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ADDENDUM

Silence is golden: gene silencing of *V. cholerae* during intestinal colonization delivers new aspects to the acid tolerance response

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ABSTRACT

Bacterial pathogens of the gastrointestinal tract alter their expression profile upon ingestion by the host and activate a variety of factors enhancing colonization and virulence. However, gene silencing during infection might be as important as gene activation to achieve full colonization fitness. Thus, we developed and successfully applied a reporter technology to identify 101 *in vivo* repressed (*ivr*) genes of the bacterial pathogen *Vibrio cholerae*. In depth analysis of the *in vivo* repressed H^+/Cl^- transporter ClcA revealed an inverse requirement along gastrointestinal colonization. ClcA could be linked to acid tolerance response required during stomach passage, but ClcA expression is detrimental during subsequent colonization of the lower intestinal tract as it exploits the proton-motive force in alkaline environments. The study summarized in this addendum demonstrates that constitutive expression of ivr genes can reduce intestinal colonization fitness of V. cholerae, highlighting the necessity to downregulate these genes in vivo.

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Introduction

Gastrointestinal infections are among the most common diseases in primary care worldwide. The WHO estimates a global morbidity of ~1.5 million people worldwide due to diarrheal disease each year. One of the most important gastrointestinal pathogens is the Gram-negative bacterium *Vibrio cholerae*, the causative agent of the severe secretory diarrheal disease cholera, responsible for about 3 – 5 million cholera cases and 120 000 deaths that occur globally every year. [1–3] Thus, cholera is still a massive threat for people, especially in areas with limited fresh water supply.

As a facultative human pathogen, *V. cholerae* constantly transits between the intestinal tract of the human host and the aquatic environment where it persists during inter-epidemic periods. Upon oral ingestion by the human host and subsequent passage to the gastrointestinal tract, *V. cholerae* alters the expression profile to accommodate to the *in vivo* requirements and induces the virulence cascade, which is mainly mediated via activation of the ToxR regulon. [4] So far, most studies investigating gene regulation of *V. cholerae* during intestinal colonization focused on gene induction to identify factors contributing to virulence and *in vivo* survival fitness. However, two factors are already known to be downregulated *in vivo*. These include the outer membrane porin OmpT, which increases bile sensitivity of the bacterial cell and the mannose-sensitive hemagglutinin type IV pilus MSHA, which has adverse effects on colonization fitness of the pathogen by a non–antigen-specific binding of host immunoglobulins. [5,6] We hypothesized that OmpT and MSHA represent just the tip of the iceberg and *V. cholerae* needs to silence many more genes to allow proper colonization and achieve full virulence *in vivo*. Thus, we designed a single cell-based reporter system to identify *in vivo* repressed (*ivr*) genes of *V. cholerae* followed by a comprehensive characterization of the H^+/Cl^- transporter ClcA, which is silenced during intestinal colonization. [7] In this addendum, we provide a

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concise summary of the reporter system and the original results along with additional commentary, further interpretations and implications related to gene silencing as an adaptational strategy of bacterial pathogens.

How the recombination-based *in vivo* expression technology (RIVET) became the tetR-controlled recombination-based *in vivo* expression technology (TRIVET)

Single-cell based reporter technologies, like the *in vivo* expression technology (IVET) and RIVET have been extremely useful to identify gene induction in complex populations according to their spatial and temporal expression [8–10], but their designs limit them to identify only genes activated in a defined condition. To identify gene silencing *in vivo* we developed a modified version of RIVET to become TRIVET. An extensive illustration of reporter-based *in vivo* technologies from RIVET to TRIVET is shown in Figure 1. The original RIVET system consists of a TnpR resolvase-mediated excision of a gene reporter cassette flanked by *res* sequences. [11] One component of RIVET is the res cassette, which can be placed in a neutral site of the *V. cholerae* chromosome via homologous recombination and confers kanamycin resistance (Kn^R) and sucrose sensitivity (Suc^S). The other component of RIVET is a promoterless *tnpR*, encoding a site-specific DNA recombinase (resolvase), which can be randomly integrated on the chromosome to generate transcriptional fusions of *V. cholerae* genes to *tnpR* by the combinatory use of homologous recombination and the pIVET suicide plasmid-library. [12] Induction of a *tnpR* fusion *in vivo* leads to excision and irreversible loss of the res cassette that can be monitored by a phenotypic change of the resistance profile (Kn^S & Suc^R).

