Ethanolamine regulates CqsR quorum-sensing signaling in *Vibrio cholerae*

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ABSTRACT

The pathogen that causes cholera, *Vibrio cholerae*, uses the cell-cell communication process known as quorum sensing (QS) to regulate virulence factor production and biofilm formation in response to changes in population density and complexity. QS is mediated through the detection of extracellular chemical signals called autoinducers. Four histidine kinases, LuxPQ, CqsS, CqsR and VpsS, have been identified as receptors to activate the key QS regulator LuxO at low cell density. At high cell density, detection of autoinducers by these receptors leads to deactivation of LuxO, resulting in population-wide gene expression changes. While the cognate autoinducers that regulate the activity of CqsS and LuxQ are known, the signals that regulate CqsR have not been determined. Here we show that the common metabolite ethanolamine specifically interacts with the ligand-binding CACHE domain of CqsR *in vitro* and induces the high cell-density QS response through CqsR kinase inhibition in *V. cholerae* cells. We also identified residues in the CqsR CACHE domain important for ethanolamine detection and signal transduction. Moreover, mutations disrupting endogenous ethanolamine production in *V. cholerae* delay the onset of, but do not abolish, the high cell-density QS gene expression. Finally, we demonstrate that modulation of CqsR QS response by ethanolamine occurs inside animal hosts. Our findings suggest that *V. cholerae* uses CqsR as a dual-function receptor to integrate information from the self-made signals as well as exogenous ethanolamine as an environmental cue to modulate QS response.

35 **IMPORTANCE**

36 Many bacteria use quorum sensing to regulate cellular processes that are important for their
 37 survival and adaptation to different environments. Quorum sensing usually depends on the
 38 detection on chemical signals called autoinducers made endogenously by the bacteria. We show
 39 here ethanolamine, a common metabolite made by various bacteria and eukaryotes, can modulate
 40 the activity of one of the quorum-sensing receptors in *Vibrio cholerae*, the etiological agent of the
 41 disease cholera. Our results raise the possibility that *V. cholerae* or other quorum-sensing bacteria
 42 can combine environmental sensing and quorum sensing to control group behaviors.

43

INTRODUCTION

Quorum sensing (QS) is used by a wide variety of bacteria to coordinate population-wide changes in behaviors in response to cell density (1). *Vibrio cholerae*, which causes the diarrheal disease cholera in the human host, uses QS to regulate virulence factor production, biofilm formation, Type VI secretion, metabolic regulation, and natural competence to maintain competitive fitness in various environments (2-10). Four parallel QS signaling systems pathways have been identified in *V. cholerae* that rely on a phosphorelay to regulate downstream gene expression (11) (Figure 1). At low cell-density (LCD), four histidine kinases CqsS, LuxPQ, CqsR, and VpsS function in parallel to phosphorylate LuxO through an intermediate phosphotransfer protein LuxU (10, 11). Phosphorylated LuxO promotes the transcription of small RNAs Qrr1-4 which promote the translation of the transcriptional regulator AphA (12, 13). Conversely, Qrr1-4 repress the translation of transcriptional regulator HapR (14, 15). Virulence and biofilm genes are expressed at LCD (4-6, 9, 11). At high cell-density (HCD), each receptor kinase detects its unique chemical messenger called autoinducer (AI) that inhibits the kinase activity upon signal binding (1). Autoinducer synthase CqsA catalyzes the production of CAI-1 (*S*-3-hydroxytridecan-4-one) which is detected by receptor CqsS (16-20) and autoinducer synthase LuxS produces AI-2 (*S*-TMHF-borate) which is detected by LuxP/Q (21-25). Thus, at HCD, when these receptors are bound to their cognate signals, kinase activity is inhibited, leading to dephosphorylation of LuxO. This prevents the transcription of Qrr 1-4 thereby inhibiting AphA production and promoting HapR translation. Type VI secretion and competence genes are expressed at HCD (3, 7). Another QS circuit was recently identified in *V. cholerae*, consisting of a cytoplasmic receptor VqmA which recognizes DPO (3,5-dimethylpyrazin-2-ol) but this system does not participate in regulating LuxO (26).

Although the kinase activity of both CqsR and VpsS is cell-density dependent, the signals that control the activity of CqsR and VpsS are unclear (11). VpsS has no predicted transmembrane domains and is thought to be cytoplasmic. Nitric oxide (NO) has been shown to regulate the kinase activity of VpsS *in vitro* through a signaling partner VpsV (27). However, *V. cholerae* does not make NO and $\Delta vpsV$ mutants has no detectable change in QS response (10, 11), so the exact role of NO sensing by VpsV in *V. cholerae* QS response remains unknown.

In contrast, CqsR is predicted to have a periplasmic CACHE domain that is known to be involved in signal sensing in many chemotaxis receptors (28). Here, by isolating constitutively active CqsR mutants, we identified several key residues within the CqsR CACHE domain required for signal binding or signal transduction. Through a chemical library screen and structure-activity relationship *in vitro* binding assay, we discovered that ethanolamine and its analogs, alaninol and serinol, bind to the CqsR CACHE domain and modulate QS response of *V. cholerae* through CqsR. We also determined that *V. cholerae* strains defective in ethanolamine biosynthesis delayed the onset of, but did not abolish, the HCD QS response. Using an infant mouse colonization model, we show that ethanolamine modulates *V. cholerae* QS response through CqsR inside animal hosts. We therefore predict that ethanolamine is used as an external cue for niche sensing and additional signals are produced by *V. cholerae* to modulate QS through CqsR.

MATERIALS AND METHODS

Bioinformatics

Conserved domain structures of *V. cholerae* CqsR (accession no.: NP_231465.1) were analyzed using a suite of online tools including CDD (29), CDART (30), InterPro (31), and TMHMM (32).

3D structural modelling of the CqsR ligand-binding domain was performed using HHPred (33) and Phyre2 (34) and compared to the known structure of the periplasmic region of Mlp37 (PDB ID: 3C8C) (35).

