Introduction

*Vibrio cholerae* is endowed with a remarkable capacity to sense diverse environmental settings and accordingly modulate disparate cellular events to maintain its sustainability in a given niche. The process of sensing and further coordination of cellular activities is largely dependent on a microbial social networking program known as quorum sensing. In recent past, an explosive growth in studies on quorum sensing of *Vibrio cholerae* is clearly illustrated the intricacy of such complex signal transduction at the molecular level and its influence on the lifestyle of this bacterium. The intricate architecture of sensory circuit has revealed the participation of myriad of factors, namely (i) two autoinducer systems (AI-2/LuxPQ and Cai-1/CqsS), (ii) one growth phase-regulated VarS/A-csrA/BCD unit, (iii) a small nucleoid protein, Fis and (iv) a cascade of small RNAs, plays a critical role in balancing the periodic appearance and the performance of master regulatory proteins LuxO and HapR [1]. Being the high cell density master regulatory protein, HapR is under the spotlight as it governs a total of nine binding domain (DBD) and dimerization domain (DD). There are three DNA binding domain. The HTH motif comprises of helices \(a_2\) and \(a_3\). The remaining six \(\alpha\)-helices are distributed in the C-terminal dimerization domain [4]. In a continuing effort, Zhu and coworkers have elegantly characterized additional novel direct targets of HapR and illustrated two distinct binding motifs (motif 1 and motif 2) in various target promoters. Motif 1 sequence contains dyad symmetry and a consensus AATAR’ (where R represents A or G) whereas motif 2 sequence lacks any dyad symmetry and contains a highly conserved consensus ‘TGT’ [5]. Though many crucial facts about the structural and functional aspects of wild type HapR are available, additional insights into virulence and survival strategy of *Vibrio cholerae* [2]. Collectively, it represses biofilm development and the production of primary virulence factors, while stimulates the production of HA/protease, promotes chitin induced competence, increases resistance to protozoan grazing, enhances the survival against oxidative stress and controls the expression of gene encoding Hcp [3]. Structurally, the protein comprises of two main domains namely DNA binding domain (DBD) and dimerization domain (DD). There are a total of nine \(\alpha\) helices where the first three helices form a putative DNA binding domain. The HTH motif comprises of helices \(a_2\) and \(a_3\). The remaining six \(\alpha\)-helices are distributed in the C-terminal dimerization domain [4]. In a continuing effort, Zhu and coworkers have elegantly characterized additional novel direct targets of HapR and illustrated two distinct binding motifs (motif 1 and motif 2) in various target promoters. Motif 1 sequence contains dyad symmetry and a consensus AATAR’ (where R represents A or G) whereas motif 2 sequence lacks any dyad symmetry and contains a highly conserved consensus ‘TGT’ [5]. Though many crucial facts about the structural and functional aspects of wild type HapR are available, additional insights into...
the contributory role of glycine 39 in DNA binding activity of various strains of natural variants of quorum sensing regulatory proteins from HapR [6] which was not evaluated in the previous studies [4].

Identification of several quorum sensing defective strains of nature. In a recent effort, Zhu and colleagues have reported the non-functional HapR variants are more frequently obtained in harboring either a single or multiple mutations in HapR [6]. Further, structural analysis reveals the mutation driven residue at position 39 (G39D; named as HapRV2) clearly elucidates functional HapR variant harboring aspartate in place of glycine studying natural variants of the same. For instance, a natural non

Structural Deformity in HapR Natural Variant

Table 1. Strains and plasmids used in this study.