The principle of an irreversible excision of the res cassette upon alteration in gene expression is conserved in TRIVET. However, the promotorless *tnpR* was replaced by a *tnpR* allele controlled by a TetR-controlled promoter, which originates from the tetracycline-resistance gene *tetA*. Thus, *tnpR*, which is integrated next to the res cassette in the *V. cholerae* genome via the suicide plasmid pTRIVET is now tightly controlled by the repressor TetR. The *tetR-phoA-cat* (*tpc*) cassette is the new element of TRIVET harbouring a

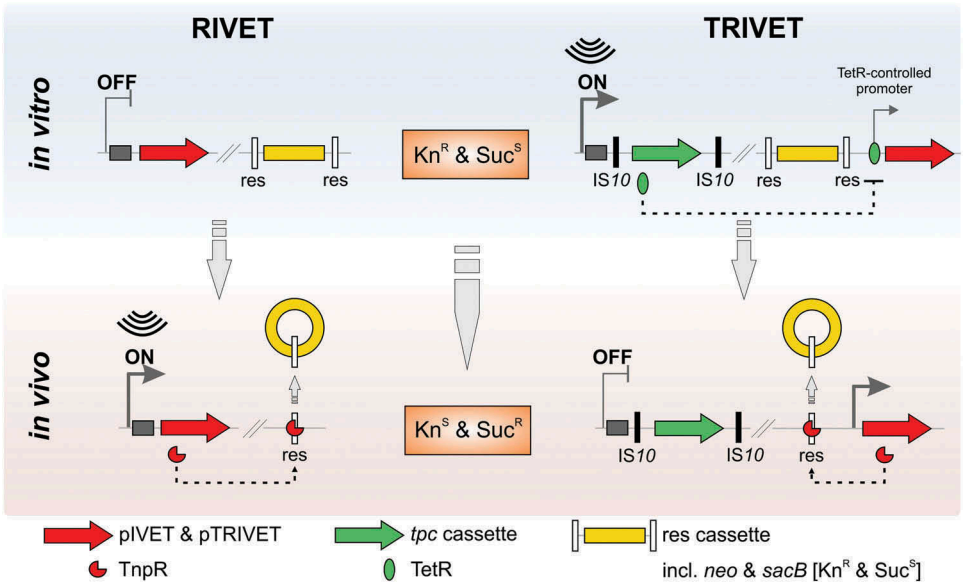

Figure 1. Comparative illustration of RIVET (left) and TRIVET (right) fused to a hypothetical *geneX* (dark grey) in the ON/OFF scenario of its promotor. Additional chromosomal sequences are highlighted in light gray, the res cassette parts in yellow, the integrated suicide vectors (pIVET and pTRIVET) harbouring *tnpR* in red, and *tetR-phoA-cat* (*tpc*) cassette in green flanked by *IS*10 sites (black). In case of RIVET, the pIVET suicide vector is integrated into *V. cholerae* hypothetical *geneX* via homologous recombination resulting in a merodiploid in which *geneX* and *tnpR* (resolvase) are transcriptionally fused and controlled by the chromosomal promotor of *geneX*. TnpR expression via activation of the *geneX* promotor results in an irreversible excision of the res cassette marked by a change in the resistance profile of the cell [kanamycin resistant (Kn^R) and sucrose sensitive (Suc^S) to kanamycin sensitive (Kn^S) and sucrose resistant (Suc^R)]. Thus, resolved strains (loss of the res cassette) can be selected by their ability to grow in sucrose.

promotorless *tetR* and *phoA*, The *tpc* cassette can by randomly integrated into the chromosome to generate transcriptional fusions of *V. cholerae* genes to *tetR* and *phoA* via the transposable element Tn*10*. TRIVET strains with sufficient *tetR* expression via the *tpc* cassette, repression of *tnpR* will keep the res cassette stably integrated and sustain the respective resistance profile (Kn^R & Suc^S). Upon silencing of a *tetR* fusion *in vivo*, insufficient expression of *tetR* leads to induction of *tnpR* resulting in excision and irreversible loss of the res cassette (Kn^S & Suc^R) allowing the identification of *in vivo* repressed genes.