Strains, media and culture conditions

All *V. cholerae* strains used in this study were derived from C6706*str2*, a streptomycin-resistant isolate of C6706 (O1 El Tor) (36). The HapR-dependent bioluminescent reporter pBB1 has been previously described (11). *V. cholerae* and *E. coli* cultures were grown with aeration in Luria-Bertani (LB) broth at 30°C and 37°C, respectively. Unless specified, media was supplemented with streptomycin (Sm, 100 µg/ml), tetracycline (Tet, 5 µg/ml), ampicillin (Amp, 100 µg/ml), kanamycin (Kan, 100 µg/ml), chloramphenicol (Cm, 5 µg/ml) and polymyxin B (Pb, 50 U/ml) when appropriate. Bacterial strains used in this study is provided in Supplementary Table 1.

DNA manipulations and strain construction

All DNA manipulations were performed using standard procedures. Deletions and point mutations were introduced into the *V. cholerae* genome by allelic exchange using the suicide vector pKAS32 or a modified version, pJT961, as previously described (11, 37, 38). Mutant strains carrying the desired mutations were screened and confirmed by PCR and sequencing.

Bioluminescence assays

Bioluminescence assays were performed as described previously (11) with slight modifications. For manual assays, single colonies were grown overnight at 30 °C in LB containing appropriate antibiotics and the cultures were diluted at least 100-fold in the same medium. Diluted cultures

were grown at 30 °C and OD₆₀₀ (1 ml of culture) and light production (0.2 ml of culture) were measured every 45–60 min until OD₆₀₀ reached ~2.0 using a Thermo Scientific Evolution 201 UV-Visible Spectrophotometer and a BioTek Synergy HT Plate Reader, respectively. For automated assays, single colonies were grown in LB medium containing appropriate antibiotics in 96- or 384-well microplates at 30°C with aeration. OD₆₀₀ and light production were measured every 30 min for at least 10 hr using a BioTek Synergy HT Plate Reader. Light production per cell was calculated from dividing light production by OD₆₀₀.

Random mutagenesis and screening of constitutively active CqsR mutants.

The region encoding the predicted ligand-binding domain of CqsR (amino acids 31-297) was amplified from pEVS143-*cqsR* (11, 39) with primers WNT0548 (GAGTTATTGGGGGCTTGAAGT) and WNT0549 (AATCCTTTTTCGATGTTGATAATTAAGTCG) and subjected to random mutagenesis using GeneMorph II EZClone Mutagenesis kit (Agilent Technologies) per manufacturer's instructions. The resulting *cqsR* random mutagenized library was transformed into XL10-Gold *E. coli* cells. A portion of the library was then conjugated into a quadruple QS receptor strain ($\Delta 4$, $\Delta cqsS$, $\Delta luxQ$, $\Delta cqsR$, $\Delta vpsS$) carrying a HapR-dependent bioluminescence reporter pBB1 (11) and the transconjugants were selected on LB/Pb/Kan/Tet2 plates and used for screening of constitutively active CqsR mutants. Colonies were picked and transferred into microplates containing LB/Kan/Tet2/IPTG medium using a colony picking robot and grown at 30°C overnight. OD₆₀₀ and luminescence were measured with a BioTek Synergy HT Plate Reader. Mutants of interest that displayed low relative bioluminescence were collected, re-screened again to confirm. The mutations in the plasmid-borne *cqsR* of these isolates were determined by sequencing. To

introduce specific amino acid changes, site directed mutagenesis on the plasmid-borne *cqsR* was performed using the QuikChange™ XL Site-Directed Mutagenesis Kit (Agilent) per manufacturer's instructions.

NMR metabolomics.

Extracellular metabolite analysis of *V. cholerae* culture supernatants was performed as described previously (8) except plain LB medium was used as reference standard. An ¹H NMR spectrum of each sample was collected at 25°C on a Bruker Avance 600 spectrometer by using 64 scans and a NOE1D pulse sequence. Data were processed and analyzed by using CHENOMX (version 8.0) for quantification of metabolites present in each sample. The average value and the standard error of the mean (SEM) were determined for at least three replicates.

CqsR LBD purification

The region encoding the periplasmic region of CqsR (CqsR-LBD, residues 35-274) was PCR amplified with primers WNT0694 (GCCTGGTGCCGCGCGGCAGCGAAGTCCCATTAGAAAAGAG) and WNT0695 (GCTTTGTTAGCAGCCGGATCTTAGATGTTGATAATTAAGTCGAAG) from *V. cholerae* genomic DNA; the vector pET28B was amplified with primers WNT0692 (GCTGCCGCGCGGCACCAG) and WNT0693 (GATCCGGCTGCTAACAAAG). The two fragments were joined together using Gibson reaction (NEB) and the resulting plasmid was transformed into *E. coli* BL21(DE3) strain (WN5327).

For CqsR-LBD purification, strain WN5327 was grown to OD₆₀₀ ~ 0.5 and induced with 1 mM IPTG at 16 °C overnight. Cells were collected by centrifugation and resuspended in binding buffer (50mM sodium phosphate buffer, pH 7.0; 300mM sodium chloride; 10mM imidazole, pH 7.7; 5%

glycerol). Resuspended cells were then lysed with a fluidizer. Insoluble materials were removed by centrifugation (10,000g, 4 °C, 1 hour) and subsequent filtering through a 0.45 µm filter. Cleared lysate was loaded onto His-Trap column (1 mL) equilibrated with binding buffer. The column was then washed with 20 mL binding buffer. Proteins were eluted with binding buffer containing 120 to 300 mM imidazole. Fractions containing CqsR-LBD were pooled, frozen in aliquots, and stored at -80°C.

Differential scanning fluorimetry

Differential scanning fluorimetry assays were performed using a BioRad CFX Connect Real-Time PCR instrument (40). For chemical screening, ligands were prepared by dissolving the contents of Biolog plates PM 1-4 in 50 µl of water (final concentration ~10–20 mM). Each 20 µl standard assay contained 1× PBS (pH 7.5), 10%(v/v) glycerol, 10 µM CqsR-LBD, and 5× SYPRO Orange. Two µl of the resuspended Biolog compounds were added to each assay. Samples were heat denatured from 20°C to 90°C at a ramp rate of 1°C min⁻¹. The protein unfolding curves were monitored by detecting changes in SYPRO Orange fluorescence. The first derivative values (–dF/dt) from the raw fluorescence data were used to determine the melting temperature (T_m). For testing other compounds, a 10× stock was prepared and 2 µl was added to each well and the experiments were conducted as described above.

