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Use of stabilized luciferase-expressing plasmids to examine *in vivo*-induced promoters in the *Vibrio cholerae* vaccine strain CVD103-HgR

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Abstract

Live, attenuated *Vibrio cholerae* vaccines can induce potent immune responses after only a single oral dose. The strategy of harnessing these strains to present antigens from heterologous pathogens to the mucosal immune system shows great promise. To fully realize this possibility, *V. cholerae* strains must be created which stably express antigens *in vivo* in sufficient quantity to generate an immune response. *In vivo*-induced promoters have been shown to increase stability and immunogenicity of foreign antigens expressed from multicopy plasmids. We report the construction of a series of genetically stabilized plasmids expressing luciferase as a heterologous protein from the following *in vivo*-induced promoters: *V. cholerae* P_{argC}, P_{fhuC}, and $P_{vca1008}$, and *S.* Typhi P_{ompC}. We demonstrate that several of these expression plasmids meet two critical criteria for *V. cholerae* live vector vaccine studies. First, the plasmids are highly stable in the *V. cholerae* vaccine strain CVD103-HgR at low copy number, in the absence of selective pressure. Second, real time bioluminescent imaging (BLI) demonstrates inducible *in vivo* expression of the promoters in the suckling mouse model of *V. cholerae* colonization. Moreover, the use of BLI allows for direct quantitative comparison of *in vivo* expression from four different promoters at various timepoints.

Keywords

Vibrio cholerae; bioluminescent imaging; promoter; vector caccine; *in vivo* expression

INTRODUCTION

Live attenuated bacterial vaccines offer a promising opportunity to deliver heterologous antigens to the immunological inductive sites involved in a wild type human infection. Several species of enteric bacteria including *Salmonella enterica* serovar Typhi (*S.* Typhi) and *Shigella flexneri* have been used to express antigens from a wide variety of unrelated pathogens. Delivering antigens in this way induces immune responses against both the bacterial host strain and the foreign antigen(s), and has the advantage of oral delivery, increasing safety and compliance.

Vibrio cholerae offers several potential advantages as a live vector vaccine: 1) *V. cholerae* is non-invasive, but induces excellent mucosal and systemic immune responses often after just one dose of live vaccine; 2) Most attenuated strains of *V. cholerae* maintain the highly immunogenic B subunit of the primary enterotoxin, cholera toxin, which has been

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demonstrated to be an excellent adjuvant for heterologous antigens (Holmgren et al., 2005); 3) Attenuated strains of *V. cholerae* expressing and secreting several antigens can elicit heterologous immunity that is protective in animal models, [reviewed in (Silva et al., 2008)]; and 4) Several live attenuated strains have been demonstrated to be safe and immunogenic in human subjects [reviewed in (Tacket and Sack, 2008; Holmgren and Kaper, 2009)], including one licensed for human use, CVD103-HgR. The safety and immunogenicity of CVD103-HgR has been established in a number of randomized, placebo-controlled, double-blind clinical trials throughout the world, which makes it an ideal vaccine platform for expression and delivery of heterologous antigens (Cryz, Jr. et al., 1990; Cryz, Jr. et al., 1992; Tacket et al., 1992; Kotloff et al., 1992; Cryz, Jr. et al., 1995; Tacket et al., 1999).

A critical balance must be maintained in a live vector vaccine whereby sufficient levels of foreign antigen are expressed to elicit an immune response without over-attenuating the bacterial host strain (Galen and Levine, 2001; Bumann, 2001). Two major factors influencing expression of genes encoding heterologous antigens are plasmid copy number and choice of promoter. Foreign genes expressed from multicopy plasmids may be unstable *in vivo*. One way to enhance the stability of plasmid-based heterologous expression systems is to use low-copy number plasmids. However, this raises concerns about producing enough heterologous protein *in vivo* to mount an immune response.

An additional factor in plasmid stability is the choice of promoter for heterologous protein expression. Several promoters have been used previously to express heterologous antigens in *V. cholerae* vaccine strains, including constitutively active *tac*, *lac* and *trc* promoters. However, constitutive expression of high levels of foreign antigen may be toxic to the live vector. The use of *in vivo*-inducible (*ivi*) promoters provides a possible solution to the problem of toxicity or metabolic burden of the heterologous antigen on the host bacterial strain. *ivi* promoters that exhibit low expression *in vitro* allow for optimal growth conditions for preparation of inocula, while maximizing expression *in vivo*. Early examples of *V. cholerae* promoters used to express foreign antigens include heat-shock-regulated *htpG* (Butterton et al., 1997; John et al., 2000), iron-regulated *irgA* (John et al., 2000), anaerobically induced *nirB* (Chen et al., 1998; Fontana et al., 2000) and the cholera toxin promoter (Fontana et al., 2000; Liang et al., 2003). Several of these strains elicited immune responses against the heterologous antigen, but plasmid instability *in vivo* was suspected to reduce immunity.

