

Novel methods for expression of foreign antigens in live vector vaccines

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Abbreviations: CVD 910-GG, CVD 910*guaBA*::*gfpuv*; CVD 910-HG, CVD 910*htrA*::*gfpuv*, CVD 910-2G, CVD 910*guaBA*::*gfpuv htrA*::*gfpuv*; GFPuv, codon optimized green fluorescent protein; LD50, 50% lethal dose

Bacterial live vector vaccines represent a vaccine development strategy that offers exceptional flexibility. In this approach, genes encoding protective antigens of unrelated bacterial, viral or parasitic pathogens are expressed in an attenuated bacterial vaccine strain that delivers these foreign antigens to the immune system, thereby eliciting relevant immune responses. Rather than expressing these antigens using low copy expression plasmids, here we pursue expression of foreign proteins from the live vector chromosome. Our strategy is designed to compensate for the inherent disadvantage of loss of gene dosage (vs. plasmid-based expression) by integrating antigen-encoding gene cassettes into multiple chromosomal sites already inactivated in an attenuated *Salmonella enterica* serovar Typhi vaccine candidate. We tested expression of a cassette encoding the green fluorescent protein (GFPuv) integrated separately into native *guaBA*, *htrA* or *clyA* chromosomal loci. Using single integrations, we show that expression levels of GFPuv are significantly affected by the site of integration, regardless of the inclusion of additional strong promoters within the incoming cassette. Using cassettes integrated into both *guaBA* and *htrA*, we observe cumulative synthesis levels from two integration sites superior to single integrations. Most importantly, we observe that GFPuv expression increases in a growth phase-dependent manner, suggesting that foreign antigen synthesis may be “tuned” to the physiology of the live vaccine. We expect this novel platform expression technology to prove invaluable in the development of a wide variety of multivalent live vector vaccines, capable of expressing multiple antigens from both chromosomal and plasmid-based expression systems within a single strain.

Introduction

Excellent progress has been made over the past 20 y in the adaptation of attenuated bacterial vaccine strains for expression of foreign antigens to create multivalent live vector vaccines. Significant effort has been devoted to the creation of expression technologies which either directly or indirectly address the important problem of metabolic stress often associated with expression of foreign immunogens.^{1,2} It is now recognized that inappropriate synthesis of high levels of foreign protein to induce an antigen-specific protective immune response can adversely affect the fitness and growth rate of an already attenuated vaccine strain, resulting in over-attenuation and loss of immunity directed at both the live vector and foreign antigen. If these target immunogens are encoded by multicopy expression plasmids, these undesirable metabolic fluxes can result in plasmid loss in the absence of selective pressure, which ultimately defeats the strategy of live vector-mediated delivery of vaccine antigens. We and others have developed effective genetic stabilization systems for enhancing the retention of multicopy plasmids encoding regulated synthesis of foreign antigens, without the further requirement to select

with antibiotics.³⁻⁵ Antigen export systems have also been developed by our group and others to reduce proteolytic degradation of foreign antigens within the cytoplasm and more effectively deliver these antigens to the immune system to enhance immunogenicity.⁶⁻⁹ It is clear that a wide variety of genetic techniques and technologies are now readily available for efficient delivery of one or more antigens using live vector vaccines. However, inclusion of all of the genes encoding these foreign antigens within a single multicopy plasmid can lead to large plasmids which ultimately prove to be genetically unstable, reducing both antigen synthesis and the ensuing immune responses.¹⁰ One approach to stable delivery of multiple antigens has been integration of foreign genes into the live vector chromosome, but the decrease in gene dosage again leads to reduced antigen synthesis and lower antigen-specific immunity.¹¹

We hypothesize that delivery of sufficiently immunogenic levels of chromosomally encoded antigens can be accomplished through strategic integration of identical gene cassettes into multiple locations within the live vector chromosome, thereby compensating for loss of copy number afforded by stable low copy plasmids while avoiding further attenuation of the vaccine strain.