MicroScale Thermophoresis (MST)

Nickel affinity resin-purified CqsR-LBD was further purified using anion exchange chromatography (Source15Q - GE Healthcare) and size exclusion chromatography (S200 – GE Healthcare). Purified CqsR-LBD was concentrated to 4.3 mg/ml and stored in 50 mM sodium phosphate, pH 7.7, and 300 mM sodium chloride. CqsR-LBD was diluted to 20 µM and labeled

using the Monolith NT Protein Labeling Ki RED-NHS (NanoTemper Technologies). Different concentrations of ethanolamine were incubated with 20 nM working stock solutions of labeled protein in the dark for 30 min at 4°C. After incubation, the samples were transferred into standard treated capillaries (NanoTemper Technologies) and read in a Monolith NT.115 Blue/Red instrument at room temperature using 20% LED and medium MST power. Binding affinities were calculated from three experiments.

Chemical Synthesis of EA Metabolites

See supplementary information for chemical synthesis of HEGly and HEHEAA.

Ethics statement

All animal experiments were done in accordance with NIH guidelines, the Animal Welfare Act, and US federal law. The infant mouse colonization experimental protocol B-2018-99 was approved by Tufts University School of Medicine's Institutional Animal Care and Use Committee. All animals were housed in a centralized and AAALAC-accredited research animal facility that is fully staffed with trained husbandry, technical, and veterinary personnel in accordance with the regulations of the Comparative Medicine Services at Tufts University School of Medicine.

Infant mouse colonization model

For *V. cholerae* infection, bacterial strains were grown aerobically overnight in LB/Sm at 30°C and diluted in LB for inoculum. Approximately 10⁶ colony forming units (CFUs) in 50 µl were introduced orogastrically to 3- to 5-day-old CD-1 mice (Charles River Laboratories). Infected animals were subsequently gavaged with 50µL 10mM ethanolamine (EA) or vehicle (LB) at 2 and 4 hr post-infection. All animals were sacrificed 8 hours after infection and their small intestines were harvested and homogenized. *V. cholerae* colonized in the small intestine was enumerated by

plating serial dilutions of intestinal homogenate on LB/Sm plates. *V. cholerae* colonization of the small intestine is presented as a single data point per mouse and data are graphed with the median. Two-tailed unpaired Student's t tests, assuming unequal variances were used for statistical analyses $**P < 0.01$.

RESULTS

CqsR is predicted to carry a periplasmic CACHE domain

Similar to other hybrid histidine kinases, CqsR possesses a cytoplasmic phosphoacceptor domain (aa 355 – 421), an HATPase domain (aa 363 – 583) and a C-terminal response regulator domain (aa 603- 720). Using various bioinformatics tools, the periplasmic region of CqsR is predicted to carry a periplasmic dCACHE_1 domain ((28, 41), aa 44-239, simplified as CACHE domain hereafter) (Figure 2A). The predicted structure contains the characteristic long N-terminal α -helix (green), a linker (yellow) that connects this helix to two globular domains (pocket I, red and pocket II, blue) with a small α -helical linker between the two pockets (orange), and a C-terminal α -helix (purple) that connects the domain to the cytoplasm (Figure 2B), strikingly similar to other CACHE proteins, such as *V. cholerae* chemoreceptor Mlp37 (35) (Figure 2B). These similarities strongly suggest that the periplasmic region of CqsR functions as a ligand-binding domain to regulate its cytoplasmic kinase activity.

Identification of residues in the CqsR periplasmic region important for signal sensing or signal transduction

CqsR kinase activity is inhibited by an uncharacterized activity present in the spent culture media from *V. cholerae* (11). We therefore hypothesized that CqsR detects a discrete chemical signal through the periplasmic CACHE domain and predicted that certain mutations in this domain could

219 impair signal sensing and signal transduction. Consequently, these mutations would turn CqsR
 220 into a constitutively active kinase even in the presence of the cognate signals. To identify these
 221 mutations, we constructed a *cqsR* plasmid library in which the whole periplasmic region was
 222 randomly mutagenized. The randomly mutagenized *cqsR* library was then introduced into a
 223 quadruple receptor mutant ($\Delta 4$) *V. cholerae* strain ($\Delta luxQ$, $\Delta cqsS$, $\Delta vpsS$, $\Delta cqsR$) that also harbors
 224 a HapR-dependent bioluminescent reporter cosmid pBB1 (11). While the quadruple receptor
 225 mutant produces light constitutively due to the constant production of HapR, in the presence of a
 226 plasmid-borne wild type (WT) copy of *cqsR*, this strain produces low bioluminescence at low cell-
 227 density (LCD) and subsequently turns on bioluminescence production at high cell-density (HCD)
 228 due to signal detection and kinase inhibition of CqsR (11). Thus, we predicted that clones carrying
 229 plasmids expressing WT *cqsR* or a null *cqsR* allele would appear bright at HCD, while clones
 230 producing CqsR variants insensitive to the presence of cognate signal would appear darker at HCD.
 231 We measured the bioluminescence of ~26,000 clones from this random library and identified 79
 232 candidates impaired for light production at HCD. Most candidates harbored multiple mutations in
 233 the plasmid-borne *cqsR*. There were 23 unique mutations in these candidates. When each unique
 234 mutation was introduced back to *cqsR* individually, only eight unique mutations in *cqsR* resulted
 235 in lower bioluminescence production at HCD (Figure 2C). These *cqsR* mutations caused changes
 236 in the two aspartate residues (D171V and D178V) mapped to pocket I of the predicted ligand
 237 binding domain, two changes in pocket II (L217S and V219E), four changes (R49S, A259V,
 238 H262Y, and L268I) in the two helical regions that either exit or enter the cellular membrane and
 239 are likely not involved in direct ligand interactions. Strikingly, both Asp171 and Asp198 residues
 240 in the pocket I of CqsR are predicted to be in the same positions as the Asp172 and Asp201 residues
 241 respectively, in Mlp37, which have been shown to be critical for binding to amino acid signals

(35). Therefore, our results suggest that, similar to Mlp37, CqsR detects its cognate signals through the periplasmic CACHE domain.

