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Attenuation of Bacterial Virulence by Quorum Sensing-regulated Lysis

Anisia J. Silva^{1,*}, Jorge A. Benitez², and Jian-He Wu¹

¹ Morehouse School of Medicine, 720 Westview Dr., SW Atlanta, GA 30310

² Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205

Abstract

Genetically attenuated pathogenic bacteria have been extensively considered as vaccine candidates. However, insufficient attenuation has been a frequent limitation of this approach. Many pathogens use quorum sensing to escape host defense mechanism. Here, we hypothesized that quorum sensing can be manipulated to diminish pathogenesis. To test this hypothesis, we modified the quorum sensing circuitry of a live cholera vaccine strain to add a second layer of attenuation. Attenuation resulted from the expression of phage PhiX174 lysis gene E on a balanced lethal plasmid from the quorum sensing-regulated *luxC* promoter. For conditional expression of quorum sensing and positive selection *in vivo*, the host strain was deleted of its *cqsA* and *thyA* genes encoding cholera autoinducer 1 (CAI-1) synthase and thymidylate synthase, respectively. A recombinant *cqsA* gene expressed from the cholera toxin (CT) promoter and an active *thyA* gene was provided *in trans*. The resulting strain expressed CAI-1 in AKI cultures (CT permissive condition) but not in LB medium. Additionally, it expressed elevated biofilm in LB medium compared to AKI conditions where CAI-1 is synthesized to repress biofilm formation. Induction of lysis gene E by quorum sensing restricted growth to a lower cell density in AKI medium, the suckling mouse intestine or LB supplemented with exogenous CAI-1. Microscopic examination revealed the presence of *V. cholerae* ghost cells at high cell density. Lysis was accompanied by the release of intracellular β -galactosidase to the culture medium. We conclude that it is possible to manipulate quorum sensing to attenuate a live vaccine vector and restrict its shedding to the environment and diminish its subsequent dissemination.

1. Introduction

Live genetically attenuated bacteria have been extensively considered as vaccine candidates to prevent diseases such as salmonellosis, shigellosis, listeriosis, tuberculosis and cholera (Detmer and Glenting, 2006; Ryan et al., 2006) and as live vectors to deliver protective antigens (Medina and Guzman, 2001; Silva et al., 2007). In general, targeted deletion of specific virulence factors and/or mutations that limit the capacity of the bacterium to survive, disseminate and/or persist in the host renders the pathogen attenuated for virulence. However, insufficient attenuation and shedding of live bacteria, which can potentially reacquire virulence genes, to the environment continue to raise concerns. Thus, the development of novel approaches to adjust the attenuation level of live vectors and limit their spread to the environment would enhance the use of live attenuated pathogens as vaccines and antigen delivery systems.

*Corresponding author: asilva-benitez@msm.edu, Phone: 404 756 6660, Fax: 404 752 1197.

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Cholera is an acute diarrheal disease that continues to be a major public health concern in densely populated regions of Asia and Africa. The current cholera pandemic is caused by *Vibrio cholerae* strains of serogroup O1 (El Tor biotype) and O139. *V. cholerae* colonizes the human small intestine and expresses a variety of virulence factors such as the toxin-coregulated pilus (TCP) required for intestinal colonization (Herrington et al., 1988) and cholera toxin (CT) which is responsible for the profuse watery diarrhea typical of cholera (Finkelstein, 1992; Kaper et al., 1995). Mutations that prevent colonization and/or CT expression lead to significant attenuation in the cholera infant mouse model and humans. Highly immunogenic and protective live genetically attenuated vaccines have been developed (Benitez et al., 1999; Garcia, L., et al., 2005, 2005a; Chowdhury et al., 2009). However, as occurred in other diseases, some of these vaccines have exhibited unacceptable clinical side effects or reactogenicity (i.e. mild to moderate diarrhea, abdominal cramps, low grade fever, etc.) (Ryan et al., 2006). These symptoms are not elicited by oral cholera vaccines consisting of heat-killed cells. Therefore, a potential approach to reduce reactogenicity is to gradually diminish the ability of the vaccine strain to multiply within the gut.

