6. Discussion
6. DISCUSSION

6.1. Different metabolic pathways and survival of the pathogen *V. cholerae*

*V. cholerae* is a human intestinal pathogen and is autochthonous to brackish water bodies. These two environments are very disparate in nature and energy sources for the survival of the pathogen. Successful utilization of carbon sources like different kinds of multiple sugars are dependent on different types of sugars, multiple specific uptake systems, and different catabolic pathways. In the intestine, the numerous ecological niches are present which are defined by availability of different types of nutrients (Peekhaus and Conway, 1998b). According to Freter’s nutrient niche hypothesis (Freter *et al.*, 1983), the mammalian intestine is similar to a chemostat in which different types of bacterial species are in equilibrium and to co-colonize within intestine, each species must use at least one limiting nutrient better than all the other species (Conway and Cohen, 2014). Therefore, *V. cholerae* should also utilize nutrients in a proficient way to maximize its colonization potential in the human intestine. It is evident that human intestine contains a variety of nutrients which includes neutral sugars, amino sugars, and sugar acids like glucose, galactose, mannose, ribose, fucose, N-acetyl glucosamine, N-acetyl galactosamine, N-acetyl glucosamine, N-acetyl glucosamine, galactosamine, N-acetylneuraminic acid, gluconate, galactonate, iodonate, ketogluconate, fructuronate, galacturonate, glucuronate (Peekhaus and Conway, 1998b). In general, most
of the bacteria of enterobacteriaceae family can use sugars via Embden-Meyerhof-Parnas (EMP), Entner-Doudoroff (ED) and Pentose Phosphate (PP) pathways. Very limited studies as yet have been made to address the role of sugar utilization in fitness or pathogenesis of *V. cholerae* (Meibom *et al*., 2004; Almagro-Moreno., 2009, Ghosh *et al*., 2011; Moisi *et al*., 2013). Earlier, gluconate (Gnt) has been shown to be an important hexonate sugar available in the intestinal environment which can be utilized through the ED pathway and can also exert signals for bacterial pathogenesis (Sweeny *et al*., 1996a; Eriksson *et al*., 2003). Recently, it was shown that Gnt utilization through the ED pathway resulted into induction of virulence phenotype in *V. cholerae in vitro* (Patra *et al*., 2012). However, role of all the components of Gnt utilization system including the ED pathway involved in overall physiology and virulence regulation in *V. cholerae* are still in grey shades. Therefore, this study was undertaken to characterize and evaluate Gnt utilization system and its functional involvement in pathogenesis of *V. cholerae*. No studies, except this attempt, were recorded in the literature regarding functionality of Gnt utilization system in *V. cholerae*.

### 6.2. Establishing the Gnt utilization system in *V. cholerae*

In this study, we used a genome wide homology based bioinformatics and *in silico* characterization to predict existence of Gnt utilization system in *V. cholerae*. It could be concluded that Gnt utilization system exists in *V. cholerae* which was shown to be constituted of *gntU*, *gntK*, *gntR* and the ED
pathway genes (edd and eda). Absence of E. coli ortholog genes, gntT, gntV, gntW and gntP, together with unique localization of the components of the V. cholerae Gnt utilization system indicated presence of a firm regulatory system in V. cholerae. Gene organization is an important aspect of transcriptional regulation. Therefore, we focused on the organization and characterization of the Gnt utilization system among different gut-pathogens as well as in commensal. Genes of the Gnt utilization system is found to be present in close proximity in case V. cholerae in comparison to other organisms. The close proximity of all genes of the Gnt utilization system in V. cholerae was unique among rest of the pathogen. Such unique organization of component genes of the Gnt utilization system might have two consequences: i) tight regulation and co-ordinate expression of complex metabolic pathways genes ii) synchronous expression of all the genes in an operon fashion leading to better operation of this system. Therefore, we analysed physicochemical characteristics of the components of the Gnt utilization system considering that this might be useful for future studies like drug targeting.

As a part of in silico characterization, phylogenetic analysis of the predicted Gnt utilization genes were performed which pointed out species specific divergent evolution within Gammaproteobacteria. Interestingly, E. coli and S. flexneri formed a monophyletic group which well corroborated to the fact of proposed similarity between these two organisms as established by hybridization studies done earlier (Brenner et al., 1972). Moreover, gntU, gntK and gntR was ubiquitously present throughout the bacterial kingdom
showing adaptive radiation of the Gnt utilization process. Interestingly, functional ED pathway was found to be absent in commensals. One logical hypothesis may be that the Gnt utilization system was omnipresent at very early stages of evolution and lost its functionality among the commensals at a certain stage of their reductive evolution. Therefore, Gnt utilization became important for those bacteria which sojourned human intestine to cause diseases.

The components of the ED pathway genes (edd and eda) (Peekhaus and Conway 1998b) were predicted to be an integral part of proposed Gnt utilization system of *V. cholerae*. Although, functionality of the edd was reported earlier from our laboratory (Patra et al., 2012), other predicted genes (eda, gntU, gntK and gntR) were never been characterized earlier for *V. cholerae*. In our previous study, it was shown that *V. cholerae* utilized glucose (Glu), Gnt or N-acetyl glucosamine (GlcNAc) as sole carbon source *in vitro*. Involvement of the EMP and ED pathway was reported for utilization of Glu and Gnt, respectively. The PP as well as other two pathways were involved for GlcNAc utilization.

### 6.3. Functional Gnt utilization system in *V. cholerae*

To ascertain the functional involvement of all genes of the Gnt utilization system, growth experiments were performed using mutants of predicted Gnt utilization genes of *V. cholerae*. Wild type *V. cholerae* N16961 grew well in minimal media supplemented with Gnt as sole carbon source.
However, mutants failed to grow in minimal media with Gnt as sole carbon source except the gntR mutant. This data confirmed that all genes *edd, eda, gntU* and *gntK* were required minimally for Gnt utilization by *V. cholerae*. In case of *E. coli*, when similar strategies were followed, mutants showed only growth retardation. Subsequent studies revealed existence of parallel mechanisms for Gnt transporter (*gntU, gntT, gntP* and *gntW*), and Gnt kinase (*gntK* and *gntV*) leading to the establishment of GntI and GntII systems (Tsunedomi *et al.*, 2003). Existence of analogous systems in *E. coli* was thought to be essential for the Gnt utilization during its intestinal survival (Tsunedomi *et al.*, 2003). However, such parallel system was not apparent in *V. cholerae*.

To characterize *V. cholerae* Gnt utilization system, structure-function modelling approaches were utilized for the GntU, the GntK and the GntR. The initial step of the Gnt metabolism in *V. cholerae* was considered for entry of Gnt into the cells. In general, proteobacteria use two major schemes to import and phosphorylate amino sugars in the cytoplasm: (i) sugar-specific phosphotransferase system (PTS) or (ii) a combination of sugar-specific permeases and kinases (Leyn *et al.*, 2012). As Gnt is a non-PTS sugar (Hogema *et al.*, 1997) thereby it should be passed through a cognate transporter present in the membrane of *V. cholerae*. It was evident from *in silico* analysis that GntU of *V. cholerae* possess more than 12 membrane spanning segments which is character of a large family of transport proteins as similar as *E. coli* Gnt permease (Henderson, 1993; Klemm *et al.*, 1996; Tong *et al.*, 1996). The GntU also possessed higher positivity in the predicted intracellular loop. According
to the positive-inside rule, which states that lysine and arginine residues to be preferably located on the cytoplasmic side of the membrane protein (von Heijne, 1992). Taken together, this *in silico* analysis clearly indicated that the GntU of *V. cholerae* to be considered as transporter despite the fact that *gntU* of *V. cholerae* showed 46% homology to *E. coli* *gntU*. Analysis of the GntK by SOSUI web based server also predicted its cytoplasmic localization (Hirokawa *et al*., 1998). Further to this, we used web based analysis of ATP binding site prediction using sequence-based template-free predictor (TargetATPsite) (Yu *et al*., 2013) which implements two important characters i) prediction of binding residues ii) prediction of binding residues dependent binding pocket in the GntK. Interestingly, no such motif was observed in remaining genes (Edd, Eda, GntU and GntR) of predicted Gnt utilization system of *V. cholerae* N16961. Therefore, it was concluded that GntK of *V. cholerae* was responsible for conversion of Gnt to gluconate-6 phosphate despite the fact that *gntK* of *V. cholerae* showed 53.7% homologies with *E. coli* *gntK*.

More than half a century ago with the pioneering works of Jacob and Monod and their colleagues on regulation of the *lac* operon opened a the then new field on transcriptional regulation in bacteria (Jacob and Monod 1961). Despite evolutionary flexibility in the operon structure, transcriptional regulation remains as a major determinant for proper functionality (Rubinstein *et al*., 2011). The RegPrecise 3.0 database search revealed that the GntR was to be considered as a LacI family protein (Novichkov *et al*., 2013). In
silico analysis and homology based modelling of the GntR revealed presence of putative LacI family helix-turn-helix (HTH) domain suggesting its DNA binding ability (Nguyen and Saier, 1995), an essential trait of a regulator molecule (Pérez-Rueda and Collado-Videsa, 2000). Inactivation of gntR did not cause depression of growth of *V. cholerae* mutant in minimal media containing gluconate. Depression of growth was expected by considering the GntR to function as negative regulator. In fact, the gntR mutant displayed weaker growth in minimal media containing Gnt as sole carbon source. We moved further by analysing transcription by Real-Time PCR. The real-time PCR based analysis confirmed up regulation of the Gnt utilization system genes in gntR mutant and thereby confirming role of GntR as negative regulator. Apparent ambiguity in growth suppression as observed in the gntR mutant in minimal media containing Gnt as sole carbon source can be explained by over activity of the *edd* for accumulation of cellular toxic product KDPG. The GntR of *E. coli* has also been represented to act as repressor with DNA binding properties (Peekhaus and Conway, 1998a). This led us to take an attempt to assess DNA binding abilities of *V. cholerae* GntR to the predicted R1 and R2 sites located within identified Gnt utilization system of *V. cholerae* through gel retardation assay. The gel retardation assay revealed that *V. cholerae* GntR had ability to bind to the R1. However, in our assay conditions non-binding of the GntR to R2 site was noted. Such observation can be explained by considering presence two consensus binding sequences with two bases overlapping were present in the R1 site that covered a stretch of 30
bases region. Presence of two overlapping binding regions in R1 may aid stronger binding affinity of the GntR to this site as compared to R2. Thus detection of non-binding phenotype of GntR to the R2 site might be attributed to weaker affinity to this site which was below the limit of detection in the in vitro assay. Interestingly, presence of Gnt inhibited the repression role of the GntR. Such observation was in tune to induce role of the Gnt to derepress the Gnt utilization system. Such de-repression was to be considered to be beneficial for V. cholerae for Gnt utilization during in vivo growth or during infection process.

6.4. The Gnt utilization system and pathogenesis of V. cholerae

Role of the Gnt utilization system in modulating V. cholerae pathogenesis was evaluated. It has been already established that in vitro optimal expression of CT and TCP was observed in AKI condition for El Tor biotype strain (Iwanaga and Yamamoto, 1985). Previous studies revealed that Gnt induced CT production in vitro and the edd mutant (non-functional ED pathway) was severely attenuated for mouse intestinal colonization as well as CT production (Patra et al., 2012). Such observation was extended through this study. Isogenic in frame mutants of the identified V. cholerae Gnt utilization system were generated and tested their ability with respect to expression of V. cholerae virulence factor. Study carried out with mutants (non-functional components of the Gnt utilization system) revealed
attenuation of the colonization potential and virulence phenotypes. This data indicated that the components of the Gnt utilization system played certain role in \textit{V. cholerae} pathogenesis. Comparative analysis of the impact as exerted by the different mutants revealed that the most attenuation was observed by the \textit{edd} mutant, a mutant defective for the ED pathway. The extent of attenuation of \textit{V. cholerae} virulence was in the following order \textit{edd}\textgreater\textit{eda}\textgreater\textit{gntU}, \textit{gntK}\textgreater\textit{gntR}. All these suggested that functional \textit{edd} and \textit{eda} to be considered more important from the point of view of \textit{V. cholerae} pathogenesis. However, basis of high degree of virulence attenuation in \textit{edd} and \textit{eda} mutants are yet to be explored. It was clear that Gnt, available in intestine (Klemm \textit{et al.}, 1996), may promote virulence regulatory pathways in \textit{V. cholerae} during \textit{in vivo} survival and virulence. Therefore, it may be said that identified Gnt utilization system genes, particularly \textit{edd} and \textit{eda} can serve as novel drug target for the treatment of cholera.

\textbf{6.5. Antimicrobial agents: todays challenges}

The invention of antibiotics catalysed a drastic medical revolution, by drastically decreasing mortality due to bacterial infections (Maxson and Mitchell, 2015). With advances in healthcare like vaccines and improved sanitation, antibiotics have contributed to an extension in the average life expectancy in many countries (Arias, 2014). However, due to the rapid emergence of resistance to antibiotics and anti-microbial agents, human civilization needs new approaches to fight bacterial infections. Resistance to
antimicrobial agents has inevitably followed with the release of newer drugs (Clatworthy et al., 2007; Walsh and Wencewicz, 2014). Thus, development of new antimicrobial agents should keep pace to cope up with the emerging of resistance to existing antimicrobials. Given the difficulties in developing new antimicrobials for safe use in human, innovative screening methods to discover new drugs has become essential. There are generally two types of antimicrobials are available i.e. broad spectrum and narrow spectrum. The use of broad-spectrum antimicrobials appears to be correlated with increased emergence of multidrug resistance (de Man et al., 2000; May et al., 2006; Dortch et al., 2011). Administration of several broad-spectrum antibiotics can have a devastating impact on the resident intestinal microbiome and recovery from such adverse effect takes months after cessation of treatment (Jernberg et al., 2010). In contrast to broad-spectrum treatment, use of narrow-spectrum drugs may slow the spread of resistance with lesser impact on the human microbiome, leading to reduced horizontal gene transfer (de Man et al., 2000; Rashid et al., 2012). Narrow spectrum drugs include pathogen specific antibacterial and/or anti-virulence drugs. Emergence of multidrug resistant (MDR) strains of *V. cholerae* have been reported from all over the world (Kitaoka et al., 2011; Pang et al., 2016; Folster et al., 2014; Jain et al., 2016). So, to identify newer drugs specific to *V. cholerae* have been considered as an important aspect. The fruitful strategies to identify novel antimicrobials by screening of small molecule natural products as well as screening from synthetic small molecules libraries have become todays need (Maxson and
Mitchell, 2015). To identify novel anti-\textit{V. cholerae} and anti-virulence compound(s), a novel high throughput screening assay (HTS) was developed. This part of the work also carried out at HZI, Germany. This was an initiation for identifying small molecule(s) suitable as drug against cholera.

### 6.6. Anti-\textit{V. cholerae} small molecules

To identify pathogen-specific narrow-spectrum antimicrobials from small molecule library at HZI, Germany, constitutively green fluorescence expressing indicator \textit{V. cholerae} O139 strain MO10 pGP13 was used. After screening of 28,300 compounds, this HTS assay identified 6 active compounds with growth inhibitory properties of \textit{V. cholerae}. These 6 compounds did not show any antibacterial effect on other human enteric pathogen. These 6 compounds were structurally different from already known compounds. Importantly, among these six compounds, the compound vz0825 exhibited lowest minimal inhibitory concentration (MIC) value and was characterized further. Mode of action of this compound identified unique kinase locus in \textit{V. cholerae}, a novel target for future studies in the areas of \textit{V. cholerae} drug development (Sergeev \textit{et al.}, 2014). The compound vz0825 was effective at a concentration of 1.6 µM to inhibit \textit{V. cholerae} growth. It showed cytotoxicity with c-IC\textsubscript{50} value of 14 µM which is very less indeed. Finally, the structure of the anti-\textit{V. cholerae} compound can provide a lead or serve as focal point for appropriate modifications by the methods of medicinal chemistry for
synthesizing new derivatives with better activities and with reduced cytotoxicities.

6.7. Anti-virulence small molecules against *V. cholerae*.

An alternative strategy for treating the disease caused by a particular bacterial pathogen is to interfere directly with pathogenesis. Such an anti-virulence strategy was planned against *V. cholerae* pathogens and a novel compound virstatin was identified (Hung *et al.*, 2005). The compound was shown to inhibit expression of most important virulence factors CT and TCP in *V. cholerae* by inhibiting dimerization of ToxT (Shakhnovich *et al.*, 2007a). Expression of CT and TCP have been shown to be linked with quorum sensing (QS) systems and the presence of multiple QS systems have already been reported in *V. cholerae* (Camara *et al.* 2002), which proceed simultaneously and have different regulatory functions, e.g. virulence gene expression, biofilm formation or protease production (Jobling and Holmes 1997; Miller *et al.* 2002; Hammer and Bassler 2003). The HapR is the central regulator of all three QS systems. The HapR negatively regulates the expression of the aphA and thereby inhibited expression of the virulence genes *ctxA* and *tcpA*, and thus connects the QS system with virulence gene expression (Kovacikova and Skorupski, 2002). The aphA has the potential to work as positive master regulator for quorum sensing. With this background, a novel HTS assay was developed in order to identify compounds with potential to suppress *V. cholerae* virulence by linking reduced aphA
expression. The selection of aphA as in this target loci HTS was based on the fact that the AphA i) is the master regulator of quorum sensing at low cell density (LCD) (Rutherford et al., 2011) ii) modulates index virulence gene expression, CT and TCP (Kovacikova and Skorupski, 1999), iii) controls many genes at LCD which include flagella synthesis, type III secretion system, pilus production etc. (Rutherford et al., 2011), and iv) is present and conserved among V. cholerae strains. The plasmid construct pAKSB was developed to confer conditional KmR under in vitro virulence inducing conditions (AKI) which served as index for the expression of aphA. The reliability of the assay was established by confirming the conditional expression of the KmR phenotype in AKI culture condition, optimal for the expression of CT and TCP by V. cholerae El Tor and O139 strains in vitro. Such KmR phenotype was absent in NB pH 8.5, a virulence repressing condition. Application of the optimized HTS assay resulted in the identification of 6 active compounds from a library of 20,338 compounds. These compounds exhibited potential to attenuate V. cholerae virulence from a total of. Among the six active compounds, vz0761, vz0852 and 53760866 were found to be the most potent by their high inhibitory index. Results from a real-time PCR based assay confirmed that the inhibitory effects of these 3 compounds were indeed linked to a reduction of aphA transcription in V. cholerae. Transcriptional analysis also confirmed concomitant reduction of the ctxA and tcpA transcripts in presence of these compounds in the growth media which
corroborated well with reported \( \text{aphA} \) dependent inhibition of \( \text{ctxA} \) and \( \text{tcpA} \) expression at the transcriptional level (Kovacikova et al., 1999).