Chemical screen identified ethanolamine as a potential CqsR ligand.

Since the periplasmic CACHE domain is critical for the signal sensing, we attempted to identify potential ligands capable of binding to the putative CqsR ligand-binding domain (CqsR-LBD), by performing a differential scanning fluorimetry screen using the common metabolites contained within the Biolog PM1-4 plates. This assay assumed that ligand binding leads to stabilization of the target protein, resulting in an increase of melting temperature (T_m) of the protein, which can be determined by measuring the dynamics of Sypro Orange fluorescence emission at different temperatures. In the absence of exogenous metabolites, the average T_m of the purified CqsR-LBD was observed to be 46 °C under the test conditions. Out of the ~400 metabolites tested, including some of the known compounds that bind CACHE domain such as amino acids, carboxylates, and polyamines, we only observed a shift in the T_m of >5 °C in the presence of one tested compound, ethanolamine (or 2-aminoethanol). The shift in T_m of CqsR in the presence of ethanolamine was dose-dependent. The largest shift in T_m of +15 °C was observed for 10 mM ethanolamine (Table 1) with smaller shifts of +9.33 °C, +6°C and +2°C for 1 mM, 100 μ M and 10 μ M ethanolamine respectively. To investigate the specificity of the interactions between ethanolamine and the CqsR-LBD, we determined how chemical modifications to the ligand affected CqsR-LBD binding (Table 1). Replacing the hydroxyl group of ethanolamine with a thiol, methyl, or carboxyl group abolished *in vitro* binding to CqsR-LBD, as indicated by the lack of change in the observed T_m in the presence of these compounds. Similarly, replacing the amine group with a thiol or methyl

group also abolished *in vitro* binding (Table 1). In addition, single or multiple methylations of the amine group of ethanolamine also abolished binding (Table 1). We therefore concluded that the presence of both the amine and the hydroxyl groups in ethanolamine are essential for efficient CqsR-LBD binding. Side-chain substitutions at the carbon atom next to the hydroxyl group (labelled i in Table 1) also abolished CqsR-LBD binding regardless of the stereochemistry of the hydroxyl group. However, substitutions on the carbon atom next to the amine group (labelled ii) appeared to be better tolerated, as indicated by the increase in T_m observed for L-alaninol and serinol (Table 1). Interestingly, stereospecificity is critical for binding activity as indicated by lower shift in T_m for D-alaninol, a stereo-isomer of L-alaninol, at the same concentration (Table 1).

A recent study showed that a specific ethanolamine derivative, N-(2-hydroxyethyl)-2-(2-hydroxyethylamino) acetamide [HEHEAA], present in commercial preparations of ethanolamine, is responsible for promotion of *pipA* transcription in *Pseudomonas sp. GM79* in response to plant exudates (42). We tested eight common compounds that accumulate during chemical synthesis of ethanolamine including HEHEAA (42, 43) for their ability to bind to CqsR and, in our hands, none of the compounds tested produced a change in T_m of the CqsR-LBD in the binding assay (Supplementary Table 2), suggesting the CqsR response to ethanolamine was not due to these common contaminants. Finally, using MicroScale Thermophoresis, we determined the binding affinity (K_d) of ethanolamine to CqsR-LBD is $\sim 0.5 \mu M$ (Supplementary Figure 1). Collectively, our data indicate that ethanolamine and some ethanolamine derivatives bind to CqsR-LBD with a high degree of specificity.

Effect of ethanolamine and its analogs in regulating *V. cholerae* quorum sensing

286 Since ethanolamine binds to the CqsR-LBD *in vitro*, we postulated that exogenous addition of
 287 ethanolamine might diminish CqsR kinase activity, leading to premature induction of HCD QS
 288 response in *V. cholerae* cells. To test this, we measured the density-dependent bioluminescence
 289 profile of a $\Delta 3$ *cqsR*⁺ ($\Delta luxQ$, $\Delta cqsS$, $\Delta vpsS$) strain that harbors the HapR-dependent
 290 bioluminescent reporter pBB1 in the presence of varying concentrations of exogenously added
 291 ethanolamine. In the absence of ethanolamine, similar to previously reported (11), the $\Delta 3$ *cqsR*⁺
 292 strain displayed the characteristic U-shaped HapR-dependent bioluminescence profile due to
 293 changes in CqsR kinase activity in different cell densities (Figure 3A). Induction of HCD QS
 294 response by ethanolamine, as observed by increased levels of light production at low cell densities,
 295 was dose-dependent (Figure 3A). In the presence of 10 mM exogenously added ethanolamine, the
 296 strain was constitutively bright at all cell densities when compared to that from the culture in LB
 297 medium without ethanolamine added (Figure 3A). Moreover, lower concentrations (1mM or
 298 0.1mM) of exogenous ethanolamine also induced a higher level of light production in this reporter
 299 strain. Consistent with our model, using a *Pqrr4-lux* reporter (11), we also determined that
 300 ethanolamine repressed *Qrr4* transcription in the $\Delta 3$ *cqsR*⁺ strain (Supplementary Figure 2). Similar
 301 QS-inducing effects were obtained for L-alaninol and serinol (Figure 3B, 3C and Supplementary
 302 Figure 2). Consistent with the observation that D-alaninol did not show any detectable *in vitro*
 303 binding activity to CqsR, 10 mM D-alaninol was inactive in inducing QS gene expression (Table
 304 I, Figure 3D, and Supplementary Figure 2). In addition, ethanolamine did not induce HapR-
 305 dependent bioluminescence in other triple QS receptor mutants where only CqsS, LuxPQ, or VpsS
 306 is present as the sole QS receptor (Supplementary Figure 3), indicating that ethanolamine induces
 307 QS in a CqsR specific manner. Finally, unlike the strain producing WT CqsR, 10 mM of
 308 ethanolamine did not induce constitutive light production in the CqsR^{D171V} and CqsR^{D198V} CACHE

domain mutants (Figure 3E and Figure 3F). Together, our results strongly suggest that ethanolamine specifically interacts with the CACHE domain of CqsR to inhibit its kinase activity and modulate QS gene expression in *V. cholerae*.