Many pathogens use quorum sensing as part of their strategy to escape host defense mechanism (i.e. by allowing bacteria to remain undetected at low cell density while unleashing their full pathogenic potential at high cell density) (Njoroge and Sperandio, 2009). Quorum sensing is a process by which bacteria communicate with one another by secreting extracellular signaling molecules termed autoinducers. In *V. cholerae*, expression of virulence is negatively regulated by quorum sensing (Zhu et al., 2002). Two autoinducer systems function in *V. cholerae*: CAI-1 and autoinducer 2 (AI-2). CqsA is responsible for the biosynthesis of CAI-1 which, at high cell density, binds its receptor CqsS (Higgins et al., 2007). AI-2 is synthesized by the activity of LuxS (Chen et al., 2002) and recognized by receptor LuxPQ. Accumulation of CAI-1 and AI-2 activate the expression of the master quorum sensing regulator HapR (Miller et al., 2002). At low cell density, CqsS and LuxPQ become phosphorylated and phosphorus is transferred to the σ^{54} -dependent activator LuxO. Phospho-LuxO negatively regulates the expression of HapR by a post-transcriptional mechanism (Lenz et al., 2004). At high cell density (in the presence of autoinducers), phosphotransfer is reversed and LuxO becomes dephosphorylated and inactive allowing the expression of HapR. Then, HapR negatively regulates the expression of the toxin co-regulated pilus (TCP), CT and biofilm formation (Zhu et al., 2002; Kovacikova and Skorupski, 2002; Zhu and Mekalanos, 2003; Hammer and Bassler, 2003; Waters et al., 2008). In parallel, HapR activates *hapA* encoding hemagglutinin (HA)/protease (Hase and Finkelstein, 1991; Jobling and Holmes, 1997), an activity that promotes *V. cholerae* detachment from the intestinal mucosa (Benitez et al., 1997; Jude et al., 2009).

Repression of TCP and CT by HapR involves the regulator AphA. At low cell density, AphA activates the expression of TcpP/H (Hase and Mekalanos, 1998; Kovacikova and Skorupski, 2001) which, in concert with a second pair of transmembrane regulators (ToxR/S) (Miller and Mekalanos, 1985; Miller et al., 1989), activates the expression of the soluble regulator ToxT (DiRita et al., 1991). ToxT acts positively at the *tcpA* and *ctxA* promoters to enhance TCP and CT production (DiRita et al., 1991). At high cell density HapR represses AphA biosynthesis to diminish the expression of TCP and CT.

Production of TCP and CT *in vitro* by *V. cholerae* strains of the El Tor biotype require special conditions known as AKI cultures (Iwanaga et al., 1986). AKI cultures, which are referred to as ToxR-permissive condition, have been suggested to mimic the environment in the small intestine conducive to virulence gene expression.

In this study we tested whether the *V. cholerae* quorum sensing pathway could be manipulated to achieve conditional expression of a factor that would limit the ability of colonizing bacteria to grow in the small intestine beyond a certain cell density. Expression of bacteriophage

PhiX174 lysis gene E (Witte et al., 1992) in *V. cholerae* has been used to produce *V. cholerae* ghosts (VCG) defined as non-living, empty bacterial cell envelopes that maintain their native surface antigenic structures (Eko et al., 1994, 1994a; Szostak et al., 1996). Here we describe the development of a *V. cholerae* plasmid-host system for quorum sensing-regulated expression of lysis gene E under ToxR-permissive conditions. Introduction of the balanced lethal plasmid in the *V. cholerae* host resulted in additional HapR-dependent attenuation in the infant mouse cholera model.

2. Materials and methods

2.1 Strains and media

V. cholerae strains used in this study are listed in Table 1. Strains were routinely grown in LB medium with shaking (250 rpm) at 37°C. For measuring CAI-1 activity, the reporter strain MM920 was incubated at 30°C to allow for the expression of Lux activity. For CT production or growth under ToxR-permissive conditions, *V. cholerae* strains were cultured in AKI medium at 30°C as described by Iwanaga et al. (1986). Plasmid DNA was introduced in *V. cholerae* by electroporation (Marcus et al., 1990). Culture media were supplemented with ampicillin for plasmid selection (Amp, 100 µg/mL); thymidine (200 µg/mL), for growth of $\Delta thyA$ mutants; X-gal (20 µg/mL), IPTG (20 µg/mL) for blue-white selection in routine cloning and, polymyxin B (PolB, 100 units/mL) to counter select *E. coli* during conjugal transfer of mutations to *V. cholerae*.

