Decoding genome-wide GadEWX-transcriptional regulatory networks reveals multifaceted cellular responses to acid stress in *Escherichia coli*

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The regulators GadE, GadW and GadX (which we refer to as GadEWX) play a critical role in the transcriptional regulation of the glutamate-dependent acid resistance (GDAR) system in *Escherichia coli* K-12 MG1655. However, the genome-wide regulatory role of GadEWX is still unknown. Here we comprehensively reconstruct the genome-wide GadEWX transcriptional regulatory network and RpoS involvement in *E. coli* K-12 MG1655 under acidic stress. Integrative data analysis reveals that GadEWX regulons consist of 45 genes in 31 transcription units and 28 of these genes were associated with RpoS-binding sites. We demonstrate that GadEWX directly and coherently regulate several proton-generating/consuming enzymes with pairs of negative-feedback loops for pH homeostasis. In addition, GadEWX regulate genes with assorted functions, including molecular chaperones, acid resistance, stress response and other regulatory activities. These results show how GadEWX simultaneously coordinate many cellular processes to produce the overall response of *E. coli* to acid stress.
Bacteria depend on maintaining pH homeostasis, as most proteins have optimal ranges of pH where they function. Diverse strategies for pH sensing and homeostasis allow bacteria to tolerate or grow at acidic external pH values. One of the most well-studied model organisms, Escherichia coli K-12 MG1655, has sophisticated acid resistance (AR) systems to survive in acidic pH environments. The entire AR system of E. coli and the complexity of its regulation have been extensively studied and well described in recent reviews –. Briefly, of the four clearly defined AR systems, AR1 is a RpoS-dependent oxidative (glucose-repressed) AR system that requires F_{1}F_{0}-ATPase, while its detailed molecular mechanism remains elusive. The other two AR systems require amino acid decarboxylation. The AR2 is a glutamate-dependent AR (GDAR) system that converts glutamate to \( \gamma \)-amino butyric acid with decarboxylases (GadA and GadB) – and imports glutamate in exchange for the \( \gamma \)-amino butyric acid by selecting among protonated substrates using a charge-based mechanism of anti-porter (GadC) –. Similarly, the AR3 and AR4 are composed of arginine decarboxylase (AdiA) with the arginine/agmatine anti-porter (AdiC) – and lysine decarboxylase (CadA) with lysine/cadaverine anti-porter (CadB) –, respectively. Consequently, this cycle pumps out cytoplasmic protons to the extracellular environment and reverses electrical membrane potential to slow proton movement into the cell, resulting in an increase of the intracellular pH to survive acidic stress. Of them, the AR2 is known to be the most effective system to allow bacteria to tolerate or grow at acidic external pH values. The entire AR system of K-12 MG1655, has sophisticated acid resistance (AR) systems to provide a comprehensive view of the coordinative genome-wide regulatory roles of these TFs under acidic stress.

**Results**

**Genome-wide binding profiles of GadEWX.** Previously, several binding sites of GadEWX have been characterized in E. coli by in vitro DNA-binding experiments and mutation analysis. However, direct measurement in vivo of GadEWX binding has not been achieved. Therefore, we employed a recently developed high-resolution ChIP-exo method to determine the in vivo genome-wide binding profiles of these three TFs with near 1-bp resolution in E. coli under acidic stress (pH 5.5).

Using a peak finding algorithm (MACE program), 16, 6 and 41 reproducible binding sites were identified for GadE, GadW and GadX, respectively, under acidic stress (Fig. 1a and Supplementary Tables 1–3). The number of binding sites of GadEWX was much lower than that of global TFs, suggesting that GadEWX may act more like local TFs to relieve acidic stress. In total, 16, 4 and 23 binding sites with multiple peaks were identified for GadE, GadW and GadX, respectively (Fig. 1b). We also found that the in vivo GadX binding sites include all of GadW binding sites as suggested in the previous comparative transcriptome study. These numbers of binding sites and their overlap suggest that GadE and GadX play broader roles than GadW and that they have distinct regulatory networks from each other. Before this study, 16 binding sites had been identified in vitro for GadEWX with strong experimental evidence, 50% (8 out of 16) of which were also detected in this study (Fig. 1c and Supplementary Table 4). It is unclear why these eight binding sites are missing from the data set obtained here. One possibility is that they are detectible only with in vitro methods and may not occur in vivo because of the interference by other regulators. Collectively, the identification of a total of 35 new binding sites was made possible by the new ChIP-exo method deployed here, significantly expanding the current knowledge of the scope of the GadEWX regulatory network.