Of the 3 most active compounds 53760866 displayed the lowest cytotoxicity in a eukaryotic cell based assay (L929 cells) done at HZI, Germany and were therefore characterized further. The compound 53760866 mediated transcriptional inhibition of the index virulence genes (\( \text{ctxA} \) and \( \text{tcpA} \)) resulting in a reduction of CT and TcpA expression. The relevance of our \textit{in vitro} observation was extended to an \textit{in vivo} evaluation. The suckling mouse colonization assay showed that 53760866 can effectively reduce intestinal colonization when co-administered with \textit{V. cholerae} NM06-058. However, limitation of our study is that the mechanism of inhibition of \( \text{aphA} \) transcription by 53760866 is still not clear. The mechanism by which this potent chemical compound diminishes expression of the \( \text{aphA} \) may be either by down regulation of \textit{qrr} small RNAs transcription or by activation of the HapR. These details are to be elaborated in the future. In conclusion, we report the development of a HTS assay targeting altered expression of the \( \text{aphA} \) as an index to identify \textit{V. cholerae} virulence suppressive compounds. Successful application of this new HTS made it possible to identify compound 53760866 with \textit{V. cholerae} virulence suppressive potential. The structure of the compound can provide a lead or serve as focal point for appropriate modifications by the methods of medicinal chemistry for synthesizing new derivatives with better activities and reduced toxicities. Despite the potential benefits of anti-virulence compounds, the fact that they don’t suppress
growth of the pathogen and therefore continuous availability of the drug at the infection site become an important issue. Some pathogenic organisms use large arsenals of virulence factors and inhibiting a single one may not adequately reduce their pathogenicity. Considering the fact that \textit{V. cholerae} utilizes two prime virulence factors CT and TCP, any drug/molecule with potential to reduce CT and TCP will be sufficient enough to treat the disease cholera.
7. References
7. REFERENCES


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8. Summary
8. SUMMERY

Salient findings of this study are given below

- For the first time, gluconate (Gnt) utilization system of *V. cholerae* has been characterized and the system has been shown to be composed of single Gnt transporter (*gntU*), single Gnt kinase (*gntK*), 6-phosphogluconate dehydratase (*edd*), 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (*eda*) and Gnt repressor (*gntR*).

- This Gnt utilization system of *V. cholerae* appeared to be unique, by genomic organization of component genes as compared to other enteric pathogens. Components genes of the identified Gnt utilization system are in close vicinity in the genome of *V. cholerae*. Nucleotide and amino acid based phylogenetic analysis using components of the Gnt utilization system of *V. cholerae* and other Gamma-proteobacteria revealed *vibrionaceae* group formed monophyletic branch while other species of proteobacteria were placed in different branches.

- *In silico* analysis and reverse transcriptase based PCR analysis showed presence of two bicistronic operon structure (*gntK-edd* and *gntU-eda*) in opposite orientation while *gntR* was transcribed in monocistronic fashion.

- Growth phenotype analysis with the mutants defective in the Gnt utilization system revealed that mutants failed to utilize Gnt as these
mutants did not grow in medium containing Gnt as sole carbon source. Such growth defect was restored upon complementation.

- **In silico** analysis of *V. cholerae* GntU identified 13 transmembrane segments, net charge of positivity (Arginine-lysine residues) in the cytoplasmic loop. These were in well agreement to proposed localization of GntU as transmembrane protein to mediate transport of Gnt from outside to inside the cell.

- Phosphorylation potential of GntK was predicted by *in silico* analysis. Analysis revealed presence of an ATP binding motif at the N-terminal region of the GntK.

- **In silico** analysis indicated that the GntR is consisted of a LacI family DNA binding helix-turn-helix (HTH) domain as well as sugar binding domain. Bioinformatics based analysis also indicated DNA binding region of the GntR located upstream of the two proposed operon (*gntK-edd* and *gntU-eda*) of the Gnt utilization system. Gel retardation assay confirmed binding of the GntR to this region. Further to this, mutation of *gntR* showed up regulation of the genes of the Gnt utilization system. All these data collectively confirmed the role of the GntR as negative regulator of the Gnt utilization system.

- Inactivation of the Gnt utilization system leads to attenuation of cholera toxin (CT) production abilities *in vitro* in AKI condition, a
condition known for optimal expression of CT by *V. cholerae* O1 El Tor and O139 strains.

- *In vitro* reduction of toxicity by the mutants was further assessed in rabbit ileal loop (RIL) model. The RIL assay showed lowering of toxicity in the mutants. Further to this, suckling mouse colonization assay was performed to have an assessment of the impact of the mutations on colonization potential. Results showed varying degree of attenuation of the mutants. Decrease of colonization potential was in the order *edd* > *eda* > *gntU* > *gntR* > *gntK* mutants. It may be said that mutations in *edd* and *eda* (the ED pathway) caused severe degree of attenuation of *V. cholerae* both *in vivo* and *in vitro*.

- A high throughput screening (HTS) assay was developed and new antibacterial compounds like vz0825, vz0500 and 1541-0004 specific to *V. cholerae* were identified at HZI, Germany. Minimal inhibitory concentration (MIC) were tested against *V. cholerae* strains N16961, El Tor, O1 Inaba, and also with the El Tor variant strain NM06-058 O1, Ogawa. Compound vz0825 showed highest antibacterial activities as evident by a lowest MIC concentration of 1.6 µM. The compound vz0825 is less cytotoxic as revealed by IC_{50} (14 µM) value. These compounds did not exert any growth inhibition against other pathogenic bacteria like *Escherichia coli*, *Shigella flexneri*, *Shigella boydii* and *Salmonella typhimurium* up to 50 µM concentration.
Another type of HTS assay was developed by targeting *aphA*, a master regulator of virulence in *V. cholerae*. Through this HTS assay, novel compounds with antivirulent properties were identified. Among other six identified compounds, the compound 53760866 displayed lowest g-IC$_{50}$ values in AKI (>100 µM) and AKI-Km (38 µM) against the HTS reporter strain MO10pAKSB and >100 µM for *V. cholerae* NM08-058. The inhibitory index value (calculated as a ratio between g-IC$_{50}$ in AKI to AKI-Km) was also very low (5.26 µM). The compound 53760866 showed lowest cytotoxicity. The compound resulted into reduction of CT production, toxin coregulated pilus (TCP) expression *in vitro*. The compound also showed reduce colonization potential in suckling mouse colonization model. To ascertain global effect by the compound 53760866, microarray based transcriptome analysis was performed which showed reduction of expressions of different metabolic pathway genes including the Gnt utilization system as well as virulence regulatory cascades of *V. cholerae*. 
9. Appendix
9. **APPENDIX**

9.1. **Microbiological media**

**Luria Bertani (LB) medium**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto Tryptone (BD Difco, USA)</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Bacto Yeast extract</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>10.0 gm</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in 1000 ml of distilled water.

Media was sterilized by autoclaving at 15 lbs for 15 min.

**Luria Bertani agar (LA)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto tryptone (BD Difco, USA)</td>
<td>10.0 gm</td>
</tr>
<tr>
<td>Bacto Yeast extract (BD Difco, USA)</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Agar (BD Difco, USA)</td>
<td>15.0 gm</td>
</tr>
</tbody>
</table>

All the ingredients were dissolved in 1000 ml of distilled water.

Media was sterilized by autoclaving at 15 lbs for 15 min and 20 ml portion of the sterilized media was poured onto 90 mm plate (Axygen, USA).
M9 minimal media

Disodium hydrogen phosphate (SRL, India) 6.0 gm
Potassium dihydrogen phosphate (SRL, India) 3.0 gm
Ammonium chloride (SRL, India) 1.0 gm
Sodium chloride 0.5 gm

All the ingredients were dissolve in 1000 ml of distilled water and autoclaved. After cool down the media, 1 ml of 1 M sterilized magnesium sulphate (SRL, India) solution and 0.1 ml of 1 M sterilized calcium chloride (SRL, India) solution were added.

AKI medium

Peptone (BD Difco, USA) 15.0 gm
Yeast extract (BD Difco, USA) 4.0 gm
NaCl (SRL, India) 5.0 gm
NaHCO₃ (SRL, India) 4.0 gm

Above ingredients except NaHCO₃ were dissolved in 950 ml of distilled water and autoclaved at 15 lbs for 15 min. 4 gm of NaHCO₃ was dissolved in 50 ml of distilled water and filtered
through 0.22 μm Millipore membrane aseptically and added to the sterile medium. pH of the medium was self-adjusted to 7.4.

9.2. Reagents

Voges-Proskauer (VP) broth

Peptone 7 gm
K$_2$HPO$_4$ 5 gm
Glucose (Sigma, USA) 5 gm
NaCl 10 gm
Distilled H$_2$O 1000 ml

Composition of Reagent A

5% α-napthol (SRL, India) in absolute ethanol (Spectrochem Pvt. Ltd, India).

Composition of Reagent B

0.3% Creatinine (SRL, India) & 40% KOH (SRL, India).

Cetyltrimethyl ammonium bromide (CTAB) solution (10% CTAB in 0.7M NaCl)

4.1 gm sodium chloride was dissolved in 80 ml distilled water and then 10 gm CTAB (Sigma, USA) was slowly added along
with continuous stirring and heating at 65°C until it was completely dissolved. The final volume was adjusted to 100 ml with distilled water.

**Proteinase K solution for DNA isolation**

20 mg of proteinase K (Sigma, USA) was dissolved in 1 ml of sterile triple distilled water and stored at -20°C.

**DNAase free RNAase**

Pancreatic RNAase (RNAase A) at a concentration of 10 mg/ml was dissolved in 10 ml of 10 mM Tris-HCl buffer, pH 7.5 containing 15 mM NaCl. The mixture was heated at 100°C for 15 min to deactivate contaminating DNAase present in it. The treated RNAase solution was allowed to cool slowly to room temperature and stored at -20°C in aliquots.

**DNA tracking dye (6X)**

- Glycerol (SRL, India) 25 ml
- SDS 0.5 gm
- Bromophenol blue (SRL, India) 0.05 gm
- Distilled water 25 ml
Above ingredients except SDS were dissolved and autoclaved at 15 lbs for 15 min. Finally to the sterile solution SDS was added and stored at 4°C.

**Biotium gel red solution for DNA**

- Biotium GelRed (10,000X) (Biotium Inc., USA) \(30 \mu l\)
- Distilled water \(100 \text{ ml}\)
- Mixed gently and stored at room temperature

**Oxidase Reagent**

- Tetramethyl-p-phenylenediamine (Sigma, USA) \(1.0 \text{ gm}\)
- Distilled Water \(100 \text{ ml}\)

**9.3. Buffers**

**Tris-EDTA (TE) buffer**

- 1 M Tris-HCl, pH 8.0 \(1 \text{ ml}\)
- 0.5 M EDTA, pH 8.0 \(0.2 \text{ ml}\)
- Milli-Q water \(98.8 \text{ ml}\)

Autoclaved and kept at 4°C in 10 ml aliquots.
Tris-acetate-EDTA (TAE) buffer (50X) pH 8.0

Tris base (Spectrochem, India) 242.0 gm
Glacial acetic acid 51.1 ml
Distilled water 900 ml

All the ingredients were mixed, to this 100 ml of EDTA solution pH 8.0 was added and autoclaved at 15 lbs for 15 min. Buffer was stored at 4°C, and 1X working solution was prepared by diluting it into sterile distilled water.

0.15 M Phosphate buffered saline pH-7.4

136.89 mM NaCl 8.00gm
1.47 mM KH₂PO₄ 0.200gm
8 mM Na₂HPO₄ (SRL, India) 1.425gm
2.68 mM KCl (SRL, India) 0.200gm

Dissolve all the ingredients in 1000 ml of distilled water and pH will be self-adjusted to 7.2-7.4.

0.1 M Citrate Buffer, pH- 4.5

0.047 mM Citric Acid (SRL, India) 0.099 gm
0.053 mM Tri-sodium dehydrate (SRL, India) 0.156 gm
Water 10.0 ml
Dissolve all the ingredients in required volume of distilled water and pH will be self-adjusted to 4.5. Just before use required amount of OPD was added and vortex to dissolve it. Next, add required amount of H₂O₂, vortex and immediately dispense 100 μl of substrate solution into each well of ELISA plate.

0.5X Tris borate EDTA (TBE)

45 mM Tris borate (pH-8.0)
1 mM EDTA (pH-8.0)

Tris buffer saline (TBS)

20 mM Tris-Cl (pH-7.5)
0.8% NaCl

9.4. Reagents for SDS-PAGE and Western blot

Sample Buffer

0.5M Tris-HCl (pH=6.8) 200μl (1M stock)
SDS 0.4 gm
Sucrose (SRL, India) 2 gm
Bromophenol Blue 0.01 gm

Dissolve all components and add bromophenol blue make the volume to 10 ml
### Solution A

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (Sigma, USA)</td>
<td>29.2 gm</td>
</tr>
<tr>
<td>Bis acrylamide (Sigma, USA)</td>
<td>0.8 gm</td>
</tr>
<tr>
<td>Water</td>
<td>100 ml</td>
</tr>
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</table>

Stored in cool and dark place.

### Solution B

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>18.2 gm</td>
</tr>
<tr>
<td>HCl (12N) (Fisher Scientific, USA)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.4 gm</td>
</tr>
</tbody>
</table>

Adjust pH to 8.8 and dissolve in 100 ml water. Stored in cool and dark place.

### Solution C

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>6.1 gm</td>
</tr>
<tr>
<td>HCl (12N)</td>
<td>4.2 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.4 gm</td>
</tr>
</tbody>
</table>

Adjust pH to 6.8 and dissolve in 100 ml water. Stored in cool and dark place.
Solution D

Ammonium persulfate (APS) (Sigma, USA) 100mg

Dissolve in 1 ml of distilled water. Prepared just before use.

Running buffer

Tris Base 1.5gm

Glycine (SRL, India) 7.2gm

SDS (0.1%) 0.5gm

Dissolve in 500 ml of water.

Fixative Solution

Methanol 50 ml

Acetic acid (SRL, India) 10 ml

Add water to make up volume 100 ml

Staining solution

Coomassie Brilliant Blue 1.25 gm

Methanol 250 ml

Acetic acid 50 ml
Add water to make up volume 250 ml. Filter sterilization of solution is needed before use.

**Destaining solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>125 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>35 ml</td>
</tr>
</tbody>
</table>

Add water to make up volume 500 ml

**Western transfer buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>2.91 gm</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.465 gm</td>
</tr>
<tr>
<td>SDS</td>
<td>18.75 gm</td>
</tr>
<tr>
<td>Methanol</td>
<td>100 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>400 ml</td>
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</table>
## Composition of the separating and stacking gel of Acrylamide gel

<table>
<thead>
<tr>
<th>Concentration</th>
<th>5%</th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>15%</th>
<th>20%</th>
<th>4.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sol A (ml)</td>
<td>3</td>
<td>4.5</td>
<td>6</td>
<td>7.5</td>
<td>9</td>
<td>12</td>
<td>0.9</td>
</tr>
<tr>
<td>Sol B (ml)</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>-</td>
</tr>
<tr>
<td>Sol C (ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
</tr>
<tr>
<td>Sol D (ml)</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.02</td>
</tr>
<tr>
<td>TEMED (ml)</td>
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<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Water (ml)</td>
<td>10.5</td>
<td>9</td>
<td>7.5</td>
<td>6</td>
<td>4.5</td>
<td>1.5</td>
<td>3.6</td>
</tr>
</tbody>
</table>
9.5. Reagents for gel retardation assay and autoradiography

5 % Native PAGE

- 30% Acrylamide-Bisacrylamide: 5 mL
- 5X TBE: 3 mL
- 10% APS: 400 µl
- TEMED: 20 µl

Add distilled water to adjust volume uptill 30 ml

Fixative solution for auto-radiography

264 gm of X-ray acid fixing salt with hardener (Kodak, USA) was mixed in 1000 ml of distilled water. Keep it at room temperature.

Developer solution for auto-radiography

- Part A (Kodak, USA): 15 gm
- Part B (Kodak, USA): 80 gm

Mix Part A and Part B (Kodak, USA) with 1000 ml of distilled water. Keep it in dark bottle at room temperature.
10. List of publications
10. PUBLICATIONS


Characterization of the gluconate utilization system of Vibrio cholerae with special reference to virulence modulation

Sambit Roy, Tapas Patra, Taniya Golder, Soniya Chatterjee, Hemanta Koley, and Ranjan K. Nandy

1National Institute of Cholera and Enteric Diseases (NICED), Kolkata 700010, India and 2Bose Institute, Kolkata 700054, India

ABSTRACT
Orthologs search identified that the Vibrio cholerae gluconate (Gnt) utilization system minimally consisted of the Entner–Doudoroff (ED) pathway (edd and eda) and three other genes, namely gntU, gntK, and gntR. This system appeared unique by genomic organization of component genes into two operons transcribed in opposite directions. In silico analysis indicated GntU as an inner-membrane protein functioning for transport and GntK as a kinase with cytosolic localization that generates Gnt6P, which is then metabolized through the ED pathway. Enzyme 6-phosphogluconate dehydratase encoded by edd converts Gnt6P to 2-keto-3-deoxy-6-phosphogluconate (KDPG), which is metabolized by the action of KDPG-aldolase encoded by eda. Transcriptional upregulation of the Gnt utilization genes in the gntR mutant matched well to a predicted repressor role of GntR. GntR displayed DNA binding to a region in the promoters of two bi-directionally transcribed operons. Growth defect of mutants in Gnt-supplemented media confirmed obligate involvement of these genes in Gnt utilization and such defect was restored upon complementation. Defective Gnt utilization resulted in attenuation of colonization potential and reduction of cholera toxin secretion in V. cholerae. The ED pathway mutants showed the highest level of virulence attenuation. Overall, this study established a minimal requirement of the V. cholerae Gnt utilization system, which played a critical role in pathogenesis.