2.2. Construction of mutants

Mutants constructed for this study were derived from the genetically attenuated strain *V. cholerae* 1333 which was derived from *V. cholerae* C6706 (O1, El Tor biotype) by deletion of the CTX Φ prophage encoding cholera toxin and insertional inactivation of *hapA* (Benitez et al., 1996; Robert et al., 1996). Strain 1333 was further modified by stepwise deletion of the chromosomal *thyA* gene encoding thymidylate synthase and *cqsA* encoding CAI-1 synthase, respectively. In order to construct 1333 $\Delta thyA$ (AJB200), chromosomal DNA fragments flanking the *thyA* gene were amplified using the Advantage 2 PCR system (Clontech Laboratories Inc., Mountain View, CA) and primer pairs ThyA47/ThyA985 and ThyA1837/ThyA2829, respectively. The PCR products were sequentially cloned in pUC19 and confirmed by DNA sequencing to yield plasmid pUC $\Delta thyA$ which contains a chromosomal DNA fragment harboring a deletion of *thyA*. The deletion fragment was transferred to the suicide vector pCVD442 containing *sacB* for selection of plasmid less segregants (Donnenberg and Kaper, 1991) to yield pCVD $\Delta thyA$ and propagated in the permissive *E. coli* strain S17-1ypir (de Lorenzo et al., 1993). The suicide vector was transferred to strain 1333 by conjugation followed by sucrose selection for segregants retaining the *thyA* deletion as described previously (Liang et al., 2007; Silva et al., 2008) to yield strain AJB200. The *thyA* deletion was confirmed by PCR and DNA sequencing. Strain AJB200 has a requirement for high level of thymidine due to the absence of thymidylate synthetase (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase) activity. In order to eliminate endogenous CAI-1 biosynthesis in strain AJB200, we used the suicide vector pCVD $\Delta cqsA$ (Liang et al., 2007) to introduce a *cqsA* deletion in strain AJB200 following the same approach used for deletion of *thyA*. The 1333 $\Delta thyA\Delta cqsA$ double mutant was designated AJB203. For intestinal colonization experiments, the suicide vector pCVD $\Delta hapR$ (Liang et al., 2007) was used to introduce a *hapR* deletion in AJB203 as described above to yield strain AJB204.

2.3. Construction of a lethal balanced plasmid vector

In order to construct a strain that would conditionally express quorum sensing *in vivo* but not under laboratory conditions (i.e. LB medium), we constructed a recombinant *cqsA* gene expressed from the *ctxA* (CT) promoter. To this end, we amplified a DNA fragment containing

ctxA transcription and translation initiation signals using primers CtxA6 and CtxA278 and cloned it as a PstI-SphI fragment downstream of the *rrnBT₁T₂* transcription terminator previously cloned as an XbaI-PstI fragment in pUC19 (plasmid pTT3, Silva et al., 2003). Then, the entire *rrnBT₁T₂-ctxA* promoter fragment was transferred as an EcoRI-HindIII fragment to pBR322 to yield plasmid pBR*ctxA*. A promoterless *cqsA* gene was amplified using primers CqsA986 and CqsA2217 and inserted as a SphI-SalI fragment in plasmid pBR*ctxA* to yield pBRS1. Next, the HapR-responsive *luxC* promoter and a promoterless PhiX174 lysis gene E were sequentially cloned in pUC19. To this end, we first amplified the *luxC* promoter from the pBB1 cosmid containing the *lux* operon (Miller et al., 2002) using primers Lux16 and Lux411 with BamHI and KpnI 5' overhangs for directional cloning. A DNA fragment encoding a promoterless PhiX74 lysis gene E was amplified from plasmid pDKL01 (Eko et al., 2003) using primers PhiX1 and PhiX261 with 5' SacI and EcoRI overhangs and inserted downstream of the *luxC* promoter. Then, an EcoRI-BamHI fragment containing the *luxC*-gene E fusion was transferred to plasmid pBRS1 to yield pBRS2. An additional DNA fragment encoding a complementing *thyA* gene was amplified from strain 1333 genomic DNA using primers ThyA694 and ThyA2045 and inserted as a SalI fragment in pBRS2 to yield the final balanced lethal vector pBRS3 shown in Fig. 1. This plasmid complemented the thymidine requirement of strain AJB203 for growth in LB and AKI medium.