Reduced colonization fitness of strain overexpressing *ivr* genes highlights the impact of gene silencing

Using a TRIVET-library comprising ~ 10,000 independent *tpc*-fusions in combination with the infant mouse model of cholera we identified 101 *ivr* genes, which can be allocated into diverse functional groups (Figure 2). Gene ontology analyses (http://pantherdb.org) which supports enrichment analysis using pathway classifications from the reactome resource [13] did not reveal any category to be significantly overrepresented in the identified *ivr* genes with regard to their abundance in the *V. cholerae* genome. This could indicate that gene silencing during infection involves global changes of several pathways and networks instead of only a few specific candidates within a defined functional group.

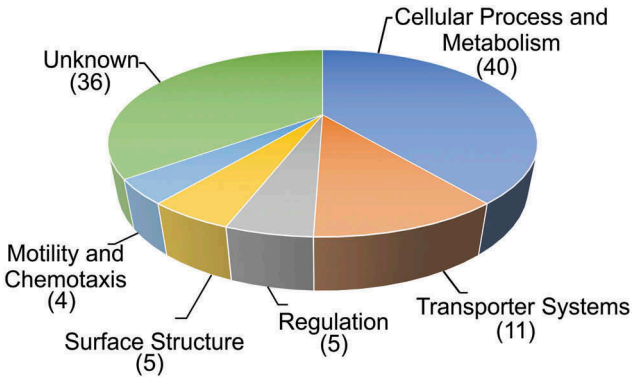

Figure 2. Functional distribution of *in vivo* repressed (*ivr*) genes of *V. cholerae*. Shown are *ivr* genes identified with the TetR-controlled recombination-based screening technology, allocated in functional groups by their proposed function according to KEGG (http://www.genome.jp/kegg/). The number of *ivr* genes in the respective group is indicated in parenthesis. In case of TRIVET, the pTRIVET suicide vector harbouring a TetR-controlled *tnpR* allele is integrated next to the res cassette. The *tpc* cassette is mobilized into the chromosome via Tn10 mutagenesis resulting in transcriptional fusion and control by the chromosomal promotor of *geneX*. Loss of TetR expression via repression of the *geneX* promotor results in de-repression of tnpR and irreversible excision of the res cassette causing the same resistance profile as described above (Kn^R & Suc^S) to (Kn^S & Suc^R).

Based on our hypothesis microbial pathogens like *V. cholerae* silence genes during *in vivo* passage to achieve full colonization fitness. To challenge this idea, we tested the impact of gene repression during murine colonization by competing *V. cholerae* strains constitutively expressing *ivr* genes against the isogenic wildtype in the infant mouse model of cholera. In six out of nine cases, constitutive expression of an *ivr* gene resulted in significant attenuation *in vivo* compared to the wildtype. Thus, silencing of *ivr* genes seems to be crucial to achieve full colonization fitness.

For most *ivr* genes, ongoing and future studies aim to elucidate the exact mechanism why silencing of these *ivr* genes is required for proper colonization. However, some *ivr* candidates already connect *in vivo* gene silencing to other important networks and pathways. To briefly touch two examples, we could show that overexpression of the VC0704–2 operon causes a 10-fold attenuation during intestinal colonization. Notably, the operon has been previously implicated to modulate biofilm formation of *V. cholerae*. [14,15] VC0704 and VC0703, also known as *nspS* and *mbaA*, have been recently reported to encode a polyamine signaling system affecting biofilm formation via modulation of c-di-GMP levels. [16,17] The current model suggests that NspS binds norspermidine and spermidine causing distinct conformational changes, which in turn affects the interaction of MbaA enhancing or decreasing its c-di-GMP phosphodiesterase activity resulting in high or low exopolysaccharide production and consequently increased or decreased biofilm formation. In *V. cholerae* levels of the second messenger need to be tightly controlled and vary along the life cycle as c-di-GMP positively regulates biofilm formation through induction of the *vps* genes [18,19], but represses motility and virulence. [20–22] Although the exact molecular mechanism causing attenuation of strain overexpressing the operon

needs to be elucidated one could easily imagine that interference in the c-di-GMP signaling might disturb coordinated virulence gene expression.