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Integration of multiple identical cassettes avoids the use of strong constitutive promoters to enhance antigen synthesis from a single gene copy, an approach which does not necessarily lead to adequate antigen synthesis or immune responses.^{12,13} In this report, we employ a synthetic codon-optimized gene cassette encoding the green fluorescent protein GFPuv as an easily quantifiable model antigen to explore expression levels after single integrations into chromosomal sites encoding synthesis of guanine nucleotide (*guaBA*), a serine protease expressed under environmental stress (*htrA*) or a cryptic hemolysin naturally found in *S. Typhi* (*clyA*). Using flow cytometry to measure GFPuv-mediated fluorescence within individual bacteria of a growing population, we compare fluorescence levels from single integrations, compared with multiple chromosomal integrations within a single vaccine strain and further examine how fluorescence varies with growth rate.

Results

Construction of CVD 910. We have previously constructed the attenuated vaccine candidate, CVD 908-*htrA*, derived from Ty2 and carrying deletions in *aroC*, *aroD* and *htrA*, which proved to be safe and highly immunogenic in Phase 2 clinical trials.¹⁴ Here, we have constructed a new vaccine strain, CVD 910, carrying deletions in *guaBA* and *htrA*. We have chosen to replace Δ *aroC* Δ *aroD* with the single deletion Δ *guaBA* for two important reasons: (1) previous work by our group has shown that Δ *guaBA* alone sufficiently attenuates Ty2, resulting in a live vector strain capable of eliciting impressive humoral immunity to a plasmid-encoded foreign antigen using the murine intranasal model of immunogenicity;¹⁵ (2) transcriptional control of the *guaBA* locus is controlled by growth rate, independent of guanine-mediated repression,¹⁶ theoretically allowing expression of properly integrated foreign gene cassettes to be increased as live vectors grow in the host. We constructed deletion cassettes, targeting *guaBA* and *htrA*, for use with the λ Red-mediated site-directed mutagenesis method,¹⁷ and have used each cassette to successfully delete either *guaBA* or *htrA* from wildtype *S. Typhi* Ty2. Introduction of both deletion mutations into a single strain resulted in the creation of CVD 910. We then performed a preliminary assessment of attenuation of CVD 910 by comparing the minimum lethal dose causing death in 50% of a group of BALB/c mice (LD50) for CVD 910 vs. CVD 908-*htrA*, using the hog gastric mucin intraperitoneal murine challenge model. For this model, we broadly follow the guidelines recommended in the Code of Federal Regulations for Food and Drugs, Title 21, Part 620.13 (*c-d*), 1986 for intraperitoneal challenge of mice with *S. Typhi*. Using this method, we have determined the LD50 for both CVD 910 and CVD 908-*htrA* to be approximately 5×10^5 CFU (data not shown), vs. an LD50 of ~ 10 CFU for wildtype Ty2.¹⁸

Chromosomal integration of *gfpuv* cassettes into CVD 910. We took advantage of both the deleted *guaBA* and *htrA* chromosomal genes of CVD 910 to express GFPuv from independently controlled cassettes in which the osmotically regulated P_{ompC} promoter was genetically fused to a promoterless *gfpuv* gene. The resulting P_{ompC} -*gfpuv* cassette was integrated into either the *guaBA* or *htrA* loci such that only the open reading frame

was replaced, but the original promoters for both chromosomal loci were preserved, as depicted schematically in **Figure 1**. For example, integration of P_{ompC} -*gfpuv* into the *guaBA* locus to create CVD 910-GG would result in transcription of *gfpuv* controlled both by osmolarity (via P_{ompC}) and growth rate (via P_{guaBA}). Similarly, integration of the same cassette into *htrA* to create CVD 910-HG would result in synthesis of GFPuv controlled both by osmolarity (P_{ompC}) and heat shock/environmental stress (P_{htrA}).¹⁹ In addition, we tested a third chromosomal integration, CVD 910-CG, in which P_{ompC} -*gfpuv* replaced *clyA*, encoding a cryptic hemolysin from Ty2 whose transcription is normally controlled by low pH.²⁰ Interestingly, when the resulting strains were grown overnight at 37°C in liquid cultures and analyzed for fluorescence by flow cytometry, observed fluorescence intensity was found to be strongly influenced by the site of integration, regardless of osmotic induction of P_{ompC} . As shown in the fluorescence histograms of **Figure 2**, under inducing conditions of 200 mM NaCl, strains with P_{ompC} -*gfpuv* integrated into either *guaBA* or *htrA* displayed remarkably uniform bacterial populations with mean fluorescence intensities of 28.65 and 21.59 respectively, while integration into *clyA* resulted in a very low mean fluorescence intensity of 7.53, barely above the background autofluorescence of 5.94 detected for CVD 910 alone. Having established substantial expression of GFPuv from two independent chromosomal loci, we then tested the hypothesis that integration of P_{ompC} -*gfpuv* into both *guaBA* and *htrA* together would result in additive expression of fluorescence. Analysis of fluorescence from the resulting strain, CVD 910-2G, revealed an uninduced (50 mM NaCl) mean fluorescence intensity of 36.01, which increased to 48.21 after induction with 200 mM NaCl. In this experiment, uninduced fluorescence intensities for CVD 910-GG and CVD 910-HG were 25.35 and 15.85 respectively, while induced fluorescence levels were 32.46 and 24.03 respectively. It is immediately evident that for overnight liquid cultures, cumulative fluorescence observed with 2 copies of *gfpuv* integrated into CVD 910-2G is approximately equivalent to the combined fluorescence levels for individual copies of integrated *gfpuv* observed in CVD 910-GG and CVD 910-HG, under both uninduced and induced osmotic conditions.