Keywords: cholera; gluconate utilization; virulence; regulation; metabolic pathways; Entner–Doudoroff pathway

INTRODUCTION
Vibrio cholerae O1 and O139 are responsible for causing the dreaded diarrheal disease cholera in man. The organism is enormously adapted to survive the disparate environments of the human intestine and aquatic settings, and maintains activation and/or deactivation of several regulatory cascades in tune to environmental stimuli for its own fitness (Reidl and Klose 2002).

Being a non-invasive pathogen, V. cholerae relies on its strong intestinal colonization potential to establish successful infection in the gut (Almagro-Moreno, Pruss and Taylor 2015). It has already been demonstrated in the literature that two different species with a preference for a particular type of growth-limiting nutrient cannot coexist together in a particular niche of the intestine; eventually one will outcompete and eliminate the other.
to establish itself as a resident of that particular niche (Sweeney et al. 1996). However, a less efficient species can overcome the odds with its higher ability to colonize within the intestine (Sweeney et al. 1996). The intestinal mucus layer is an important source of various types of carbohydrates constantly providing growth support to the commensal and sojourn of pathogenic organisms that arrive through the ingestion of contaminated food or water.

Bacterial growth within the lumen of the gut is dependent on its utilization capacity of different types of growth-limiting nutrient. The carbohydrates derived from intestinal mucosa include N-acetylglucosamine, N-acetylgalactosamine, galactose, fucose, sialic acids, etc. and lesser amounts of glucurionate and galacturonate (Peekhaus and Conway 1998b). Studies on Escherichia coli strain MG1655 showed that glucosonate (Gnt) was the major carbon source for large intestinal survival and Gnt utilization was important for both initiation and maintenance stages of colonization when assessed in a mouse model (Chang et al. 2004). Extensive studies have been made on the Gnt utilization system of E. coli. Gnt uptake and initial catabolism to Gnt6P in E. coli through GntI and GntII systems have been well documented (Tsunedomi et al. 2003). During the initial stage of catabolism, Gnt gets converted to Gnt6P, which further metabolizes through the Entner–Doudoroff (ED) or the pentose phosphate (PP) pathway to pyruvate. The two key enzymes distinctive to the ED pathway are: (i) 6-phosphogluconate dehydratase (Edd; encoded by edd) that catalyzes dehydration of 6-phosphogluconate to form 2-keto-3-deoxy-6-phosphogluconate (KDPG), and (ii) KDPG aldolase (Eda; encoded by eda) that cleaves KDPG to pyruvate and glyceraldehyde 3-phosphate, the latter being further catabolized through the EMP pathway and tricarboxylic acid (TCA) cycle. The GntI system, the main system, of E. coli comprises gntT, gntU, gntK and gntR genes encoding high- and low-affinity permease, thermo-resistant Gnt kinase and regulatory molecule, respectively. The genes gntW and gntV encode another high affinity permease and another thermosensitive Gnt kinase, respectively, and form the GntII system in E. coli (Tsunedomi et al. 2003). Regulation of Gnt uptake and initial catabolism in E. coli is tightly regulated through temporal expression of some of these genes. GntII system genes are positively regulated by the cyclic AMP (cAMP)–cAMP receptor protein (CRP) complex and negatively regulated by GntR (Peekhaus and Conway 1998a). In a recent study, the dual role of gntH to activate genes of the GntII system with concurrent repression of the GntI system has been reported, helping the organism to switch between the two systems during different phases of cell growth in Gnt-containing media (Tsunedomi et al. 2003). It is an intriguing situation to note the existence of several alternative mechanisms of uptake and initial catabolism of Gnt by E. coli. Although the physiological basis of the presence of multiple systems in E. coli is largely unknown, deactivation of gntR resulted in altered specificity towards colonizing the intestinal niche in an animal model (Sweeney et al. 1996). Escherichia coli strains defective in eda showed a severe decrease in their ability to utilize Gnt and colonize the intestine in a mouse model (Sweeney, Laux and Cohen 1996). It was reported earlier that an inability to utilize GlcNac or Gnt caused severe virulence attenuation in V. cholerae when tested in appropriate animal models (Ghosh et al. 2011; Patra et al. 2012). Thus, utilization of certain sugars may be considered to have enough influence on V. cholerae survival and pathogenesis in vivo. The intestinal mucus layer is rich in GlcNac while a primary source for Gnt in the intestine has been suggested to be Gnt6P derived from sloughed dead epithelial cell contents (Sweeney et al. 1996). Presumptive Gnt6P-specific phosphatases present in the mucus layer subsequently convert Gnt6P to Gnt.

Although the E. coli Gnt utilization system has been much studied, very little is known about the Gnt utilization system in V. cholerae, an important gut pathogen causing cholera in man. Our earlier work demonstrated obligate involvement of the ED pathway (edd and eda) in V. cholerae for Gnt utilization, and deactivation of the edd resulted in severe attenuation in intestinal colonization and cholera toxin (CT) secretion both in vivo and in vitro (Patra et al. 2012). Although a likely role of Gnt utilization by V. cholerae in mediating pathogenesis was evident, to date the functionalities of Gnt utilization genes in V. cholerae are largely unknown. The present study was undertaken on the Gnt system of V. cholerae to address (i) the components of V. cholerae Gnt utilization, and (ii) the role of Gnt utilization to modulate V. cholerae pathogenesis. The significance of our findings on functional attributes of the Gnt utilization system in V. cholerae is presented and to the best of our knowledge this is the first study to shed light on the minimal requirements of the Gnt utilization system in V. cholerae.

**MATERIALS AND METHODS**

**Bacterial strains, media and antibiotics**

This study was carried out with different bacterial strains and plasmids (see Table S1 in the online supplementary material). Strains were streaked onto Luria–Bertani (LB) (BD, Sparks, MD, USA) agar plates and incubated overnight at 37°C. Strains were also allowed to grow in M9 minimal media [6 g Na2HPO4, 3 g KH2PO4, 1 g NH4Cl, 0.5 g NaCl, 1 mL of 1 M MgSO4 and 100 μL of 1 M CaCl2 in 1000 mL of the medium (SRL, Mumbai, India)] and sterilized by filtration through a 0.22 μm pore size filter (Millipore Corporation, Billerica, MA, USA) with individually supplemented 0.2% (w/v) of either Gnt (Sigma, St Louis, MO, USA), or glucose (Glu) (Sigma) (Patra et al. 2012). For El Tor biotype strain, AKI [(0.5% NaCl (SRL), 0.4% yeast extract (BD), 1.5% Bacto Peptone (BD), and 0.3% NaHCO3 (SRL)], pH 7.4 at 37°C media conditions, was used for CT induction (Iwanaga et al. 1986). Antibiotics such as ampicillin (Amp, 50 μg mL−1), kanamycin (Kan, 30 μg mL−1), chloramphenicol (Cm, 6 μg mL−1), polymyxin B (PolB, 50 U mL−1) and streptomycin (Str, 50 μg mL−1) (Sigma) were also supplemented into appropriate media as and when required.

**Orthologs analysis in predicting Gnt utilization system in V. cholerae**

Well characterized components of the Gnt utilization system of E. coli were used as a template for an orthologs search against the V. cholerae N16961 genome using the KEGG server (http://www.genome.jp/kegg). For this analysis, edd, eda, gntK, gntU, gntT, gntR, gntW, gntV and gntG genes as established for the E. coli Gnt utilization system were considered. Prediction of V. cholerae Gnt utilization genes in operon structures was made using the DOOR2 database (Mao et al. 2009). The PSORTb v3.0 web server (Yu et al. 2010) was used to predict the cellular locations of transcribed proteins of the Gnt utilization genes, and this was further independently supported by the SOSUI web server (Hirokawa, Boon-Chieng and Mitaku 1998). The putative RNA polymerase Sigma 70 binding sites in the upstream of V. cholerae Gnt utilization component genes were predicted by the BPROM server (Solovyev and Salamon 2011). Putative structures supporting localization as membrane protein and ATP binding sites were
predicted by TopPred II (Claros and von Heijne 1994) and TargetATPsite server (Yu et al. 2013), respectively. Protter server was used to draw the 2D figure of membrane protein (Omasits et al. 2014).

**Generation of in-frame deletion mutants**

In-frame deletion mutants of *eda*, *gntU*, *gntK* and *gntR* were constructed using a tri-parental mating procedure with suicide vector construct pDS132 (Philippe et al. 2004). Overlap extension PCR was performed to generate a DNA fragment with in-frame internal deletion of the target gene and sub-clone the fragment into the positive selection suicide vector pDS132. The resulting construct was mobilized into *V. cholerae* N16961 by conjugal transfer and trans-conjugants with integrated vector (integrants) were selected on plates on 6 μg mL⁻¹ Cm (Sigma) and 50 U mL⁻¹ PoiB (Sigma) that allowed the growth of integrants but not the donor or the recipient strain. These integrants were subjected to passages on 10% sucrose (SRL) containing plate to recover the double cross-over mutant with internal deletion of the target gene (Patra et al. 2012). PCR amplicon size-based analysis was performed for an initial identification of target gene deletion event followed by nucleotide sequencing analysis of the PCR amplicon to confirm in-frame deletion. Target genes were *eda*, *gntU*, *gntK* and *gntR* and the corresponding deletion mutants as generated were Δ*edan16961*, Δ*gntun16961*, Δ*gntkn16961* and Δ*gntrn16961*, respectively. Complementation of the mutated genes was performed after incorporation of suitable vectors carrying functional copy in the target gene. Details of these constructs and properties have been described in Table S1 in the online supplementary material.

**Real-time PCR assay**

Total RNA was extracted by the TRIZOL method (Patra et al. 2012) from *V. cholerae* cells grown under different cultural conditions and RNase free DNase I treatment was performed to eliminate contaminating DNA, if any, present in the preparation. Absence of contaminated DNA in the preparation was confirmed by PCR amplicon negativity when performed with extracted RNA as the PCR template. RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis using 1.5 μg of total RNA and with the random hexamers. Real-time PCR assay to detect effects of culture conditions and/or mutation(s) on the relative levels of target gene transcripts was performed with SYBR Green I master mix (Life Technologies, Carlsbad, CA, USA) in an HT Fast real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Target gene specific real-time PCR primers are described in Table S2 in the online supplementary material. Normalization of the data was done using the *recA* transcript as reference and fold changes were estimated (Pfaffl 2001).

**Purification of GntR and gel retardation assay**

The plasmid construct pBADgntR was developed by putting gntR structural gene under the control of the arabinose-inducible promoter of pBAD/HisB (Invitrogen, Carlsbad, CA, USA). Overexpression of His-tagged GntR was achieved by the inclusion of arabinose in the growth media. Cells with overexpressed His-tagged GntR were harvested, lysed by ultrasonic vibration, and cell-free extract after removal of all the debris was made by centrifugation. Over-expressed protein was purified through a Ni²⁺-NTA agarose column according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Eluted protein concentration was estimated using a Bradford assay and purity of the preparation was checked by analyzing the protein on SDS-PAGE. A mixture of marker proteins with known subunit molecular mass was included in a separate lane to estimate the molecular mass of purified His-tagged GntR.

Virtual Footprint server (Munch et al. 2005) analysis was performed to identify putative GntR DNA binding consensus sequences where a positional weight matrix option for *E. coli* GntR was used. Analysis identified two putative DNA binding regions R1 and R2. PCR amplicons were generated spanning identified GntR DNA binding sites and used in gel retardation assay to validate binding of the GntR, if any, in vitro (Mandal et al. 2007; Karisa, Grube and Tamayo 2015) with minor modification. Amplification was performed with γ-³²P labelled primers against *V. cholerae* N16961. Briefly, primer end-labeling was performed by kinasing of 10 pmol of the forward primer with [γ-³²P]ATP (BRIT, Hyderabad, India) and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) and the labelled primer following heat inactivation was used for PCR. Labelled amplicon was purified using a PCR purification kit (Qiagen) and considered as a probe. DNA and protein binding assay reaction was performed in 30 μL total reaction volume which contained varying amounts of purified protein and 1 μg of salmon sperm DNA (Sigma). A separate reaction was also performed that contained 10 mM Gnt and 3 μM of purified GntR. In all cases, the reaction mixture contained 10 000 c.p.m. of labelled DNA in binding buffer which was composed of 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 M KCl, 0.1 mM DTT, 5% v/v glycerol and 0.1 mg ml⁻¹ BSA. Following an incubation of 1 h at 25°C mixtures were electrophoretically separated onto 5% native polyacrylamide gel in TBE (50 mM Tris/borate, 1 mM EDTA, pH 8.0) buffer. The gel was subjected to a pre-run for 1 h at 4°C before samples were loaded. Following electrophoresis, electrophoretic mobility of probe DNA was recorded by autoradiography.

**GM₁ enzyme-linked immunosorbent assay**

The ability to produce CT by *V. cholerae* strains in vitro was assayed by GM₁ enzyme-linked immunosorbent assay (ELISA) (Holmgren 1973) with minor modifications (Patra et al. 2012). The *V. cholerae* strains were grown in varied cultural conditions and cell-free culture supernates were used for the assay. The amount of CT present in the culture supernatant of the *V. cholerae* strain was estimated with the help of a standard curve generated using known amounts of purified CT (Sigma) included in the assay. The ability to secrete CT by *V. cholerae* strains was expressed as ng of CT ml⁻¹ (opacity unit)⁻¹ of the culture determined at 600 nm.

**Rabbit ileal loop assay**

The overall virulence potential of *V. cholerae* strains was determined using a rabbit ileal loop (RIL) assay (Patra et al. 2012). Rabbits were anesthetized; a surgical incision was made to take out intestine for a brief period of time and a maximum of six ligated ileal loops (∼10 cm in length) per rabbit were made. Each of the ligated intestinal loops received either 1 mL of the culture suspension comprising ∼10⁹ colony forming units (CFUs) or normal saline. Next, ligated intestine was placed back and a suture was done for the survival of the rabbits. After 18 h of the challenge, rabbits were sacrificed and the loops were examined for fluid accumulation (FA) ratio. Experiments were done in triplicates where the results were expressed by taking the mean
volume (mL) of fluid accumulated per unit length (cm) of the loop. An increase of FA ratio served as an index for increasing virulence and vice versa. Assessment of negative and strong positive responses was based on FA ratio &lt;0.2 and ≥1.0, respectively (Syngkon et al. 2010).

Suckling mouse colonization assay

The colonization potential of V. cholerae wild-type and isogenic mutants defective in components of the Gnt utilization system was evaluated in a suckling mouse model (Chatterjee et al. 2009). A group of mice were orally challenged with &sim;10^6 CFU of bacterial cells. The mice were sacrificed post 18 h of challenge, intestines were recovered and washed. Next, intestinal homogenates were made using a tissue tearer (Polytron PT 1600E, Thermo Fisher Scientific); recovery counts of V. cholerae were estimated by the dilution plating method and expressed per mouse intestine. Colonization potential was expressed as ratio of recovered counts to that of the feeding dose.

Statistical analyses

Statistical significance was performed by one-way ANOVA analysis and Dunnett’s multiple comparison test using Prism 6.1 (GraphPad, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Establishing components of the Gnt utilization system in V. cholerae

Despite recent advances in molecular understanding, the interaction between metabolic pathways to overall physiology and virulence regulation in V. cholerae have not been well addressed. Our earlier work has established obligate involvement of the ED pathway for Gnt utilization by V. cholerae and deactivation of the ED pathway caused severe virulence attenuation (Patra et al. 2012). However, the basis of such virulence attenuation is still not clear. For a better understanding of the Gnt utilization system of V. cholerae an in silico approach was initiated as a comparative basis with the Gnt system of E. coli. Analysis showed that the Gnt utilization system of V. cholerae is composed of eda, gntU, gntK, edd and gntR (Fig. 1a). It may be mentioned here that functional edd and eda formed the ED pathway (Peekhaus and Conway 1998b). Absence of E. coli ortholog genes, gntT, gntV, gntW and gntP, together with unique localization of the components of the V. cholerae Gnt utilization system, indicated the presence of a very rigid regulatory system in V. cholerae. Comparative analysis of the Gnt utilization genes among other organisms also confirmed the organizational uniqueness of the V. cholerae Gnt system (Fig. S1 in the online supplementary material). Gnt utilization genes were located within a ~5 kb region of the larger chromosome of V. cholerae (Fig. 1a). Though an orthologs search using the E. coli Gnt utilization system as a reference helped in predicting the V. cholerae Gnt utilization system, Clustal Omega-based multiple sequence alignment analysis failed to show a good percentage match (~50% identity) between V. cholerae and E. coli orthologs (Table 1). Thus, speculations ran high whether or not the V. cholerae Gnt utilization components as identified in this study were really participating in Gnt utilization. To confirm participation of the identified loci in Gnt utilization by V. cholerae, isogenic deletion mutants of these genes were generated. The growth properties of these mutants in the presence of Gnt as sole carbon source were assessed. The summarized

![Figure 1](image_url)
Table 1. Homology analysis (% homology present in *V. cholerae* as compared to genes of *E. coli* origin) of Gnt utilization system of *V. cholerae* N16961 compared with *E. coli* K-12 MG1655.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide</th>
<th>Amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>edd</td>
<td>57.3%</td>
<td>55.6%</td>
</tr>
<tr>
<td>eda</td>
<td>52.8%</td>
<td>55.6%</td>
</tr>
<tr>
<td>gntU</td>
<td>42.8%</td>
<td>45.6%</td>
</tr>
<tr>
<td>gntK</td>
<td>53.7%</td>
<td>54.7%</td>
</tr>
<tr>
<td>gntR</td>
<td>50.2%</td>
<td>54.7%</td>
</tr>
</tbody>
</table>

Figure 2. Growth of *V. cholerae* wild-type N16961 and in-frame deletion mutants when allowed to grow in M9 media supplemented either with glucose (Glu) (lane 2) or gluconate (Gnt) (lane 3). In-frame deletion mutants of edd, eda, gntU, gntK and gntR are indicated. Growth of the mutants upon complementation are shown in lane 4. Number below each of the tubes denotes optical density measured at 600 nm (OD600) upon overnight incubation. Presence or absence of Glu or Gnt in the media are indicated by ‘+’ and ‘−’, respectively.