2.4. Measurement of CAI-1 activity

Cell-free supernatants of *V. cholerae* cultures were used as a source of CAI-1 activity. To this end, single colonies of the strain to be tested were inoculated to 5 ml of LB medium and incubated 16 h at 37°C with shaking. The cultures were centrifuged at $17,400 \times g$ for 10 min and the clear supernatant filtered through a 0.22 μ m disposable syringe filter. Cell-free culture supernatants were tested for the presence of CAI-1 activity by inducing light production in the *V. cholerae* reporter strain MM920 containing the cosmid pBB1, which carries the *V. harveyi* *lux* operon (Miller et al., 2002). This reporter strain does not produce CAI-1 and does not respond to AI-2. Briefly, the reporter strain was grown overnight with shaking at 30°C, diluted 1:10 in fresh medium, and 70 μ L aliquots transferred to an opaque wall 96 well microtiter plate. Cell-free culture fluids were added to a final concentration of 30 % (v/v). The plates were incubated at 30°C with agitation and light production measured at 30 min intervals in a BioTek Synergy 2 plate reader. The data was reported as peak fold light induction compared to a sterile medium control.

2.5. Autoinducer cross feeding experiments

Cross feeding experiments were conducted to test the effect of exogenous CAI-1 on our quorum sensing-regulated lysis system. To this end, we prepared conditioned media consisting of a filter-sterilized supernatant of a *V. cholerae* N16961 culture grown in LB. The resulting conditioned media contained 16,722 fold induction units of CAI-1 activity. The effect of exogenously supplied CAI-1 was determined by reconstituting cells in LB containing 60 % (v/v) of conditioned media.

2.6. Biofilm assay

Biofilm formation was measured by the crystal violet staining method and results were normalized for growth and expressed as the OD₅₇₀/OD₆₀₀ ratio (Zhu and Mekalanos, 2003).

2.7. Enzymatic and toxin assays

β -Galactosidase activity was measured as described previously (Miller, 1971) using the substrate o-nitrophenyl- β -D-galactopyranoside (ONPG). Specific activities are given in Miller units [$1000 (OD_{420} / t \times v OD_{600})$] where t is reaction time and v is the volume of enzyme

extract per reaction. CT was determined by GM₁ ELISA using a standard curve of pure CT (Sigma-Aldrich, Saint Louis, MO) as described previously (Silva et al., 2006).

2.8. Transmission electron microscopy

Strain AJB203 containing pBRS3 was incubated with conditioned media containing exogenous CAI-1 (60 % v/v) or fresh LB medium for 90 min. The cells were centrifuged and resuspended in phosphate-buffered saline pH 7.4 (PBS). Samples were adhered to a carbon-coated grid and stained with 1% uranyl acetate before microscopy.

2.9. Infant mouse colonization assay

Five 4-to-5 days old CD-1 mice were starved for 6-h and inoculated orally through a 21-gauge feeding needle with 10⁶ CFU of each challenge strain in 50-μL or PBS. Mice were maintained for 16 h in a Styrofoam box at 30°C. The animals were sacrificed by cervical dislocation and the entire small intestine removed, weighed and homogenized in 5 ml of PBS in an ultraturrax T8 homogenizer. Colonizing vibrios were enumerated by plating in LB agar and the bacterial load expressed as colony forming units (CFU) per g tissue.

3. Results

3.1. Expression of CAI-1 in strain AJB203 containing the balanced lethal plasmid pBRS3 mimics the regulation of cholera toxin expression

In this study we provide a new approach to adjust the attenuation of a vaccine vector by manipulating quorum sensing to limit the ability of the vector strain to grow within the host. A basic requirement of our approach was to prevent *V. cholerae* from expressing HapR and lysis gene E in LB medium (so it could be grown to high cell density) while allowing the expression of quorum sensing in AKI medium and *in vivo* to activate lysis. Permissive and non permissive conditions for the expression of CT and other ToxR-regulated virulence factors have been well established (Childers and Klose, 2007; Matson et al., 2007). CT is expressed in AKI cultures (Iwanaga et al., 1986) (ToxR-permissive condition) but not in LB cultures (ToxR-non permissive condition) (Childers and Klose, 2007; Matson et al., 2007). Since CAI-1 is the major quorum sensing signal that activates HapR under laboratory conditions (Zhu et al., 2002; Zhu and Mekalanos, 2003; Liang et al., 2007), we decided to place CAI-1 biosynthesis under ToxR regulation by expressing the *cqsA* gene from the *ctxA* ToxR-responsive promoter. To this end, we constructed plasmid pBRS1 (Table 1). Strain 1333 and its derivatives have been deleted for CT genes so it was not possible to test for co-regulation of CT and CAI-1 biosynthesis in this genetic background. To solve this problem, we introduced plasmid pBRS1 in the toxigenic $\Delta cqsA$ mutant AJB61 described previously (Liang et al., 2007). The transformant was grown in LB and AKI cultures to measure production of CT and CAI-1 production. The transformant produced 1.86 ± 0.07 μg/mL/OD₆₀₀ unit of CT in AKI cultures (n = 3) versus 0.24 ± 0.05 ng/mL/OD₆₀₀ unit in LB (n = 3). In parallel, production of CAI-1 in AKI cultures reached 855 ± 103 fold induction (n = 3) versus 1.22 ± 0.07 in LB. This result demonstrates that CAI-1 biosynthesis and expression of the quorum sensing regulator HapR could be tightly regulated in a ToxR-dependent manner from a plasmid vector expressing a *ctxA-cqsA* fusion. This result prompted us to construct a host-vector system that would conditionally express lysis gene E at high cell density and be suitable for propagation *in vivo* as a live vaccine. The host-vector system consists in an attenuated cholera strain that does not make CAI-1 and is auxotrophic for thymidine (AJB203) and the balanced lethal plasmid pBRS3 (Fig. 1). As shown in Fig. 2A the thymidine auxotrophy had no impact on CAI-1 biosynthesis while the $\Delta thyA \Delta cqsA$ double mutant expressed negligible CAI-1 activity. As in our previous study using a toxigenic strain, plasmid pBRS3 complemented the $\Delta cqsA$ mutation of the host only in ToxR-permissive AKI cultures.