We next assessed the widths and the genomic locations of the GadEWX-binding sites using currently available genome annotation. The widths of binding sites of each TF were 29 ± 3.6, 30 ± 7.6 and 28.7 ± 8.0, respectively (Supplementary Fig. 1). The majority of GadEWX-binding sites (88%, or 38 out of 43) were observed within regulatory regions (that is, upstream of promoters, promoters and 5′-proximal to coding regions). The remaining 12% were found in intragenic regions or between two coding regions of convergent genes (Supplementary Tables 1–3). These results show a strong preference of GadEWX-binding site location being within noncoding intergenic regions with relatively fixed binding widths. To identify DNA sequence motifs of these newly identified GadEWX-binding sites, we used the sequence of each binding peak in the motif search procedure. The identified sequence motif of GadE from 16 binding peaks (TTARGAWWWWAWAAATA) was mostly consistent with the previously characterized asymmetric GadE-binding site (TTAGGATCGTATTATTAT) (Fig. 1d). The motif search of GadW and GadX from 6 and 41 binding peaks, respectively, also yielded a sequence (AKGKCTGWTWTTWTYT) that resembled the previously reported binding motif. This same motif is due to the binding regions of GadW being overlapped with those of GadX as revealed in the previous study.

**Identification of GadEWX regulons and causal relationships.** Currently, a total of 16 genes in 10 transcription units (TUs) (GadE: 12 genes in 7 TUs; GadW: 6 genes in 3 TUs; and GadX: 10
genes in 6 TUs) have been characterized with strong evidence as members of GadEWX regulons in *E. coli* [15–17,26,28–36]. Based on our ChIP-exo data sets, we can significantly expand the size of the GadEWX regulons to comprise 45 target genes in 31 TUs (GadE: 20 genes in 15 TUs; GadW: 6 genes in 4 TUs; GadX: 29 genes in 19 TUs), several genes are co-regulated (Supplementary Tables 1–3). Functional analysis with clusters of orthologous groups (COG) categories was performed to look for any functional enrichment of GadEWX regulon genes (Supplementary Fig. 2). The COG category for amino acid metabolism and transport (E) was found to be statistically overrepresented (hypergeometric test P-value = 4 × 10^{-6}) and it includes *gadA*, *gadBC*, *gltBD* and *ybaST*, whose reactions are closely related with glutamate metabolism. Besides, a diverse range of COG functional categories indicate that GadEWX may play complicated roles beyond regulating genes in AFI to coordinate associated cellular processes in *E. coli* K-12 MG1655 as GadE has multiple roles in other pathogenic *E. coli* strains [38–40]. In addition, the *gadA* and *gadBC* genes in the GDAR system were regulated by three additional TFS (H-NS, FliZ and AdiY) [42,43]. This result represents the importance of complicated regulation of these genes according to the changes of the environments. The remaining 24 genes were not directly regulated by other TFS, meaning that GadEWX exclusively regulates these genes in response to acid stress.

To determine the causal relationships between the binding of GadEWX and changes in RNA transcript levels of genes in the GadEWX regulon, we compared transcript levels between the wild-type strain and that of each deletion mutant (Δ*gade*, Δ*gadW* and Δ*gadX*) grown under acidic stress conditions. Overall, a total of 351 genes were differentially expressed in at least one mutant. Genes with expression changes with log2 fold change ≥ 0.5 and false discovery rate < 0.01 were defined as differentially expressed. Only 25 out of 351 were differentially expressed in all mutants (Supplementary Fig. 3 and Supplementary Data 1–3). This unexpectedly small overlap in the transcriptional response suggests that each TF has a specific transcriptional response and may have a distinct role in regulating the AR system.