| Strains/plasmids          | Description                                                                 | Source/reference          |
|---------------------------|-----------------------------------------------------------------------------|----------------------------|
| **V. cholerae strains**   |                                                                             |                            |
| V2                        | Non-O1, non-O139, Serogroup O37                                             | Ranjan K Nandy, National Institute of Cholera and Enteric Diseases (NICE), India |
| V2s-C                     | Non-O1, non-O139, Serogroup O37, hapR:pCD, Cm' (17 μg ml⁻¹), Ap'(100 μg ml⁻¹) | Dongre et al. 2011         |
| V2e-RV2G                  | Non-O1, non-O139, Serogroup O37, hapR:pCD having pSV2G, Cm' (17 μg ml⁻¹), Ap'(100 μg ml⁻¹) | Dongre et al. 2011         |
| V2e -RV2G-E117K           | Non-O1, non-O139, Serogroup O37, hapR:pCD having pSV2G-E117K, Cm' (17 μg ml⁻¹), Ap'(100 μg ml⁻¹) | This study                 |
| V2e -RV2G-FLAG            | Non-O1, non-O139, Serogroup O37, hapR:pCD having pSV2G-FLAG, Cm' (17 μg ml⁻¹), Ap'(100 μg ml⁻¹) | This study                 |
| V2e -RV2G-E117K-FLAG      | Non-O1, non-O139, Serogroup O37, hapR:pCD having pSV2G-E117K-FLAG, Cm' (17 μg ml⁻¹), Ap'(100 μg ml⁻¹) | This study                 |
| **E.coli strains**        |                                                                             |                            |
| Nova blue                 | *E. coli* K-12, recA endA, lacI, lacY                                      | Novagen                    |
| BL21(DE3)                 | *E. coli* B, F ompT lon, with a λ prophage carrying the T7 RNA polymerase  | Novagen                    |
| **Plasmids**              |                                                                             |                            |
| pKK177-3R1                | Ap'                                                                         | Giesla Stroz, National Institute of Health, U.S.A. |
| pET15b                    | Ap', N-terminal 6His-tag expression vector                                   | Novagen                    |
| HapR-V2PET15b             | 612 bp fragments of hapR (ORF) containing D39 (V2) was cloned into NdeI-BamHI site of pET15b | Dongre et al. 2011         |
| HapR-V2GPE117K PET15b     | 612 bp fragments of hapR (ORF) containing G39 (V2G) was cloned into NdeI-BamHI site of pET15b | Dongre et al. 2011         |
| HapR-V2GPE117K-E117K PET15b | 612 bp fragments of hapR (ORF) containing K117(V2G-E117K) was cloned into NdeI-BamHI site of pET15b | This study                 |
| pSV2G                     | 612 bp fragment of functional hapR (ORF) cloned into SmaI-HindIII site of pKK177-3R1 | This study                 |
| pSV2G-E117K               | pSV2G with lysine at position 117                                           | This study                 |
| pSV2G-FLAG                | hapR tagged with FLAG. FLAG and hapRl fragments were amplified from the p3XFLAG-CMV-10 Expression Vector (Sigma Aldrich) and pSV2G with primer pairs O L FLAG F/ HindIII FLAG R and SmaI HapR F/O L FLAG R, respectively. The products were purified and overlapping PCR was performed with primer pair SmaI HapR F/HindIII FLAG R. The final product was cloned into SmaI/HindIII sites of pKK177-3R1. | This study                 |
| pSV2G-E117K-FLAG          | hapRl (E117K) tagged with FLAG. FLAG and hapRl fragments were amplified from the p3XFLAG-CMV-10 Expression Vector and pSV2G-E117K with primer pairs O L FLAG F/ HindIII FLAG R and SmaI HapR F/O L FLAG R, respectively. The products were purified and overlapping PCR was performed with primer pair SmaI HapR F/HindIII FLAG R. The final product was cloned into SmaI/HindIII sites of pKK177-3R1. | This study                 |

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the functionality of this molecule can further be explored by studying natural variants of the same. For instance, a natural non-functional HapR variant harboring aspartate in place of glycine residue at position 39 (G39D; named as HapRV2) clearly elucidates the contributory role of glycine39 in DNA binding activity of HapR [6]. Further, structural analysis reveals the mutation driven shape change in the DNA binding domains, thereby explaining the loss in DNA binding ability of this HapR variant. In short, molecular analysis of HapRV2-G39D natural variant illustrates the importance of a conserved glycine residue in the performance of HapR [6] which was not evaluated in the previous studies [4].

A flurry of research articles evidences the preponderance of natural variants of quorum sensing regulatory proteins from various strains of *Vibrio cholerae* [6–10]. Of these, strains having non-functional HapR variants are more frequently obtained in nature. In a recent effort, Zhu and colleagues have reported the identification of several quorum sensing defective strains of *Vibrio cholerae* harboring either a single or multiple mutations in HapR protein [10]. Though, these natural variants of HapR have been categorized as non-functional based on certain phenotypic traits, further molecular analysis is required to understand the cause of their functional inertness. In this work, we have chosen to investigate the molecular basis of the functional impairment of a HapR natural variant harboring substitution of a conserved glutamate residue (E117) at position 117 with lysine in the dimerization domain. In literature, similar substitution where glutamate is replaced by lysine could result in mutant proteins either with a compromise in DNA binding ability or dimer stability. For example, glutamate to lysine substitution in many cases such as E62K and E 95K severely affect the DNA binding stability. For example, glutamate to lysine substitution in many cases such as E62K and E95K severely affect the DNA binding stability. For example, glutamate to lysine substitution in many cases such as E62K and E95K severely affect the DNA binding stability.
Table 2. Primers used in this study.