Several genomic-level screens have identified new genes that are activated *in vivo* after *V. cholerae* infection of animals and humans (Chiang and Mekalanos, 1998; Lee et al., 2001; Merrell et al., 2002a; Merrell et al., 2002b; Hang et al., 2003; Osorio et al., 2005; Larocque et al., 2005; Larocque et al., 2008). One recent *in vivo* expression technology (IVET) study conducted in humans led to the identification of several previously unreported and highly inducible *ivi* promoters with great potential for use in a foreign antigen expression system (Lombardo et al., 2007). Here we exploit several of these novel *ivi* promoters for expression of bioluminescence using genetically stabilized expression plasmids, originally developed for use in attenuated *S*. Typhi and *S. flexneri* live vectors (Galen et al., 1997; Galen et al., 1999; Altboum et al., 2001; Altboum et al., 2003; Stokes et al., 2007). Bioluminescent imaging (BLI) is a highly sensitive technique used previously in mice to follow colonization dynamics non-invasively, leading to the identification of previously unknown sites of pathogen colonization (Hardy et al., 2004; Wiles et al., 2006). Here we use this powerful imaging technique to quantitatively compare the *in vivo* induction of several *V. cholerae ivi* promoters previously identified by IVET to be induced in human volunteers.

METHODS

Bacterial strains and growth conditions

CVD103-HgR (O1, Classical biotype) was used for all expression studies. N16961 (O1, El Tor biotype) was used as a template for PCR amplification of promoter sequences. Cells were grown in 1× M9 salts plus 20% glucose as a carbon source, LB, MOPS or EZ-Rich (Teknova). Kanamycin was used at a concentration of 50 μg mL^{-1} to maintain plasmids *in vitro*, except where noted.

Construction of luciferase expression plasmids

Construction of pOMP15lux, pOMP5lux, and pOMP60lux—All plasmids are listed in Table 1. Because our intention was to insert several different promoters upstream of the *lux* operon, our first goal was to create an easily replaceable cassette to move promoters into and out of this plasmid. To this end, pGEN-*lux*CDABE (a generous gift from M. Lane and H. Mobley) was digested with *Eco*RI and *Sna*BI to remove the EM7 promoter, which was replaced by P_{ompC} from pSEC84. To accomplish this, P_{ompC} was removed from pSEC84 by digestion with *Spe*I, end-filled with T4 DNA polymerase and subsequent digestion with *Eco*RI. This P_{ompC} fragment was then ligated into pGEN-*lux*CDABE to create pOMP15lux. We then subcloned the entire P_{ompC}-lux fragment from pOMP15lux as a ~6500bp *Eco*RI - *Nhe*I cassette into appropriately digested pSEC10 (*ori*101) and pSEC84 (*ori*E1) to create pOMP5lux and pOMP60lux, respectively.

Construction of pCM10—To reduce background luminescence resulting from read-through transcription into the *lux* operon, a copy of the *rrnB* terminator was PCR amplified from pSE380 (Invitrogen) using primers K5917 and K5918 (Table 2) to construct a cassette flanked at the 5′-terminus by *Mfe*I and at the 3′-terminus with *Eco*RI and *Bam*HI sites. The resulting PCR product was ligated into pOMP5lux digested with *Eco*RI and *Bam*HI, creating the 5-copy pCM10 promoterless negative control plasmid, which was confirmed to exhibit extremely low background luminescence.

Construction of 5- and 60-copy expression plasmids—P_{argC}, P_{fhuC}, and $P_{vca1008}$ promoters were PCR amplified from wildtype *V. cholerae* N16961 genomic DNA to create cassettes with 5′-terminal *Eco*RI-*Not*I sites and *Bam*HI at the 3′ terminus. Primers used are listed in Table 2. Promoter fragments were inserted as *Eco*RI - *Bam*HI cassettes into pCM10 digested with the same enzymes, thereby creating 5-copy plasmids with the *argC* (pCM11), *fhuC* (pCM13), and *vca1008* (pCM14) promoters. The *ompC* promoter was also removed from pSEC84 as an *Eco*RI - *Bam*HI cassette and inserted into appropriately digested pCM10 to create the 5-copy pCM17. The terminator-promoter cassette from pCM10, pCM11, and pCM14 was then removed by digestion with *Xho*I and *Bam*HI and ligated into pOMP60lux digested with the same enzymes to create the 60-copy isogenic plasmids pCM18, pCM19, and pCM22 respectively. The expected nucleotide sequence of all expression plasmids was verified by sequence analysis.

In vitro induction studies

Expression plasmids were introduced into *V. cholerae* by electroporation as previously described (Marcus et al., 1990). For experiments reported in Figure 1, plasmid-bearing *V. cholerae* live vectors were subcultured 1:500 from overnight cultures (using 2–3 colonies from freshly streaked plates) into fresh medium and added in triplicate (200 μL per well) to an optical 96-well plate. Absorbance and luminescence of 200 μL cultures grown at 37°C in an optical 96-well plate were measured at 20 min intervals using a Synergy HT luminometer. Triplicate or quadruplicate samples were assayed for each strain and medium condition.

For experiments reported in Figure 2, overnight cultures grown from 2–3 freshly isolated colonies were diluted 1:100 into fresh LB and grown to mid-log phase (OD_{600} ~0.4). Several 1 mL portions of each culture were pelleted and resuspended in an equal volume of minimal medium supplemented either with 0.5 mM arginine, 50 μM DIP, or 50 μM $FeSO_4$ and added in triplicate (200 μL per well) to an optical 96-well plate. Bioluminescence and absorbance were again measured every 20 min using a Synergy HT luminometer.

In vitro stability studies

In vitro stability experiments were performed essentially as described by Fang et al. (Fang et al., 2008). Briefly, 2–3 freshly isolated colonies were used to establish starter cultures grown at 37°C in LB without selection. 24 hours later the cultures were diluted and plated onto non-selective medium (Day 0) and re-inoculated into fresh LB at a1:100 dilution. This process was repeated each day for up to 5 days. Plate images were acquired using an IVIS 200 imaging system at small binning for 10–30 sec to determine the percentage of luminescent colonies.