Furthermore, strains constitutively expressing VC2137, also known as *flrA*, exhibit a 7-fold attenuation during intestinal colonization. The transcriptional activator FlrA is the key class-I regulator for flagella gene expression in *V. cholerae*. [23] Importantly, ON/OFF-control of the polar flagellum of *V. cholerae* and virulence regulation are connected during intestinal colonization in a spatio-temporal manner. In the initial phase of infection, the flagellar motility is required to attach to and penetrate through the intestinal mucosal layer. [24,25] During this mucosal penetration the flagella breaks, which results in secretion of the anti-sigma factor FlgM and consequently releases the alternative sigma factor FliA, belonging to class II flagella genes and essential to activate flagella class IV genes for assembling a functional flagellum. [26,27] Once *V. cholerae* has digged into the mucus, flagellar motility becomes dispensable and flagellar gene expression is tuned down by a Lon-dependent proteolysis of FliA to allow full virulence gene expression. [28–30] At the late stage of infection *V. cholerae* activates a RpoS-dependent mucosal escape response to again induce flagella and chemotaxis genes, which facilitates detachment from the epithelium and transition into the aquatic environment. [8,31] Our data suggests that constitutive expression of FlrA can be as detrimental for colonization fitness as presence of FliA, which is a potent inhibitor for virulence gene expression. [28] Thus, tight spatio-temporal control of the flagellar gene cascade seems to be crucial for proper colonization fitness.

Overexpression of VCA0526 (ClcA, H^+/Cl^- transporter, CLC family) also resulted in a pronounced 50-fold colonization defect *in vivo*. In general, the CLC family comprises several integral membrane proteins involved in the translocation of chloride ions through biological membranes. Moreover, these chloride channel proteins were previously connected to the acid tolerance response (ATR) against hydrochloric acid in bacteria. [32] Interestingly, the ATR was already identified to be induced during infection and crucial for survival in acidic conditions, which are encountered during passage of the stomach. [33,34] Intrigued by these facts, ClcA was prioritized for a more detailed analysis to reveal molecular explanation for required downregulation during intestinal colonization.

H^+/Cl^- transporter of *V. cholerae* requires different regulation and temporal expression through gastrointestinal tract

Gastrointestinal pathogens, such as *V. cholerae*, have to pass the acidic stomach barrier to reach their primary colonization sites in the intestine. Thus, bacteria have evolved protective mechanisms known as ATR to enhance survival under low pH conditions.[35,36] The most common ATR pathway of bacteria is the amino acid-dependent decarboxylation system, found in several gastrointestinal pathogens, including *Escherichia coli, Shigella flexneri, Salmonella typhimurium, Listeria monocytogenes* and *V. cholerae*. [37] Excessive protons are detoxified by cytoplasmic amino acid-decarboxylases converting glutamate, arginine or lysine to CO_2 and γ-amino butyric acid, agmatine or cadaverine, respectively (Figure 3). This is coupled with an antiporter importing the amino acid in exchange to the amine. Thus, bacteria can stabilize the intercellular pH for survival conditions from life-threatening acidic environment. Two open questions arise: First, the antiporter process is electrogenic and effectively moves a positive charge outward (e.g.: $glutamate^-$ exchange for GABA°, $arginine^{1+}$ exchange for $agmatine^{2+}$, or $lysine^{1+}$ exchange for $cadaverine^{2+}$). Hence, an electrical shunt is required to prevent the excessive inner-membrane hyperpolarization. Second, the counterion of the acid, e.g. chloride in case of hydrochloric acid, accumulates and needs to be removed. H^+/Cl^- transporters of the CLC family were implicated to fulfil these roles, especially in the context of the hydrochloric acid-rich stomach, by export of two chloride ions for one proton. [32]