3.2. The balanced lethal plasmid pBRS3 complemented the biofilm phenotype of a *cqsA* mutant under ToxR-permissive conditions

The above results predicted that expression of CAI-1 biosynthesis in a ToxR-dependent manner should translate into similar regulation of HapR and HapR-regulated phenotypes. It is well established that expression of HapR at high cell density leads to repression of biofilm formation (Hammer and Bassler, 2003; Zhu and Mekalanos, 2003; Waters et al., 2008). As a consequence, *hapR* mutants exhibit an enhanced biofilm forming phenotype. Thus, we predicted that expression of CAI-1 biosynthesis in a ToxR-dependent manner should also lead repression of biofilm formation in a ToxR-dependent dependent fashion. To test this prediction, we examined the biofilm formation capacity of strain AJB203 containing pBRS3 and its precursor. As shown in Fig. 2B, thymidine auxotrophy had no effect on biofilm formation while deletion of *cqsA* enhanced biofilm formation in both LB and AKI cultures due to lower expression of the HapR repressor. However, expression of *cqsA* from pBRS3 restored wild type biofilm formation only in AKI cultures (Fig. 2B).

3.3. Growth of strain AJB203 containing the balanced lethal plasmid pBRS3 is arrested at high cell density in ToxR-permissive conditions

Expression of lysis gene E in *V. cholerae* has been consistently shown to lead to the formation of VCG (Eko et al., 2003; Eko et al., 1994, 1994a; Szostak et al., 1996). The common assay for gene E activity is to measure the induction of bacterial cell lysis. Expression of lysis gene E from the *luxC* HapR-dependent promoter in plasmid pBRS3 is expected to occur as the culture increases its cell density. Therefore, we would expect that as cell density increases and HapR is induced, expression of gene E would lead to an overall growth arrest. To test this prediction, we determined the cell density reached by strain AJB203 containing pBRS3 and its precursor grown under three conditions: LB (ToxR-non permissive), AKI (ToxR-permissive *in vitro*) and in the suckling mouse intestine (ToxR-permissive, *in vivo*). As shown in Fig. 3, a significant reduction in cell yield was only apparent in strain AJB203 containing pBRS3 in AKI cultures. Similarly, the titer of *Vibrios* recovered from the suckling mouse intestine in AJB203 containing pBRS3 was significantly lower compared to the precursor strain 1333 (Fig. 3). To exclude the possibility that plasmid replication rather than expression of gene E could be responsible for lower bacterial recovery, we introduced pBRS3 in strain AJB204 containing a deletion of *hapR* to silence the *luxC* promoter. It has been previously shown that *hapR* is epistatic to the *cqsA* mutation and has no effect on intestinal colonization (Miller et al., 2002; Zhu et al., 2002). As shown in Fig. 3, silencing of the *luxC*-gene E fusion by elimination of *hapR* led to enhanced recovery of viable *V. cholerae* from the infant mouse intestine. Taken together, these results show that quorum sensing or HapR-dependent expression of lysis gene E from a balanced lethal plasmid added an additional layer of attenuation to strain 1333 in this animal model.