Characterization of the Gnt utilization system in *V. cholerae*

It was interesting to note that the components of the ED pathway genes (*edd* and *eda*) (Peekhaus and Conway 1998b) formed an integral part of the predicted Gnt utilization system of *V. cholerae*. Functionality of the *edd* and *eda* (Patra et al. 2012) and other predicted genes (*gntU*, *gntK* and *gntR*) has never been reported to date for *V. cholerae*. As this was the first study, to the best of our knowledge, to identify the *V. cholerae* Gnt utilization system, the structure–function modeling approach was survival (Tsunedomi et al. 2003). However, such a parallel system was not evident in *V. cholerae*.
Figure 3. Schematic representation of membrane topology of *V. cholerae* GntU (a) and ATP binding motif of the GntK (b). Predicted 13 transmembrane helices of the GntU are indicated by numbers. Predicted ATP binding sites of *V. cholerae* GntK and *E. coli* GntK are indicated by red letters. Topology prediction of the GntU and ATP binding sites in GntK were made using TopPred II and TargetATPsite web servers, respectively. IM represents inner membrane. ‘∗’, ':' and '.' represent conserve, semi-conserve and similar type of amino acid replacement.

utilized to characterize GntU, GntK and GntR. Proteobacteria use two major strategies to import and phosphorylate amino sugars into the cytoplasm, namely (i) sugar-specific phosphotransferase (PTS) systems, or (ii) a combination of sugar-specific permeases and kinases (Leyn et al. 2012). As Gnt is a non-PTS sugar (Hogema et al. 1997), it should pass through a cognate transporter present in the membrane which was identified as GntU. Server-based analysis using PSORTb and SOSUI predicted the localization of GntU in the membrane. Bioinformatics analysis failed to identify any N-terminal signal sequence in GntU and it was found to be composed of a high percentage of hydrophobic amino acid residues (44%) considering I, L, V, M, W, Y and F as an index for hydrophobicity (Klemm et al. 1996). Further, hydrophobic amino acid residues were interspaced by hydrophilic residues. Escherichia coli Gnt permease protein GntP, present in the inner membrane, also possessed similar features (Klemm et al. 1996). Analysis also indicated the presence of 13 transmembrane segments in *V. cholerae* GntU, with a high number of arginine–lysine residues (Fig. 3a), which corroborated well with the large family of transport proteins (Henderson 1993; Klemm et al. 1996) as well as with *E. coli* Gnt permease (Klemm et al. 1996; Tong et al. 1996). Similar analysis on *V. cholerae* Edd, Eda, GntK and GntR did not show more than 12 peaks on the upper cut-off value leading to exclusion of these proteins as transporters.

GntK, a putative kinase, was considered to phosphorylate Gnt into Gnt-6P for subsequent metabolism through the ED pathway and/or PP pathway as in the case of *E. coli* (Cohen 1951; Eisenberg and Dobrogosz 1967). In the case of *E. coli*, at the N-terminal portion of the GntK, a consensus sequence of ATP-binding, VSNGSKSA, has been reported (Walker et al. 1982; Izu, Adachi and Yamada 1996; Tong et al. 1996). TargetATPsite software (Yu et al. 2013)-based analysis showed the presence of CACGKST for *V. cholerae* GntK, which weakly matched the *E. coli* cognate counterpart (Fig. 3b). The presence of this motif in *V. cholerae* GntK explained well the potential to phosphorylate Gnt after its cellular entry leading to generation of Gnt-6P, an important event for non-PTS sugar before entering into the metabolic cycles. No such motif was observed in the remaining component genes of the predicted Gnt utilization system of *V. cholerae*.

More than half a century ago the pioneering works of Jacob and Monod, and their colleagues, on the regulation of the lac operon, opened the field of transcriptional regulation in bacteria (Jacob and Monod 1961). Despite evolutionary flexibility in the operon structure, transcriptional regulation remains as a major determinant for proper functionality (Rubinstein et al. 2011). To have a regulatory function, generally protein will have a DNA binding region as well as sugar binding sites. Amino acid analysis using Clustal Omega revealed that *V. cholerae* GntR possessed 24.79% similarity with the LacI protein of *E. coli* whereas *E. coli* GntR has 23.77% similarity with LacI. InterProScan version 5.0 enabled us to identify the LacI family Helix-turn-Helix (HTH) motif at the N-terminal region spanning 8–53 amino acids of the *V. cholerae* GntR and a putative periplasmic sugar binding domain at the 65–321 amino acid region. The presence of the LacI family HTH domain served as sign of a typical negative regulator protein. Collectively, these data indicated that the GntR is to be considered as a negative regulator for the Gnt utilization system in *V. cholerae*. 
**Vibrio cholerae** Gnt utilization system operates through two bicistronic operons

This study predicted five open reading frames (ORFs) in the *V. cholerae* Gnt utilization system, of which three ORFs were in the same transcriptional direction and the remaining two ORFs were in the opposite direction (Fig. 1a). In contrast to *E. coli*, the *V. cholerae* Gnt utilization system, as predicted in this study, has two operon structures *edd-gntK* and *gntU-eda* under negative control by the GntR, which is encoded from the *gntR* locus, that corroborated well with data DOOR2 database analysis. Operon structures are widespread in the prokaryotic kingdom and necessary for a concerted bacterial gene regulation. In case of the *V. cholerae* Gnt utilization system, the *gntU* and *eda* were predicted to be transcribed in the same direction as *gntR* (Fig. 1a). An upstream 153 bp region was identified as a putative promoter by the presence of two consensus sites for Sigma70 RNA polymerase i.e. −10 bp (TAACAT) and −35 bp (TTATCT). It was also identified that the reverse complement of promoter for *gntU-eda* served as putative promoter for *gntK* and *edd* genes together. Sigma70 binding sites containing −10 bp (TAACAT) and −35 bp (CTCACA) having a 4 bp and 3 bp match with the *E. coli* consensus −10 bp (5′-TATAAT-3′) and −35 bp (5′-TACACA-3′), respectively, were identified in this region. Using web-based ARNold finding terminators (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php) software, GC-rich intrinsic terminator sequences (5′-GACCAACGCTCCGTTTTG-3′ for *gntU-eda* and 5′-GACCAACGCTCCGTTTTG-3′ for *gntK-edd*) were identified downstream of the two operons. These proposed intrinsic terminator sequences confirmed co-transcription status of the genes present in the respective operons. With regard to the predicted bicistronic operon structure of the *V. cholerae* Gnt utilization system, we extended our study to obtain experimental evidence on this prediction using PCR-based analysis. Target primer binding locations used for the detection of the intergenic region between *gntK-edd* (region II) and *gntU-eda* (region III) are shown in Fig. 1a. Whole-cell lysate as a source of genomic DNA (gDNA) and random hexamer-based cDNA synthesized from total RNA using reverse transcriptase assay were used as a template for PCR. It is evident from Fig. 1b that PCR analysis with gDNA as well as cDNA as a template resulted in expected-sized amplicons of 408 and 1017 bp against regions II and III, respectively. PCR-based analysis by the targeting of region I, spanning gntR-edd, failed to produce an amplicon when the cDNA preparation served as a template (Fig. 1b, lane 1) but gave an amplicon of 678 bp against the gDNA template (lane 2). These data were in good agreement with the proposed existence of two bicistronic operons, i.e. *gntK-edd* and *gntU-eda* for the *V. cholerae* Gnt utilization system. The presence of an operon structure is needed for the synchronicity of the Gnt utilization system during particular sugar utilization, here, Gnt. Further analysis showed that the expression of *gntR* is monocistronic.
GntR negatively regulates the *V. cholerae* Gnt utilization system

Real-time PCR-based GntR regulatory genes (*edd, eda, gntU* and *gntK*) transcript analysis between wild-type (N16961) and dysfunctional GntR mutant (Δ*gntR*) revealed a relative increase of target transcripts in the Δ*gntR* strain (Fig. 4). Arabinose-induced overexpression of GntR from the pBADgntR construct resulted in significant reduction of the transcripts in the Δ*gntR* strain (Fig. 4). It was also observed that the presence of the pBADgntR construct even without induction caused reduction of target gene transcripts. This observation may be considered as leaky expression since the presence of vector alone showed comparable levels of transcripts to that of the mutant. *In vitro* assays were useful for an initial prediction of GntR-mediated negative impact on the expression of *edd, eda, gntU* and *gntK*. In the case of *E. coli*, negative regulation by the GntR was due to DNA binding properties (Peekhaus and Conway 1998a) and the same may be true for the *V. cholerae* system. Virtual Footprint server-based analysis revealed the presence of putative GntR binding consensus sequences R1′ 5′-AAGTTACCGGTAACAT-3′ with two bases overlapping R1″ 5′-ATGTTACCCATAACAT-3′ upstream of predicted bicistronic operons *gntU-eda* located in opposite strands. We have considered the region comprising R1′ and R1″ as R1 and another consensus sequences R2 (5′-ATGTTACTCATAACAT-3′) was also identified upstream to *gntR* (Fig. 1a). Bioinformatics-based predictions were further addressed through *in vitro* DNA binding assays using purified His-tagged GntR and amplicons spanning R1 and R2 consensus sequences. Gel retardation assay showed migration retardation of the DNA fragment spanning R1 and the extent of retardation was proportional to the GntR concentration used in the binding assay (Fig. S2a in the online supplementary material). All these results experimentally confirmed the binding of the GntR to the DNA fragment spanning the R1 consensus sequence. The reversal of such binding was indicative in the presence of Gnt in the reaction mixture (Fig. S2a, lane 6). Such de-repression is to be considered beneficial for *V. cholerae* utilizing Gnt when present as the sole carbon source. However, the same protocol failed to demonstrate any binding affinity to the DNA fragment spanning R2 (Fig. S2b). Two consensus binding sequences with two bases overlapping were present in an R1 site that covered a stretch of 30 bases. The presence of two overlapped binding regions in R1 may aid stronger binding affinity of GntR to this site as compared to R2. Thus detection of a non-binding phenotype of GntR to the R2 site may be attributed to a weaker affinity to this site which was below the detection limit of the *in vitro* assay. Therefore, it may be concluded that GntR of *V. cholerae* also acted as a negative regulator of the Gnt utilization system.

The Gnt utilization system and virulence modulation in *V. cholerae*

Earlier work demonstrated that inactivation of *edd* resulted in loss of Gnt utilization properties with concurrent virulence attenuation in *V. cholerae* (Patra et al. 2012). So, a connection between virulence mechanism and Gnt utilization in the case of *V. cholerae* was evident. Therefore, extended studies were carried out to reveal the contribution of other Gnt utilizations genes, per se, towards *V. cholerae* virulence modulation. Mutants (defective in either *eda, gntU, gntK* or *gntR*) were tested for their ability to secrete CT in *vitro*. Varying degrees of reduction in CT secretion ability were evident (Fig. 5a). Among the mutants,
edd deactivation caused most attenuation. In vitro reduction in toxicity was further validated through the in vivo toxicity assessment model of rabbit ileal loop (RIL) assay. *Vibrio cholerae* N16961 exhibited strong enterotoxic responses in the ligated loop (Fig. 5b) with a FA ratio of 1.1 ± 0.1, whereas among the mutants a negative response was evident for the edd mutant. The other mutants, *eda*, *gntU*, *gntK* and *gntR*, displayed somewhat lower responses as compared to wild-type N16961 when assayed under similar conditions. These results indicated that the functional Gnt utilization system positively modulated the toxigenicity of *V. cholerae* in vitro and in vivo with most impact by the involvement of *edd*. It was identified that GntR operated as a negative regulator for the Gnt utilization system in *V. cholerae*. Thus reduction of toxicity in the gntR-negative mutant was not expected. This apparent ambiguity can be explained by considering stepwise events: (i) in the absence of GntR, regulation caused overexpression of the *edd*; (ii) overexpression of *edd* caused intracellular accumulation of the toxic intermediate metabolite KDPG (Fuhrman et al. 1998) from Gnt metabolism; and (iii) accumulation of KDPG caused a bacteriostatic effect leading to overall reduction of CT secretion per unit of the viable cells. The abovementioned hypothesis has been supported by an observed growth suppression in vitro in N16961 with arabinose-induced overexpressed Edd from the construct pTGeedd2.2 (Fig. S3 in the online supplementary material). Studies were further extended by evaluating the impact of individual Gnt utilization gene components towards intestinal colonization properties. Suckling mouse assay with parent strain N16961 showed good colonization potential (Fig. 5c) while mutants in the defective Gnt utilization system displayed a significantly lesser level of colonization potential. It was interesting to note that mutants of the ED pathway (*edd* and *eda*) displayed the lowest level of colonization potential as compared to the mutated components of the other Gnt utilization genes. Thus it may be said that the functional Gnt utilization system included a functional ED pathway and the genes of this system function to modulate *V. cholerae* pathogenesis. An understanding of the basis of such modulation is underway.

**SUPPLEMENTARY DATA**

Supplementary data are available at FEMSPD online.

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**Conflict of interest.** None declared.

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Targeting \textit{aphA}: a new high-throughput screening assay identifies compounds that reduce prime virulence factors of \textit{Vibrio cholerae}

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A high-throughput screening (HTS) assay was developed for identifying compounds with inhibitory effect on \textit{aphA}, one of the key regulators positively controlling \textit{Vibrio cholerae} pathogenesis. An inhibitory effect on \textit{aphA} was expected to lead to attenuation in the secretion of the major pathogenicity factors of \textit{V. cholerae}, cholera toxin and toxin co-regulated pilus. The plasmid construct pAKSB was developed with a kanamycin resistance (Km\textsuperscript{R}) gene under the control of the \textit{aphA}-like promoter for conferring a Km\textsuperscript{R} phenotype under \textit{aphA}-expressing conditions. The HTS assay was performed to identify compounds with inhibitory effect on the growth of O139 \textit{V. cholerae} MO10 carrying the construct pAKSB in growth medium containing Km (30 g ml\textsuperscript{-1}), but not in its absence. Of 20 338 compounds screened, six compounds were identified to inhibit the pAKSB-induced Km\textsuperscript{R} phenotype and these compounds caused transcriptional inhibition of \textit{aphA} in \textit{V. cholerae} O139 strain MO10 as well as variant \textit{V. cholerae} O1 El Tor strain NM06-058. Of the three most active substances, compound 53760866 showed lowest half-maximal cytotoxicity in a eukaryotic cell viability assay and was characterized further. Compound 53760866 caused reduction in cholera toxin secretion and expression of TcpA in vitro. The \textit{in vitro} virulence attenuation corroborated well in a suckling mouse model \textit{in vivo}, which showed reduction of colonization by \textit{V. cholerae} NM06-058 when co-administered with 53760866. The screening method and the compounds may lead to new preventive strategies for cholera by reducing the pathogenicity of \textit{V. cholerae}.

INTRODUCTION

\textit{Vibrio cholerae} is a Gram-negative, rod-shaped bacterium belonging to the genus \textit{Vibrionaceae}. Strains belonging to the serogroups O1 and O139 are responsible for causing acute waterborne diarrhoeal epidemics known as cholera (Kaper \textit{et al}., 1995). The disease can cross continental boundaries to cause large pandemics. To date, human civilization has witnessed several pandemics; the current is the 7th pandemic, which began in 1961 and is still ongoing (Kaper \textit{et al}., 1995; WHO, 2010). In 2013, 47 countries reported a total of 129 064 cases of cholera including 2102 deaths, resulting in a case-fatality rate of 1.63 % (WHO 2014). In fact, cholera is still a major concern today with a high degree of morbidity and mortality among people living in developing or underdeveloped countries who lack access to safe drinking water and good hygienic conditions. A recent estimate shows that 36 % of the global population lacks access to improved sanitation facilities (Brocklehurst, 2014). Oral rehydration therapy and antibiotics are available for cholera treatment, but a growing concern is the emergence of multidrug-resistant...
V. cholerae strains belonging to O1 and O139 serogroups (Kitaoka et al., 2011). Due to this development, the availability of novel therapeutic approaches is urgently needed (Ghosh & Ramamurthy, 2011).

Much attention has been attributed to the development or identification of new antimicrobial compounds or drugs that target component(s) of regulatory cascades (Clatworthy et al., 2007; Raju et al., 2012; Anthouard & DiRita 2013; Zahid et al., 2015). A promising compound, ‘virstatin’, was identified, having the potential to attenuate CT expression by V. cholerae without altering growth of the target organism (Hung et al., 2005). In an era when the occurrence of multidrug resistance is common among different human pathogens, identification of virulence-specific and safe drugs will be advantageous. Virulence inhibitors may have the potential to be employed in situations where an infection has not (yet) occurred and when vaccinations are not available or would not be able to build up a timely protection. To identify such virulence-specific active compound(s), the major impediment is non-availability of HTS assays for Infection Research (HZI) were also included: (i) the NCH collection (Gerth et al., 2003); (ii) the peptide library (VAR), containing at the time of this study 1936 synthetic organic molecules that were provided by various collaborators; and (iii) the library Various Sources (VAR), consisting of 154 secondary metabolites isolated from myxobacteria, short linear and cyclic peptides, and synthetic small molecules, were screened to identify active compounds with potential to attenuate V. cholerae virulence.