In vivo BLI

Freshly isolated colonies were resuspended in 10 mL of PBS and adjusted to an OD_{600} of 0.2 (~1×10^8 CFU mL^{-1}). Evans blue dye (40 μL) was then added to 1 mL of each culture. 4–5 day old CD-1 mice were isolated one hour prior to inoculations. Mice were individually anesthetized using 3% isoflurane, intragastrically inoculated with 50 μL of culture through flexible silicon tubing, and imaged immediately for 1 min with medium binning in an IVIS 200 imaging system. Luminescence within the mice was calculated by generating an identically-sized Region of Interest (ROI) circle for all mice using Living Image 3.0 software. Total photon flux (photons sec^{-1}) was used for all calculations. At various time points, the mice were again anesthetized and imaged individually with the same parameters. At the final time point, mice were euthanized immediately after imaging by cervical dislocation and their intestines dissected and submerged in 2 mL PBS + 10% glycerol. After homogenization, the intestinal samples were diluted and plated to determine colony forming units (CFU). Colony plates were imaged as described above to confirm *in vivo* stability of plasmids. This animal protocol was approved by the Institutional Animal Care & Use Committee at the University of Maryland School of Medicine.

RESULTS AND DISCUSSION

Construction of plasmids expressing luciferase under *ivi* promoter control

To assess the expression of *ivi* promoters *in vivo*, we elected to use *lux*-mediated bioluminescence as a reporter. Bioluminescence is a useful and quantitative tool for studying gene expression of pathogens *in vivo* in animal models of infection [reviewed in (Contag, 2008)]. Foreign antigens delivered by live vectors are usually expressed using multicopy plasmids such that high levels of antigen are presented to the immune system. However, such plasmids are often unstable and we therefore elected to express the *lux* operon from genetically stabilized expression plasmids. These plasmids encode a maintenance system comprising the Hok-Sok post-segregational killing function and two plasmid partitioning loci to enhance plasmid inheritance (Galen et al., 1999; Lane et al., 2007). The plasmid pGEN-*lux*CDABE (P_{em7},~15 copies/cell, (Lane et al., 2007)) was re-engineered for this study to create a removable promoter cassette and a copy of the *rrnB* terminator was inserted upstream of the promoter cassette to eliminate background luminescence observed in the absence of the terminator (data not shown). This re-engineered terminator-*Eco*RI-P_{neg}-*Bam*HI-*lux* operon cassette was then introduced into low copy (~5-copies/cell) and high copy (~60-copies/cell) genetically stabilized plasmids creating promoterless negative controls ($P_{neg}5$ and $P_{neg}60$).

We then replaced P_{neg} with one of three *ivi* promoters identified by IVET in human volunteers (Lombardo et al., 2007). Two of the promoters, P_{argC} and $P_{vca1008}$, had also been previously identified in a suckling mouse IVET study (Osorio et al., 2005; Lombardo et al., 2007). These two promoters are induced in the suckling mouse model at levels of 700- and 770-fold, respectively, compared to growth *in vitro* in rich broth medium. We chose a third promoter from the human IVET study, P_{fhuC}, because a strain with a mutation in this gene was shown in follow-up experiments to display a colonization defect in the suckling mouse intestine. A fourth promoter, P_{ompC}, from *E. coli* was also included in our studies. OmpC is a porin that is present in many gram-negative bacteria, but not in *V. cholerae*. In *E. coli*, *ompC* expression is induced by high osmolarity and it encodes one of the most highly expressed outer membrane proteins. P_{ompC} has been used successfully in both attenuated *S.* Typhi and *Shigella* strains to express heterologous antigens and has been shown to induce immune responses in animal models (Altboum et al., 2001; Altboum et al., 2003; Galen et al., 2004; Stokes et al., 2007).

All plasmids were electroporated into a spontaneous streptomycin-resistant derivative of the attenuated *V. cholerae* strain CVD103-HgR. The safety and immunogenicity of CVD103-HgR has been tested in thousands of individuals, including children and persons with HIV, making it an ideal *V. cholerae* vector vaccine [Reviewed in ((Tacket and Sack, 2008)].

Evaluating *in vitro* expression

A major goal in live vector vaccine construction is to limit expression of the heterologous antigen *in vitro* to minimize selective pressure for plasmid loss, while maintaining high levels of expression *in vivo* to induce an immune response. The three *V. cholerae ivi* promoters were previously shown to have low levels of expression *in vitro* on rich medium, but expression was detected from a single chromosomal copy, using a very different indirect reporter assay (Osorio et al., 2005; Lombardo et al., 2007). Thus, luciferase expression from the plasmid constructs was first assessed *in vitro*. Expression from all bioluminescent reporter plasmids was initially monitored using a defined minimal medium (MOPS) versus a rich medium (EZ-Rich). Light output was measured and normalized to optical density in a 16-hour kinetic study. As expected, the promoterless 5- and 60-copy plasmids ($P_{neg}5$ and $P_{neg}60$) showed low background luminescence in both media (Figure 1A and B). Strains expressing luciferase under the control of P_{ompC} displayed constitutively high expression compared to the promoterless negative control in all media tested (Figure 1A and B). All strains showed a dramatic decrease in expression upon entering stationary phase. This phenomenon, observed by several groups, is termed Abrupt Decline of Luciferase Activity (ADLA) (Koga et al., 2005; Galluzzi and Karp, 2007). The cause of ADLA seems to be a decrease in the availability of reduced flavin mononucleotide ($FMNH_2$), which is the direct electron donor for the bacterial luciferase reaction.