Growth-phase regulated expression of GFPuv in CVD 910-2G. We have previously hypothesized that regulated but sustained expression of foreign antigens delivered by live vectors will reduce any metabolic burden associated with antigen synthesis, thereby allowing live vectors to persist longer in immunized hosts and prolong delivery of candidate vaccine antigens to the immune system.²¹ However, we have suspected that despite recent improvements, tightly regulated and appropriately timed antigen expression using plasmid-based expression technologies still remains elusive in many cases, with leaky expression potentially contributing to over-attenuation of live vector vaccine strains. Therefore, one of the goals of the current work was to investigate the feasibility of linking foreign antigen expression to the growth phase of the live vector, such that expression would be reduced when bacteria are adapting to a significant change in environmental conditions (i.e., lag phase), but would be strongly induced after bacteria have successfully adapted their metabolism to new

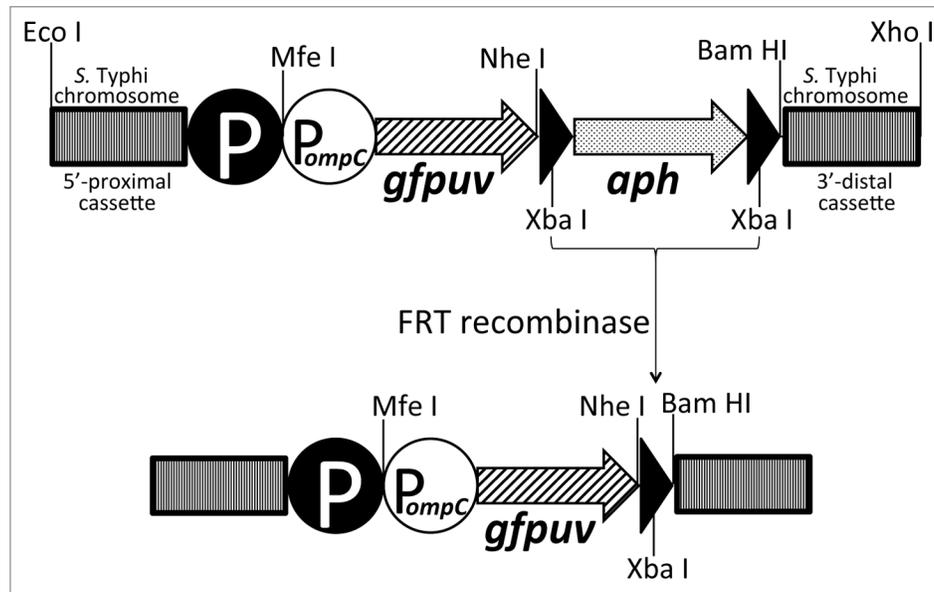


Figure 1. Schematic depiction of the strategy for chromosomal integration of the foreign antigen cassette P_{ompC} -*gfpuv*, encoding the model fluorescent antigen GFPuv. Details of this approach are thoroughly presented in the Materials and Methods. Briefly, an osmotically controlled GFPuv-encoding cassette (tandem white circle and hatched thick arrow) was constructed and linked to an *aph* marker encoding resistance to kanamycin (shaded thick arrow), flanked by FRT recombination sites (black triangles). The incoming P_{ompC} -*gfpuv*-*aph* cassette was integrated into the live vector chromosome using the λ Red recombination system, followed by removal of the *aph* marker using FLP recombinase, to yield the final live vector strain bearing no genes encoding resistance to antibiotics. The bacterial chromosome is represented by 5'-proximal and 3'-terminal darkened rectangles, and the black circle labeled with a "P" represents the wildtype chromosomally encoded promoter of the deleted target open reading frame (i.e. either *guaBA* or *htrA*).