METHODS

Strains and cultural conditions. The strains used in this study are listed in Table 1. V. cholerae O139 strain MO10 (Nandy et al., 1999) carrying the construct pAKSB (see below) served as reporter strain during development of the HTS assay for identifying active compounds that have the potential to attenuate virulence phenotypes. For the construction of the reporter strain, WT MO10 was electroporated with the plasmid construct pAKSB containing ampicillin resistance (AmpR) for selection and kanamycin resistance (KmR) under the control of the promoter of the apha-like gene of V. cholerae. V. cholerae O1 El Tor variant clinical strain NM06-058 (Sergeev et al., 2014) was also included to validate the effectiveness of the compounds on this variant (Raychoudhuri et al., 2008), which is currently prevalent in many parts of the world. For virulence-inducing conditions, strains were grown in AKI (Iwanga & Yamamoto, 1985), pH 7.4 at 37°C, and nutrient broth [NB (peptone 15 g l⁻¹, yeast extract 3 g l⁻¹, sodium chloride 6 g l⁻¹, D (+)-glucose 1 g l⁻¹)] pH 8.0 was used for virulence-suppressive conditions. For the HTS assay, the reporter strain MO10 pAKSB was grown in AKI with or without 30 µg kanamycin (Km) ml⁻¹. Ciprofloxacin (Cip; 100 µM) was used as a positive control in the HTS assay while medium containing 1% DMSO served as negative control. Assessment of the compounds to attenuate virulence was performed under AKI growth conditions.

Generation of plasmid construct pAKSB with KmR phenotype under control of the apha-like gene promoter. The 200 bp upstream region of the apha-like gene of N16961 (Heidelberg et al., 2000) (GenBank accession no. AE003852) and the 816 bp KmR structural gene from pUC4K were amplified separately with high-fidelity Taq polymerase (AmpFlaQ Gold, Applied Biosystems). A PCR fragment-joining method was used for fusing these amplicons to generate a 1039 bp fragment where the KmR structural gene was under control of the apha-like gene promoter. Briefly, a reverse primer targeting upstream of the apha-like gene and a forward primer of the KmR structural gene were designed with 15 bases of complementary identity towards the 5′ end of the primers (Table 1). Both amplicons were mixed together at equimolar ratio, and PCR was performed with a combination of apha-F1 and Km-R1 primers to generate a PCR-ligated amplicon where the promoter of the apha-like gene was fused upstream of the KmR structural gene. The amplified product was cloned into the EcoRV site of pBluescript SK+ after adding one additional T overhang. Ready-to-use electro-competent Escherichia coli host strain DH10B (Invitrogen) was used to generate transformants that were selected on 50 µg ampicillin (Amp) ml⁻¹ plates. The transformants were characterized initially by PCR to confirm the presence of the appropriately sized insert of the cloned fragment followed by nucleotide sequence analysis to confirm the regularity of the sequence. The construct, named pAKSB, was electroporated into the O139 strain MO10 (sensitive to both Amp and Km) and the transformants were selected on plates containing 50 µg Amp ml⁻¹.

Compound libraries. Three commercially available libraries were either completely or partially used for the screening: (i) the LOPAC collection of pharmacologically active compounds with 1408 entities (Sigma); (ii) the Echaz Microcollection with 7304 compounds (EMC Microcollections); and (iii) the CDI collection with approximately 17 000 compounds (Chemical Diversity Lab), a commercially available collection assembled by members of the ChemBioNet consortium (Lisurek et al., 2010). Three additional unique libraries of the Helmholtz Centre for Infection Research (HZI) were also included: (i) the NCH collection consisting of 154 secondary metabolites isolated from myxobacteria (Gerth et al., 2003) and with known antimicrobial activities (Weissman & Müller 2009); (ii) the library Various Sources (VAR), containing at the time of this study 1936 synthetic organic molecules that were provided by various collaborators; and (iii) the peptide library containing 1045 short linear or cyclic peptide sequences synthesized at the HZI (Tegge et al., 2007). All test compounds were utilized as stock solutions in DMSO.

HTS of the compounds. This assay was carried out in either 96 or 384-well clear, flat-bottom microtitre plates (MTPs) using MO10 pAKSB grown in 100 µl or 50 µl (96- or 384-well plate, respectively) AKI-Km (30 µg ml⁻¹) medium with an OD₃00 of 0.1 (1×10⁶ c.f.u.). Addition of the compounds was made into individual wells from DMSO stock. Plates were incubated statically in a humid atmosphere for 24h at 37°C, and growth of the cells was recorded (OD₆00) using a Fusion Universal
Table 1. Strains, cells, plasmids and primers used in this study

<table>
<thead>
<tr>
<th>Strain, cell line, plasmid, primer</th>
<th>Relevant description/sequence</th>
<th>Reference or purpose</th>
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<tr>
<td><strong>V. cholerae strain</strong></td>
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<tr>
<td>MO10</td>
<td>WT, serogroup O139; Amp&lt;sup&gt;+&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
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Amp<sup>+</sup>, sensitivity to ampicillin; Km<sup>R</sup>, sensitivity to Km; Km<sup>CR</sup>, culture condition-dependent resistance to Km.

Underlined bases indicate complementary overlap.

Microplate Analyzer (PerkinElmer). Screening was performed with a total of 20,338 compounds in one fixed concentration between 20 and 50 µM. Accurate dispensing into the wells of the MTP was achieved with the help of a pipetting robot (equipped with either a pintool or pipetting tips). As negative and positive controls, DMSO (1%) and Cip (100 µM) were used in duplicate, respectively. Z-values were calculated using the negative and positive controls (Zhang et al., 1999). Compounds of the initial screening that caused at least 50% reduction of growth were tested in a second run at the same concentration. In order to identify and to eliminate active compounds with non-specific growth-suppression activities, the same assay was repeated in AKI medium without Km. The compounds that exhibited growth-inhibitory properties in AKI-Km but not in AKI medium without Km were selected for further characterization.

**Growth inhibitory half-maximal concentration (g-IC<sub>50</sub>) against MO10 pAKSB.** The g-IC<sub>50</sub> values of the active compounds were determined by growing reporter strain MO10 pAKSB in 96-well MTP in the presence of serially diluted compounds. Seed inoculum (100 µl) with an OD<sub>600</sub> of 0.1 (1 x 10<sup>5</sup> c.f.u.) was added to each of the wells containing appropriately diluted active compounds in either AKI or AKI-Km media. Active compounds were used at concentrations from 0.1 µM to 100 µM. The plates were incubated statically for 24 h at 37°C, and growth of the cells was recorded (OD<sub>600</sub>) using a Fusion Universal Microplate Analyzer. Based on the extent of growth corresponding to different concentrations of a compound, the g-IC<sub>50</sub> value was determined by visual inspection of the growth curves generated with the program Excel (Microsoft) after normalization to the DMSO negative control. The g-IC<sub>50</sub> value of a compound was separately determined for AKI and AKI-Km media.

**GM<sub>1</sub> assay to estimate CT.** V. cholerae O1 El Tor variant strain NM06-058 was used as a test strain to estimate CT production when grown in the presence of various amounts of the active compounds following methodologies as described by Patra et al. (2012). Briefly, the strain was grown in AKI for 18 h in the presence of serially diluted active compounds, intact cells and debris were pelleted by centrifugation and cell-free culture supernatants containing CT were collected. A GM<sub>1</sub> ELISA was performed with the cell-free supernatants to quantify the secreted CT. Readings were taken in an automated ELISA reader with filter set to 492 nm after background corrections at 620 nm (Multiskan EX, Thermo Scientific). Varied dilutions of known amounts of purified CT (Sigma) were included in each of the assays to generate a standard curve for the estimation of CT in the culture supernatants. Wells containing cell-free supernatants of cultures grown in the presence of up to 1% DMSO served as negative controls. Amounts of CT secreted by V. cholerae were expressed as pg (ml culture)<sup>-1</sup> per unit opacity at 600 nm.
**Quantitative real-time PCR (qRT-PCR).** *V. cholerae* O1 variant El Tor strain NM06-058 was used to detect the effects of compounds vz0852, vz0761 and 53760866 on transcription of the virulence genes *ctxA* and *tcpA* and the regulatory element *apfA*. Cells were grown for 12 h in 2 ml AKI in the presence of the compounds vz0852 at 3.1 µM, vz0761 at 12.5 µM and 53760866 at 25 µM concentration, respectively. Following 12 h of growth, OD_{600} was determined, the concentration adjusted to 5.5 × 10^7 c.f.u. (1 OD_{490} corresponded to ~1×10^8 c.f.u. ml^-1) and a twofold volume of RNAprotect Bacteria reagent (Qiagen) was added. The mixture was incubated for 5 min at room temperature and the cells were recovered as pellets by 10 min of centrifugation at 5000 g. Total RNA was extracted using the TRIZOL method (Patra et al., 2012). Briefly, total RNA thus extracted was further treated with RNase-Free DNase (Qiagen) to remove contaminating traces of DNA, if present. A reverse transcription was carried out with 1 µg of isolated RNA obtained from cells grown under different culture conditions and in the presence of random hexamer. For this, a RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas) was used and cDNA synthesis was performed in a PCR machine (model 2720, Applied Biosystems). This cDNA was utilized for qRT-PCR analysis with SYBR Green I Master mix (Life Technologies) in a HT Fast real-time PCR machine (Applied Biosystems). The gene-specific primers used in the real-time PCR assay are listed in Table 1. Results were recorded and normalized with respect to C values for *recA* and compared to respective values obtained from cells that were grown in the absence of any active compound. Comparative data were expressed as a relative percentage of the target mRNA. Cells grown separately in the presence of 1 % DMSO served as a negative control.

**Cytotoxicity assay.** The mouse fibroblast cell line L929 was utilized to investigate the cytotoxicity of the active compounds in an MTT assay according to a standard protocol (Mosmann, 1983), with some modifications (Sasse et al., 2002). Varied amounts of the compounds were used to detect the effects of cytotoxicity and to determine half-maximal inhibitory concentration (c-IC_{50}). Cells were observed following incubation for 24 h to detect acute toxicity. Incubation was continued for 5 days mainly to record the inhibition of cell proliferation and sub-acute toxicity.

**Western blot analysis.** *V. cholerae* NM06-058 was grown in AKI, pH 7.4 at 37°C for 18 h under static conditions. Cells were grown in media containing 1 % DMSO or varied amounts of compound 53760866. Cells were harvested, washed, and lysate was prepared using SDS-PAGE sample buffer. The lysate was separated by SDS-PAGE (15 % gel) and transferred to PVDF membranes using a Transblot apparatus (Bio-Rad). A polyclonal anti-TcpA serum was raised against the peptide KVSADEAKNPFTGTAMG with commercial support (Thermo Scientific). The blots were subsequently probed with polyclonal rabbit anti-TcpA serum followed by treatment with horseradish-peroxidase-conjugated goat anti-rabbit IgG. The blots were developed with 3,3’-diaminobenzidine in the presence of hydrogen peroxide.

**RESULTS**

**Characterization of construct pAKSB conferring a conditional KmR phenotype in V. cholerae MO10**

The pAKSB construct was developed that contained the KmR structural gene under the control of the *aphA*-like gene promoter of *V. cholerae* N16961. Presence of the construct in the *V. cholerae* O139 reporter strain MO10 resulted in an AmpR phenotype. The AKI condition promotes optimal expression of the virulence factors by O1 El Tor and O139 strains in *vitro* and thus this condition was considered to favour *aphA* expression (Lin et al., 2007). In contrast, lowered expression of *aphA* was considered in the NB culture condition that suppresses virulence factors in *vitro*. Therefore, it was assumed that the conditional KmR phenotype would be induced in MO10 pAKSB (KmR under the *aphA*-like gene promoter) under the AKI condition, to serve as a surrogate marker for the expression of *aphA*, and as such the KmR phenotype would be abolished in the NB culture condition. Validation of the conditional KmR phenotype was established by observing the growth of MO10 pAKSB in AKI but not in NB when both media contained 30 µg Km ml^-1 (Table 2). The parent strain MO10 failed to grow in the presence of Km but grew well in the absence of Km.

#### Table 2. Km (30 µg ml^-1) susceptibility patterns of *V. cholerae* strains with or without plasmid constructs under two different culture media conditions when allowed to grow at 37°C for 18 h under static conditions

<table>
<thead>
<tr>
<th><em>V. cholerae</em> strain</th>
<th>Growth of <em>V. cholerae</em> in:</th>
<th>(\text{AKI, pH 7.4, medium}^{*})</th>
<th>(\text{NB, pH 8.5, medium}^{†})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Km</td>
<td>With Km</td>
<td>Without Km</td>
</tr>
<tr>
<td>M010</td>
<td>+ (0.34)</td>
<td>− (0.04)</td>
<td>+ (0.51)</td>
</tr>
<tr>
<td>M010 pAKSB</td>
<td>+ (0.28)</td>
<td>+ (0.22)</td>
<td>+ (0.55)</td>
</tr>
<tr>
<td>M010 pUC4K</td>
<td>+ (0.25)</td>
<td>+ (0.28)</td>
<td>+ (0.28)</td>
</tr>
</tbody>
</table>

+, positive growth; −, no growth. OD_{600} values of cultures are indicated in parentheses.

*Culture condition known for optimal expression of CT and TCP by O1 El Tor background strains.

†Culture condition known to suppress CT and TCP expression by O1 El Tor background strains.
Km. Expectedly, the presence of pUC4K containing the KmR structural gene conferred the KmR phenotype to MO10 pUC4K in both AKI and NB media.

**HTS assay to identify active compounds**

The new HTS assay was developed to screen active compounds from six different libraries consisting in total of 20,338 compounds. After optimizing screening parameters, growth inhibitory assays were performed using the MO10 pAKSB indicator strain. The Z-values of the initial screen were between 0.5 and 0.9, indicating reliable performance of the assay (Zhang et al., 1999). The threshold for the selection of active compounds in the initial screening was defined as a growth reduction of more than 50% in Km-containing media. The small NCH collection of 154 compounds had on average the most active molecules (statistically, 45.5 per 1000). The VAR library consists of molecules with predominantly unknown activities and had 19.1 antibacterial compounds per 1000 molecules. The other sources contained rather low amounts of active compounds, with the LOPAC collection scoring at 2.8 per 1000. This compound collection contains several antimicrobial agents that are in clinical use, which were reliably identified in the assay. The new HTS assay identified 56 active compounds with growth-inhibitory properties of *V. cholerae* in AKI containing Km. None of these compounds were reported previously to have bacterial growth inhibitory properties. Any compound with inhibitory potential on the promoter of the aphA-like gene will result in a loss of the KmR phenotype of MO10 pAKSB in the AKI condition. Thus, repeat assays were performed with the 56 growth inhibitory compounds in the absence of Km in AKI growth medium. Fig. 1 shows that compounds in certain wells (Fig. 1a, marked by circles) failed to display growth inhibition in the assay with AKI growth medium (absence of Km) (Fig. 1b), whereas other compounds (Fig. 1a, b, marked by squares) caused growth inhibition in both the presence and absence of Km.

The analysis identified six active compounds which caused growth inhibition of MO10 pAKSB in AKI-Km but not in AKI media at the same concentration. The chemical structures of these six compounds are shown in Fig. 2. The virulence inhibitor virstatin could not be used as a positive control for the inhibition of aphA, because its target is localized downstream of this gene.

**Characterization of active compounds**

The six active compounds were from the VAR collection, but from two different providers. They have in common a heterocyclic five-membered pyrazole (vz0761 and vz0852), thiazole (53760866 and 53761430) or oxazole (53761172 and 53761188) ring to which one or two further aromatic systems are linked, plus halogens. For none of these six active compounds have antibacterial activities previously been described. The database SciFinder (ACS) was scanned for compounds with structure similarities. Among the compounds that displayed at least 70–80% structural similarity to the six active compounds, no antibacterial activities could be identified. Non-antibacterial functionality of these six active compounds matched well as all these compounds

![Fig. 1](image-url)
failed to exert anti-\textit{V. cholerae} growth activities when tested at levels between 20 and 50 µM.

Reassessment of the six active compounds identified was made to reveal their growth inhibitory potential, if any, at higher concentrations on O139 indicator strain MO10. Growth inhibitory potential of these compounds at higher concentrations was evident, and g-\textit{IC}_{50} values in AKI medium varied widely. The g-\textit{IC}_{50} values of these compounds are presented in Table 3. Comparative analysis showed lower g-\textit{IC}_{50} in AKI-Km as compared with the AKI growth condition. A marked reduction of the g-\textit{IC}_{50} values in the AKI-Km condition may be due to compound-mediated inhibition of the Km\textsuperscript{R} phenotype under the control of the \textit{aphA}-like promoter.

The g-\textit{IC}_{50} values ranged between 6 and 100 µM. In AKI without Km the g-\textit{IC}_{50} values ranged between 38 and >>100 µM. The inhibitory index of these compounds was estimated as the ratio of g-\textit{IC}_{50} in the absence of Km in the culture medium to that of the same compound in the presence of Km in the culture medium. Based on the inhibitory index values, the three most active compounds, namely vz0761, vz0852 and 53760866, were identified. Similarly, g-\textit{IC}_{50} values were also determined against the study strain NM06-058 in Mueller–Hinton broth and these were 27, 61 and >>100 µM for the compounds vz0761, vz0852 and 53760866, respectively.

![Fig. 2. Codes and chemical structures of the active compounds.](image-url)

### Table 3. g-\textit{IC}_{50} determined against compounds for the growth of \textit{V. cholerae} O139 strain MO10 pAKSB and cytotoxicity values (c-\textit{IC}_{50}) as determined with the cell line L929

<table>
<thead>
<tr>
<th>Compound</th>
<th>g-\textit{IC}_{50}(µM)</th>
<th>c-\textit{IC}_{50}(µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AKI-Km</td>
<td>AKI</td>
</tr>
<tr>
<td>vz0761</td>
<td>10</td>
<td>&gt;100</td>
</tr>
<tr>
<td>vz0852</td>
<td>6</td>
<td>&gt;50</td>
</tr>
<tr>
<td>53760866</td>
<td>38</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>53761172</td>
<td>100</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>53761188</td>
<td>14</td>
<td>38</td>
</tr>
<tr>
<td>53761430</td>
<td>75</td>
<td>&gt;&gt;100</td>
</tr>
<tr>
<td>virstatin</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Ratio of amount required (>50 µM, >100 µM and >>100 µM was considered as 50 µM, 100 µM and 200 µM, respectively) for 50 % reduction of growth in absence and presence of Km in growth media.
The cytotoxicity potential (c-IC₅₀) of these compounds was evaluated in an MTT assay using the mouse fibroblast cell line L929. Expectedly, the c-IC₅₀ values were higher after 24 h of incubation as compared with 5-day observations. Summarized results are presented in Table 3. The three most active compounds (vz0761, vz0852 and 53760866) showed quite different cytotoxicity, with 53760866 displaying the lowest activity.