The role of intrinsically produced ethanolamine in QS

We showed above that exogenous ethanolamine inhibits CqsR kinase activity in *V. cholerae*, we then tested if endogenously made ethanolamine also plays a role in controlling CqsR. In *E. coli*, ethanolamine is derived from the degradation of glycerol-3-phosphoethanolamine to glycerol-3-phosphate and ethanolamine (44, 45). This reaction is predicted to be catalyzed by two *V. cholerae* glycerophosphodiester phosphodiesterases VCA0136 (GlpQ homolog) and VC1554 (UgpQ homolog). We used ¹H NMR to determine the ethanolamine level in the cell-free supernatants derived from both the $\Delta 3$ *cqsR*⁺ and the $\Delta 3$ *cqsR*⁺ Δ *vca0136* Δ *vc1554* strains. We detected ~25 μ M ethanolamine in the supernatants harvested from the $\Delta 3$ *cqsR*⁺ strain but the level of ethanolamine was below the detection limit in the supernatants from the $\Delta 3$ *cqsR*⁺ Δ *vca0136* Δ *vc1554* strain.

After confirming the double phosphodiesterase mutants did not make any ethanolamine, we used the HapR-dependent bioluminescence pBB1 reporter to measure the QS response of these strains. We expected the mutant is impaired in expressing the HCD QS response due to the lack of ethanolamine to repress the CqsR kinase activity. However, the double phosphodiesterase mutant still exhibited density-dependent U-shaped bioluminescence profile similar to the $\Delta 3$ *cqsR*⁺ strain. Nevertheless, the onset of the transition to HCD bioluminescence production was delayed in the double phosphodiesterase mutants (Figure 4), suggesting the inhibition of CqsR kinase activity and HapR production occur at a higher cell density. Consistent with the above results, using a *Pqrr4-lux* reporter, light production at LCD was higher in the $\Delta 3$ *cqsR*⁺ Δ *vca0136* Δ *vc1554* strain

when compared to that of the $\Delta 3$ *cqsR*⁺ strain (Supplementary Figure 4), indicating transcription of Qrr sRNAs is increased in the absence of endogenous ethanolamine production in the double phosphodiesterase mutants, resulting in a delayed HapR production. It should be noted that, even in the absence of endogenous ethanolamine production, repression of Qrr transcription and induction of HapR production still occur, indicating the presence of additional signals inhibiting CqsR kinase activity at HCD (Figure 4 and Supplementary figure 4). Moreover, addition of 10 mM ethanolamine increased light production in $\Delta 3$ *cqsR*⁺ as well as in the $\Delta 3$ *cqsR*⁺ Δ *vca0136* Δ *vc1554* carrying the pBB1 HapR-dependent reporters (Figure 4) and decreased light production in the same strains carrying the *Pqrr4-lux* reporters (Supplementary figure 4), indicating CqsR sensing is functional in responding to exogenously supplied ethanolamine in these strains. Together, our results indicate that while endogenously made ethanolamine participates in QS gene regulation, *V. cholerae* produces additional signals that are capable of activating QS through CqsR.

Ethanolamine modulates CqsR QS signaling inside animal hosts

Production of the Toxin-Coregulated Pili, which is critical for *V. cholerae* host colonization, is repressed by the HCD QS response (5, 6). We previously showed that the $\Delta 3$ *cqsR*⁺ strain, but not the $\Delta 4$ strain, can colonize the small intestines of infant mice (11). To determine if exogenous ethanolamine influences CqsR signaling *in vivo*, we assayed the ability of the $\Delta 3$ *cqsR*⁺ strains to colonize the host in the presence of exogenous ethanolamine. While the $\Delta 3$ *cqsR*⁺ strain showed a ~5-fold decrease in mouse small intestine colonization in the presence of ethanolamine compared to the group given the vehicle control, the $\Delta 3$ *cqsR*^{D171V} strain showed no significant impairment in the ability to colonize the infant mouse in the presence of ethanolamine (Figure 5). These results indicate that high levels of ethanolamine in the gut can prematurely induce the HCD QS response

in the $\Delta 3$ *cqsR*⁺ strain and decrease colonization. Importantly, the effect of ethanolamine is dependent on the interaction between the compound and the CACHE domain of the CqsR.

DISCUSSION

In this study, we demonstrated that the periplasmic CACHE domain of CqsR QS receptor is involved in signal sensing; and we also identified a common metabolite ethanolamine as a signal that regulates the CqsR QS signaling pathway in *V. cholerae*. The periplasmic CACHE domain is common in many chemotaxis receptors and other membrane bound regulators (28); and CACHE domain proteins have been shown to interact with a variety of chemical compounds (28, 35, 41, 46-48). While CACHE domain has been shown to interact with quaternary ammonium including choline (an ethanolamine analog that CqsR does not interact) to control chemotaxis (49, 50), our study provided an example where a CACHE domain interacts with an amino alcohol (i.e., ethanolamine) to regulate QS gene expression. Of note, the CACHE domain of CqsR shares no homology to that of another ethanolamine-sensing histidine kinase HK17 (EutW) in *Enterococcus faecalis* or the cytoplasmic transcriptional regulator EutR that detects ethanolamine in *Salmonella* (51, 52).

Although ethanolamine binds to CqsR-LBD with sub-micromolar affinity ($k_d \sim 0.5 \mu M$), millimolar level of ethanolamine is needed to fully induce the HCD QS response in *V. cholerae*. This apparent difference is not uncommon for signaling pathways dependent on CACHE receptor proteins (e.g., (50, 53)). This is in stark contrast to the typical effective concentration for canonical autoinducers, such as CAI-1 and AI-2, which are in micromolar levels (16-19, 24, 25). Nonetheless, interaction of ethanolamine to CqsR is highly specific and modifications of almost any functional groups in ethanolamine result in total loss of activity. We reasoned that while ethanolamine binds to CqsR-LBD with high affinity *in vitro*, the binding affinity to the full length

CqsR inside *V. cholerae* cells could be different. Moreover, the import mechanism of ethanolamine into the *V. cholerae* periplasmic space is unclear. Indeed, *V. cholerae* is not known to metabolize ethanolamine as either carbon or nitrogen source (54) and it lacks the known ethanolamine transporters for importing the compound into the cytoplasm (55, 56). In addition, since the mutants defective in ethanolamine synthesis are still able to express a delayed HCD QS response, additional signals must be present and detected by CqsR, and these molecules could compete with ethanolamine for CqsR binding.