| Primer name                     | Primer sequence (5’-3’)                      |
|--------------------------------|-----------------------------------------------|
| HapR HindIII (cloning and sequencing) | CCCAAGCTCTAGTGTCTGATCCAGTAC                      |
| HapR E117K-F (mutagenesis primer) | GGCCTCAAGTCTGGTTAATGTCGGTCTCAACCC               |
| Ndel HapR (cloning and sequencing) | GGGAACTTTAGTATGGACCGCATCATCAGGAAAAC             |
| BamHI HapR (cloning and sequencing) | CCGCGATCCTGTTTCTTAGATACACAG                      |
| pcv0900 F (promoter vc0900)      | TGTTTTAGCGAGACTCGAGCAG                         |
| pcv0900 R (promoter vc0900)      | GAGTTAGGGCAGCAAGTACATG                         |
| YF13 (promoter aphA)             | GATCGGGATTCGAATGCGCAATATCGTGTATAC              |
| YF12 (promoter aphA)             | GATCGGGATTCGAATGCGCATGACTTCG                  |
| phapA F (promoter hapA)          | GGAATTCGACTCATGGGGACCCTG                      |
| phapA R (promoter hapA)          | GGAATTCGACTCATGGGGGACCCTG                      |
| O L FLAG F                       | GTGATTCTGATAGGACGATCACAGAGGACGTAGC             |
| O L FLAG R                       | CATGGCTTCTGGTGCTCGTCTTAGATACAC                |
| HindIII FLAG R                   | CCCAAGCTTTTACGTGCATCGATC                       |

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Effect of replacement of a conserved salt bridge glutamate residue by lysine (E117K) on the stability as well as DNA binding activity of HapRV2G-E117K variant. Herein, our data reveals the intactness of dimeric status in HapRV2G-E117K variant with a compromise in DNA binding activity. To correlate the loss of function to its global shape, SAXS experiments were carried out. Data analysis and structure reconstruction revealed that though HapRV2G-E117K variant remains dimeric, its DNA binding domains are significantly open compared to the wild type functional protein. Overall, our work offers clear evidence to support a proposal that the widening of space between the DNA binding regions impairs the ability of HapRV2G-E117K to effectively bind to its cognate promoter regions.

Materials and Methods

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. Vibrio cholerae strains were derived from a non-O1, non-O139 strain V2, serogroup O37. Strains were maintained at −70°C in Luria-Bertani (LB) medium containing 20% glycerol. Escherichia coli BL21 (DE3) (Novagen) was used for the overexpression of proteins. All strains were propagated at 37°C in liquid with agitation or on solid (1.5% agar) in Luria Broth unless otherwise mentioned. For protease assay, V. cholerae strains were grown with aeration at 37°C in tryptic soya broth without dextrose (TSB-D). When appropriate, the growth medium was supplemented with ampicillin (100 µg ml⁻¹) and chloramphenicol (17 µg ml⁻¹). All antibiotics were purchased from Sigma-Aldrich and media ingredients from Himedia and Difco.

Protease assay

Protease activity was measured using an azocasein assay as described earlier [7]. Brieﬂy, the recombinant derivatives of V. cholerae strain V2s (Table 1) were grown in tryptic soya broth without dextrose (TSB-D), containing chloramphenicol (17 µg ml⁻¹) and ampicillin (100 µg ml⁻¹) accordingly, with agitation to stationary phase at 37°C. 100 µl of stationary phase culture supernatant was incubated with 100 µl azocasein (5 mg ml⁻¹ in 100 mM Tris, pH 8.0) for 1 h at 37°C. The reaction was stopped by the addition of 400 µl of 10% trichloroacetic acid. After centrifugation, supernatant was transferred to 700 µl of 525 mM NaOH, and the optical density was determined at 442 nm. One azocasein unit was defined as the amount of enzyme producing an increase of 0.01 OD units per hr.

Site-specific mutagenesis

Glutamate 117 to lysine (E117K) mutation in the functional hapR ORF was generated on plasmid pSV2G (Table 1) using quick change mutagenesis kit from Stratagene as per manufacturer’s guidelines. The primers are listed in Table 2. Positive clones were analyzed by sequencing in their entirety to confirm the desired mutation. One of such mutant clone designated as pSV2G-E117K was further transformed into V2s, and the recombinant strain was designated as V2s-R V2G-E117K.