RpoS, one of the alternative sigma factors in *E. coli*, is known to be crucial for expression of GadEWX and GDAR as well as performing general stress management in association with EvgS/EvgA and PhoQ/PhoP [44–46]. To investigate the involvement of RpoS in regulating GadEWX regulons, we additionally performed ChIP-exo to determine the *in vivo* binding profile of RpoS under acid stress. Combining our GadEWX- and RpoS-binding maps with a GadEWX-dependent transcriptome, we could determine the causal relationships between the binding of GadEWX and the changes in transcript levels of genes in GadEWX regulons under acidic stress as well as RpoS involvement for expression (Fig. 2). Among 45 target genes identified from ChIP-exo analysis of GadEWX, we determined that 19 genes in 13 TUs (GadE, 11 genes in 7 TUs; GadX, 10 genes in 7 TUs; gadBC are overlapped)
(43%) were directly regulated by GadEWX under acidic stress (Fig. 2a, Supplementary Fig. 4 and Supplementary Tables 1–3). In addition, the expression of 62% of GadEWX regulon members (28 out of 45) was involved in the association of RpoS (Supplementary Fig. 4 and Supplementary Tables 1–3). Among the binding events of RpoS on GadEWX regulon members, 64% of them (18 out of 28) were novel (Supplementary Fig. 4 and Supplementary Tables 1–3). This result confirms the importance of RpoS recruitment for acid-stress response and expands the current knowledge of RpoS involvement in E. coli.

For example, the divergent promoters upstream of hdeA and hdeD TUs were extensively occupied by GadE and RpoS, and this GadE binding significantly increased transcript level of downstream genes under the acidic condition (Fig. 2b). Likewise, GadE acted as an activator on other promoters (Supplementary Table 1). The calculation of relative distance from GadE bindings to transcription start site showed that GadE binding sites are localized to either further upstream or downstream of the promoter region (–35 and –10 boxes) (Supplementary Table 1). This observation suggests that GadE may prevent binding of other repressors or directly activate transcription through another mechanism, such as recruiting RNAP to activate transcription. However, unlike GadE, GadX acted as a dual regulator either to activate or repress the transcription of target genes. For instance, the association of GadX and RpoS on gadB promoter increased transcript level under acidic stress (Fig. 2c). However, the binding of GadX on ydeN promoter decreased transcript level and RpoS association was not observed (Fig. 2d). Although GadW bound to the promoter regions (Supplementary Table 2), we were not able to observe significant changes in transcript levels upon gadW knockout as shown in the previous transcriptome study. Also, the remaining 59% of the genes (including GadW target genes) lacked significant changes in transcript levels despite the binding of corresponding TFs. These results indicate that the changes in their transcript levels may require additional regulatory signals such as association or dissociation of other TFs.

**Genome-wide roles of GadEWX regulons.** To determine how GadEWX coherently regulate gene expression in response to acidic stress, we reconstructed the genome-wide GadEWX regulatory network in E. coli. First, we looked into the regulatory circuit that GadEWX comprises. Although we detected bindings of each TF on either itself or other TFs as known previously, we were not able to observe changes of transcript levels by association of these TFs except for the repression of gadW by GadX (Fig. 3a). Since we compared transcript levels between wild-type and each of deletion mutant (GadE, GadW or GadX) to determine causal relationships, we are not able to examine the autoregulation of TFs. A previous study showed that the transcription of gadE is activated by GadW and GadX when cells were grown to stationary phase at pH 5.5 in LB glucose media. However, a different growth phase or growth media could have contributed to different transcriptional regulation on gadE. (Fig. 3a and Supplementary Tables 2 and 3). We believe that multiple reiterative-control circuits consisting of other TFs...
regulators still activate GadE, which is crucial in the GDAR system for AR, even if GadW or GadX is deleted. A recent kinetic study of promoters and regulators in the AR2 system claimed that GadX (gadWX double mutant) did not affect the kinetics of the promoters of core genes (gadA and gadBC) in the GDAR system. However, another comparative transcriptome analysis suggested that these promoters are significantly activated by GadX. Interestingly, our study observed that both GadE and GadX activate transcription of these core genes, but with a different activation ratio (over 50-fold activation by GadE but less than 2-fold by GadX; Supplementary Data 1 and 3). These results demonstrate that GadE is a much stronger activator than GadX and consequently GadX cannot fully compensate for the loss of activation of the GDAR system caused by gadE deletion. Similarly, physiological studies showing that the gadE mutant was more sensitive to acidic challenge than the gadX mutant also supported this observation.