energy sources and environmental conditions (i.e., exponential growth transitioning into stationary phase). We first compared chromosomally encoded GFPuv expression in CVD 910–2G to a previously described live vector CVD 908-*htrAssb*(pGEN206),³ in which GFPuv is expressed independently of growth phase from a low copy (~5 copies per chromosomal equivalent) stabilized expression plasmid. Overnight starter cultures of CVD 910–2G and CVD 908-*htrAssb*(pGEN206) were grown at 37°C for approximately 16 h and then diluted 1:100 into 100 ml of fresh medium in 250 ml baffled flasks. To reduce the influence of osmolarity on growth phase and more clearly establish any link between observed fluorescence and induction of P_{guaBA} and P_{htrA} during growth, all strains were grown under non-inducing conditions of 50 mM NaCl. Fresh cultures were incubated at 37°C/ 250 rpm, and 5 ml aliquots were removed every hour for 7 h to measure both OD₆₀₀ and fluorescence intensities by flow cytometry. As expected, plasmid-based expression in CVD 908-*htrAssb*(pGEN206) significantly slowed the growth kinetics of the live vector when compared with either CVD 910 or CVD 910–2G, even under non-inducing conditions of 50 mM NaCl (Table 1). Initial fluorescence intensities in lag phase started out quite high at 1262.66, dipped during exponential phase to 686.27, and then rose again to 1131.59 in stationary phase. In sharp contrast, the kinetics of GFPuv expression in CVD 910–2G was closely linked to the growth phase of the culture, with a low mean fluorescence intensity of 81.19 measured in the lag phase, which gradually increased with cell density to a maximum fluorescence intensity of 200.06 as the culture reached stationary phase. We stress here that the observed variation of fluorescence

with growth phase, as quantitated by flow cytometry, is not an aggregate effect of increasing cell numbers, but instead reflects the level of GFPuv synthesis within individual bacteria in a growth-rate dependent manner. These data support the feasibility of chromosomal expression of a foreign antigen from multiple integration sites, and the possibility of antigen expression synchronized with growth-rate, a possibility not supported by plasmid-based expression in these experiments.

We then repeated this experiment to compare GFPuv expression from double integrations in CVD 910–2G to single integration expression levels in CVD 910-GG and CVD 910-HG. As summarized in Table 2, we again observed growth phase-dependent expression of fluorescence intensity, increasing from an initial lag phase level of 38.31 to a high of 161.65 in stationary phase. Interestingly, fluorescence levels during the 3 h lag phase for the double integration did not reflect the sum of fluorescence observed with single integrations during this period, but became additive as the cultures progressed into exponential and stationary phases. Fluorescence intensities from single integrations did not seem to reflect the same dependence on growth phase as observed for the double integration; intensities for the *guaBA* integration in CVD 910-GG progressed from 74.94 to 96.31 during growth while *htrA*-controlled fluorescence in CVD 910-HG progressed from 32.90 to 68.94. Despite this anomaly, the data reported here suggest that integration of foreign antigen cassettes into multiple loci within a live vector chromosome can be accomplished without further attenuation of the vaccine strain, and that this multiple integration strategy results in superior expression levels of foreign antigens vs. conventional integration into a single locus.

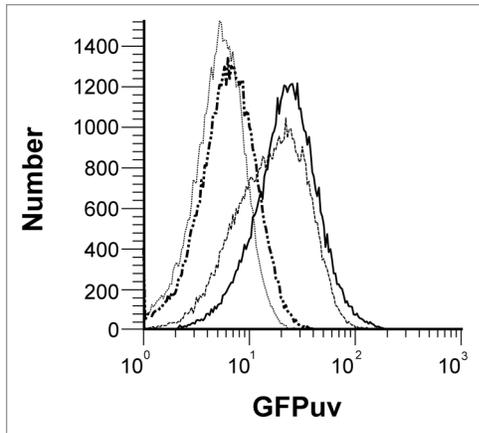


Figure 2. Flow cytometry histograms of GFPuv-mediated fluorescence encoded by P_{ompC} -*gfpuv* gene cassettes integrated into either the *guaBA* (thick solid line), *htrA* (thin hatched line) or *clyA* (thick broken line) sites of the attenuated *S. Typhi* live vector vaccine candidate CVD 910, compared with the vaccine strain alone (thin dotted line). Fluorescence intensities are measured for individual bacterial cells grown under inducing conditions of 200 mM NaCl in rich medium at 37°C/250 rpm for 16 h.