In concordance to this proposed mechanism, we can show that a loss-of-function mutant of the H^+/Cl^- transporter (Δ*clcA*) is attenuated during passage of the acidic stomach as well as *in vitro* conditions with low pH due to high levels of hydrochloric acid. Moreover, our study reveals a tight spatial regulation of the ClcA-system *in vivo*. *V. cholerae* silences *clcA* in alkaline environments,

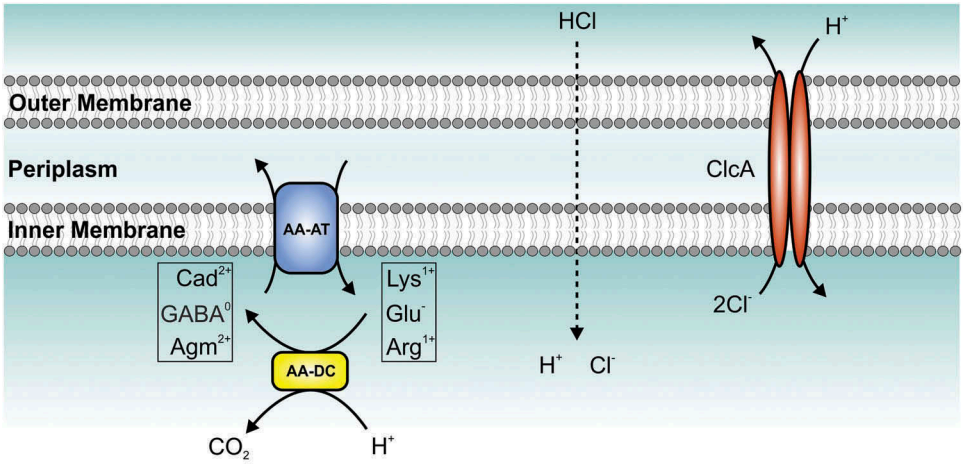

Figure 3. Model of the amino acid-dependent decarboxylation system and ClcA (H^+/Cl^- transporter) within the bacterial acid tolerance response. Upon exposure to hydrochloric acid (HCl), bacteria activate the amino acid-dependent decarboxylation system: amino acid decarboxylase (AA-DC, yellow) convert amino acids [lysine (Lys^{1+}), glutamate (Glu^-) or arginine (Arg^{1+})] to their decarboxylated versions [cadaverine (Cad^{2+}), γ-aminobutyric acid (GABA°), or agmatine (Agm^{2+})] thereby consuming a proton (H^+) and producing carbon dioxide (CO_2). A coupled, electrogenic amino acid-amine antiporter (AA-AT, blue) located in the membrane removes the product and provides new substrate via exchange of Cad^{2+} and Lys^{1+}, GABA° and Glu^- or Agm^{2+} and Arg^{1+}, respectively. The H^+/Cl^- transporter (ClcA, red) exports two chloride-ions (Cl^-) in exchange of one proton (H^+) to detoxify the chloride and acts as electrical shunt to prevent excessive inner-membrane hyperpolarization, which would paralyze the system. However, under alkaline conditions an active H^+/Cl^- transporter would exploit the proton motive force thereby depleting the energy of the bacterial cell. Thus, expression of *clcA* is high in the stomach, but *V. cholerae* represses *clcA* during colonization of the intestine representing an alkaline environment.

such as the intestinal tract representing the primary site of colonization for *V. cholerae*, allowing its identification as *ivr* gene along the study. In such alkaline environments an active H^+/Cl^- transporter exploits the proton-motive force of the bacterium resulting in severe energy depletion. Concordantly, our data indicate that constitutive expression of *clcA* becomes detrimental under alkaline conditions, e.g. during colonization of the lower gastrointestinal tract (Figure 4).