The arginine biosynthesis pathway is well characterized in *E. coli* [Reviewed in (Cunin et al., 1986; Charlier and Glansdorff, 2004)]. The *argCBH* operon, encoding proteins involved in arginine biosynthesis is negatively regulated by the ArgR repressor, which binds to ARG boxes within the promoter sequence. Additionally, like many other amino acid biosynthesis genes, the arginine regulon is positively regulated by the stringent response, a multifaceted physiological response in the face of starvation [Reviewed in (Cunin et al., 1986; Cashel et al., 2009)]. Thus the full gamut of arginine regulation in *V. cholerae* can be predicted as follows: full expression in the absence of arginine and under starvation conditions, intermediate expression in the presence of arginine and under starvation conditions, and complete repression in the presence of arginine in rich medium. Consistent with this model, in our kinetics experiments we saw the highest luciferase activity in both P_{argC}-lux5 and P_{argC}-lux60 in minimal medium (Figure 1A). In additional experiments, cultures were initially grown in rich medium, pelleted and resuspended in various media. For the 5-copy

plasmid, repression is very quickly alleviated by growth in minimal medium, but is maintained with as little as 0.5 mM arginine in minimal media (Figure 2A), whereas for the 60-copy plasmid, addition of the same amount of arginine only partially repressed bioluminescence (Figure 2B). Increasing the amount of arginine to 5 mM did not increase the repression (data not shown). However, full repression of the 60-copy plasmid was achieved in rich medium (Figure 2B). These data confirm previous findings that *V. cholerae* P_{argC} exhibits low expression *in vitro*.

The *fhuCDB* operon is involved in ferrichrome iron utilization and is part of the Fur regulon (Rogers et al., 2000). At high concentrations in the bacterium, iron binds to Fur, which then binds to Fur boxes in the promoter regions of iron-regulated genes and prevents transcription. The consensus sequence of the Fur box in *V. cholerae* is similar to that of *E. coli* (Mey et al., 2005). In our experiments, the P_{fhuC} promoter was only examined using the 5-copy replicons, and showed relatively constant expression *in vitro* in minimal and rich media that was higher than the promoterless negative control strain (Figure 1A and B). Kinetic experiments in other media showed high expression in minimal medium containing 50 μM 2,2-dipyridyl, which limits iron availability, and low expression in minimal medium containing 50 μM $FeSO_4$ (data not shown). Additionally, in induction experiments, despite high basal level expression in rich LB medium, P_{fhuC} was repressed by the addition of 50 μM FeSO4 to minimal medium (Figure 2C). The design of the IVET study allowed for the inclusion of promoters with low activity *in vitro,* with higher expression *in vivo* (Lombardo et al., 2007). Because we are studying the promoter in a multicopy plasmid instead of the single chromosomal copy identified in IVET, we are more likely to see increased activity *in vitro*.

VCA1008 is a putative outer membrane protein that is necessary for infection of suckling mice (Osorio et al., 2004). The precise regulation of VCA1008 is unknown. Previous studies showed that *vca1008* is not induced *in vitro* in either minimal M9 or rich LB medium, but was induced *in vivo* in suckling mice using IVET techniques (Osorio et al., 2004). Our initial kinetic studies confirm these findings. Regardless of plasmid copy number or growth conditions, *in vitro* expression of bioluminescence from $P_{vca1008}$ was not detectable above promoterless control levels (Figure 1A and B). Expression could not be demonstrated in any other media tested, including M9 minimal medium, LB, DMEM tissue culture medium (low and high glucose), nor under ToxR-permissive conditions (LB pH 6.5 at 30°C) (data not shown). Additionally, expression could not be seen after infection of T84 intestinal cells (data not shown).

In vitro plasmid stability

Expression of heterologous proteins at high levels can decrease plasmid stability (Londono et al., 1996). Luciferase-expressing plasmids have had limited use *in vivo* until recently because of issues with stability. The pGEN-*lux*CDABE plasmid was shown by Lane et al. to be relatively stable in UPEC *in vitro* for up to 3 days with ~13% plasmid loss (Lane et al., 2007). We examined the *in vitro* stability of our plasmids in CVD103-HgR, expressing the *lux* operon from pGEN-*lux*CDABE under the transcriptional control of P_{argC}, P_{fhuC}, $P_{vca1008}$, and P_{ompC} from high or low copy plasmids. For live vectors passaged in liquid medium in the absence of antibiotic selection, the 5-copy expression plasmids all maintained 100% stability over a 5 day period, whereas the 60-copy plasmids were quickly lost after 2 days (Figure 3). Plasmid loss did not appear to correlate with high luciferase expression because all three 60-copy plasmids (no promoter, P_{argC}, and $P_{vca1008}$) express minimal levels of luciferase, while some of the 5-copy plasmids exhibit high levels of expression in rich media, including P_{fhuC} and P_{ompC} (Figure 1B). We therefore attribute plasmid loss to high copy-number, which has previously been suggested to decrease the fitness of the

bacterial vector strain due to the greater amount of energy required to maintain the DNA within the host cell (Glick, 1995).

Evaluating *in vivo* promoter induction

Suckling mice provide a model for colonization with *V. cholerae* strains, but their immune systems are too immature to examine the immunogenicity of vaccine strains [Reviewed in (Klose, 2000)]. Several groups have utilized various adult mouse models to study the immunogenicity of *V. cholerae* live vector vaccines with inconsistent results (Butterton et al., 1996; Chen et al., 1998). In this study we focused only on quantifying *in vivo* induction of *ivi* promoters controlling luciferase expression in suckling mice. Because the suckling mice are removed from their mothers, colonization studies are limited to 24 hours. We compared expression of the promoters at an early (4-hour) and late (24-hour) timepoint.