The comprehensive genome-wide reconstruction of the GadEWX regulatory system in E. coli allowed us to extend the scope of its roles under acidic stress. After functional classification of 43 genes out of 45 GadEWX regulons excluding gadEW, we observed that the functions of 23% (10) of those genes were mainly involved in proton consumption, generation, and transport. Under acidic stress, only GadX-repressed genes involved in proton generation and influx such as speG (serpemide acetyltransferase) and dtpA (proton-dependent oligopeptide transporter) (Fig. 3c). On the other hand, both GadE and GadX activated several genes, such as gadA and gadB that encode enzyme consuming protons. We could not observe any proton-efflux systems regulated by GadEWX in E. coli K-12 MG1655. From this analysis, we believe that GadEWX connect any proton-efflux systems regulated by GadEWX in GadE and GadX activated several genes, such as ygeH, yhiD and stress17,49,50 (Fig. 3d and Supplementary Fig. 4). Based on genome-wide binding properties, GadX was likely to participate in the regulation of general stress response (uspA, uspB, uspC, and uspD), indicating that GadEWX may play more complex genome-wide roles in association with other TFs. Several genes with predicted or unknown functions (yjbQ, yjbR, yjjU, yjtD, yjiS, yjfb, yjal, yqeK, ygeF and ygeH) were also regulated by GadE and GadX (Fig. 3d). Recent studies revealed that expression of genes of the locus of enterocyte effacement (LEE) for pathogenesis of E. coli O157:H7 was indirectly regulated by GadE and GadX. In E. coli K-12 MG1655, we found GadX binding peaks (yqeK, ygeF and ygeH) within a remnant of a pathogenicity island (Supplementary Table 3). Since it was found that GadE and GadX indirectly regulate LEE in E. coli O157:H7 (ref. 39), investigation of genome-wide in vivo bindings of these regulators in pathogenic strains might be able to reveal regulatory cascades in LEE that cause pathogenicity. To further examine the physiological roles of GadEWX regulon members with predicted or unknown functions under acidic stress, we chose five genes (yjbQ, yjbR, yjjU, yjtD and yjiS) that either were activated or showed no causal relationship upon single TF knockout. Acid-stress resistance assays of knockout strains of these genes revealed that they were not directly related to GDAR, but three of them (yjjU, yjtD and yjiS) were implicated in general AR (Supplementary Fig. 5). This result indicates that GadEWX contribute to generate more global acid-resistance responses in E. coli beyond the regulation of the GDAR system.