Given *V. cholerae* does not use ethanolamine as a nutrient source, it is unclear why CqsR detects this specific metabolite out of many secreted molecules. However, ethanolamine has been implicated in niche recognition in several pathogens such as enterohaemorrhagic *E. coli* (EHEC), *S. typhimurium* (57), *E. faecalis* (58), and *Clostridium difficile* (59). Our results suggest that ethanolamine signaling may only operate in environments where ethanolamine is found in high abundance. Because ethanolamine is derived from the membrane phospholipid turnover, this compound is particularly prevalent in the gastrointestinal tract, where it can be found at concentrations over 2 mM per some reports (60). Thus, ethanolamine might act primarily as an environmental cue as opposed to a *bona fide* QS signal in *V. cholerae*. In *Salmonella*, ethanolamine metabolism genes and Type 3 secretion system genes are activated with high and low level of ethanolamine, respectively (57, 61). Similarly, in EHEC, exogenous ethanolamine increases the expression of virulence genes (51). However, unlike these examples in which ethanolamine sensing promotes virulence gene expression, ethanolamine detection through CqsR instead represses virulence gene expression by promoting HCD QS response in *V. cholerae* (11). This is especially relevant to the life cycle of *V. cholerae* since the major colonization site of this pathogen is the small intestine while ethanolamine may be more abundant in the lower gastrointestinal (GI)

tract because it cannot be metabolized by the majority of commensal species (62), thus, ethanolamine sensing may prevent *V. cholerae* from colonizing in an undesirable niche.

It is puzzling why *V. cholerae* QS circuit is composed of four functionally-redundant pathways, we previously suggest that the “many-to-one” arrangement is important for the robustness of the system to prevent signal perturbation and the HCD QS response is only expressed when all four receptors are bound with the cognate signals (11). Our results here suggest that the four QS receptors integrate information derived from different sources. It has been suggested CAI-1 is used for inter-*Vibrio* signaling (16, 18) and AI-2 is for inter-species signaling (63, 64); perhaps CqsR serves as a dual-function receptor that senses an unknown self-made signal (e.g., autoinducer) and an exogenous metabolic by-product as an environmental cue. A combination of different signals ensures *V. cholerae* only expresses the HCD QS response to repress virulence factor production and biofilm formation at the most appropriate time and environment. This kind of dual detection of cognate AIs and other chemical signals by a single QS receptor is not commonly observed. QseC is one of the known QS receptors that detects both an AI signal (AI-3) and other chemical cues (epinephrine and norepinephrine) to control virulence gene expression in *E. coli* and *Salmonella* (69). It is proposed that such unique QS receptor can be used for communication between the pathogens and the hosts (52). Further work is required to elucidate the role of ethanolamine sensing in inter-kingdom QS signaling and virulence control in *V. cholerae*.

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TABLE 1 Effects of ethanolamine and its analogs on the melting temp of CqsR-LBD.

Compound	Structure ^a	T _m (°C) ^b
None	-	46
Ethanolamine [2-aminoethanol]		61
Cysteamine [2-aminoethanethiol]		46
1-propanamine		46.33 [1.15]
β-alanine [2-Carboxyethylamine]		46
Mercaptoethanol [2-Hydroxyethanethiol]		46
1-Propanol		43.67 [0.58]
2-Methylaminoethanol		49.67 [0.58]
2-Dimethylaminoethanol		48
Choline [(2-Hydroxyethyl)trimethyl ammonium]		46

(S)-(+)-1-Amino-2-propanol		48.67 [0.58]
(R)-(-)-1-Amino-2-propanol		48
L-alaninol [(S)-(+)-2-Amino-1-propanol]		60
D-alaninol [(R)-(-)-2-Amino-1-propanol]		45
Serinol [2-Amino-1,3-propanediol]		53.67 [0.58]

^aFunctional groups displayed in red are the ones that vary from the base compound ethanolamine.

^bData shown as average of 3 replicates in the presence of 10 mM tested compounds. Square brackets indicate standard deviation where applicable.

Figure 1 Quorum-sensing circuit in *Vibrio cholerae*

Quorum sensing in *V. cholerae* is controlled by four histidine kinases CqsS, LuxPQ, CqsR and VpsS. At low cell density, these receptors act predominantly as kinases and phosphorylate LuxO through LuxU. Phosphorylated LuxO activates transcription of small RNAs Qrr1-4 which inhibit HapR translation and promote AphA translation, thereby resulting in a low cell density expression profile. At high cell density, when the cognate signals are bound, the kinase activity of these receptors is repressed. This leads to dephosphorylation of LuxO, preventing Qrr1-4 transcription. Therefore, HapR translation is induced and AphA translation repressed, leading to a high cell density gene expression pattern. Autoinducers CAI-1 (produced by CqsA) and AI-2 (produced by LuxS) have been previously characterized in regulating the kinase activity of CqsS and LuxPQ respectively.

Figure 2

The CqsR CACHE domain is important for signal sensing and signal transduction.

(A) CqsR is predicted to contain all the conserved cytoplasmic domains (phosphoacceptor (PA), histidine kinase (HK), and response regulator (RR) domains) of a hybrid histidine kinase with an N-terminal periplasmic CACHE domain flanked by two transmembrane helices (TM). (B) The predicted structure of the periplasmic CACHE domain of CqsR (right) is similar to that of Mlp37 (left). The residues identified as important for signal sensing and signal transduction are highlighted in cyan in the predicted structure. See main text for further details. (C) Eight periplasmic CqsR residues are involved in signal sensing and signal transduction. Relative light production (lux/OD₆₀₀) was measured in quadruple QS receptor ($\Delta 4$) strains carrying a plasmid producing CqsR with single amino changes as well as a HapR-dependent bioluminescence reporter. Alteration in the CqsR helix domain (R49S), CACHE pocket 1 region (D171V, D198V), CACHE pocket 2 region (L217S, V219E), or the transmembrane proximal regions (A259V, H262Y, L268I) impaired bioluminescence production at high cell-density (OD₆₀₀ > 1.5). Average values and standard errors from at least three independent replicates are shown.

Figure 3 Effects of ethanolamine and its analogs on CqsR quorum-sensing response.