To study the induction of P_{argC}, P_{fhuC}, $P_{vca1008}$ and P_{ompC} *in vivo*, we inoculated 5-day old CD-1 mice with ~1×10^7 CFU of each strain and imaged the mice at 0, 4, and 24 hours post inoculation using an IVIS 200 imaging system. Mice were anesthetized and imaged individually for the best results. Representative images from each time point of a single mouse in each group inoculated with 5-copy plasmids are shown in Figure 4. Because the amount of bacteria per mouse was different at each time point, the total flux (photons sec^{-1}) was normalized to CFU mL^{-1} of either the initial inoculum (0-hour) or intestinal homogenates (4- and 24-hour). All strains tested colonized mice at similar levels (Figure 5).

Since the *argCBGH* operon was identified as being *in vivo*-induced by IVET studies in both suckling mice and human studies, displaying a 700-fold induction level in the suckling mouse intestine (Osorio et al., 2005), it appeared to be an excellent candidate for *in vivo* bioluminescence studies. In the suckling mouse intestine, P_{argC} in the 5-copy plasmid showed very low expression at the 0-hour timepoint and was induced 36-fold above that of the inocula at 4 hours and a further 544-fold above the inocula at 24-hours (Figure 6). The 60-copy plasmid had a higher background bioluminescence at the 0-hour time point but achieved one of the highest total expression levels of all constructs, reaching 42.3 vs. 5.1 $photons^{-1}$ sec^{-1} CFU^{-1} mL^{-1} for the 5-copy plasmid at 24-hours (Figure 6). Despite high levels of expression in the mouse intestine, the lack of stability *in vitro* in the absence of selective pressure (Figure 3) may ultimately detract from using P_{argC} with 60-copy plasmids for antigen expression in *V. cholerae* live vectors. The 5-copy plasmid, however, shows great promise for use in *V. cholerae* live vector strains because of its tight regulation and stability *in vitro* and high induction in the mouse intestine.

The *fhuACDB* operon was also identified as being *in vivo*-induced by IVET studies in both suckling mice and human studies, displaying induction late in the infection of suckling mice (Schild et al., 2007). By measuring bioluminescence, we demonstrated that there was substantial induction of P_{fhuC} in the suckling mouse intestine, with a 24-fold increase in expression above the inocula, but the induction was only statistically significant at the 24-hour time point (Figure 6), confirming the previous findings of Schild et al. Although expression of the *fhuC* promoter can be repressed *in vitro* by adding $FeSO_4$ to the medium, the basal level of expression is still quite high compared to P_{argC} and $P_{vca1008}$ (see below), and therefore may not be useful for the expression of possibly toxic antigens in *V. cholerae* live vectors.

As with P_{argC} and P_{fhuC}, the *vca1008* promoter was shown by IVET to be inducible *in vivo* in both suckling mice and human studies, exhibiting the highest induction of all promoters studied in the suckling mouse model (Osorio et al., 2005). Although we were unable to detect expression of luciferase from the *vca1008* promoter *in vitro*, consistent with previous findings (Osorio et al., 2004), we observed excellent induction *in vivo*. This promoter is

strongly induced in suckling mice using both the 5- and 60-copy replicons. Activation of the *vca1008* promoter occurred by 4-hours post infection and remained elevated at 24-hours (Figures 4 and 6). In additional experiments, expression from $P_{vca1008}$ was shown to occur as early as one hour post-inoculation, the earliest time point tested (data not shown). Interestingly, the maximum amount of bioluminescence was equivalent for both the 5- and 60-copy plasmids. We conclude that regulation of this promoter is exquisitely sensitive to the *in vivo* environment, as it exhibits virtually no leakiness of expression *in vitro* even when present in high-copy number, but shows excellent induction *in vivo*. $P_{vca1008}$ represents an intriguing candidate for achieving tightly regulated expression of heterologous antigens in *V. cholerae* live vector vaccines. In the case of expression of antigens that are toxic to the host strain, maximal repression could be attained *in vitro* during preparation of the strain for immunizations, possibly ensuring better immunogenicity due to improved fitness of the live vector.

As discussed above, since the *E. coli* OmpC protein does not have an orthologue in *V. cholerae*, we were uncertain as to whether P_{ompC} would be induced *in vivo* in *V. cholerae*. Surprisingly, we observed a 100-fold induction of P_{ompC} in the sucking mouse intestine. Indeed, P_{ompC} showed the highest total expression of bioluminescence for the 5-copy replicons, with an average of 44.8 photons^{-1} sec^{-1} CFU^{-1} mL^{-1}, a level equivalent to expression levels from P_{argC} carried by 60-copy plasmids (Figure 6). As with P_{argC}, use of P_{ompC} with 5-copy replicons shows great promise for use in *V. cholerae* live vector strains because of its *in vitro* stability and high induction in the mouse intestine.

Concluding remarks

In summary, this study has identified three excellent candidate promoters, P_{argC} $P_{vca1008}$, and P_{ompC}, for use in regulated expression of heterologous antigens in attenuated *V. cholerae* live vector vaccine strains. Since the promoters were originally identified in an El Tor strain and then studied here using bioluminescence in a Classical strain it is likely that they will function well in both biotypes. The rational identification and direct demonstration of *in vivo* expression of these promoters has identified useful tools for advancing the development *V. cholerae* multivalent live vector vaccines.