Concluding remarks

Reporter-based systems, such as IVET and RIVET, delivered valuable insights how bacterial pathogens adapt to *in vivo* conditions during host colonization. In our recent study, we extended the application of these single-cell expression technologies to identify gene silencing during *in vivo* colonization of *V. cholerae*. The necessity to silence these genes *in vivo* became evident by subsequent analyses, which revealed that constitutive expression of *ivr* genes can be detrimental for the colonization fitness of *V. cholerae*. Our results highlight the potential of studying gene silencing by bacterial pathogens during infection, which has been so far fairly neglected in the field. It is quite likely that gene silencing has a similar impact for other bacterial pathogens. For example, repression of flagellins at later stages of intestinal colonization seems to be a common theme for several gastrointestinal pathogens, although flagellar motility is generally important at early stages to initiate infections. In addition to *V. cholerae*, *L. monocytogenes* and *Salmonella enterica* serovar Typhimurium downregulate their flagellar motility to avoid host detection, as their flagellins are potent stimulators of the human immune system. [38,39] Moreover, it might be worthwhile to study gene silencing during other stages of the pathogen's lifecycle. For example, we recently adapted RIVET to identify genes induced during biofilm formation of *V. cholerae*, representing its aquatic lifestyle. [40] Amongst other interesting candidates, extracellular nucleases were found to be induced during biofilm formation, which resulted in the characterization of extracellular DNA as a novel matrix component of *V. cholerae* biofilms. [41] Similarly, identification of

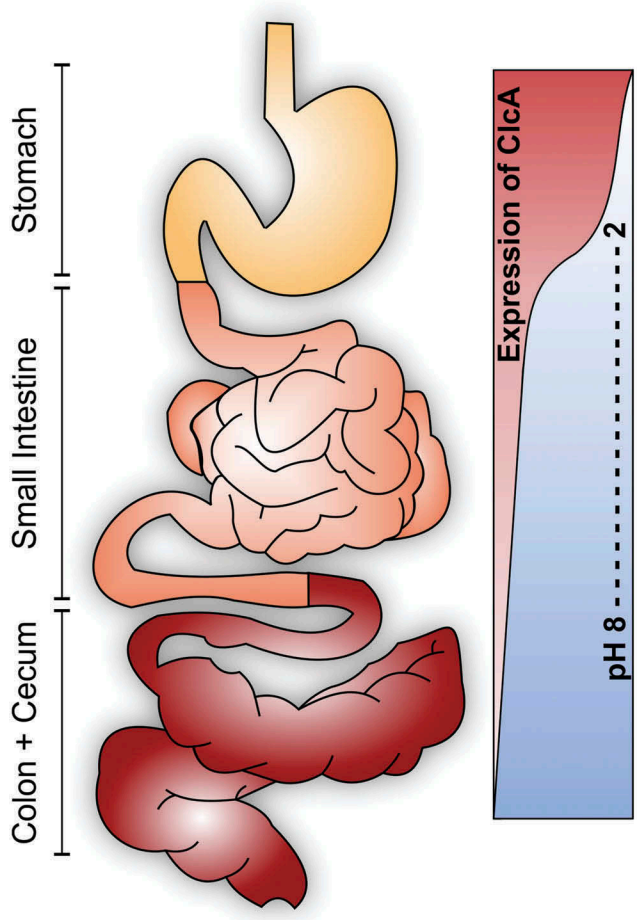

Figure 4. Differential requirement of ClcA (H^+/Cl^--transporter) along the human gastrointestinal tract. Upon oral ingestion, *V. cholerae* has to pass the acidic stomach barrier. ClcA facilitates survival in presence of hydrochloric acid via detoxification of chloride and acts as an electrical shunt to prevent membrane hyperpolarization. Once *V. cholerae* reaches the alkaline environment of the intestine an active ClcA would exploit the proton motive force thereby disrupting the energy metabolism. Concordantly, *clcA* is induced during the stomach passage, but silenced during colonization of the intestine.

genes silenced during biofilm formation could provide new physiological insights in this persistence mode. The design of the reporter-technology TRIVET offers the possibility for such investigations in the future, as it could be adapted to different conditions and other genetically engineerable bacterial pathogens.

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