By employing the OSIRIS Property Explorer software (an online tool provided by www.openmolecules.org) for the most promising compound, 53760866 a partition coefficient between n-octanol and water (logP) of 3.27 and further very drug-like properties with an overall drug score of 0.83 (on a scale of 0 to 1) were predicted. These toxicity predictions correspond well with our experimental findings (Table 3). Compound 53760866 thus appeared most promising from the in silico prediction as well as from the experimental data obtained in this study. Virulence inhibitors that are designed to reduce the secretion of bacterial toxins in the intestine will preferentially be utilized to further improve the activity of the active compounds.

Impact of the most active compounds on CT secretion by V. cholerae O1

The ability of V. cholerae to secrete CT in vitro was assessed by GM₃ ELISA. The variant El Tor O1 strain (Raychoudhuri et al., 2008) NM06-058, a representative of the presently circulated biotype, was used for this assay. Table 4 shows that in the absence of any active compound (in the presence of 1% DMSO), NM06-058 produced 36 ng of CT (unit opacity)⁻¹ ml⁻¹ in the AKI condition known for optimal expression of El Tor and variant El Tor biotypes (Ghosh-Banerjee et al., 2010). Assays were performed in parallel with two different concentrations of active compounds. It is evident from Table 4 that the presence of the active compounds caused reduction in the secretion of CT by V. cholerae as compared with the DMSO control. The highest concentration of the compound used in the assay was based on 25% of the amount required for g-IC₅₀ as determined separately for NM06-058. Expectedly, virstatin also showed a reduction in CT secretion.

**Table 4. In vitro CT production by V. cholerae O1 variant El Tor strain NM06-058 when grown in AKI conditions in the absence and presence of varied amounts of the compounds vz0761, vz0852 and 53760866.**

<table>
<thead>
<tr>
<th>Growth in presence of</th>
<th>Concentration of compounds (µM)</th>
<th>CT in cell-free supernatant [ng (unit opacity)⁻¹ ml⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% DMSO*</td>
<td>NA</td>
<td>36.0</td>
</tr>
<tr>
<td>vz0761</td>
<td>6.3</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>3.7</td>
</tr>
<tr>
<td>vz0852</td>
<td>1.6</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>53760866</td>
<td>25</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1.3</td>
</tr>
<tr>
<td>virstatin</td>
<td>100</td>
<td>2.0</td>
</tr>
</tbody>
</table>

NA, not applicable.

*Final DMSO concentration in growth media in all cases.

**Impact of the most active compounds on virulence genes (ctxA and tcpA) and regulatory element aphA transcripts**

An assessment was made to determine the inhibitory potential of the three most active compounds on the transcription level of ctxA, tcpA and aphA, which are involved in pathogenicity or regulation of pathogenicity in V. cholerae. The qRT-PCR analysis of the relative mRNA content was estimated after normalization with the values for the housekeeping gene recA. In the presence of the active compounds vz0761, vz0852 and 53760866 at 12.5, 3.1 and 25 µM, respectively, the relative transcript levels of the target genes ctxA, tcpA and aphA dropped below 50% as compared with the DMSO control (Fig. 3). A similar inhibitory effect was obtained with virstatin for the ctxA and tcpA transcripts. However, virstatin failed to exert any inhibitory effect on the aphA transcript level as compared with the DMSO control, as expected. These results indicated that the most active compounds identified in this study had either a direct influence on the expression of aphA or acted further upstream of this virulence gene activator.

![Fig. 3. Relative transcript levels of the genes ctxA, tcpA and aphA in the presence of different concentrations of the compounds vz0852, vz0761, 53760866, virstatin and the solvent DMSO only. Bar represents standard deviations.](image-url)
Impact of compound 53760866 on TcpA expression and mouse intestinal colonization

Compound 53760866 showed the lowest cytotoxicity among the three most active compounds (Table 3) and was therefore selected for further characterization. *V. cholerae* NM06-058 was grown in AKI medium in the presence of varied concentrations (12.5, 25, 50 and 100 µM) of compound 53760866 for 18 h. Western blot analysis was carried out to detect the impact of the compound on the expression of TcpA in *vitro*. It is evident from Fig. 4 that *V. cholerae* NM06-058 expressed TcpA when grown in the presence of 1% DMSO only. Addition of compound 53760866 caused a concentration-dependent gradual decrease of TcpA expression. This corroborates well with the transcriptional inhibition of tcpA observed in cells grown in the presence of 53760866. The inhibition of TcpA expression observed in *vitro* was further investigated to reveal any alteration of the intestinal colonization potential in a suckling mouse model. Orogastrically inoculated ~6×10⁵ c.f.u. of *V. cholerae* NM06-058 per mouse was increased by 45-fold post-18 h of challenge due to mouse intestinal colonization of *V. cholerae*. Co-administration of 100 µM of compound 53760866 (10 nmol) along with ~6×10⁵ c.f.u. of *V. cholerae* drastically affected the colonization with *V. cholerae*. In fact, presence of the compound caused ~80% reduction of colonization. In a separate experiment, two doses with concentrations of 50 µM (5 nmol each) of the compound were given separated at an interval of 3 h. A similar level of inhibition to that of the single dose of 100 µM was obtained (P values for single dose application 4.033×10⁻⁵ and for two applications 3.896×10⁻⁵).

DISCUSSION

Expression of CT and TCP has been shown to be linked with quorum sensing (QS) systems, and the presence of multiple QS systems has already been reported in *V. cholerae* (Camara et al., 2002), which proceed simultaneously and have different regulatory functions, e.g. virulence gene expression, biofilm formation or protease production (Jobling & Holmes, 1997; Miller et al., 2002; Hammer & Bassler, 2003). The central regulator, which all three QS systems have in common, is HapR. HapR regulates negatively the expression of aphA and thereby inhibits expression of the virulence genes ctxA and tcpA, and thus connects the QS system with virulence gene expression (Kovackova & Skorupski, 2002). Considering that virulence regulation in *V. cholerae* is highly complex and linked to other regulatory pathways including QS, the index gene aphA has the potential to work as a positive master regulator. With this background, a novel HTS assay has been developed in order to identify compounds that suppress *V. cholerae* virulence by linking reduced aphA expression to growth suppression. The selection of aphA in this HTS was based on the fact that the transcription factor AphA (i) is the master regulator of QS at low cell density (LCD) (Rutherford et al., 2011), (ii) modulates index virulence gene expression, CT and TCP (Kovackova & Skorupski, 1999), (iii) controls nearly 300 genes at LCD which include flagella synthesis, type III secretion system, pilus production, etc. (Rutherford et al., 2011) and (iv) is present and conserved among *V. cholerae* strains. The plasmid construct pAKSB was developed to confer conditional KmR under *in vitro* virulence-inducing conditions (AKI), which served as an index for the expression of aphA. The reliability of the assay was established by confirming the conditional expression of the KmR phenotype in the AKI culture condition, which is known to stimulate CT and TCP expression by *V. cholerae in vitro*, but not in NB, pH 8.5, a virulence-repressing condition (Table 2). Application of the optimized HTS assay resulted in the identification of six active compounds with the potential to attenuate *V. cholerae* virulence from a total of 20 338 compounds. The g-IC₅₀ values of the six compounds were markedly different when compared between AKI and AKI-Km conditions (Table 3). The differences can be considered as an eloquent testimony of the KmR phenotype being linked to an inhibitory effect on the aphA-like gene promoter. Among the six active compounds, vz0761, vz0852 and 53760866 were found to be the most potent by their high inhibitory index (Table 3). Results from a real-time PCR assay confirmed that the inhibitory effects of these three compounds were indeed linked to a reduction of

Fig. 4. Western blot analysis using anti-TcpA peptide antibody against whole-cell lysate of NM06-058 grown in AKI with or without different concentrations of 53760866 stated in micromolar. The arrow indicates detection of TcpA.

Fig. 5. Mouse intestinal colonization in fold exhibited by 100 µl *V. cholerae* NM06-058 per animal when co-administered with either 0.5% DMSO or compound 53760866 either as a single dose of 100 µM or two doses of 50 µM, each separated by an interval of 3 h. Mean and standard deviations are indicated.
aphA transcription in *V. cholerae* (Fig. 3). Transcriptional analysis also confirmed concomitant reduction of the *ctxA* and *tcpA* transcripts in the presence of these compounds in the growth media (Fig. 3), which corroborates well with the aphA-dependent inhibition of *ctxA* and *tcpA* expression at the transcriptional level (Kovacikova et al., 1999).

Of the three most active compounds, 53760866 displayed the lowest toxicity in a eukaryotic cell-based assay (L929 cells) and was therefore characterized further. The compound 53760866 mediated transcriptional inhibition of the index virulence genes (*ctxA* and *tcpA*), resulting in a reduction of CT and TcpA expression (Table 4, Fig. 4). The relevance of our *in vitro* observation was extended to an *in vivo* evaluation. A suckling mouse colonization assay showed that 53760866 can effectively reduce intestinal colonization when co-administered with *V. cholerae* strain NM06-058 (Fig. 5). A similar extent of the inhibitory effect was evident between the regimens of a 100 µM single dose and two separate 50 µM doses 3 h apart (*P*<0.05 in both cases). This observation may be due to the stability of the compound within the host, although further characterizations are needed to gain conclusive evidence. Another limitation of our study is that the mechanism of inhibition of aphA transcription by 53760866 is not clear. The mechanism by which this potent chemical compound diminishes expression of aphA may be either by down-regulation of qrr small RNA transcription or by activation of HapR. These details are to be elaborated in the future.

In conclusion, we report the development of a HTS assay targeting altered expression of aphA as an index to identify *V. cholerae* virulence-suppressive compounds. Successful application of this new HTS made it possible to identify compound 53760866 with *V. cholerae* virulence-suppressive potential. The structure of the compound can provide a lead or serve as a focal point for appropriate modifications by the methods of medicinal chemistry for synthesizing new derivatives with better activities and reduced toxicities. Such compounds may be useful in reducing the pathogenicity of *V. cholerae*, especially in situations where the inoculum is low, thus providing species-specific protection on a short time-scale without the need to apply broadly acting antibiotics that could perturb the normal intestinal microbiome.

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V. cholerae virulence inhibitors


High-throughput screening and whole genome sequencing identifies an antimicrobially active inhibitor of Vibrio cholerae

Galina Sergeev1, Sambit Roy2, Michael Jarek1, Viktor Zapolskii3, Dieter E Kaufmann3, Ranjan K Nandy2* and Werner Tegge1*

Abstract

Background: Pathogenic serotypes of Vibrio cholerae cause the life-threatening diarrheal disease cholera. The increasing development of bacterial resistances against the known antibiotics necessitates the search for new antimicrobial compounds and targets for this pathogen.

Results: A high-throughput screening assay with a Vibrio cholerae reporter strain constitutively expressing green fluorescent protein (GFP) was developed and applied in the investigation of the growth inhibitory effect of approximately 28,300 structurally diverse natural compounds and synthetic small molecules. Several compounds with activities in the low micromolar concentration range were identified. The most active structure, designated vz0825, displayed a minimal inhibitory concentration (MIC) of 1.6 μM and a minimal bactericidal concentration (MBC) of 3.2 μM against several strains of V. cholerae and was specific for this pathogen. Mutants with reduced sensitivity against vz0825 were generated and whole genome sequencing of 15 pooled mutants was carried out. Comparison with the genome of the wild type strain identified the gene VC_A0531 (GenBank: AE003853.1) as the major site of single nucleotide polymorphisms in the resistant mutants. VC_A0531 is located on the small chromosome of V. cholerae and encodes the osmosensitive K+ channel sensor histidine kinase (KdpD). Nucleotide exchange of the major mutation site in the wild type strain confirmed the sensitive phenotype.

Conclusion: The reporter strain MO10 pG13 was successfully used for the identification of new antibacterial compounds against V. cholerae. Generation of resistant mutants and whole genome sequencing was carried out to identify the histidine kinase KdpD as a novel antimicrobial target.

Keywords: Vibrio cholerae, Small molecules, Histidine kinase inhibitor, KdpD, whole genome sequencing

Background

Vibrio cholerae, a Gram-negative rod-shaped bacterium belonging to the family Vibrionaceae, induces the acute diarrheal disease cholera. Cholera has pandemic properties and appears mainly in third world countries with estimated 3–5 million cases and more than 100,000 deaths per year [1]. The major pathogenic strains belong to the serogroups O1 and O139. Infections are treated by oral or intravenous rehydration therapy, which is complemented in severe cases with antibiotics to shorten the duration of the clinical symptoms and to reduce the spreading. Long-term and extensive use of antibiotics has led to resistance development. A growing problem is the emergence of multidrug resistant pathogenic V. cholerae strains against which therapeutic options are more and more limited [2]. Due to this development the availability of novel therapeutic options is urgently needed.

In the present study we have developed a high-throughput screening (HTS) assay that utilizes a V. cholerae reporter strain constitutively expressing green fluorescence protein and screened approximately 28,300 compounds from six different chemical structural groups in a growth inhibition assay. Several active molecules were identified
which are active in suppressing growth of *V. cholerae* *in vitro*. *V. cholerae* mutants resistant to the most potent molecule were generated. Whole-genome sequencing and comparative analysis of the mutant to the wild type strain was carried out. The apparent target of the most active compound was identified to be the osmosensitive K⁺-channel sensor histidine kinase KdpD that apparently exerts certain essential function in this pathogen.

**Results**

**HTS assay for inhibitors of *V. cholerae* viability**

Green fluorescence producing plasmid pG13 was electroporated into *V. cholerae* strain MO10 and the transformants were selected on LB agar plates containing kanamycin (Km, 30 μg/ml). Transfer of the plasmid pG13 conferred green fluorescence phenotype in *V. cholerae O139* strain MO10. The screening assay was optimized in 96- and 384-well microtiter plates (MTP). To differentiate between active and non-active compounds and as controls for the functionality of the assay, ciprofloxacin (Cip, 100 μM) and dimethyl sulfoxide (DMSO, 1%) were included on each plate. DMSO had no growth reducing effect at concentrations up to 1%. The evaluation of the effect of compounds on the growth of strain MO10 pG13 was carried out after 24 h of incubation, with measurement of absorbance at 600 nm in combination with fluorescence determination (Figure 1). In the screening campaigns of the six different substance collections with 28,300 compounds in total, Z’-values between 0.5 and 0.9 with a mean of 0.8 were obtained, which is an indication of a reliable performance of the assay [3].

The six groups of screening compounds consisted of: i) the commercially available LOPAC library (a collection of pharmaceutically active compounds); ii) and iii) the EMC (Echaz Microcollection) and CDI collections (Chemical Diversity Lab), which contain small organic molecules that were mainly generated by combinatorial synthesis; iv) the VAR collection (natural compounds), which is unique at the HZI and consists of purified secondary metabolites from myxobacteria. It included potent agents with already known antimicrobial or antiproliferative activity, e.g. epothilon, which has been developed into a therapeutic agent against breast cancer [4,5]; and finally vi) collections of linear and cyclic peptides with a length of seven or eight D- or L-amino acids were investigated [6]. The compounds were used in one defined concentration between 20 to 50 μM in the initial screening. An overview of the growth-reducing activities of the six different substance collections is shown in Figure 2 and in Table 1. The threshold for active compounds was defined at a minimum growth reduction of

![Figure 1 HTS assay.](http://www.biomedcentral.com/1471-2180/14/49)
50% in comparison to the DMSO control, which resulted in a suitable initial hit rate. The smallest of the six collections, the NCH collection of 154 compounds, showed the most active molecules with 32.5 hits per 1,000 substances. Several of these molecules displayed antibacterial activities that have been known before [7]. The VAR library consists of molecules with predominantly unexplored activities and contained 8.8 antibacterial compounds per 1,000 molecules. With 17 hits this collection contained the highest number of antibacterial molecules in total.

In total 42 hits were identified in the initial screening campaign. These initial hits were reevaluated in different concentrations by using *V. cholerae* strains and several other Gram-positive and Gram-negative pathogenic bacteria. After these reevaluations, the number of active compounds was reduced to three most promising agents with the designations vz0825, vz0500 and 1541–0004. The former two compounds are derived from the VAR library, the last one from the commercially available CDI library. The chemical structures are shown in Figure 3.

### MIC and MBC values of the most active substances

The two pathogenic *V. cholerae* O1 type stains N16961 and NM06-058 were used to determine the MIC and MBC values for the compounds vz0825, vz0500 and 1541–0004 (Table 2). *V. cholerae* N16961 belongs to biotype El Tor which caused the seventh pandemic [8] and was isolated in 1971. *V. cholerae* NM06-058 was isolated in 2006 in Kolkata from a cholera patient and represents the altered El Tor biotype. The active compounds inhibited growth of both strains equipotent at low micromolar concentrations with MIC values of 1.6 μM, 3.1 μM and 6.3 μM, respectively. In order to obtain reliable data, bactericidal activities were determined after 2, 6 and 24 hours. All three compounds killed the bacteria at low micromolar concentrations, only slightly above the respective MIC values (Table 2). Further nine *V. cholerae* strains belonging to the O1, O139 and non O1/O139 serogroups (Table 3) (three strains of each serogroup) were tested with compound vz0825, which is active against all tested strains with MIC values between 0.4 and 3.1 μM. Overall vz0825 was the most active substance.

#### Table 1 Summary of the screening for growth-reducing compounds

<table>
<thead>
<tr>
<th>Substance collection (number of compounds)</th>
<th>Number of active compounds at different growth reduction rates</th>
<th>∑</th>
<th>Hit rate/1,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCH (154)</td>
<td>0</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Peptide (1,045)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LOPAC (1,408)</td>
<td>2</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>VAR (1,936)</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>EMC (7,304)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CDI (16,608)</td>
<td>5</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>28,324</td>
<td>42</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>
The antibacterial specificity of the active substances was investigated with different Gram-positive and Gram-negative pathogenic bacteria, which are able to induce serious gastrointestinal infections in humans (Table 4). Apparently, the antimicrobial activity of the three substances was limited to *V. cholerae*, only compound 1541–0004 also displayed a moderate activity against *S. aureus* with an MIC of 6.3 μM.