Evolutionary aspects of GadEWX regulons. A recent survey on conservation analysis of the gadBC system in mostly enteric bacteria suggested that the presence and expression of this operon in pathogenic bacteria has been strongly connected to the requirement to survive under extremely acidic conditions but not necessarily in other non-pathogenic bacteria. We further expanded our investigation of how E. coli GadEWX and their entire regulons have been evolved in the proteobacteria phylum including 134 γ-proteobacteria, 40 β-proteobacteria and 58 α-proteobacteria. We first compared the conservation of the genes encoding the regulators (gadEWX). They were almost conserved in Escherichia and Shigella, but not in Salmonella, Yersinia and even further distant enterobacteria (Fig. 4). As stated in the previous studies12,51, the presence of the GDAR system (gadA, gadB and gadC) in these bacteria would allow them to survive in an extreme acidic environment (pH 2) in the presence of glutamate. Interestingly, gadX as well as gadAB are conserved in some of β- and α-proteobacteria whereas gadE and gadW are not. Probably, GadX alone would be able to regulate the GDAR system in part when GadE is absent in these classes. Genes related to the proton consumption/generation (gltBD, gcvHTP, ybaS and so on) exhibited higher conservation ratio across proteobacteria even in the species that do not have the GDAR system (Fig. 4). These genes may be regulated by other TFs and participate in controlling intracellular proton flow.

By maintaining pH homeostasis under alkaline condition, the Na\(^+\)/H\(^+\) anti-porter plays a crucial role in adaptation under alkaline challenge4. It should be noted that nhaR encoding Na\(^+\)/H\(^+\) anti-porter activator was strongly bound by GadX and RpoS under acidic stress (Supplementary Table 3). Since the deletion of GadX did not significantly affect the transcription level of nhaR, additional signal and/or TF associations may be required in regulation of this activator under acidic condition so that it can be activated only under alkaline condition. Unlike the GDAR system, it seems like the involvement of cation–proton anti-porter is a commonly used strategy for alkaline challenge across proteobacteria given the high conservation rate of nhaR (Fig. 4).

Discussion
We comprehensively reconstructed the GadEWX regulons at the genome scale in E. coli by combining genome-wide GadEWX-binding maps and GadEWX-dependent transcriptome dauner acidic stress. We identified (i) a total of 45 genes in 31 TUs that belong to GadEWX regulons and associations of RpoS for 28 of those genes; (ii) 19 genes in 13 TUs showing causal relationships upon single TF knockout; (iii) the negative-feedback loop pairs of proton transporters and utilization enzymes by GadEWX for pH homeostasis; and (iv) the additional roles of GadEWX regulatory networks beyond the GDAR system.

Using active proton transport8 is a major strategy for bacterial pH homeostasis. They include the proton-pumping respiratory chain complexes such as cytochrome bd terminal oxidase (cbo) and NADH:ubiquinone oxidoreductase (ndh). Under conditions of acid challenge, E. coli increases expression of those complexes that pump protons out of the cell. Our recently developed genome-scale model of metabolism and gene expression (ME-Model) in E. coli52 predicted that these proton-pumping complexes are more efficient for relieving the acid stress than other amino acid decarboxylase-anti-porter pairs (AR2 and AR3) (Supplementary Fig. 6). Nevertheless, AR2 and AR3 are certainly
required by cells for survival when passing through extreme acidic environments such as the stomach as they secrete cytoplasmic protons to the extracellular environment. 

Recently, two studies showed interesting observations on AR in E. coli. One study revealed that polyamines such as spermidine, putrescine and cadaverine induce multiple components in GDAR and they are important for protection against acidic stress. Surprisingly, we also found that GadX represses RpoS-involved transcription of speG (spermidine acetyltransferase), which consequently controls the polyamine concentration by degradation (Fig. 3c and Supplementary Fig. 4). This reaction also generates protons during acetylation of spermidine which agrees with our hypothesis that proton-generating enzymes will be repressed for the control of proton flow. By doing so, cells can regulate the level of intracellular proton and polyamine concentration, so that they can survive under acidic stress. The other study reported that enzymatic release of ammonia by ybaS encoding glutaminase neutralizes protons, resulting in elevated intracellular pH under acidic environment. We identified that GadW, GadX and RpoS bound to the promoter region of ybaS while we could not observe significant changes in transcript level upon single TF knockout (Supplementary Fig. 4). However, a previous microarray-based study showed that the double knockout strain (ΔgadXW) showed significant changes in transcript level of ybaS compared to wild type, indicating that transcription of ybaS can be activated in the presence of either GadW or GadX. In addition, the promoter region of gcvH encoding glycine cleavage system, which is known to release ammonia similar to ybaS, was also occupied by GadX (Supplementary Table 3). Given the properties of the ybaS system, it is plausible that this system may also contribute to AR by releasing ammonia. In summary, we have described the complex roles of the GadEWX regulatory network in E. coli under acidic stress by a systems approach that integrates various types of cutting-edge genome-scale experimental data. Future efforts on revealing the precise molecular role of small RNAs in GadEWX transcriptional regulatory networks by recently developed techniques such as ribosome profiling would expand acid-response regulatory networks including the post-transcription level. Understanding AR in microbes has important implications for prevention and clinical treatment as well as health-care applications such as probiotics and even for engineering microbes to produce organic acids. Further, similar to how the genome-scale model of E. coli metabolism with protein structures (GEM-PRO) successfully predicted thermostability of proteomes, the inclusion of structural information under acidic stress with these comprehensive operon structures into the current model would expand the predictive capability of the model on future complex phenotypes.