Protein purification and Electrophoretic gel mobility shift assay with promoter regions of aphA, hapA and vc0900

HapRV2G and HapRV2G-E117K proteins were purified by Ni²⁺-Nitrilotriacetic acid chromatography as described previously [6]. Both wild type and variant HapR were cloned into NdeI-BamHI site of the pET15b vector (Novagen) to generate N-terminal His-HapR fusion proteins. All the clones were confirmed by sequencing and transformed into E. coli BL21 (DE3). After induction with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), HapR proteins were purified through QiaGen Ni²⁺-nitrilotriacetic acid columns and subjected to overnight dialysis in a solution of buffer A containing 10 mM Tris-HCl, pH 7.9, 300 mM KCl, 0.1 mM EDTA. Gel mobility shift assay was done essentially as described earlier [6]. Briefly, three fragments of 399, 665 and 467 bp corresponding to promoter regions of aphA, hapA and vc0900 respectively, were amplified with primer pairs as listed in Table 2. The fragments were gel-purified and end labeled with [γ-32P] dATP using T4 polynucleotide kinase (New England Biolabs). The binding reaction was carried out with 4 µg of labeled fragment in 10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 1 mM DTT, 60 mM KCl, 10% glycerol, 5 µg BSA and 1 µg poly (dl-
dC) in a 20 µl reaction volume for 20 min at 26°C. The reaction mixture was applied to a 5.5% polyacrylamide gel and subjected to electrophoresis in 1X Tris-Acetate-EDTA, pH 8.5, at 4°C. The gel was dried and autoradiographed to examine the shift of the band.

Circular Dichroism (CD) measurement
Both HapR_{V2G} and HapR_{V2G-E117K} were examined by CD using a Jasco J-810 spectropolarimeter. Measurements in the far ultraviolet region (250–200 nm) were performed on protein solutions (0.2 mg/ml) employing a cell with path length of

Figure 1. Analytical molecular sieve chromatography to determine the molecular weight of the functional (HapR_{V2G}) and non-functional proteins (HapR_{V2} and HapR_{V2G-E117K}). (A) A calibration curve of column was obtained by plotting the Kav of the protein standards against the Mr of the standards. Six GE-Healthcare gel filtration molecular mass markers were used: Blue dextran (200 kDa), Conalbumin (75 kDa), Ovalbumin (44 kDa), Carbonic anhydrase (29 kDa), Ribonulcease A (13.7 kDa) and Aprotinin (6.5 kDa). Each marker along with its Mr is represented by a unique colour. Kav values for all the proteins obtained from the Eq. 1 were fitted to calibration curve to estimate the molecular weight of proteins (~52 kDa). (B) The elution profiles of the functional (HapR_{V2G}) and non-functional proteins (HapR_{V2} and HapR_{V2G-E117K}). Column was equilibrated in 10 mM Tris, pH7.9, 100 mM KCl, and 0.1 mM EDTA. Elution profile of Carbonic anhydrase (29 kDa) is shown in dotted line for comparison.

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0.1 cm at 25°C. The mean residue ellipticity, [θ], was calculated using a mean residue molecular mass for both the proteins. Each spectrum reported is an average of 10 scans.

**Molecular weight determination**

HapR\textsubscript{V2G}, HapR\textsubscript{V2} and HapR\textsubscript{V2G-E117K} were dialyzed overnight in buffer A. All proteins were subjected to molecular sieve chromatography using a Superdex 200 5/150 GL analytical column coupled to an “AKTA-Purifier” chromatography system (GE-Healthcare). The molecular weight (\(M_r\)) of all the proteins were evaluated according to a calibration curve generated with the gel filtration of molecular mass markers (GE-Healthcare) including blue dextran (200 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) (Figure 1A). These standards were loaded independently at the concentrations recommended by GE-Healthcare. The elution volume of blue dextran was used to determine the void volume (\(V_0 = 1.215\) ml) and the total volume (\(V_t = 3\) ml) was provided by the product instruction manual. The peak elution volumes (\(V_e\)) were calculated from the chromatogram. \(K_a\) values were calculated using the following equation:

\[
K_a = \frac{V_e}{V_t - V_0}
\]
The calibration curve was determined by plotting the $K_{av}$ of the protein standards against the $M_r$ of the standards. Elution volumes were determined by monitoring the absorbance at 280 nm. All proteins were eluted at the same volume. $K_{av}$ values for all the proteins were calculated based on elution volumes and fitted to a calibration curve to estimate the size of the proteins.