Our series of luciferase-expressing stabilized plasmids could also be useful for the study of other enteric pathogens. For bacteria with more robust mouse colonization models, strains expressing *luxCDABE* from the *ompC* promoter could be very useful for studying colonization dynamics, without having to create chromosomal *lux* fusions. Our plasmids can be transformed into other bacterial strains, including *S.* Typhi. After transforming 5-copy replicons expressing P_{ompC}- controlled luciferase into *S.* Typhi and inoculating mice intranasally, we were able to track both colonization and tissue distribution over a 5-day period (data not shown). These plasmids could also be used to examine *in vivo* induction of other predicted *in vivo*-induced promoters in *V. cholerae* or other bacteria. More detailed studies of induction kinetics or *in situ* induction studies could be performed with enteric bacteria since the gastrointestinal tract can be removed from colonized mice and quantitatively imaged. Additionally, bacteria expressing luciferase have been used to target tumor cells and non-invasively follow tumor growth and metastasis over time without the need for exogenous luciferin injections, as is necessary when imaging tumor cells that have been engineered to express the firefly *luc* gene. Min et al. have recently used *E. coli* expressing luciferase from a plasmid stabilized using the *asd* balanced-lethal host-vector system to image a variety of tumors in living mice (Min et al., 2008a; Min et al., 2008b). Our system could easily be adapted for such use.

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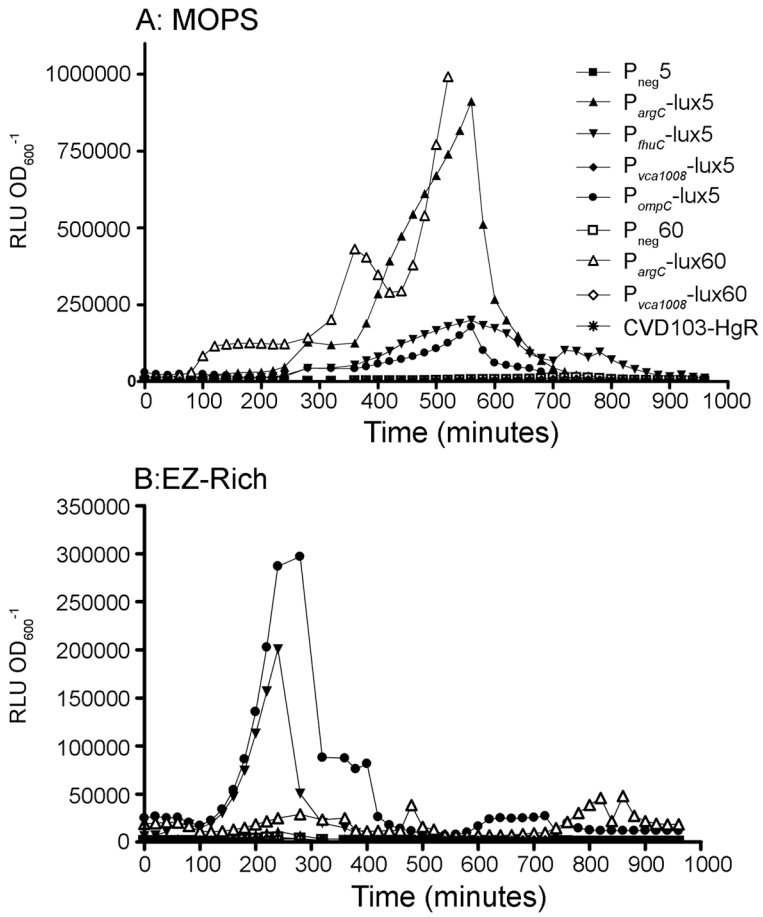

Figure 1.

Luciferase kinetics and stability. The symbols shown in A are used for both panels. The symbols for 5-copy plasmids are solid and those for 60-copy plasmids are open. A) and B) show strains grown in 96-well plates in minimal and rich media respectively. Each value is the mean of triplicate cultures and is representative of at least 3 experiments. The values of 60-copy P_{argC} strain are cut-off at 500 minutes; this strain goes on to achieve a peak level of 3.94×10^6 at 780 minutes and then rapidly declines like the other strains.

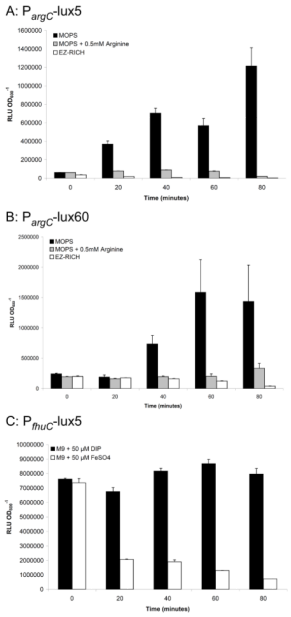

Figure 2.

In vitro regulation of promoters. Strains were grown in LB to mid-log phase and then resuspended in permissive or non-permissive medium. A and B) show the relief of repression of the *argC* promoter by the removal of exogenous arginine in the 5- (A) or 60-copy (B) plasmids. C) shows repression of the *fhuC* promoter by the addition of $FeSO_4$. Each value is the mean of triplicate cultures, error bars show standard deviation. Each graph is representative of at least three experiments.