Discussion

Live vectors engineered for delivery of foreign antigens to the host immune system have performed exceedingly well in experimental animal models, but have been only modestly successful in clinical trials.²¹ Given the advances in the development of powerful plasmid-based expression technologies designed to deliver ample levels of foreign protein, it is unlikely that the lack of antigen-specific immunity observed in clinical trials is due to insufficient antigen synthesis following immunization. To the contrary, we hypothesize that inappropriate antigen synthesis occurring *in vivo* results in sufficient shock to the metabolism of the live vector to over-attenuate the strain and destroy immunogenicity. Although various attempts have been made to control the timing of foreign protein synthesis, using tightly regulated promoters to control transcription of genes in response to host environmental signals, improved immunogenicity in animals has not translated into improvements in clinical trials.^{13,22,23} Here we present an alternative to this dilemma, in which we attempt to circumvent over-attenuation by linking antigen synthesis with the growth rate of the live vector vaccine, such that synthesis is initially reduced after immunization, but steadily increases as the vaccine strain adjusts to prevailing environmental conditions and undergoes limited replication within the host.

We are pursuing an expression strategy designed to facilitate efficient expression of multiple foreign antigens within a single live vector vaccine strain, which complements plasmid-based methods by distributing the location of foreign gene cassettes between the chromosome and an expression plasmid. This approach offers the flexibility of independently adjusting the copy number of potentially toxic foreign genes by integrating a designated number of copies into the chromosome. By appropriate integration of foreign genes into chromosomal loci whose

induction of expression is intimately associated with the physiology and growth rate of the vaccine strain, it becomes theoretically possible to “tune” foreign antigen synthesis to the metabolic state of the live vector.

To investigate this approach, we examined expression of the model fluorescent antigen GFPuv, and compared plasmid-based expression from a low copy genetically stabilized expression plasmid to stable expression from both the *guaBA* and *htrA* sites in the chromosome of an attenuated *S. Typhi* live vector vaccine strain. High levels of plasmid-encoded fluorescence were observed in live vectors, which adversely impacted the growth rate even under non-inducing conditions. In contrast, gradually increasing levels of fluorescence were observed for chromosomally encoded GFPuv, which correlated well with growth rate and exerted no apparent effect on fitness when compared with the growth of empty live vector CVD 910. As expected, chromosomal expression of GFPuv from two sites was not equivalent to expression from a plasmid with a copy number of five per chromosomal equivalent, but our results clearly suggest that with appropriate integration of additional copies of *gfpuv*, we may be able to achieve high level expression of a foreign antigen which remains linked to growth rate without further attenuating the live vector. Toward this goal, we have integrated a third copy of *gfpuv* into the *rpoS* site (which is naturally inactivated in the parent Ty2 *S. Typhi* strain used to construct CVD 910); initial results show an additional increase in GFPuv fluorescence which continues to correlate well with growth rate without further attenuation of the vaccine (data not shown). In addition, we have applied these observations to development of a multivalent live vector vaccine against plague, in which one key protective antigen LcrV (an essential type III secretion virulence protein) is integrated into both *guaBA* and *htrA* while the remaining protective capsular antigen F1 is expressed from a low copy plasmid. Mouse immunogenicity and challenge experiments are underway to determine if a multivalent live vector vaccine can be successfully engineered using both chromosomal and plasmid-based expression of foreign antigens to confer protection against potentially devastating infections with *Yersinia pestis*.

Materials and Methods

Bacterial strains and culture conditions. The attenuated *S. enterica* serovar Typhi live vector vaccine strain CVD 910 used in these studies is an auxotrophic derivative of wild-type strain Ty2, with deletions in *guaBA* and *htrA*. To improve the clinical acceptability of our live vector vaccine strains, all genetic and bacteriologic manipulations of the live vectors were performed using an animal product-free medium equivalent to Luria-Bertani medium, comprised of 10 g/liter of Soytone (Teknova; S9052), 5 g/liter Hy-Yest 412 (Sigma; Y1001) and 3 g/liter NaCl (American Bioanalytical; AB01915), supplemented with 0.002% guanine (Sigma; G6779).