$K_{av} = (Ve - Vo)/(Vt - Vo)$  \hspace{1cm} (1)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

Western blot analysis was done to check the expression of HapR. Briefly, FLAG- tagged recombinant derivatives of *V. cholerae* strain V2s were grown overnight in TSB-D medium with agitation. Protein samples were prepared from equal amount of bacterial cells and separated on 13.5% gel by SDS-PAGE. For immunoblotting, the proteins were transferred to Immobilon-P membrane (Millipore) at 75 mA for 1 h. Membrane was subsequently blocked in PBS-T with 5% skim milk at 37°C for 2 h and then shifted to 4°C for overnight with shaking. Blot was then washed in PBS-T five times for 10 min each, incubated in monoclonal HRP-conjugated Anti-flag (Sigma Aldrich) at a dilution of 1:5000 in PBS-T with 2% skim milk for 1 h and again washed in PBS-T five times for 10 min each. Proteins were
Small Angle X-ray Scattering Data Acquisition, Analysis and Structure Reconstruction

All SAXS intensity profiles were acquired at the X9 beam line (National Synchrotron Light Source, Brookhaven National Laboratory). Scattered X-rays were recorded as images on Pilatus detectors, corrected for slit smearing, circularly averaged about the beam centre, and the contribution from buffer components were subtracted using Python script based programs written by Dr. Lin Yang (X9 Beam line, NSLS). For each experiment, 120 μl of protein solutions (HapR<sub>VG</sub> and HapR<sub>VG-E117K</sub>) and their matched buffers from FPLC were exposed to X-rays in a quartz capillary flow cell. Using programmable liquid handling robotics at X9 beam line, the collection of entire SAXS data described in this study were carried out in triplicate and averaged during processing. To calibrate the beam intensity at zero angles and estimate the actual concentration of protein samples, the SAXS datasets were collected under identical conditions on a series of hen egg white lysozyme (in NaOAc buffer containing 150 mM NaCl, pH 3.8) and recombinant gelsolin (in Tris-EGTA, buffer pH 0) with their predetermined concentrations. Image processing provided the scattering intensity [I] as a function of momentum transfer vector, Q (Q = 4πsinθ/λ), where λ and θ are the wavelength of X-ray and the scattering angle, respectively. Kratky plots (I(Q) Q² vs. Q) were generated to interpret globular scattering nature of the wild type and variant protein in solution. The Guinier approximations of the low Q region were carried out using PRIMUS software [14] presuming globular and rod-like scattering shape which provided the radius of gyration (R<sub>G</sub>) and radius of cross-section (R<sub>C</sub>) of the predominant shape of protein molecules. Using the relationship between R<sub>G</sub> and R<sub>C</sub> [Eq. 2], we estimated the linear dimension of the molecules with following equation:

\[
L = \left(12\left(\frac{R_G^2}{R_C^2}\right) - 1\right)^{1/2}
\]  

Further, indirect Fourier transformation of the SAXS data (Q range: 0.008–0.25 Å⁻¹) was performed using GNOM45 program [15] to obtain probability of finding various pairwise vectors arising from the scattering shape of the protein molecules. During P(r) curve estimation, the probability of finding a pairwise vector of length equal to 0 Å and equal to the maximum linear dimension (D<sub>max</sub>) of the molecule was considered to be zero. This analysis also provided an estimate of the R<sub>G</sub> and scattering intensity at zero scattering angles, I<sub>0</sub>. To visualize the three dimensional shapes of wild type and the variant HapR protein, ten independent models of each were generated using the DAMMINIQ program and the respective SAXS I(Q) profile as reference [16]. No symmetry or shape bias was employed during each shape reconstruction. For each protein, the independent structural models were averaged using DAMAVER suite of programs [17] which resulted in a structural model capable of best-representing the solution shape of the proteins in solution. The SAXS data based dummy model and the available crystal structure of HapR (PDB ID: 2PBX) were overlaid by aligning inertial axes of the models using SUPCOMB20 program [18]. The open source VMD version 1.9.1 and Chimera 1.6.1 were used for graphical analysis and figure generation.

Results and Discussion

Molecular weight and secondary structure determination of non-functional HapR<sub>VG-E117K</sub>

Like other members of TetR family proteins, HapR also acts as a dimer [4]. Analysis of the dimerization domain and interface reveals the significance of critical residues in the formation and stabilization of dimer. One such residue glutamate at position 117 (E<sup>117</sup>) contributes to the stability of the HapR dimer by forming a salt bridge with arginine residue at position 123 (R<sup>123</sup>) of the other monomer [4]. There is a preponderance of data elucidating the crucial role of salt bridge in stabilizing protein structure. For example, substitution of a glutamate residue at position 99 with
lysine (E99K) in case of *Vibrio harveyi* flavin reductase FRP completely abolishes the dimer formation. It should be noted that E99 makes salt bridge with R133 and R225 of the other monomer in FRP [13]. In our study, we were driven by a desire to understand the molecular basis of the non-functionality of previously identified HapR variant harboring substitution of a conserved glutamate residue at position 117 (E117) with lysine. As described in the preceding section, E117 of a functional wild type HapR participates in the formation of salt bridge, it is therefore conceivable that substitution of this residue by lysine in non-functional HapR

![Figure 7](https://doi.org/10.1371/journal.pone.0076033.g007)

**Figure 7.** SAXS data analysis from the samples of HapR_{V2G} and HapR_{V2G-E117K}. (A) SAXS I(Q) profiles are plotted versus Q for both the samples (HapR_{V2G}, magenta; HapR_{V2G-E117K}, gray). Inset shows the linear region of the Guinier analysis done presuming globular nature of the protein molecules in solution. (B) Kratky analysis from individual SAXS data sets confirmed the globular nature of proteins in solution. (C) Real space information of the predominant scattering species computed by indirect Fourier transformation are plotted between P(r) and R.