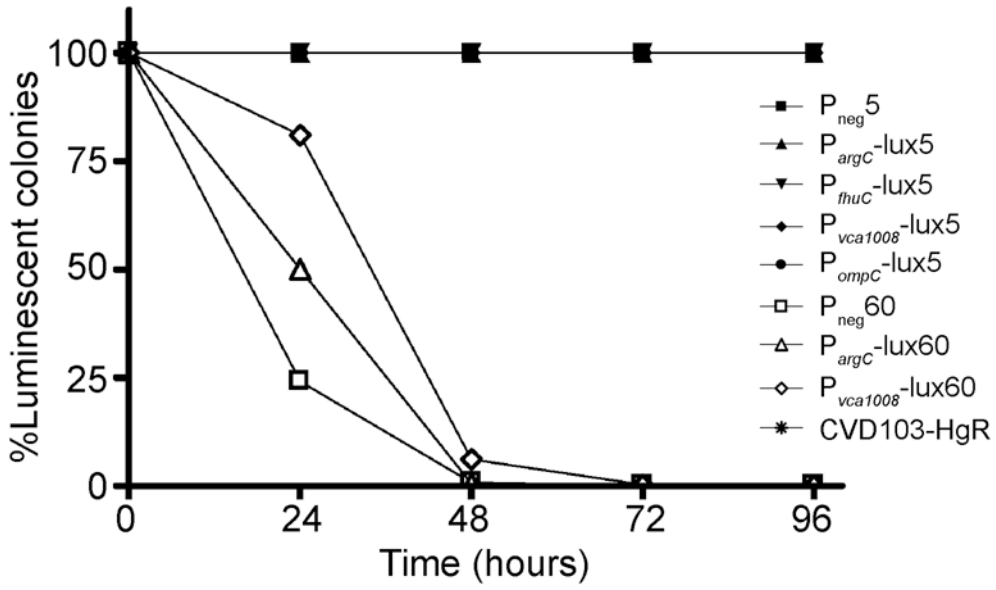

Figure 3.

Plasmid maintenance in the absence of selective pressure *in vitro*. Strains were serially passaged at 1:100 dilutions over 5 days in rich medium without antibiotics. The first time point (0 hours) is the first plating after 24 hours of growth in liquid medium without selection. The symbols for 5-copy plasmids are solid and those for 60-copy plasmids are open.

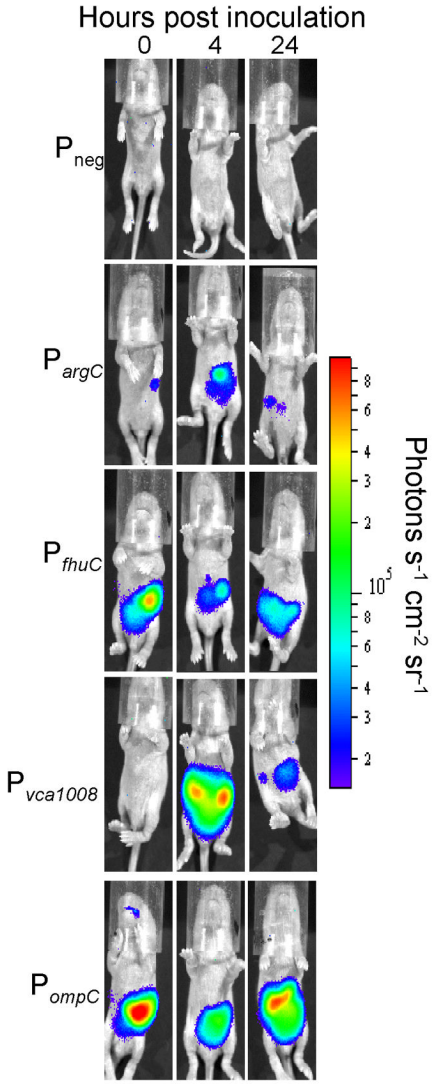

Figure 4.

Representative images demonstrating *in vivo* promoter induction from 5-copy plasmids. 5-day old CD-1 mice were inoculated with CVD103-HgR carrying luciferase-expressing plasmids (dose ~10^7). 1-minute images were acquired immediately after inoculation (0 hour) and at 4- and 24-hours p.i. Note that the images represent raw luciferase activity that is not normalized to CFU mL^{-1} as in Figure 6.

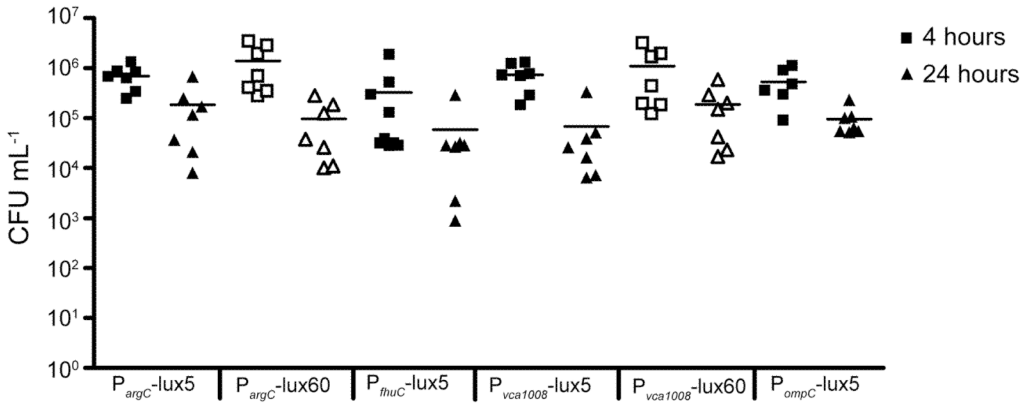

Figure 5.