HapR-dependent bioluminescence profiles (lux/OD₆₀₀) were measured in a $\Delta 3$ *cqsR*⁺ strain in the presence of 10 mM, 1 mM, 0.1 mM A) ethanolamine, B) serinol, C) L-alaninol, D) D-alaninol. Blank indicates LB medium without ethanolamine added. HapR-dependent bioluminescence profiles (lux/OD₆₀₀) were measured in E) $\Delta 3$ *cqsR*^{D198V} strain and F) $\Delta 3$ *cqsR*^{D171V} strain in the presence of 10 mM, 1 mM, 0.1 mM concentrations ethanolamine respectively. Each figure shows a representative profile of each condition with two biological replicates. Each experiment was performed independently at least two times.

Figure 4 Effect of glycerophosphodiesterase deletions on CqsR quorum-sensing response.

HapR-dependent bioluminescence profiles (lux/OD₆₀₀) were measured in a $\Delta 3 \text{ cqsR}^+$ strain and an isogenic strain with deletions in loci *vc1554* and *vca0136*, encoding the two putative glycerophosphodiester phosphodiesterases in LB medium and in the presence of 10 mM ethanolamine. The figure shows a representative profile of each condition with two biological replicates. Each experiment was performed independently at least two times.

Figure 5 Effect of exogenous ethanolamine on CqsR quorum-sensing response inside animal hosts.

CFU counts per small intestine homogenate collected from mice ($n = 7-8$) infected with a $\Delta 3$ *cqsR*⁺ or $\Delta 3$ *cqsR*^{D171V} mutant strain. To assess the effect of ethanolamine on strain colonization, mice were gavaged with 10mM ethanolamine (EA) or vehicle at 2 and 4 hours post infection. Each symbol represents the CFU enumerated from an individual mouse and the horizontal lines indicate the median for each group. ** $P < 0.01$ (unpaired t test).

SUPPLEMENTARY INFORMATION

Supplementary Table 1. List of strains used in this study

Strain Number	Organism	Genotype	Ab Resistance ^a	Source
WN3176	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS$	Sm	(11)
WN3208	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS$ / pBB1	Sm Tet	(11)
WN3357	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta cqsR$ /pBB1	Sm Tet	(11)
WN3649	<i>V. cholerae</i>	$\Delta cqsS \Delta cqsR \Delta vpsS$ /pBB1	Sm Tet	(11)
WN3651	<i>V. cholerae</i>	$\Delta luxQ \Delta cqsR \Delta vpsS$ /pBB1	Sm Tet	(11)
WN3354	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$	Sm	(11)
WN4899	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> / pBB1	Sm Kan Tet	This study
WN5267	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / <i>pEVS143-cqsR</i> ^{R49S} pBB1	Sm Kan Tet	This study
WN5275	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> ^{D198V} / pBB1	Sm Kan Tet	This study
WN5277	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> ^{L217S} / pBB1	Sm Kan Tet	This study
WN5279	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> ^{V219E} / pBB1	Sm Kan Tet	This study
WN5281	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> ^{A259V} / pBB1	Sm Kan Tet	This study
WN5285	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> ^{H262Y} / pBB1	Sm Kan Tet	This study
WN5318	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> ^{F268I} / pBB1	Sm Kan Tet	This study
WN5354	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta cqsR$ / pEVS143- <i>cqsR</i> ^{D171V} / pBB1	Sm Kan Tet	This study
WN5781	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta vc1554 \Delta vca0136$	Sm	This study
WN5784	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta vc1554 \Delta vca0136$ /pBB1	Sm Tet	This study
WN5886	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS cqsR$ ^{D198V}	Sm	This study
WN5887	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS cqsR$ ^{D171V}	Sm	This study
WN5890	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS cqsR$ ^{D198V} /pBB1	Sm Tet	This study
WN5891	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS cqsR$ ^{D171V} /pBB1	Sm Tet	This study
WN5976	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS$ /pBK1003 (<i>Pqrr4-lux</i>)	Sm Cm	This study
WN5979	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS \Delta vc1554 \Delta vca0136$ / pBK1003	Sm Cm	This study
WN5980	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS cqsR$ ^{D198V} / pBK1003	Sm Cm	This study
WN5981	<i>V. cholerae</i>	$\Delta cqsS \Delta luxQ \Delta vpsS cqsR$ ^{D171V} / pBK1003	Sm Cm	This study
WN048	<i>E. coli</i>	<i>S17-1</i> λ pir / pBB1	Tet	(11)
WN3705	<i>E. coli</i>	<i>S17-1</i> λ pir / pEVS143- <i>cqsR</i>	Kan	(11)
WN5233	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{V219E}	Kan	This study
WN5236	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{A259V}	Kan	This study
WN5239	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{H262Y}	Kan	This study

WN5242	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{R49S}	Kan	This study
WN5248	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{D198V}	Kan	This study
WN5250	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{L217S}	Kan	This study
WN5318	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{F268I}	Kan	This study
WN5326	<i>E. coli</i>	BL21DE3/ pET28b <i>cqsR</i> -LBD	Kan	This study
WN5344	<i>E. coli</i>	XL10-Gold / <i>pEVS143-cqsR</i> ^{D171V}	Kan	This study
WN5745	<i>E. coli</i>	S17 λ pir DAP- / pKAS::Kan <i>Δvc1554</i>	Kan	This study
WN5746	<i>E. coli</i>	S17 λ pir DAP- / pKAS::Kan <i>Δvca0136</i>	Kan	This study
WN5880	<i>E. coli</i>	S17 λ pir DAP- / pKAS::Kan <i>cqsR</i> ^{D198V}	Kan	This study
WN5881	<i>E. coli</i>	S17 λ pir DAP- / pKAS::Kan <i>cqsR</i> ^{D171V}	Kan	This study

^a Sm = Streptomycin, Kan = Kanamycin, Cm = Chloramphenicol, Tet = Tetracycline

Supplementary Table 2

Binding activity of CqsR to common byproducts of ethanolamine

Compound	T _m ^a
None	47.00
N-(2-hydroxyethyl)-acetamide	48.00
N,N'-Bis(2-hydroxyethyl)-ethanediamide	49.00
N-(2-hydroxyethyl)-2-[(2-hydroxyethyl)amino]-acetamide	47.67 [0.57]
N-(2-hydroxyethyl)ethylenediamine	47.67 [0.57]
N-(2-hydroxyethyl) imidazolidone	47.00
N-(2-hydroxyethyl)-glycine	47.33 [0.57]
N-(2-hydroxyethyl)-imidazole	47.33 [0.57]
N-(2-hydroxyethyl)-formamide	50.00

^aData shown as average of 3 replicates in the presence of 1 mM tested compounds. Square brackets indicate standard deviation where applicable.