Construction of chromosomal integrations. We constructed deletion cassettes for use with the λ Red-mediated site-directed mutagenesis method¹⁷ to delete either *guaBA*, *htrA* or *clyA* from wildtype *S. Typhi* Ty2. Cassettes encoding upstream and

Table 1. Chromosomal vs. plasmid-based expression of GFPuv in attenuated *Salmonella* Typhi live vectors

Time (hr)	CVD 910		CVD 910-2G (<i>guaBA::gfpuv htrA::gfpuv</i>)		CVD 908 <i>htrAssb</i> (pGEN206S2)	
	OD ₆₀₀ ^a	MFI ^b	OD ₆₀₀	MFI	OD ₆₀₀	MFI
0	0.04	ND ^c	0.04	ND	0.04	ND
1	0.07	ND	0.08	81.19	0.06	1262.66
2	0.27	ND	0.3	96.77	0.14	1196.59
3	0.71	ND	0.71	105.59	0.38	721.34
4	1.36	ND	1.36	182.77	0.72	686.27
5	1.88	ND	1.86	ND	1.25	ND
6	2.18	6.34	2.18	169.87	1.67	891.53
7	2.29	ND	2.29	200.06	1.95	1131.59

Table 2. Growth-phase regulated chromosomal expression of GFPuv in CVD 910 attenuated *Salmonella* Typhi live vectors

Time (hr)	CVD 910		CVD 910-GG (<i>guaBA::gfpuv</i>)		CVD 910-HG (<i>htrA::gfpuv</i>)		CVD 910-2G (<i>guaBA::gfpuv htrA::gfpuv</i>)	
	OD ₆₀₀ ^a	MFI ^b	OD ₆₀₀	MFI	OD ₆₀₀	MFI	OD ₆₀₀	MFI
0	0.04	ND ^c	0.04	ND	0.03	ND	0.02	ND
1	0.09	ND	0.09	74.94	0.06	32.9	0.06	38.31
2	0.33	ND	0.3	71.03	0.24	49.08	0.24	72.53
3	0.81	ND	0.72	70.58	0.68	56.12	0.6	95.41
4	1.45	ND	1.31	75.26	1.29	60	1.36	121.95
5	1.96	ND	1.86	84.81	1.84	66.55	1.86	138.01
6	2.24	5.87	2.17	96.31	2.19	68.94	2.16	161.65

^aCultures grown under non-inducing conditions in 50 mM NaCl; ^bMean Fluorescence Intensity; ^cNot Determined.

downstream flanking chromosomal sequences were constructed using primer pairs listed in Table 3 and purified chromosomal DNA from Ty2 as the template DNA. These cassettes were used to exchange chromosomal targets with a Tn5 neomycin phosphotransferase cassette (*aph*), encoding resistance to kanamycin and recombined into the chromosome using the λ Red recombination system encoded by pKD46. Final removal of the kanamycin resistance cassette was accomplished using FLP recombinase encoded by pCP20. We confirmed the integrity of the intended chromosomal deletion mutations by DNA sequence analysis of the chromosomal locus from each strain using PCR primers listed in Table 3. For chromosomal expression of GFPuv, we chose to integrate a gene cassette in which an osmotically regulated *ompC* promoter ($P_{ompC}^{ref.24}$) was linked to *gfpuv* and inserted 5'-proximal to the *aph* resistance marker of chromosomal deletion cassettes. As shown in Figure 1, care was taken to preserve the natural chromosomal promoters controlling transcription of chromosomally encoded targets, with the intent that synthesis of GFPuv would ultimately be controlled both by osmolarity (via P_{ompC}) as well as growth rate in the case of the *guaBA* locus,¹⁶ heat shock/environmental stress in the case of the *htrA* locus,¹⁹ or possibly low pH for *clpA*.²⁰