Colonization levels in suckling mice. 5-day old CD-1 mice were inoculated with CVD103-HgR carrying luciferase-expressing plasmids (dose ~10^7). Groups were split into two for enumerating bacteria in the intestine at 4- or 24-hour. Each symbol represents a single mouse and the bar represents the mean. The 4-hour time point is represented by squares and 24-hours by triangles. Closed symbols are used for 5-copy replicons and open symbols for 60-copy replicons.

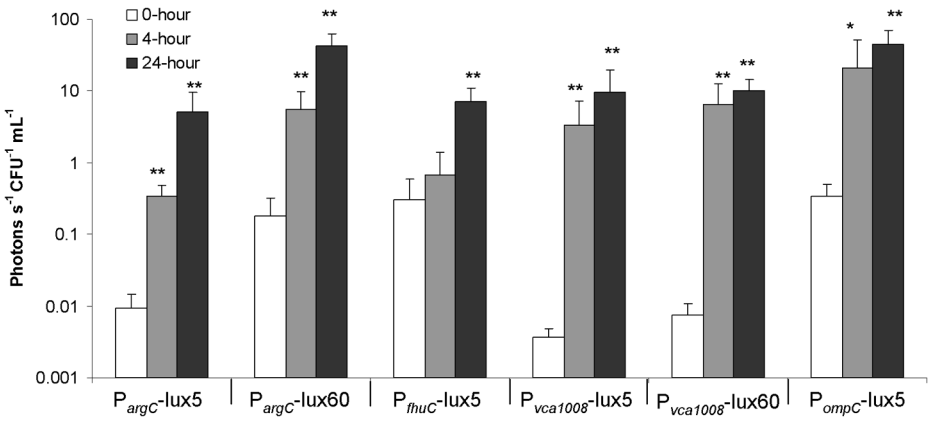

Figure 6.

Quantified *in vivo* bioluminescence. ROIs were calculated for each mouse using Living Image 3.0 software, giving a unit of photons sec^{-1}. The relative bioluminescence per bacteria was calculated for each promoter construct by normalizing the total flux to CFU mL^{-1} from either the calculated inocula (0-hour) or intestinal homogenates (4- and 24-hour). N=7–10 mice. Significant differences in luminescence between the 0-hour and 4- or 24-hour were determined by two-tailed Student's *t*-test. *, P<0.05. **, P<0.005.

Table 1

Plasmids used in this study with relevant features noted.

Plasmid	Approx. copy #	Promoter	Relevant characteristics	Source or reference
pGENlux	15	P_{em7}	*ori*15A l*uxCDABE bla hok-sok par parA*	Lane et al., 2007
pSEC10	5	P_{ompC}	*ori*101 *clyA aph hok-sok par parA*	Stokes et al., 2007
pSEC84	60	P_{ompC}	*ori*E1 *clyA aph hok-sok par parA*	Galen et al., 2004
pOMP5lux	5	P_{ompC}	*ori*101 *luxCDABE aph hok-sok par parA*	This work
pOMP15lux	15	P_{ompC}	*ori*15A *luxCDABE bla hok-sok par parA*	This work
pOMP60lux	60	P_{ompC}	*ori*E1 *luxCDABE aph hok-sok par parA*	This work
pCM10	5	P_{neg}	*ori*101 *luxCDABE aph hok-sok par parA rrnB*	This work
pCM11	5	P_{argC}	*ori*101 *luxCDABE aph hok-sok par parA rrnB*	This work
pCM13	5	P_{fhuC}	*ori*101 *luxCDABE aph hok-sok par parA rrnB*	This work
pCM14	5	$P_{vca1008}$	*ori*101 *luxCDABE aph hok-sok par parA rrnB*	This work
pCM17	5	P_{ompC}	*ori*101 *luxCDABE aph hok-sok par parA rrnB*	This work
pCM18	60	P_{neg}	*ori*E1 *luxCDABE aph hok-sok par parA rrnB*	This work
pCM19	60	P_{argC}	*ori*E1 *luxCDABE aph hok-sok par parA rrnB*	This work
pCM22	60	$P_{vca1008}$	*ori*E1 *luxCDABE aph hok-sok par parA rrnB*	This work

Table 2

Primers used in this study. Restriction enzymes sites are underlined.

Primer #	Template	Cassette created	Direction	Oligonucleotide sequence (5′ → 3′)
K4125	N16961 genomic DNA	P_{argC}	forward	caggatcctaagtggtgaaagagagcgaaca
K4126	N16961 genomic DNA	P_{argC}	reverse	tgggaattcgcggccgctatctttgctcctgctattgacagagtg
K4245	N16961 genomic DNA	$P_{vca1008}$	forward	gaattcgcggccgctaatttcgattcctcatcactcaccacggcg
K4246	N16961 genomic DNA	$P_{vca1008}$	reverse	ggatcctattagtttgattgggctttac
K5512	N16961 genomic DNA	P_{fhuC}	forward	gaattcgcggccgcggtgccttaatgaaagtaattg
K5513	N16961 genomic DNA	P_{fhuC}	reverse	ggatccattgcgcatgataaaacctctgc
K5917	pSE380 (Invitrogen)	*rrnB*-P_{neg}	forward	caattgataaaacagaatttgcctgg
K5918	pSE380 (Invitrogen)	*rrnB*-P_{neg}	reverse	ggatccattcttatgaattcgagtttgtagaaacgcaaaaag