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| Proteins          | Mass(KDa) | Guinier Analysis | Indirect Fourier Transformation | Actual Conc. (mg/ml) |
|-------------------|-----------|------------------|---------------------------------|----------------------|
|                   |           | R_G(Å) | R_C(Å) | L(Å) | D_{max}(Å) | R_G(Å) | I_0  |
| **Samples with predetermined concentrations** |           |        |        |      |           |        |      |
| Lysozyme          | 14.2      | 14.1   | 7.7    | 41   | 44        | 14.2   | 20   |
| EGTA Gelsolin     | 82        | 30.4   | 16.4   | 88   | 100       | 30.1   | 115  |
| **HapR Proteins (Dimer)** |           |        |        |      |           |        |      |
| HapR_{V2G}        | 46        | 23.4±0.3 | 10.1   | 73   | 78        | 23.9±0.2 | 175  | 2.7  |
| HapR_{V2G-E117K}  | 46        | 27.1±0.1 | 8.3    | 89   | 93        | 27.5±0.1 | 162  | 2.5  |

*The I_0 values were estimated from predetermined dilution series of lysozyme (four samples in the range of 0.7–3.2 mg/ml) and recombinant plasma gelsolin (four samples in the range of 0.5–2.1 mg/ml).

*It is important to mention here that an error remained in our previous publication (Dongre et al., 2011) during description of results from Guinier analysis: on page 15049: left column line 1 and 2 “HapR_{V2G} and HapR_{V2G-E117K} provided an R_G of” should be read as “HapR_{V2G} and HapR_{V2G-E117K} provided an R_G of”.

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variant (HapRv2G-E117K) may alter dimer stability, thereby affecting its functionality. To ascertain, we estimated the molecular weight of proteins by size-exclusion chromatography. The $K_{av}$ values of all the proteins were obtained using Eq. 1 and put in equation $x = y - (0.746)/-4E-06$ to calculate their respective molecular weights (Figure 1A). In addition to the wild type functional protein as control, we also kept a previously identified non-functional HapR variant (HapRv2) that is defective in DNA binding but forms a stable dimer [6] as a second control in gel filtration chromatography experiment (Figure 1B). Intriguingly, no significant difference was observed in the gel filtration elution profiles of the purified His-tagged HapR functional (HapRv2G) protein, DNA binding defective variant (HapRv2) and dimerization domain variant (HapRv2G-E117K). We also carried out CD data analysis to examine any alteration in the secondary structure (Figure 2). K2D2 analysis of the CD data (in the range of 250–
200 nm) suggested that only a minor loss of α helical content (<4%) occurred in case of HapR V2G-E117K. Taken together, our results indicate that the hydrodynamic property of HapR V2G-E117K variant is similar to that seen for the dimeric wild type HapR V2G suggesting the intactness of dimeric status of HapR V2G-E117K variant.

Evaluate DNA binding ability of variant HapR V2G-E117K

HapR justifies its role as a master regulator by interacting with a range of cognate promoter sequences. To assess the DNA binding ability of HapR V2G-E117K, a gel shift assay was employed with hapA, aphA and vc0900 promoter regions. It should be noted that the promoter region of vc0900 contains motif 1 binding site while the promoter regions of hapA and aphA contain motif 2 binding site. Unlike its wild type functional counterpart HapR V2G, HapR V2G-E117K fail to bind to any of these promoter regions, thereby indicating a compromise in DNA binding ability (Figure 3).

Combining gel filtration and gel retardation results, we conclude that substitution of a salt bridge glutamate residue with lysine in the dimerization domain abrogates DNA binding activity without affecting dimeric status of HapR variant.