Flow cytometry. GFPuv-expressing strains were grown overnight at 37°C on rich solid medium supplemented with guanine. 2–3 fluorescing colonies were then inoculated into 20 ml of supplemented liquid medium and incubated with shaking at 250 rpm overnight at 37°C. Overnight starter cultures were then

diluted 1:100 into fresh supplemented liquid medium and incubated at 37°C, 250 rpm. For growth curve studies, 5 ml volumes were periodically removed from incubating cultures, from which bacteria from 4 ml were pelleted, while the remaining 1 ml volume was used to measure the optical density at 600 nm (OD₆₀₀). Pelleted bacteria were resuspended in 1 ml of PBS, and cells then diluted 1:1,000 in PBS prior flow analysis. Quantitation of GFPuv fluorescence was analyzed using a MoFlo Legacy flow cytometer/cell sorter system (Beckman Coulter) with the argon laser exciting bacteria at 488 nm and emissions detected at 525 nm. Forward vs. side light scatter, measured with logarithmic amplifiers, was used to gate on bacteria. A minimum of 50,000 events were acquired from each sample at a collection rate of approximately 3,500 events per second. The mean fluorescence intensity was determined using Summit software (Beckman Coulter). Background autofluorescence was determined using the negative control *S. Typhi* vaccine strain CVD 910.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Table 3. Primers used in the construction and testing of live vector strains expressing chromosomally encoded GFPuv

Primer	Sequence ^a
5guaBA-for	5'- <u>GAA TTC</u> TAG CTG CTC ATA CTT CTG CTG CA -3'
5guaBA-rev	5'- <u>GCT AGC CAA TTG</u> GGG CAA TAT CTC ACC TGG -3'
3guaBA-for	5'- <u>GGA TCC</u> ACT AGT GTC GAT AAC CCT TCC TGT GT -3'
3guaBA-rev	5'- <u>CTC GAG</u> ACA GCA CCT ACA AGT CTG GCA TG -3'
guaBA PCR-for	5'- GCG CTG ACC ACC GGA ATA CGG CTG -3'
guaBA PCR-rev	5'- CAT GGC ATG GAT GAG GCA ACC GCG AAG C -3'
5htrA-for	5'- <u>GAA TTC</u> GTA CCT TCA ATC AGG CGT TAC TGG AAG ATG -3'
5htrA-rev	5'- <u>GCT AGC CAA TTG</u> CGA TTA ACA GGT AAC GCA AAA TTG CTG TGT ACG TCA G -3'
3htrA-for	5'- <u>GGA TCC</u> ACT AGT CTG CGT AAG ATT CTC GAC AGC AAG CCG TCG GT -3'
3htrA-rev	5'- <u>CTC GAG</u> CCA GCA TCA TTT CGG CAG TCA TAC ACA CCA GTT CGC -3'
htrA PCR-for	5'- GTG TCG CCG ATC TTG AAG ACG CGG TAG AG -3'
htrA PCR-rev	5'- CTA TCG ACG CCA AGC TGG CCG CTG TCG AC -3'
5clyA-for	5'- TAG TAA TGA <u>GAA TTC</u> GCT GGT ATT GAT CGG CTC TCC GGT AGA GAT TAG CGA -3'
5clyA-rev	5'- <u>GCT AGC CAA TTG</u> TGC CTC TTT AAA TAT ATA AAT TGC AAT TAA GTA CCT G -3'
3clyA-for	5'- <u>GGA TCC</u> ACT AGT GAT ACA TTT TCA TTC GAT CTG TGT ACT TTT AAC GCC CGA TAG CG -3'
3clyA-rev	5'- TGA TAG TAA <u>CTC GAG</u> ACA ATC CAT AAG AAA GGT CAG GCA CAC TGG GAA GGC GAC ATC -3'
clyA PCR-for	5'- CAT GAT GGT ATC CAG TAT GGC ACA AGC -3'
clyA PCR-rev	5'- GTA ATC GAC AAC ATG CTA CAT CCA TCG -3'
5FRT-aph-for	5'- <u>GAA TTC GCT AGC</u> GCT GGA GCT GCT TCG AAG TTC -3'
3FRT-aph-rev	5'- <u>CTC GAG TTC CGG GGA TCC</u> GTC GAC CTG CAG TTC -3'
5gfpuv	5'- <u>CAA TTG</u> TGT GGT AGC ACA GAA TAA TGA AAA GT -3'
3gfpuv	5'- <u>GCT AGC</u> TCA TTA TTT GTA GAG CTC ATC CAT -3'

^aRelevant restriction sites are underlined.

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