3.4. Growth arrest and ghost formation can be induced under ToxR-non permissive conditions by exogenous CAI-1

In order to confirm that growth inhibition at high cell density is due to biosynthesis of CAI-1 accumulation and HapR induction, we conducted a cross feeding experiment to demonstrate that growth arrest can be induced under ToxR-non permissive condition by addition of exogenous CAI-1. To this end, we prepared conditioned media for CAI-1 cross feeding to AJB203 and AJB203/pBRS3. In this experiment, each strain was grown in LB medium to OD₆₀₀ 0.3, the cells collected by centrifugation and resuspended in 0.25 volumes of fresh LB medium. Each cell suspension was diluted 1:10 in fresh LB medium and LB containing 60 % conditioned media. As shown in Fig. 4, both fresh LB and conditioned media supported further exponential growth of strain AJB203 indicating the absence of any bacteriostatic or bactericidal factor in the conditioned media. However, growth of strain AJB203 containing pBRS3 was

arrested when incubated with conditioned media. This result indicates that the presence of CAI-1 in the conditioned media becomes toxic to *V. cholerae* cells that contain the balanced lethal plasmid expressing lysis gene E at high cell density. We also measured if incubation with conditioned media led to the release of intracellular β -galactosidase activity. To this end, we measured β -galactosidase activity using the method of Miller (1971) but without permeabilizing cells by treatment with chloroform and sodium dodecyl sulfate (SDS). As shown in Fig. 4, incubation of AJB203/pBRS3 with conditioned media lead to significant leakage of β -galactosidase to the medium compared to untreated cells.

We next considered if growth arrest at high cell density correlated with the formation of VCG. VCG can be clearly distinguished by their lower electron density and often collapsed or round morphology in transmission electron micrographs (Eko et al., 1994). Thus, we examined samples of strain AJB203/pBRS3 grown in LB medium in the absence and presence of exogenous CAI-1. As shown in Fig. 5, VCG were present in treated (panel B) but not in untreated cultures (panel A).

In summary, our results demonstrate that lysis gene E is expressed in AJB203 containing pBRS3 at high cell density leading to growth arrest under *in vitro* conditions that mimic the intestinal milieu (AKI cultures) or in the suckling mouse intestine. The engineered CAI-1-gene E signaling cascade leads to additional attenuation of virulence as demonstrated by diminished recovery of viable *Vibrios* from the suckling mouse intestine.

4. Discussion

The starting strain used in this study was a live attenuated *V. cholerae* vaccine candidate shown to be highly immunogenic in the cholera volunteer model (Garcia, H.M., et al., 2005, 2005a). The totality of volunteers that received 10^8 CFU of strain 1333 shed live *Vibrios* in their stool (geometric mean, 3×10^4 CFU per g stool) (Garcia, H.M., et al., 2005). The potential long-term consequences of shedding live genetically-modified organisms to the environment have been a source of major concerns (Frey, 2007). Here we provide an approach to add an additional level of attenuation to a live vaccine vector by quorum sensing-regulated expression of phage PhiX174 lysis gene E. Expression of *cqsA* from the *ctxA* promoter provided tight regulation of quorum sensing in a ToxR-dependent manner preventing expression in LB and allowing auto induction *in vivo*. Cross feeding experiments confirmed that expression of gene E from the HapR-dependent *luxC* promoter and formation of VCG is indeed induced by the accumulation of CAI-1 activity in the medium at high cell density. Quorum sensing-regulated expression of lysis gene E led to growth arrest under ToxR-permissive conditions *in vitro* and lower recovery of live bacteria from the suckling mouse intestine. The infant mouse colonization assay is a widely accepted cholera animal model that closely mimics conditions in the human small intestine (Klose, 2000). Therefore, reduced recovery of live *Vibrios* in this model would be predictive of lower colonization and shedding of the vaccine strain to the environment in humans. The use of recombination-based *in vivo* expression technology (RIVET) has demonstrated that the *ctxA* promoter is silent in LB, expressed in AKI cultures and turned on in the infant mouse intestine 3–5 h post-infection in a ToxR-dependent manner (Lee et al., 1999). Expression of lysis gene E in AJB203 containing pBRS3 clearly paralleled the above ToxR regulatory pattern and explains the reduced recovery of AJB203 containing pBRS3 from the suckling mouse intestine.