### Cytotoxicity determination via MTT-assay

*In vitro* cytotoxicity determination by MTT test with mammalian cells is one of the standard procedures for the evaluation of new active agents [10]. The well established assay was carried out with the permanent mouse fibroblast cell line L929 according to a published procedure [11] with some modifications [12]. In the assay cell viability is determined by the reduction of the yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid) to the violet formazan by the action of ER- and mitochondrial enzymes. Concentrations of the active compounds vz0825, vz0500 and 1541–0004 from 0.003 to 370 μM were used and effects on the fibroblasts were analyzed after 24 hours and 5 days of incubation. The IC₅₀ values are shown in Table 5. The two most active compounds vz0825 and vz0500 showed cytotoxic (inhibition after 24 hours of incubation) and anti-proliferative (inhibition after 5 days of incubation) IC₅₀ values at low micromolar concentrations. Compound 1541–0004 is less cytotoxic, but has also a strong antiproliferative activity.

### Generation of resistant mutants against vz0825

Mutants against vz0825 were generated by selection of variants of the wild type strain NM06-058 that are able to grow on agar plates containing 8 μM vz0825. After one round of selection, 15 resistant mutants were picked and analyzed individually. They displayed 4–16 fold reduced sensitivities (MIC 6.3 - 25 μM) against vz0825 compared to the wild type strain. In order to obtain an indication if vz0825 has a mode of action that is different from standard antimicrobials, eight established antibiotics against the major different antibacterial targets were tested with the resistant mutants. The addressed targets and their inhibitors were i) cell wall synthesis (ampicillin), ii) protein biosynthesis (tetracycline), iii) DNA-replication (ciprofloxacin), iv) DNA-dependent RNA polymerase (rifampicin), v) translation (chloramphenicol, erythromycin) and vi) synthesis of folic-acid (trimethoprim/sulfamethoxazol).

The *V. cholerae* wild type strain NM06-058 and resistant mutants did not show differences in their MIC values against all tested antibiotics (data not shown), suggesting that vz0825 has a mode of action that is different from the classical antibiotics.

### Target identification

This result initiated a further investigation of the mode of action of vz0825 by the comparative genome sequence analysis approach. The method makes use of whole genome sequence analysis of resistant mutants that were generated against an active compound and the comparison of the genome of the wild type and the mutant strain [13]. The genomes of the 15 resistant *V. cholerae* mutants were isolated, pooled and analyzed via paired-end sequencing. In parallel, the genome of the wild type strain from which

---

**Table 2 MIC and MBC values for the most active compounds against *V. cholerae***

<table>
<thead>
<tr>
<th><em>V. cholerae</em> strain</th>
<th>Incubation time</th>
<th>Concentration [μM]</th>
<th>vz0825</th>
<th>vz0500</th>
<th>1541-0004</th>
</tr>
</thead>
<tbody>
<tr>
<td>N16961</td>
<td>MIC 24 h</td>
<td>1.6</td>
<td>3.1</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MBC 2 h</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>12.5</td>
<td>6.3</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>6.3</td>
<td>6.3</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>NM06-058</td>
<td>MIC 24 h</td>
<td>1.6</td>
<td>3.1</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MBC 2 h</td>
<td>50</td>
<td>50</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>12.5</td>
<td>6.3</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1.6</td>
<td>6.3</td>
<td>6.3</td>
<td></td>
</tr>
</tbody>
</table>

---

**Figure 3 Chemical structures.** Most active compounds of *V. cholerae* growth inhibition. Panel A: compound vz0825; Panel B: compound vz0500; Panel C: compound 1541-0004.
<table>
<thead>
<tr>
<th>Strain, cell, plasmid, primer</th>
<th>Relevant description/sequence</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. cholerae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MO10 pG13</td>
<td>O139 containing pG13</td>
<td>This study</td>
</tr>
<tr>
<td>N16961</td>
<td>Wild type, O1, El Tor, Inaba</td>
<td>Makassar (1971), clinical isolate [8]</td>
</tr>
<tr>
<td>NM06-058</td>
<td>Wild type, O1, El Tor, Ogawa</td>
<td>Kolkata (1996), clinical isolate</td>
</tr>
<tr>
<td>NM06-058 T283M</td>
<td>Contains a point mutation in gene VC_A0531 on AA position 283</td>
<td>This study</td>
</tr>
<tr>
<td>RKI-ZBS2-A310-3</td>
<td>Isolate, O1, El Tor, Inaba</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-A310-12</td>
<td>Isolate, O1, El Tor, Ogawa</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-A198-1</td>
<td>Isolate, O1, El Tor, Ogawa</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-A310-25</td>
<td>Isolate, O139, El Tor</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-A186-9</td>
<td>Isolate, O139, El Tor</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-186-10</td>
<td>Isolate, O139, El Tor</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-A220-1</td>
<td>Isolate, Non O1/O139</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-A222-1</td>
<td>Isolate, Non O1/O139</td>
<td>RKI</td>
</tr>
<tr>
<td>RKI-ZBS2-A227-1</td>
<td>Isolate, Non O1/O139</td>
<td>RKI</td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>ATCC 30007</td>
<td>DSMZ</td>
</tr>
<tr>
<td>E. coli</td>
<td>ESBLE, 5044257621-1</td>
<td>HZI</td>
</tr>
<tr>
<td>E. coli</td>
<td>ETEC</td>
<td>NICEDE</td>
</tr>
<tr>
<td>E. coli</td>
<td>S17-1</td>
<td>HZI</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>50219455</td>
<td>HZI</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>90013687</td>
<td>HZI</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td></td>
<td>NICEDE</td>
</tr>
<tr>
<td>Shigella boydii</td>
<td></td>
<td>NICEDE</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td></td>
<td>NICEDE</td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ATCC 20212</td>
<td>HZI</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>MRSA, N315</td>
<td>HZI</td>
</tr>
<tr>
<td><strong>Cell line</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L929</td>
<td>Mouse fibroblastic cell line</td>
<td>Derived from commercial source, DSMZ: ACC 2</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pG13</td>
<td>Plasmid containing the constitutive expressing G13 promoter- and gfp-gene sequence, ligated in pFPV27 vector, (Km)</td>
<td>[9]</td>
</tr>
<tr>
<td>pEX18Ap</td>
<td>Plasmid containing AmpR gene β-lactamase, the sacB gene encoding the levansucrase</td>
<td>HZI</td>
</tr>
<tr>
<td><strong>Oligonucleotide primer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VC_A0531_forw2</td>
<td>TCACGAACCAACAGGATTAAG</td>
<td>Used for colony PCR and sequencing of the products</td>
</tr>
<tr>
<td>VC_A0531_rev2</td>
<td>CGTTAAGGGGCTAGAGCAGAGAGG</td>
<td>Same as above</td>
</tr>
<tr>
<td>Mut_forw_1</td>
<td>ACATCATCTAGAGCGAGCAGCAACACACAGAG (XbaI)</td>
<td>Used for generation of the point mutation</td>
</tr>
<tr>
<td>Mut_rev_1</td>
<td>ATCCGCGCCAAGGCGCCATTTTAGATCG</td>
<td>Same as above</td>
</tr>
<tr>
<td>Mut_forw_2</td>
<td>CGATCTAAAAATGCCGCTTGGCCGAGAT</td>
<td>Same as above</td>
</tr>
<tr>
<td>Mut_rev_2</td>
<td>ACATCAAGGTTAACATGCGGCCACCCAGAC (HindIII)</td>
<td>Same as above</td>
</tr>
<tr>
<td>kdpD_del_forw_1</td>
<td>ACATCATCTAGAGGAATCCATCAAGAAA (XbaI)</td>
<td>Used for generation of the deletion mutation of kdpD</td>
</tr>
</tbody>
</table>
the resistant mutants have been generated was also sequenced by the same method. The alignment and annotation of both probes was based on the published genome of *V. cholerae* strain N16961 (chromosome 1: AE003852, chromosome 2: AE003853 in NCBI) [14]. As shown in Table 6, approximately 98% and 94% of the fragments from the mutant-pool and the wild type, respectively, could be aligned. The alignment was carried out via the application of CLC Genomics Workbench V. 4.7.2 software. The algorithm to search for crucial distinctions were parameters like single nucleotide polymorphism (SNP) and deletion and insertion polymorphism (DIP), where one nucleotide was affected with a minimal mutation frequency of 30%.

Under those conditions, the comparison of the wild type and the pooled sequences from the mutants showed only one significant mutation, this was located at position 848 in gene VC_A0531 and was present in about 30% (precisely 29.1%) of the sequenced fragments. These mutants have the nucleobase thymine instead of cytosine on position 848. The point mutation of this nucleobase leads to an exchange of threonine to methionine on position 283 (T283M) of the expressed protein.

The gene VC_A0531 (GenBank: AE003853.1) is located on the small chromosome of *V. cholerae* and encodes a sensor histidine kinase, which is the homologous to KdpD of *E. coli* and is responsible for osmotic potassium regulation in the bacterial cell [15]. In addition to the whole genome pool sequencing, the gene VC_A0531 (*kdpD*) of the 15 mutants was analyzed individually by PCR amplification. 4 of the 15 mutants, corresponding to 26.7%, had the same mutation on reference position 848 of the gene *kdpD* that was identified in the whole genome pool sequencing. Another four of the mutants showed point mutations at other positions of the *kdpD* gene (Table 7).

### Sensitivity of strain NM06-058 T283M against vz0825

A strain containing the point mutation T283M in the *kdpD* gene was generated by site-directed mutagenesis. Successful cloning was verified by a PCR amplification of the affected gene and the sequencing of the fragment. The mutant was selected on LB-agar plates containing vz0825 at 16 μM concentration, which is 10-times higher than the MIC of the wild type strain. A growth analysis with this strain was carried out in vz0825 supplemented LB-medium and in T-medium with different potassium and sodium ion concentrations (Figure 4). Overall, growth of the T283M mutant was much less effected by vz0825 in comparison to the wild type strain. Sensitivity of the T283M mutant against compounds vz0500 and 1541–0004 did not differ from the wild type strain NM06-058 (data not shown).

### Attempts to construct a *kdpD* knockout mutant

For a further elucidation of the effect of vz0825, the construction of a *V. cholerae kdpD* knockout mutant was attempted. If KdpD is a major target of compound vz0825, the *V. cholerae kdpD* knockout mutant should be insensitive to the compound, unless the protein itself and its function are essential for the viability of the bacteria. The cloning procedure delivered the expected plasmid construct according to sequencing. The plasmid was successfully transformed into the *E. coli* strain S17-1, according to the acquirement of ampicillin resistance, which is located on the plasmid pEX18Ap and also according to PCR amplification of the construct. The conjugation of the

### Table 4 MIC values of active compounds for different pathogenic bacteria

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>vz0825</th>
<th>vz0500</th>
<th>1541-0004</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>50</td>
<td>&gt; &gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td><em>Escherichia coli</em>, ESBL</td>
<td>&gt; 100</td>
<td>&gt; &gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td><em>Escherichia coli</em>, ETEC</td>
<td>&gt; &gt; 50</td>
<td>&gt; &gt; 50</td>
<td>&gt; 50</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>100</td>
<td>&gt; 100</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>&gt; &gt; 100</td>
<td>&gt; &gt; 100</td>
<td>&gt; &gt; 100</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>&gt; &gt; 50</td>
<td>&gt; &gt; 50</td>
<td>&gt; &gt; 50</td>
</tr>
<tr>
<td><em>Shigella boydii</em></td>
<td>&gt; &gt; 50</td>
<td>&gt; &gt; 50</td>
<td>&gt; &gt; 50</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>&gt; &gt; 50</td>
<td>&gt; &gt; 50</td>
<td>&gt; &gt; 50</td>
</tr>
<tr>
<td><strong>Gram-positive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>50</td>
<td>&gt; &gt; 100</td>
<td>&gt; 100</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em>, MRSA</td>
<td>50</td>
<td>100</td>
<td>6.3</td>
</tr>
</tbody>
</table>

### Table 5 Cytotoxic (24 h) and antiproliferative (5 d) activity of the most active compounds according to MTT test with L929 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 [μM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>vz0825</td>
<td>14</td>
</tr>
<tr>
<td>vz0500</td>
<td>3</td>
</tr>
<tr>
<td>1541-0004</td>
<td>170</td>
</tr>
</tbody>
</table>
transformed E. coli with V. cholerae and the following selection on LB agar plates supplemented with carbenicillin (Carb) and Km did not lead to clones with a deleted VC_A0531 gene, even after several modifications of the protocol. A possible explanation is that the gene product KdpD is indeed essential for V. cholerae, in agreement with KdpD being a prime target of vz0825.

**Discussion**

A HTS assay for small molecule inhibitors of V. cholerae was developed and validated using a viability phenotype of V. cholerae that constitutively expresses green fluorescence. The assay is reliable, reproducible and simple to perform. During the development of the reporter strain, two reference strains of O1 serogroup belonging to bio-types O395 (classical) and N16961 (El Tor) were included along with the O139 strain MO10. The green fluorescence producing plasmid pG13 was electropropated into the three strains. During initial standardization experiments it was observed that the strain MO10 pG13 produced much greater level of green fluorescence as compared to other two strains (data not shown). For this reason strain MO10 pG13 was used in the screening experiments.

A data bank search in SciFinder for the most active compounds, we have used the same strain to create resistant mutants against vz0825. The V. cholerae strain NM06-058 was isolated from hospitalized diarrhea cases during 2006 at Kolkata, India. This strain along with other V. cholerae strains isolated during 2006 was studied for the expression of cholera toxin (CT) and it was identified that NM06-058 is capable of producing a higher amount of CT in vitro compared to other strains and to reference V. cholerae O1 El Tor strain N16961. Based on the high virulence expression, this strain was selected for our investigations. Clinical V. cholerae O1 strains isolated at Kolkata during and after 1995 belonged to altered El Tor biotypes [19]. Thus it can be considered that strain NM06-058 represents the altered V. cholerae El Tor biotype, which is still the prevailing type among cholera cases.

The generation of mutants that were resistant against vz0825 was straightforward in this study by plating the wild type strain on agar plates containing the active compound at 5-times the MIC value of the wild type. The successfull generation of resistant mutants with only one

**Table 7 Modifications detected in gene VC_A0531 (kdpD) by PCR analysis of 15 resistant mutants (AA, amino acid)**

<table>
<thead>
<tr>
<th>Nucleotide pos.</th>
<th>Ref. allele</th>
<th>Mut. allele</th>
<th>Number of mutants</th>
<th>Codon old</th>
<th>Codon new</th>
<th>AA pos.</th>
<th>AA old</th>
<th>AA new</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>218</td>
<td>T</td>
<td>C</td>
<td>1</td>
<td>CUCA</td>
<td>73</td>
<td>Leu</td>
<td>Pro</td>
</tr>
<tr>
<td>2</td>
<td>848</td>
<td>C</td>
<td>T</td>
<td>4</td>
<td>ACCG</td>
<td>AUG</td>
<td>283</td>
<td>Thr</td>
</tr>
<tr>
<td>3</td>
<td>1,022</td>
<td>C</td>
<td>A</td>
<td>1</td>
<td>CCUG</td>
<td>CAU</td>
<td>341</td>
<td>Pro</td>
</tr>
<tr>
<td>4</td>
<td>1,177</td>
<td>G</td>
<td>A</td>
<td>1</td>
<td>GAA</td>
<td>AAA</td>
<td>393</td>
<td>Glu</td>
</tr>
<tr>
<td>5</td>
<td>1,178</td>
<td>A</td>
<td>G</td>
<td>1</td>
<td>GAA</td>
<td>GGA</td>
<td>393</td>
<td>Glu</td>
</tr>
</tbody>
</table>

In bold the major statistically significant mutation is highlighted.
passage indicates a single essential molecular target of vz0825. The aligned sequences of the wild type genome and the mutant genome pool were compared with each other. For the identification of significant mutations the minimal frequency in the mutant genome pool was defined at 30%. A lower frequency would deliver too many non-relevant mutations. In the genome pool of the 15 resistant mutants only the gene with the code number VC_A0531, which corresponds to the homologue \textit{kdpD} in \textit{E. coli}, showed a significant mutation under the chosen parameters with frequency of 29.1%. The sequencing of the 15 resistant mutants showed, that 4 of them (26.7%) possess this particular modification. Another four mutants also possess point mutations at other positions of the gene (shown in Figure 5). All of those mutations lead to an exchange of one particular amino acid in the expressed protein, two of which are located in the N-region (position 1,177 and 1,178) lead to the exchange of glutamic acid 393 to lysine or glycine, respectively (Table 7 and Figure 5). Thus, 8 of 15 mutants possess a mutation in the \textit{kdpD} gene.

A comparison of known protein domains in the database Pfam Protein Families [21] resulted in the localization of the affected amino acid in the dimerization/phosphor acceptor domain. Histidine kinase dimers are formed by parallel association of two domains creating 4-helix bundles; usually these domains contain a conserved histidine residue and are activated via trans-autophosphorylation by the catalytic domain [22]. They subsequently transfer the phosphoryl group to the aspartic acid acceptor residue of a response regulator protein. Based on the comparison of conserved regions in a number of bacterial histidine kinases [20], the localization could be specified more precisely between the H–region and the N-region (Figure 5). The H–region is the most variable sequence of histidine kinases in bacteria and contains the histidine that is phosphorylated in the signal transduction process. The N–region shuttles the gamma-phosphate from ATP to the histidine residue. The mutated amino acid is localized between the conserved H- and N-region (Figure 5) and thus in a part of the protein that shows high interspecies variation [23], which could explain the specificity of vz0825 against \textit{V. cholerae}.

In the two-component system of signal transduction, the histidine kinase transfers the signal to a response regulator. The \textit{V. cholerae} protein VC_A0531 is the homolog of KdpD in \textit{E. coli}, the response regulator of which is KdpE [24]. The signal transduction system KdpABC, regulated by KdpD and KdpE, is part of the osmoregulation machinery in bacteria [15]. Compound vz0825 may exert its mode of action by binding to the histidine kinase KdpD and thereby inhibiting signal transduction. This would lead to a deficient uptake of potassium. If this mechanism leads to the observed reduction of bacterial viability remains to be elucidated.