Functional examination and stability of variant HapR V2G-E117K under in vivo condition

From in vitro gel shift assay, inability of the HapR variant (HapR V2G-E117K) to shift promoter region of gene encoding HA/protease (hapA) is evident. It is therefore expected that this variant will remain non functional to stimulate protease production in a...
protease negative strain of Vibrio cholerae. To ascertain its in-vivo functionality, recombinant constructs of hapR<sub>V2G</sub> (pSV2G, wild type functional HapR control) and hapR<sup>E<sup>117K</sup></sup> (pSV2G-E<sup>117K</sup>) were transformed into a protease negative Vibrio cholerae strain V<sub>2</sub><sup>S</sup> (hapR disrupted strain of V<sub>2</sub>, Table 1) to generate strains V<sub>2</sub><sup>S</sup>-R<sub>V2G</sub> and V<sub>2</sub><sup>S</sup>-R<sub>V2G-E<sup>117K</sup></sub>. In contrast to V<sub>2</sub><sup>S</sup>-R<sub>V2G</sub> and V<sub>2</sub><sup>S</sup>-R<sub>V2G-E<sup>117K</sup></sub> the HapR<sup>E<sup>117K</sup></sup> remains protease negative (Figure 4A). The protease data recapitulates in vitro gel shift data with promoter hapA where protease production is directly proportional to the functional level of HapR. In addition to the protease production, we have also checked the rugosity pattern of V<sub>2</sub><sup>S</sup>-strains harboring HapR proteins. It has been observed that strain having non-functional HapR develops rugosity (Figure 5). This data is in agreement with that of Wang et al, who demonstrated rugosity in the strain harboring such mutant HapR protein[10].

It has been documented that substitution of an amino acid often results in an increased susceptibility of mutants to proteases, if critical for global stability of the folded protein [19]. It is thus possible that substitution of glutamate by lysine may affect the in vivo stability of HapR<sub>V2G-E<sup>117K</sup></sub> variant. To investigate the stability, FLAG epitope was inserted in the C-terminal domain of wild type HapR<sub>V2G</sub> and variant protein (Table 1). The recombinant constructs were transformed into a protease negative Vibrio cholerae strains V<sub>2</sub><sup>S</sup> (hapR disrupted strain of V<sub>2</sub>, Table 1) to generate strains V<sub>2</sub><sup>S</sup>-R<sub>V2G-FLAG</sub> and V<sub>2</sub><sup>S</sup>-R<sub>V2G-E<sup>117K</sup>-FLAG</sub>. The recombinant strains were then subjected to western blot analysis to examine the stability of each protein. Our western blot analysis clearly indicated the intactness of lysine (K<sup>117</sup>) variant along with wild type HapR protein (Figure 6A). The protease assay with FLAG containing recombinant constructs further confirmed no functional alteration upon FLAG insertion (Figure 4B). Until now results support that substitution of a salt bridge contact residue glutamate by lysine abrogates DNA binding stability of HapR<sub>V2G-E<sup>117K</sup></sub> variant.

**Structural deformity of HapR<sub>V2G-E<sup>117K</sup></sub> variant as evidenced by SAXS data analysis and structure restoration**

In the preceding section, our characterization of hydrodynamic, secondary structural and stability analysis of the proteins ruled out a grossly misfolded shape of HapR dimerization domain HapR<sub>V2G-E<sup>117K</sup></sub> variant, but lacked in explaining the loss of DNA binding function. In this scenario, high resolution structural analysis may shed further light on the global architecture, thereby enabling us to understand the loss of DNA binding activity of these dimerization domain variant proteins. To further delve into the structural details, SAXS analysis was performed as described previously [6]. SAXS scattering data lacking an upturned profile in low Q region assured lack of aggregation in samples during data collection (Figure 7A). Considering globular scattering nature of the protein, Guinier analysis indicated that both wild type and variant HapR adopted a monodisperse profile in solution. Steeper slope of the linear fit for the HapR<sub>V2G</sub>-E<sup>117K</sup> variant protein (compared to wild type) indicated a bigger R<sub>G</sub> variant (Figure 7A inset). In fact, the R<sub>G</sub> values were 23.4 Å and 27.1 Å for the HapR<sub>V2G</sub> (functional) and HapR<sub>V2G-E<sup>117K</sup></sub> protein, respectively (Table 3). Presuming rod-like scattering shape for the protein molecules provided the R<sub>G</sub> values for the two proteins to be 10.1 Å and 8.3 Å. Using the Eq. 2, the L values for wild type and HapR variant were estimated to be 73 Å and 89 Å, respectively. Interestingly, peak-like Kratyk plot of the wild type protein confirmed a globular scattering nature of HapR<sub>V2G</sub> molecules in solution, a wider peak with LQ<sup>2</sup>*Q not dampening to zero as a function of increasing Q for the mutant suggested relatively higher inherent disorder in the HapR<sub>V2G-E<sup>117K</sup></sub> protein molecules (Figure 7B). Increased dimensions of the variant protein were also seen from the indirect Fourier transformation of the SAXS datasets (Figure 7C). The wild type functional protein HapR<sub>V2G</sub> showed P(r) curve with D<sub>max</sub> and R<sub>G</sub> about 78 Å and 23.9 Å, respectively [6]. At the same time, the P(r) analysis for HapR<sub>V2G-E<sup>117K</sup></sub> protein provided D<sub>max</sub> and R<sub>G</sub> close to 93 Å and 27.5 Å, respectively. The computed P(r) profile for the HapR<sub>V2G-E<sup>117K</sup></sub> variant suggested an extended curve seen for shapes with tail-like features.