**Methods**

**Bacterial strains and growth conditions.** All strains used are E. coli K-12 MG1655 and its derivatives. The E. coli strains harbouring GadE-8myc, GadW-8myc and GadX-8myc were generated by a λ Red-mediated site-specific recombination system targeting C-terminal region of each gene. Deletion mutants (ΔgadX, ΔgadW, ΔgadX and other y-genes) were also constructed by a λ Red-mediated specific-recombination system. Glycerol stocks of E. coli strains were inoculated into fresh 70 ml of M9 minimal medium (47.8 mM Na2HPO4, 22 mM KH2PO4, 8.6 mM NaCl, 18.7 mM NH4Cl, 2 mM MgSO4 and 0.1 mM CaCl2) supplemented with 0.2% (w/v) glucose in 500 ml flask and cultured overnight at 37°C with 250 r.p.m. For acidic stress, the overnight cultures were inoculated into the fresh 70 ml of M9 minimal medium at pH 5.5 (adjusted with HCl) in 500 ml flask and continued to culture at 37°C with 250 r.p.m. To OD600 = 0.3 ± 0.02. At the sampling point, the pH was measured to be 5.28 ± 0.03.

**ChIP-exo.** To identify GadEWX- and RpoS-binding maps in vivo, we isolated the DNA bound to each protein from formaldehyde cross-linked E. coli cells by ChIP with the specific antibodies that specifically recognizes myc tag (9E10, Santa Cruz Biotechnology) and RpoS (1RS1, Neoclone) with 1:50 dilutions, and Dynabeads Pan Mouse IgG magnetic beads (Invitrogen) were also used to capture specific antibody followed by stringent washings. With ChIP materials (chromatin-beads), we performed on-bead enzymatic reactions of the original ChIP-exo.

**Figure 4** | Evolution of GadEWX regulons. Conservation rates of GadEWX regulons across γ-, β-, α-proteobacteria are presented based on the ortholog calculation. The genes are subdivided by their functional categories.
method with following modifications as shown in our previous study. Briefly, the sheared DNA of chromatin-beads was end-repaired, de-tailed and ligated with the first adapter (5' phosphorylated). After that, nick sites of each fragment were repaired before Lambda exonuclease and RecF exonuclease treatment. The protein-DNA crosslink was reversed by overnight incubation at 65 °C with RNAs and proteins removal. DNA samples were used to perform primer extension and second adaptor ligation. The size-selected DNA sample was amplified and quantified. Prepared DNA library was sequenced using MiSeq Illumina in accordance with the manufacturer's instructions. ChiP-exo experiments were performed in biological duplicate. Sequence reads generated from ChiP-exo were mapped onto the reference genome (NC_000913.2) using bowtie with default options with library type of dUTP RNA-seq. The RNA-seq data generated by our Cufflinks and Cuffdiff were run with default parameters to calculate fragments per kilobase of exon per million fragments and P-value determined by performing one-tailed Fisher's exact test (Hypergeometric test), and RAST output.

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