin C (Sigma) were used at final concentrations of 10 μ g ml⁻¹, 20 μ g ml⁻¹, 30 μ g ml⁻¹, 5 μ g ml⁻¹ and 2 μ g ml⁻¹ respectively. The selective media used in obtaining sucrose resistant derivatives was LB media without NaCl supplemented with 20% sucrose (Sigma) and 50 μ g ml⁻¹ kanamycin.

General strain construction methods

Relevant strains are listed in Table 1. Except where indicated, all strain constructions utilized the λ Red based recombination system (Court *et al.*, 2002; Thomason *et al.*, 2003). The following is a brief outline of the procedure. Sequences to be recombined were amplified by PCR with primers that included on their 5' ends 35–40 bp of homology to the regions flanking the target insertion site. The sequences of primers used in strain construction are listed in *Supplementary material*, Table S1. Following column purification (Quaigen), these amplicons were electroporated into derivatives of strain DY378, which carries a defective λ prophage. This prophage encodes the *cI857 ts* repressor allele and therefore expresses the lambda recombination functions at high temperature. Transient expression of these recombinational

Table 1. Bacterial strains.

Strain	Relevant genotype	Source
DY378	See <i>Experimental procedures</i>	D. Court
DY406	W3110 (λ cI857 <i>N-kil::cat-sacB</i>)	D. Court
K7523	C600(H-19B)	A. O'Brien
K8443	C600(933 W)	A. O'Brien
K9723	DY378(H-19B $\Delta N::kan$)	This laboratory
K9725	DY378(H-19B)	This laboratory
K9834	MC1000(H-19B)	This laboratory
K9979	K9981 <i>galk::res</i>	This laboratory
K9981	DY378(H-19B) pKan	This laboratory
K9988	DY378(H-19B $\Delta N::kan \Delta OP::amp$)	This laboratory
K9989	K9981 <i>galk::res-tet-res</i> pKan	This laboratory
K10045	MC1000(mult H-19B) <i>galk::res-tet-res</i>	This laboratory
K10373	DY378 <i>galk::cat</i>	This laboratory
K10376	DY378 <i>galk::resC-tet-resC</i>	This laboratory
K10447	MC1000(mult H-19B) <i>galk::resC-tet-resC</i>	This laboratory
K10448	MC1000(H-19B $\Delta N::kan cro-tnpR \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10449	MC1000(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10450	MC1000(H-19B $\Delta N::kan cro-tnpR184 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10484	MC1000(λ)(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10487	MC1000(H-19B $\Delta N::kan cl-ind^+ cro-tnpR \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10488	MC1000(H-19B $\Delta N::kan cl-ind^+ cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10490	MC1000(H-19B)(H-19B $\Delta N::kan cro-tnpR \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10491	MC1000(H-19B)(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10492	MC1000(H-19B)(H-19B $\Delta N::kan cro-tnpR184 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10493	DY378(H-19B $\Delta N::kan cl::cat-sacB cro-tnpR184 \Delta OP::amp$)	This laboratory
K10494	DY378(H-19B $\Delta N::kan cl-ind^+ cro- cat-sacB \Delta OP::amp$)	This laboratory
K10556	MC1000(λ cI857 Ind ⁺)(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10559	MC1000(933 W)(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10579	MC1000(933 W $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10583	MC1000(P21)(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10637	MC1000 <i>galk::res-tet-res</i>	This laboratory
K10638	MC1000(λ imm434)(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory
K10640	MC1000(λ O _R 323)(H-19B $\Delta N::kan cro-tnpR168 \Delta OP::amp$) <i>galk::resC-tet-resC</i>	This laboratory

tion factors shortly before transformation allows for efficient recombination between the linear fragment and target sequences. Recombinants with a transferred antibiotic resistance gene were selected by plating transformed cultures on LB agar plates supplemented with antibiotics at concentrations noted above. Recombinants in which the amplicon had replaced the *cat-sacB* cassette were selected on sucrose media then screened for sensitivity to chloramphenicol. Insertion of the amplicon in the proper location was confirmed by PCR using one insert-specific primer and one primer specific to sequence outside the site of insertion.

Table S1 listing sequences of all primers is found in the *Supplementary material*.