To gain insight into the shape of the two proteins, ab initio modeling approaches were followed using dummy residues. The model solved for the predominant solution shape of HapR<sub>V2G</sub> agreed well with the crystal structure of the HapR protein (PDB ID: 2PBX), as published earlier (Figure 8A). In contrast, the model solved for HapR<sub>V2G-E<sup>117K</sup></sub> variant protein revealed a much more open structure, particularly the DNA binding domains (comparing with the HapR<sub>V2G</sub>). Overlay of the two models confirmed that while the shape profile of dimerization domain of the variant remained similar to wild type protein, the DNA binding domains in mutant somewhat “fell-apart”. Moreover, this deviation in the global shape of the mutant occurred only in dimension (please see the side-views in Figure 8B). To realize how this single point mutation is causing such a deleterious effect, we analyzed the polar interactions surrounding E<sup>117</sup> in the crystal structure of the HapR<sub>V2G</sub> (PDB ID: 2PBX) (Figure 8C). It brought forth that alongside interacting with R<sup>223</sup>, HapR<sub>V2G-E<sup>117K</sup></sub> indirectly positions other residues in vicinity viz. K<sup>117</sup> and some water molecules. A mutation to lysine, not only reverses the local electrostatic surface potential which would alter the intricate network of interactions resolved for the native protein, but also the extended length of lysine side-chain compared to that of glutamate would find it difficult to accommodate in the groove formed between the upper portion of the dimeric interface. Replacement of two glutamates in the dimer (in the zone highlighted in Figure 8C) by two lysines would cause a substantial remodeling of the local topography. Very likely, the adjustments are done by parting away of the upper portion (since lower part of molecule is engaged in dimerization). A culmination of local changes eventually results in eventual separation of the upper domains, a geometry which has evolved for binding different double stranded DNA (Figure 8D).

**Conclusions**

There is a growing body of evidence that elucidates how substitution of a single amino acid could affect the functionality of a DNA binding protein by altering its DNA binding activity, dimeric status and overall stability by increasing susceptibility to proteases [6,11,13,19–22]. Keeping this view in mind, we wanted to examine the underlying cause of non-functionality of a natural HapR variant designated as HapR<sub>V2G-E<sup>117K</sup></sub> where a salt bridge glutamate residue is replaced by lysine moiety. It has been documented that similar substitution where glutamate is replaced by lysine could result in mutant proteins either with a compromise in the DNA binding ability or dimer stability [11–13]. A combined in vivo and in vitro analysis helped us to establish that HapR<sub>V2G-E<sup>117K</sup></sub> variant protein forms a stable dimer with a compromise in its DNA-binding activity. As evidences suggest salt bridges not only maintain the proper orientation as well as stability of dimer interface, but also controls the wedge angle between DNA-binding domain and the dimerization domain, thereby mediating interaction between the protein molecules and their cognate DNA.
sequences [23,24,25]. In our case, replacement of a salt bridge contact residue glutamate by lysine neither affects the dimer stability nor overall protein stability, but it introduces a deformity in the DNA binding domain as revealed by SAXS analysis, thereby offering a plausible explanation of lack of DNA binding activity and overall cellular performance of HapR<sub>234G</sub> variant. Based on our previous work and present observation, it is seemingly apparent that HapR adopts a particular “Y” shape that facilitates its interaction with cognate promoters ([6]; Figure 8). Any deviations from its typical “Y” shape either too close [6] or too wide [Figure 8] substantially compromises its functionality. Interestingly, glutamate (E<sub>117</sub>) residue is highly conserved in the HapR homologues of other Vibrio species (Figure 9). It would be interesting to examine the effect of such mutation on the functionality of HapR homologues of different Vibrio species. Studies being pursued in our laboratory will possibly shed more insight into the encoded balance between the sequence content, global fold and functionality of HapR homologues, and our current work will be interesting to community involved in shape-function studies of proteins, particularly Vibrio species.

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Author Contributions

Conceived and designed the experiments: SRC. Performed the experiments: RS NSS YSR. Analyzed the data: SRC RS YSR NSS NP A. Contributed reagents/materials/analysis tools: RS YSR NSS NP. Wrote the paper: SRC A.

Structural Deformity in HapR Natural Variant