In previous studies, lysis gene E has been expressed using the *Pm/xyIS* promoter-repressor system of the *Pseudomonas* TOL plasmid (Eko et al., 2003a) leading to quantitative *in vitro* conversion of the *V. cholerae* population to ghosts. It is clear from our data that not all *Vibrios* are converted to ghosts upon quorum sensing-induced gene E expression. This could be advantageous as *Vibrios* are provided with limited colonization capacity to induce a level

of immune stimulation higher than what can be achieved with completely inactivated oral cholera vaccines. There could be several explanations for the incomplete lysis observed in our host-vector system. First, selection of the *ctxA* promoter to control quorum sensing was based on its differential expression between *in vitro* and *in vivo* conditions rather than on promoter strength. It is likely that a more effective lysis would require a stronger promoter driving increased accumulation of protein E. Moreover, at high cell density expression of HapR could diminish expression of the *ctxA-cqsA* fusion by repressing the upstream regulator AphA (Kovacikova and Skorupski, 2002). It is also well established that the medium and timing of lysis gene E induction significantly influences the extent of lysis. Lysis is more effective on bacteria actively dividing in rich medium (Young and Young, 1982; Lubitz et al., 1984). In our system, the time window for gene E to induce lysis (i.e. from the point the culture attains a sufficiently high cell density to induce HapR to cessation of growth) might be narrow. The use of a stronger *in vivo* expressed promoter to increase the rate of CAI-1 biosynthesis could expand this time window by allowing cells to activate HapR and gene E at a lower cell density. Thus, our approach can be optimized in this direction in cases where minimal shedding of live bacteria or biological containment is a priority.

Several inducible lysis systems have been previously described. One such system so far tested *in vitro* exploited the expression of lambda phage S and R genes in *V. cholerae* and *Salmonella enterica* serovar Typhimurium (Jain and Mekalanos, 2000). Another approach has been to construct strains that can be grown *in vitro* in the presence of a specific inducer but upon entry into the inducer-free host environment cease to express cell wall biosynthesis genes and lyse (Kong et al., 2008). The system described in this study is a valid alternative to previous inducible lysis systems. First, lysis is induced upon expression of a single phage gene. Second, the system can be down- or up-regulated by controlling the rate of autoinducer synthesis to allow more or less colonization. Third, the product of gene E activity – the VCG – has consistently been shown to exhibit high immunogenicity and adjuvanticity (Eko et al., 2003; Eko et al. 1994, 1994a; Szostak et al. 1996).

It has been shown that expression of gene E causes the formation of a transmembrane tunnel structure through the cell envelope of Gram-negative bacteria leading to the expulsion of cytoplasmic material through the lysis tunnel (Szostak and Lubitz 1991, Eko et al 2000). Leakage of intracellular β -galactosidase activity to the medium upon incubation of cells with CAI-1 (conditioned media) is fully consistent with the above protein E mechanism of action. This suggests that quorum sensing-induced *in vivo* lysis could be also exploited to release foreign antigens expressed in the bacterium's cytoplasm eliminating the need for its effective secretion.

In summary our data shows for the first time that a quorum sensing regulatory circuit can be manipulated to modulate bacterial propagation in host tissues. The system is amenable to further optimization (as we continue to identify stronger expression signals specifically induced in the host during infection) in the direction of allowing more colonization or enhancing lysis for biological containment. Finally, lysis can be additionally exploited to deliver foreign antigens at mucosal surfaces.

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Fig. 1. Structure of balanced lethal plasmid for quorum sensing-regulated lysis

Abbreviations: Amp^R, ampicillin resistance; bla, β-lactamase gene; ORI, colE1 origin of replication.

Fig. 2. A. Production of CAI-1 activity in wild type and recombinant *V. cholerae* strains Strains 1333 (WT) and mutants were grown to stationary phase in LB medium at 37°C with agitation (□) or AKI medium at 30°C (■). The CAI-1 activity present in the clear supernatants was measured using a bioassay as described in methods. Error bars indicate the standard deviation (n = 3). **B. Biofilm formation by wild type and recombinant *V. cholerae* strains.** Strains 1333 (WT) and mutants were grown in LB medium at 30°C for 16 h and diluted 1:100 in fresh LB medium (□) or AKI medium (■). The cultures were transferred to 96-well microtiter plates. Biofilms were allowed to develop at 30°C for 24 h and measured by crystal violet staining as indicated in methods. Error bars indicate the standard deviation (n = 3).

Fig. 3. Growth of wild type and recombinant strains under different conditions

A. Strain 1333 (WT) and mutants were grown to saturation in LB (□) or AKI medium (■) (n = 3). **B.** Strain 1333 (WT) and mutants were orally fed (10^5 cells) to 2–5 days-old suckling mice (n = 5). Mice were incubated 16 h, euthanized by cervical dislocation, the small intestine dissected and homogenized in PBS. The final cell density (or titer of colonizing *Vibrios*) was determined by dilution plating on LB agar. Error bars indicate the standard deviation.