Construction of *galK::cat::resC-tet-resC*

In constructing *galK::cat::resC-tet-resC*, the chromosomal *galK* gene of strain K9981 (DY378-H-19B-pKan) was replaced by the *cat-sacB* cassette, which subsequently was replaced with the *resC* site. Subsequent steps in construction are elaborated in Fig. 8. The general strategy of using recombination rather than PCR amplification to create the *resC-tet-resC* construct was adopted to avoid the problems inherent in PCR amplification of direct repeats, such as primer dimers and the amplification of one of the *resC* sites rather than the complete amplicon resulting from hybridization of both *resC*-specific primers to the same direct repeat. P1 transduction of *galK::cat::resC-tet-resC* to strain K9834 yielded strains K10447.

Construction of the SIVET lysogens and SIVET lysogen derivatives carrying a second, intact H-19B prophage

The essential features of the SIVET prophage are pictured in Fig. 2. The *N* gene of the H-19B prophage of strain K9725 was replaced with a *kan* gene yielding strain K9723. The H-19B *OP* genes of K9723 were replaced with an *amp* gene yielding strain K9988. Insertion of the *tnpR* gene required two recombination events. First, the *cat-sacB* cassette was placed downstream of the *cro* gene of the H-19B prophage in K9988. Replacement of the *cat-sacB* cassette by the wild-type *tnpR*, *tnpR168*, or *tnpR184* genes yielded a prophage with the *tnpR* gene downstream of the P_R promoter. The specific nucleotide changes carried in the RBS of *tnpR168* and *tnpR184* have been previously reported (Lee *et al.*, 1999). The H-19B prophages with $\Delta N::kan$, $\Delta OP::amp$ and *cro-tnpR* constitute the SIVET H-19B prophages. P1 transduction of the SIVET^{WT} prophage to strain K10045 (MC1000 *galK::res-tet-res*) yielded strain K10637. The SIVET^{WT}, SIVET¹⁶⁸, and SIVET¹⁸⁴ prophages were also P1 transduced to strain K10447 (MC1000 *galK::cat::resC-tet-resC*). Strain K10447 carries multiple tandem copies of the wild-type H-19B prophage. This transduction of the SIVET prophages yielded two distinct classes of lysogens. Strains K10448, K10449, K10450, in which the SIVET prophage had replaced all of the wt H-19B prophages, carry only a single SIVET prophage. Strains K10490, K10491, and K10492, in which recombination of the SIVET lysogen had failed to remove all tandem copies of the wild-type prophage carry both a SIVET prophage and at least one wild-type H-19B prophage. These

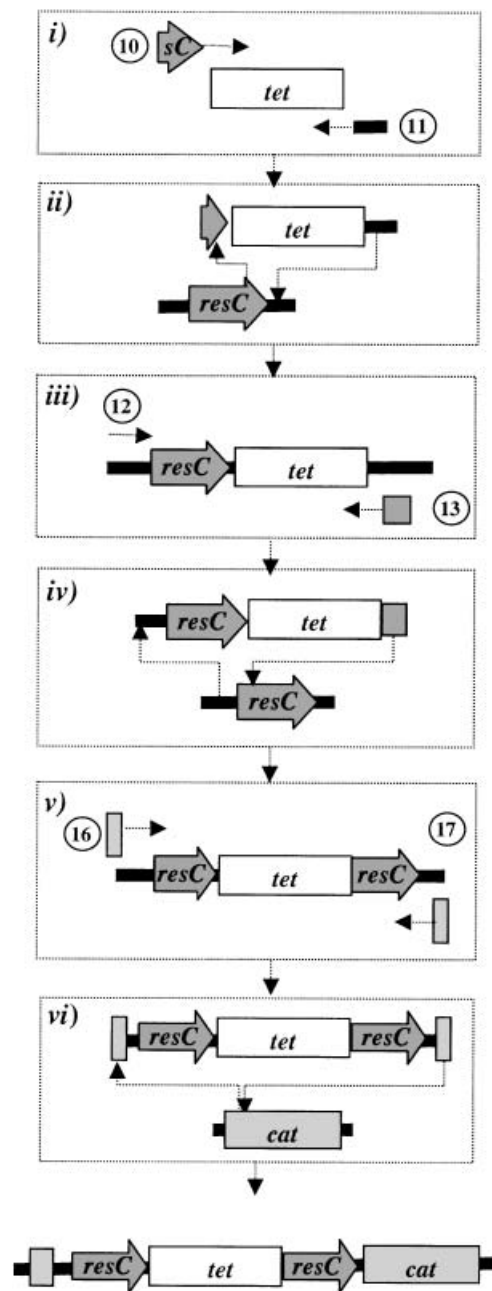


Fig. 8. Construction of *galK::cat::resC-tet-resC*. Primer sequences can be found in Table S1 in *Supplementary Material* (i) Primers 10 and 11 were used to fuse *resC* and *galK* sequences, respectively, to the *tet^r* gene. (ii) The *tet* gene was recombined directly 3' to the chromosomally inserted *resC* site. (iii) The *resC-tet* insertion was amplified using primers 12 and 13. (iv) The resulting amplicon was recombined 5' of *galK::resC* yielding strain K10376. (v) The *resC-tet-resC* construct was amplified from strain K10376 using primers 16 and 17 and (vi) recombined between the sixth and seventh codon of the chromosomally inserted *cat* gene in strain K10373 to produce *galK::cat::resC-tet-resC*.