Due to a lack of specific information about the potassium regulation in \textit{V. cholerae}, we compared our findings with results that have been obtained with \textit{E. coli}. \textit{E. coli} possesses in addition to the KdpABC potassium regulatory system several further K⁺ dependent signal transduction systems. The K⁺ regulatory systems Trk and Kup are active at physiological K⁺ concentrations [15]. The expression of KdpD and consequently of the KdpABC system in \textit{E. coli} is induced at low potassium concentrations (<60 mM) [25]. In \textit{E. coli} KdpD is not essential at a potassium concentration >115 mM, as mutants with truncated forms of KdpD are viable under these conditions, but in media with <15 mM K⁺ those strains do not grow [25]. \textit{V. cholerae} wild type strain NM06-058 (A) and the T283M exchange mutant (B) in the presence of vz0825 in media with different K⁺ and Na⁺ concentrations.
**V. cholerae** also possesses these three potassium regulatory systems for the adaptation to changing osmotic conditions [26,27].

The *V. cholerae* mutant strain T283M grows well in media with high and low K+ and Na+ concentrations in absence of vz0825 as shown in Figure 4. Even at 4 mM K+ growth is not diminished. This figure also shows the difference between the tolerance of the wild type and the T283M strain against vz0825. Our findings that T283M grows well in K+ reduced medium indicates that the inhibition of KdpD may have profound influence on some other, hitherto undefined, regulatory function of this protein in *V. cholerae*. The influence of vz0825 on KdpD may appear in different ways, e.g. reducing the binding of ATP to the histidine kinase, inhibiting the transfer of gamma-phosphate to the histidine residue, or to the asparagine residue of the response regulator. Like other histidine kinases KdpD also has phosphatase activity [28], which may be disturbed by vz0825. The mutated amino acid on position 283 is located between the H-region and N-region. Mutations that alter this motif, which is termed the X-region, have been shown to alter the conformation of the histidine kinase EnvZ and significantly reduce its phosphatase activity [29]. EnvZ is a membrane receptor kinase-phosphatase, which modulates porin expression in *E. coli* in response to medium osmolarity. It shares its basic scheme of signal transduction with many other sensor-kinases [29].

If KdpD is the major target of compound vz0825, the deletion construct ΔkdpD should be insensitive to the substance in media with physiological K+ concentration – provided that it is still viable. The construction of the required plasmid for the generation of this construct, its transformation into *E. coli* S17-1 and the conjugation from *E. coli* into *V. cholerae* were successful in this study, but several attempts to induce the homolog recombination within *V. cholerae* NM06-058 failed. None of the analyzed clones showed a loss of the *kdpD* gene. The apparent growth reducing effect of vz0825 and its targeting of KdpD in *V. cholerae* suggests a more important role of KdpD in *V. cholerae* than in *E. coli*. Further experiments are required in order to corroborate the effect of vz0825 on KdpD, like functional assays with the expressed protein, in which the kinase- and phosphatase activities of the wild type and mutated forms in the presence of vz0825 are compared. It would also be desirable to carry out expression profiling of the transcriptome of vz0825 sensitive and resistant *V. cholerae* strains. This procedure could help to determine how relevant the expression of KdpD in *V. cholerae* is and whether the expression of other genes is reduced or induced in the resistant strains.

**Conclusions**

In a high-throughput screening assay with 28,300 compounds the synthetic small molecule vz0825 was identified as the most active antibacterial substance against *V. cholerae* with an MIC of 1.6 μM and an MBC of 3.2 μM. Whole genome sequencing was carried out with resistant mutants and the two-component histidine kinase KdpD was identified as the prime target of the substance. Further investigations should address the inhibitory mechanism in more detail and corroborate on the possibility of an essential function of KdpD in *V. cholerae*. Histidine kinase inhibitors are in principal promising antimicrobial drug candidates [30] and compounds like vz0825 may lead to new treatment options.

**Methods**

**Strains, media and plasmids**

The strains used in this study are listed in Table 3. Reporter strain MO10 pG13 was generated from the

**Figure 5 Sequence of KdpD from V. cholerae.** Amino acids labeled in green in the regions H, N, G1, F, G2 are conserved in different species [20]. Labeled in red is threonine 283 which is exchanged by methionine in the dominant mutations of the resistant strains. Amino acids labeled in blue indicate the positions that are modified in four additional mutants (L73P, P341H, E393K and E393G).
pathogenic wild type strain MO10, serogroup O139, which was electroporated with the plasmid construct pG13 containing a kanamycin resistance gene (Km) and was selected on a plate containing 30 μg/ml Km. *V. cholerae* strains were grown in LB medium (pH 7.0) at 37°C. LB medium containing Km (30 μg/ml) was used for HTS and Cip (100 μM) was used for positive control. To determine the MIC and MBC values, Mueller-Hinton (MH) broth (pH 7.4) was used as growth medium. Susceptibility to ampicillin (Amp), tetracycline (Tet), Cip, rifampicin, chloramphenicol, erythromycin, sulfamethoxazole, and trimethoprim/sulfamethoxazole (SXT) was determined in 96-well MTP containing MH medium supplemented with varied amounts (1 to 1,024 μg/ml) of each antibiotic separately and varied amounts of SXT (0.13/2.38 to 8/152 μg/ml). Supplemented LB medium with Amp (50 μg/ml), Km (30 μg/ml) and Carb (100 μg/ml) was used during the procedures of site-directed mutagenesis and in T medium pH 7.4. T medium was prepared by adding 17 g tryptone, 3 g neutralized soy peptone, 10 g glucose, 50 mM MOPS, 3 g neutralized NaCl, 2 mM KCl and 2 mM CaCl2 in 1 l of water. For homolog recombination NaCl-free (for increased sucrose sensitivity [31]) LB medium or T medium with 10% sucrose (for induction of pEX18Ap plasmid excision, carrying the sacB gene) was used. Cultivation of the mouse fibroblasts cell line L292 was carried out in DMEM with 10% FBS (Lonza).

**Substance collections**

Three commercially available substance collections were used in the screening campaigns: i) the LOPAC collection of pharmacologically active compounds with 1,408 entities (Sigma-Aldrich); ii) the Echaz Microcollection with 7,304 compounds (EMC Microcollections GmbH, Tübingen, Germany); and iii) the CDI collection with approximately 17,000 compounds (Chemical Diversity Lab, Inc., San Diego, USA), this commercially available collection has been assembled by members of the ChemBioNet consortium [32]. Three additional libraries that were used are unique at the HZI: iv) the NCH collection consisting of 154 unique at the HZI; iv) the NCH collection consisting of 154 secondary metabolites from myxobacteria [33]; v) the library *Various Sources* (VAR) contained at the time of this study 1,936 synthetic organic molecules that were provided by various collaborators; and vi) the Peptide library contained 1,045 short linear or cyclic peptide sequences synthesized at the HZI [6]. All test compounds were utilized as stock solutions in DMSO.

**Growth assay**

50 μl or 25 μl of LB-Km medium were inoculated in clear flat-bottom 96-well or 384-well MTP, respectively. Test compounds were added from DMSO stocks in amounts that resulted in assay concentrations between 20 and 50 μM. 50 μl or 25 μl of bacterial culture in LB-Km medium with an absorbance of 0.2 at 600 nm (OD600) (Ultraspec 2100 Pro photometer, Pharmacia, GE Healthcare, Chalfont St Giles, UK) were added to the 96-well or 384-well MTP, respectively. The seeding of bacteria and addition of the compounds was carried out with the pipetting system Evolution P3 (PerkinElmer, Waltham, USA). Stationary incubation of the plates for 24 h at 37°C under moist conditions was carried out, followed by determination of absorbance at 600 nm and fluorescence at 485/535 nm (Fusion Universal Microplate Analyzer, PerkinElmer, Waltham, USA). As negative and positive controls DMSO (1%) and Cip (100 μM) were used, respectively. During the initial screening, approximately 28,300 compounds were investigated with single determinations. Compounds that reduced bacterial growth by at least 50% were retested in a second campaign and the most active substances were reevaluated at different concentrations between 0.1 and 100 μM.

**MIC and MBC values determination**

The determination of MIC and MBC values was carried out with *V. cholerae* wild type strains and several Gram-negative and Gram-positive bacteria (Table 3) following standardized protocol [34] in broth dilution assays. Starting inocula of 2·8×105 colony forming units/ml (CFU/ml) in MH medium at 37°C were used and serial dilutions were carried out in 96-well MTP in duplicate. At 2, 6 and 24 h of incubation, 10 μl of the cultures were plated on LB agar plates. After an incubation of the plates for 24 h at 37°C, CFU/ml were determined and used for the determination of MBC, which is defined as minimum concentration of the substance required for 99.9% reduction of CFU after an incubation period of 6 h. The 2 h and 24 h measurements were used for additional correlation. MIC values were determined after 24 h of incubation.

**Cytotoxicity assay**

The mammalian cell line L929 was utilized to investigate the cytotoxicity of the active compounds in a MTT assay according to a modified protocol of Mosmann [11,12]. Following 24 h of incubation, acute toxicity was determined based on the extent of cell viability and after incubation for 5 d mainly the inhibition of cell proliferation and subacute toxicity were measured (absorption at 595 nm) (Wallac Victor 1420 Multilabel counter, PerkinElmer, Waltham, USA). IC50 is the concentration that reduces the viability of the cells by 50%.

**Generation of resistant mutants against vz0825**

The protocol for the generation of resistant mutants was the same as used in the publication of Bielecki et al. [13]. *V. cholerae* strain NM06-058 was plated at a cell number of 1×108 CFU on LB agar plates containing 8 μM vz0825 (5-times the MIC value). After incubation for 24 h at 37°C,
micro-colonies were visible. 15 colonies were picked and preserved as mutants against vz0825.

Isolation of genomic DNA and sequencing of genome-pool
Isolation of the genomic DNA was performed according to the protocol of the DNeasy Blood and Tissue Kit (Qiagen). Briefly, the 15 resistant mutants were inoculated individually in 5 ml LB medium and incubated for 6 h at 37°C with shaking at 180 rpm. In parallel, the wild type strain was cultivated under identical conditions. Based on the OD_{600} measurements of the cultures, the 15 mutants were pooled in equal amounts. After adjusting the cell number at $2 \times 10^9$ CFU the pooled mutants and the wild type strain were collected by centrifugation. The cell pellets were lysed by addition of ATL buffer and proteinase K for 1 h at 56°C. RNA was removed by addition of 4 μl RNase A (100 mg/ml) and incubation for 2 min at RT. 200 μl AL buffer and afterwards 200 μl of ethanol were added with mixing. The mixture was transferred to DNeasy Mini spin columns and centrifuged at $\geq 6,000 \times g$ for 1 min. Washing was carried out with 500 μl AW1 buffer followed by centrifugation for 1 min. A second washing step was carried out with 500 μl AW2 buffer. The tubes were centrifuged for 3 min at 20,000 × g and the genomic DNA was eluted from the membranes with 200 μl AE buffer.

Whole genome sequencing, alignment and annotation were carried out in the sequencing facility of the HZI (head Dr. Robert Geffers). Libraries of DNA fragments with an average length of 300 bp were prepared according the manufacturer's instructions “Preparing Samples for Sequencing Genomic DNA” (Illumina). Sequencing was carried out with the Illumina Cluster Station and the Genome Analyzer IxX. The resulting data was transformed into FastQ-format. Sequencing of the DNA library resulted in a total base count of 855,825,664 and 2,546,713,435 for wild type and resistant mutants genome pool, respectively. This corresponds to a calculated average coverage of 214 for the wild type and for each resistant mutant to a coverage of 42. The published complete genome has a total base number of 4,033,460 (Table 6, [14]).

The sequencing procedure resulted in 11,260,862 and 35,196,596 reads for wild type and resistant mutants genome pools, respectively, which were mapped to the reference genome of the annotated V. cholerae strain N16961 [14] by the application of the Read Mapper Tool and the Probabilistic Variant Caller as part of CLC Genomics Workbench V. 4.7.2 software. The Read Mapper Tool maps reads and calculates average coverage at single nucleotide resolution. The Probabilistic Variant Caller identifies variants by using a probabilistic model built from read mapping data. Based on a combination of a Bayesian model and a Maximum Likelihood approach the algorithm calculates prior and error probabilities for the Bayesian model. By using the Probabilistic Variant Caller software and defining various parameters, such as sequence frequency, size of mutated areas and mutation abundance, lists of SNPs and DIPs were created. A frequency of more than 30 reads was required for all fragments. The maximum number of allel-variations was restricted to two, and the threshold of the frequency of the allel-variations was set at a minimum of 30%. These lists were compared for the wild type strain and the pooled resistant mutants, and SNPs that are unique for the mutants were identified.

Colony PCR and sequencing
The 15 resistant mutants were analyzed individually to determine whether they carry the point mutation on position 848 of the kdpD gene. Individual colonies were heated in 36,5 μl of water for 5 min at 95°C. 1 μl of dNTPs (stock solution 10 mM), 2.5 μl of primers VC_A0531_forw2 and VC_A0531_rev2 (stock solution 100 pmol/μl), 5 μl 10× PCR buffer and 2.5 μl RED Taq polymerase (1 U/μl) were added. After the PCR procedure, the products had the expected size of 915 bp. They were purified and sequenced in the sequencing facility of the HZI using the above primers.

Construction of the point-mutant KdpD T283M in strain NM06-058
The gene VC_A0531 has a size of 1,494 base pairs (coding for 497 amino acids plus stop codon). The base cytosine, which was changed to tyrosine in the predominant resistant mutants, is located on position 848. Site-directed mutagenesis was used for the incorporation of this modification into the wild type strain NM06-058. Two overlapping amplicons with a size of 525 and 616 bp were generated from the gene of the wild type strain NM06-058. Fragment one was amplified using the primer pair (i) Mut_forw_1/Mut_rev_1, and the second fragment was amplified with primer pair (ii) Mut_forw_2/Mut_rev_2. The primers Mut_rev_1 and Mut_forw_2 carried the point-mutation (Table 3, bold nucleobases). Primers Mut_forw_1 and Mut_rev_2 contained specific recognition nucleotide sequences for the restriction enzymes XbaI and HindIII. Both amplicons were mixed at equimolar ratio and a re-PCR was performed with the primers Mut_forw_1 and Mut_rev_2 to generate an amplicon with a size of 1,114 bp. This amplicon and the plasmid pEX18Ap were restricted with XbaI and HindIII. Insert and plasmid were ligated and transformed into chemically competent E. coli strain S17-1. Amp (100 μg/ml) was incorporated into the agar of the plate for selection of pEX18Ap containing transformants. PCR based analysis of the transformants followed by nucleotide sequencing analysis confirmed the proper insert into the vector, which was subsequently used for the conjugation assay.
Conjugation was carried out on LB agar plates overnight with a bacterial proportion of 4:1 of *E. coli* containing conjugative plasmid (donor) and *V. cholerae* as recipient strain. Bacterial cultures (mixed *E. coli* and *V. cholerae*) were plated on LB agar plate containing Carb (100 μg/ml) and Km (30 μg/ml) for selection of *V. cholerae* transconjugants carrying the plasmid. The removal of vector backbone from *V. cholerae* genome was achieved by favoring the homologous recombination and use of lethal *sacB* gene while passaging the transconjugants in sodium chloride free LB medium supplemented with 10% sucrose.

**Attempts for construction of a kdpD knockout mutant using *V. cholerae* strain NM06-058**

The gene VC_A0531 encodes for the histidine kinase KdpD in *V. cholerae* and is flanked by the genes VC_A0530 encoding pyruvate-flavoredoxin oxidoreductase and VC_A0532 encoding response regulator KdpE homologue of *E. coli*. To generate a VC_A0531 deletion mutant, two fragments were amplified from the small chromosome of the wild type strain NM06-058 using two primer pairs (i) *kdpD_del_forw_1 / kdpD_del_rev_1* and (ii) *kdpD_del_forw_2 / kdpD_del_rev_2*. Using the first primer pair an approximately 600 pb fragment of gene VC_A0530 was amplified with a 24 bp homolog overhang to the start region of the VC_A0532 at the C-terminus. The second primer pair was used to amplify an approximately 400 bp fragment of the gene VC_A0532 with a 16 bp overhang homolog to the end region of the VC_A0530 at the N-terminus. Both amplicons were mixed together at equimolar ratio and a re-PCR was carried out with a combination of primers *kdpD_del_forw_1* and *kdpD_del_rev_2* to generate an amplicon with a size of approximately 1,000 bp. The restriction of vector pEX18Ap and the insert was carried out with *XbaI* and *PstI*. After ligation and transformation into *E. coli* S17-1, a conjugation into the wild type *V. cholerae* strain NM06-058 was mediated according to the protocol described above. The cloning strategy was successful until transconjugation according selection on Carb / Km agar plates and sequencing, but homolog recombination attempts with *V. cholerae* strain NM06-058 did not yield viable strains with deleted *kdpD* gene.

**Abbreviations**

AA: Amino acid(s); Amp: Ampicillin; Carb: Carbenicillin; Cip: Ciprofloxacin; CFU: Colony-forming unit; CT: Cholera toxin; DIP: Deletion and insertion polymorphism; DMSO: Dimethyl sulfoxide; ER: Endoplasmic reticulum; GFP: Green Fluorescent Protein; HTS: High-throughput screening; Km: Kanamycin; MBC: Minimal bactericidal concentration; MIC: Minimal inhibitory concentration; MTP: Microtiter plate; NKI: Robert Koch Institute; SNP: Single nucleotide polymorphism; SXT: Trimethoprim/sulfamethoxazole; Tet: Tetracycline.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

GS performed experiments, including assay development, screening, hit evaluation and the first target analysis using genome sequencing of resistant mutants. MJ is member of the sequencing facility at the HZI and carried out and interpreted the genome sequencing. SR developed the reporter strain MO10 pcl3 which was used for the screening. Compounds showing activity against *V. cholerae* were conceived and synthesized by DT and VAZ. RKN and WT conceived the study, participated in its design and coordination and helped to draft or revise the manuscript. All authors read and approved the final manuscript.

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