Fig. 4. Cross feeding of CAI-1. Panel A. Growth arrest

Strains were grown to mid-exponential phase, centrifuged and resuspended in fresh LB or LB containing 60 % conditioned media as described in methods. Symbols: (□), AJB203 in fresh LB; (■), AJB203 in conditioned media; (▲), AJB203 containing pBRS3 in fresh LB; (◆), AJB203 containing pBRS3 in conditioned media. **Panel B. Leakage of intracellular activity.** β -Galactosidase activity of cell suspensions was determined with and without permeabilization with chloroform and SDS. Strain AJB203 containing pBRS3 was incubated 90 min in fresh LB or LB plus conditioned media. Unfilled bar, fresh LB without permeabilization (no treatment); grey bar, conditioned media without permeabilization; black bar conditioned media with permeabilization. Each bar is the mean of three independent experiments. Error bars indicate the standard deviation.

Fig. 5. Transmission electron microscopy

Samples of AJB203 containing pBRS3 incubated 90 min in fresh LB media or LB containing conditioned media were examined for the presence of VCG. VCG are indicated with arrows.

Table 1*V. cholerae* strains, plasmids and primers

Strains		
Name	Description	Reference
1333	Δ CTX Φ , <i>hapA::celA</i> mutant derived from C6706 (O1, El Tor)	Benitez et al. (1996), Robert et al. (1996)
AJB200	1333 Δ <i>thyA</i> (thymidine auxotrophic mutant)	This study
AJB203	1333 Δ <i>thyA</i> Δ <i>cqsA</i>	This study
AJB204	1333 Δ <i>thyA</i> Δ <i>cqsA</i> Δ <i>hapR</i>	This study
MM920	CAI-1 reporter (Δ <i>cqsA</i> Δ <i>luxP</i> containing <i>lux</i> operon on pBB1 cosmid (Tet ^R))	Miller et al. (2002)
AJB61	CqsA-defective mutant derived from C7258 (O1 El Tor)	Liang et al. (2007)
Plasmids		
Name	Description	Reference
pUC Δ <i>thyA</i>	DNA fragment harboring a chromosomal deletion of <i>thyA</i> in pUC19	This study
pCVD Δ <i>thyA</i>	DNA fragment harboring a chromosomal deletion of <i>thyA</i> in pCVD442	This study
pCVD Δ <i>cqsA</i>	<i>V. cholerae</i> DNA containing a <i>cqsA</i> deletion in pCVD442	Liang et al. (2007)
pCVD Δ <i>hapR</i>	<i>V. cholerae</i> DNA containing a <i>hapR</i> deletion in pCVD442	Liang et al. (2007)
pTT3	<i>rnnBT</i> ₁ T ₂ transcription terminator in pUC19	Silva et al. (2003)
pBRctxA	<i>rnnBT</i> ₁ T ₂ transcription terminator and <i>ctxA</i> promoter in pBR322	This study
pBRS1	pBRctxA containing a promoterless <i>cqsA</i> gene cloned downstream of <i>ctxA</i> promoter.	This study
pBRS2	pBRS1 containing a <i>luxC</i> -gene E fusion.	This study
pBRS3	pBRS2 containing a complementing <i>thyA</i> gene (Fig. 1)	This study
Primers		
Name	Sequence (5' → 3')	
CqsA986	GAAGCATGCCTAGGATATATTGCGA	
CqsA2217	GAAGTCGACTTATGCAAGGAAACGT	
CtxA6	GGACTGCAGTTTGTGAAACCGAGAG	
CtxA278	GCTGCATGCCTCCCTTTGTTTAAC	
Lux16	GCAGGATCCTTGTGGTTTCTTATGA	
Lux411	GGAGGTACCCATATCAAGAGCTTCT	
PhiX1	AAGAGCTCATGGTACGCTGGA	
PhiX261	AAAGAATTCTCACTCCTTCCGCACG	
ThyA47	TATGAGCTCCTAAACTTGCCACCCA	
ThyA985	GCCGGATCCCTAACTACCTATTTT	
ThyA1837	GTTGGATCCCCGTTTTTCAGTCTAA	
ThyA2829	GAAGCATGCATTGTTGGTCTCGTTG	
ThyA694	TTCGTCGACCCATCCTTCACAGCT	
ThyA2045	GTTGTCGACTTAAAGGTGCTGAGCT	

Restriction sites used for cloning are underlined