Supplementary Figure 1

MST quantification (FNorm; normalized fluorescence) for ethanolamine binding to CqsR. Ethanolamine was titrated between 200 μ M and 0.0061 μ M with 20 nM His6-tagged CqsR. Ethanolamine binds to CqsR with a K_d of 0.478 ± 0.076 μ M. Binding affinity was calculated from three independent experiments.

Supplementary Figure 2

Effect of ethanolamine and its analogs on *qrr4* transcription.

Normalized bioluminescence production (lux/OD₆₀₀) using a *Pqrr4-lux* reporter was measured in a $\Delta 3$ *cqsR*⁺ strain in the presence of 10 mM ethanolamine, L-alaninol, D-alaninol, or serinol. Blank indicates LB medium without added compound.

Supplementary Figure 3

Ethanolamine induces high cell density QS response in a CqsR-specific manner

HapR-dependent bioluminescence profiles (lux/OD₆₀₀) were measured in A) $\Delta 3 \text{ luxQ}^+$, B) $\Delta 3 \text{ cqsS}^+$, C) $\Delta 3 \text{ cqsR}^+$, and D) $\Delta 3 \text{ vpsS}^+$, in LB medium and LB medium containing 10 mM ethanolamine. Each figure shows a representative profile of each condition with two biological replicates. Each experiment was performed independently at least two times.

Supplementary Figure 4

Effect of ethanolamine on *qrr4* transcription in the double phosphodiesterase mutants.

Normalized bioluminescence production (lux/OD₆₀₀) using a *Pqrr4-lux* reporter was measured in a $\Delta 3 \text{ cqsR}^+$ and the isogenic $\Delta 3 \text{ cqsR}^+ \Delta \text{vc1554} \Delta \text{vca0136}$ strains with and without 10 mM ethanolamine. Each figure shows a representative profile of each condition with three biological replicates. Each experiment was performed independently at least two times.

Detail of chemical synthesis of HEGly and HEHEAA

Unless otherwise noted, all reactions were performed in flame-dried glassware under an atmosphere of nitrogen using dried reagents and solvents. All chemicals were purchased from commercial vendors and used without further purification. Anhydrous solvents were purchased from commercial vendors. Flash chromatography was performed using standard grade silica gel 60 230-400 mesh from SORBENT Technologies or was performed using a Biotage Flash Purification system equipped with Biotage silica gel or C18 columns. Analytical thin-layer chromatography was carried out using Silica G TLC plates, 200 μm with UV₂₅₄ fluorescent indicator (SORBENT Technologies), and visualization was performed by staining and/or by absorbance of UV light. NMR spectra were recorded using a Varian Mercury Plus spectrometer (400 MHz for ¹H-NMR; 100 MHz for ¹³C-NMR). Chemical shifts are reported in parts per million (ppm) and were calibrated according to residual protonated solvent. Mass spectroscopy data was collected using an Agilent 1100-Series LC/MSD Trap LC-MS or a Micromass Quattro micro with a Waters 2795 Separations Module LC-MS with acetonitrile containing 0.1% formic acid as the mobile phase in positive ionization mode. All final compounds were evaluated to be of greater than 90% purity by analysis of ¹H-NMR and ¹³C-NMR unless otherwise indicated.

2-((2-Hydroxyethyl)amino)acetic Acid, HEGly. This compound was synthesized following the reported procedure (65) with modifications. Briefly, to a solution of ethanolamine (3.15 mL, 52.2 mmol) in anhydrous THF (53 mL) in a oven-dried flask was added *i*-Pr₂NEt (9.4 mL, 54.0 mmol). The resulting solution was cooled to 0°C and was treated with ethyl bromoacetate (5 mL, 45.2 mmol) dropwise. The mixture was stirred overnight with warming to room temperature and was concentrated *in vacuo* to remove solvent and was purified by silica gel flash chromatography to provide the ester, ethyl (2-hydroxyethyl)glycinate (3.5 g, 23.8 mmol, 53% yield). The ester,

ethyl (2-hydroxyethyl)glycinate (3.5g, 23.8 mmol) was dissolved in a solution of acetone (119mL) and water (119mL) and was treated with LiOH (4.99g, 119.01mmol) and stirred at room temperature overnight. The reaction was quenched by the addition of 119mL of 1M HCl and was concentrated to dryness *in vacuo*. The residue was purified by slow recrystallization from H₂O/EtOH at 5°C to provide 2-((2-Hydroxyethyl)amino)acetic Acid, HEGly (1.10g, 9.2mmol, 39% yield). Spectral data was consistent with previously reported data (65).

***N*-(2-hydroxyethyl)-2-((2-hydroxyethyl)amino)acetamide, HEHEAA.** 2-((2-Hydroxyethyl)amino)acetic Acid (HEGly, 87 mg, 0.73 mmol) was dissolved in H₂O (200 uL) with warming. The solution was diluted with ACN (1.5 mL) and was treated sequentially with *i*-Pr₂Net (250 uL, 1.46 mmol), 2-aminoethanol (88 uL, 1.46 mmol), 1-Hydroxybenzotriazole (HOBt, 197 mg, 1.46 mmol) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 279 mg, 1.46 mmol). The resulting mixture was heated to 55 °C and was stirred overnight. The reaction mixture was concentrated to dryness and purified using C18 flash chromatography to provide *N*-(2-hydroxyethyl)-2-((2-hydroxyethyl)amino)acetamide, HEHEAA. The product HEHEAA co-eluted with 1-(3-(dimethylamino)propyl)-3-ethylurea and was characterized and used in biological studies as an inseparable ca. 1:1 mixture based on ¹H-NMR and ¹³C-NMR.

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