two groups were distinguished using PCR primers flanking the site of *tnpR* insertion, with SIVET lysogens giving one band and SIVET(wt-H-19B) lysogens producing a second, smaller band.

To construct the H-19B *clind*⁻ SIVET prophages, the conserved serine codon residue in the *cl* gene identified as potentially required for autocleavage (J.W. Little, pers. comm.) was replaced by the *cat-sacB* cassette in the SIVET¹⁸⁴ prophage to produce strain K10493. The *cat-sacB* cassette was then replaced with a ssDNA oligo encoding an alanine codon at the position of the conserved serine codon flanked by 36 and 42 bp of *cl* homology, respectively, on each side. This yielded strain K10494. The *cl* genes of two of these recombinants were PCR amplified and sequenced to confirm the presence of the designed mutation and to insure the absence of secondary mutations. To construct the *clind*⁻ derivatives of the SIVET^{WT} and SIVET¹⁶⁸ prophages, the *tnpR184* gene in the prophage in strain K10494 was replaced by the *cat-sacB* cassette, which was subsequently replaced by either *tnpR*^{WT} or *tnpR168*. P1 transduction was used to move the *clind*⁻ SIVET prophages to strain K10447, yielding the SIVET strains K10487, K10488 and K10489 with non-inducible prophages.

Construction of the 933 W SIVET¹⁶⁸

The *N* gene of the 933 W prophage in strain K9857 was replaced with *kan*. Replacement of the 933 W *OP* genes with *amp* and insertion of the *tnpR* gene was achieved in two steps. First, the *cat-sacB* cassette was placed downstream of the *cro* gene in the 933 W $\Delta N::kan$ prophage yielding the 933 W $\Delta N::kan$ *cro-cat-sacB* prophage. Second, primers with homology to the 933 W *cro* gene and to sequence downstream of the 933 W *P* gene, respectively, were used to amplify the *tnpR* gene and adjoining $\Delta OP::amp$ sequence from SIVET¹⁶⁸. This amplicon was used to replace *cat-sacB* and the *O* and *P* genes in the 933 W $\Delta N::kan$ *cro-cat-sacB* prophage, yielding the 933 W SIVET¹⁶⁸ prophage. This prophage was transduced using P1 into strain K10636 to yield the 933 W SIVET lysogen.

Construction of SIVET¹⁶⁸ lysogens carrying a second intact prophage other than H-19B

Top agar (2.5 ml) containing approximately 50 μ l of SIVET¹⁶⁸ overnight culture was overlaid on LB + tetracycline plates. One hundred microlitres of the appropriate phage lysate was spotted on the bacterial lawn and the plate was incubated overnight at 37°C (32°C for λ cl857*ind*⁺). Bacteria from the zone of lysis were struck out on LB + tetracycline and grown overnight. Colonies were tested for the second phage by determining if they were immune to infection by a clear derivative of the second phage, but sensitive to a phage with the same adsorption specificity but a different immunity (Gottesman and Yarmolinsky, 1968).

Measuring the frequency of SIVET induction

To measure spontaneous induction of SIVET lysogens, overnight cultures grown in LB supplemented with tetracycline, kanamycin and ampicillin were diluted 1 : 100 in LB and grown overnight at 32 or 37°C. Dilutions of these cultures were transferred to LB agar plates supplemented with either

kanamycin and ampicillin (to select for all SIVET lysogens) or kanamycin, ampicillin, and chloramphenicol (to select for induced SIVET lysogens). Plates were incubated at 32 or 37°C for 48 h. To measure induction or survival following exposure to mitomycin C, one ml cultures of LB inoculated with 10 μ l of overnight culture were grown to exponential phase, diluted 1 : 2 in LB supplemented with mitomycin C and grown for an additional 2–4 h. The bacteria were then pelleted, washed twice in LB, resuspended in 4 ml LB, grown for 4 h at 37°C, and plated at appropriate dilutions on selective plates as described above.

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Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi3934/mmi3934sm.htm>

Table S1. Sequences and reference numbers of primers used for strain construction.

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