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Bacterial Homologs of Progestin and AdipoQ Receptors (PAQRs) Affect Membrane Energetics Homeostasis but Not Fluidity

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ABSTRACT Membrane potential homeostasis is essential for cell survival. Defects in membrane potential lead to pleiotropic phenotypes, consistent with the central role of membrane energetics in cell physiology. Homologs of the progestin and AdipoQ receptors (PAQRs) are conserved in multiple phyla of Bacteria and Eukarya. In eukaryotes, PAQRs are proposed to modulate membrane fluidity and fatty acid (FA) metabolism. The role of bacterial homologs has not been elucidated. Here, we use Escherichia coli and Bacillus subtilis to show that bacterial PAQR homologs, which we name “TrhA,” have a role in membrane energetics homeostasis. Using transcriptional fusions, we show that E. coli TrhA (encoded by yqfA) is part of the unsaturated fatty acid biosynthesis regulon. Fatty acid analyses and physiological assays show that a lack of TrhA in both E. coli and B. subtilis (encoded by yplQ) provokes subtle but consistent changes in membrane fatty acid profiles that do not translate to control of membrane fluidity. Instead, membrane proteomics in E. coli suggested a disrupted energy metabolism and dysregulated membrane energetics in the mutant, though it grew similarly to its parent. These changes translated into a disturbed membrane potential in the mutant relative to its parent under various growth conditions. Similar dysregulation of membrane energetics was observed in a different E. coli strain and in the distantly related B. subtilis. Together, our findings are consistent with a role for TrhA in membrane energetics homeostasis, through a mechanism that remains to be elucidated.

IMPORTANCE Eukaryotic homologs of the progestin and AdipoQ receptor family (PAQR) have been shown to regulate membrane fluidity by affecting, through unknown mechanisms, unsaturated fatty acid (FA) metabolism. The bacterial homologs studied here mediate small and consistent changes in unsaturated FA metabolism that do not seem to impact membrane fluidity but, rather, alter membrane energetics homeostasis. Together, the findings here suggest that bacterial and eukaryotic PAQRs share functions in maintaining membrane homeostasis (fluidity in eukaryotes and energetics for bacteria with TrhA homologs).

KEYWORDS bacteria, fatty acid biosynthesis, membrane energetics, membrane potential, PAQRs

Biological membranes are semipermeable barriers composed of proteins, which impart their biological functions, and lipids, which modulate biophysical properties (1). Maintaining membrane homeostasis is essential for cell survival. The lipid composition of membranes varies with cell type, organelle, and environmental conditions (2, 3). Membrane lipid composition, in particular its fatty acid moiety, imposes constraints on the physicochemical properties of the bilayer and controls membrane protein insertion, folding, and function (3, 4). Defects in membrane lipid homeostasis are associated...
with multiple cellular stresses and metabolic changes (5, 6). Organisms regulate the lipid composition of membranes using diverse mechanisms that target phospholipids and fatty acids (1, 7–9) to adjust membrane function to changes in the biophysical properties of the membrane bilayer (10).

The physicochemical properties of membranes are also essential for the maintenance of concentration and electrical gradients that comprise the membrane potential ($\Delta \psi$) and power physiological work (11). The establishment of gradients across membranes directly results from their differential permeability to ions and charged molecules. Changes in $\Delta \psi$ have been best described in excitable cells such as neurons (12). However, recent experimental evidence indicates that most "nonexcitable" animal cells (12), as well as bacteria (13–15), have a dynamic $\Delta \psi$. In bacteria, dynamic $\Delta \psi$ generates electrical signals that regulate intra- and interspecies communications in biofilms and serve as signals for mechanosensing (14, 16, 17).

Like eukaryotes, the bacterial $\Delta \psi$ is established through the activity of ion channels and transporters of monovalent/divalent cations and charged organic molecules (18–21). The bacterial transmembrane potential powers diverse physiological functions, including ATP synthesis, motility, pH homeostasis, electrical communication, resistance to antibiotics, cellular growth and division, and molecular transport (11). The transmembrane proton gradient ($\Delta p$) and the transmembrane electrical potential ($\Delta W$) comprise the proton motive force (PMF), which plays an essential role in bacterial physiology (11). Homeostasis of the PMF is maintained by altering either the $\Delta p$ or $\Delta W$; if the $\Delta W$ is collapsed, bacteria compensate for this loss by increasing $\Delta p$ (22). In both eukaryotes and bacteria, voltage-gated ion channels modulate their transport activity in response to changes in the electrical gradient across the membrane and are major regulators of the transmembrane electrochemical gradients (18, 23).

Here, we show that a membrane protein of unknown function in two Escherichia coli (encoded by yqfA) strains and the distantly related Bacillus subtilis (encoded by yplQ) modulates the ability of cells to maintain optimal (i.e., wild-type [WT]-like) membrane energetics, as reported by measuring alterations in dye fluorescence reflecting the changes in the membrane potential, which we refer to as membrane energetics homeostasis. We named this protein TrhA for “transmembrane homeostasis” protein A. TrhA is not related to voltage-gated channels or to transporters of ions, metals, or organic molecules but is related to the progestin and AdipoQ receptor (PAQR) family of proteins (24). PAQRs are conserved across multiple phyla of Bacteria and Eukarya (24). In eukaryotes, the PAQR superfamily includes three classes of membrane receptors (24). Class I PAQRs are related to the human adiponectin receptors (AdipoRs) and respond to adiponectin (25, 26). The PAQR homologs in humans and Caenorhabditis elegans function to maintain membrane fluidity and membrane fatty acid composition in response to conditions which rigidify the membrane (27–30). Class II PAQRs respond to progestereone (31–33). Class III PAQRs are phylogenetically closely related to bacterial TrhA/PAQR homologs (24). These proteins are proposed to function as hemolysin-III proteins because expression of recombinant bacterial homologs induces cytolysis of eukaryotic cells (34, 35). Results reported here implicate bacterial TrhA homologs in the maintenance of membrane energetics homeostasis, with membrane potential measurement as a reliable read-out.

RESULTS

The gene coding for TrhA is part of the E. coli FabR regulon. TrhA in E. coli (TrhAEC) is encoded by yqfA. In E. coli, several studies suggested that FabR, the repressor of unsaturated fatty acid synthesis genes, binds to the promoter of yqfA (trhAEC), in addition to the promoters of fabA and fabB (36, 37). The position of the FabR binding site relative to the transcription start site of trhAEC (38) is consistent with FabR acting as a repressor (Fig. 1A). However, the regulation of trhAEC by FabR in vivo suggested by global transcriptome analyses (39) was not confirmed in a subsequent study (36), maybe because the experiment in this later study was performed in the BW25113
strain reported to contain a mutation in fabR (39). We used transcriptional fusions with green fluorescent protein (GFP) (40) to compare the expression of trhAEC in the presence or absence of FabR in a ΔfadR genetic background to isolate the FabR regulation effects as previously described (36). The expression of trhAEC was upregulated in the ΔfabR strain as efficiently as for fabB (Fig. 1B). Mutations of three nucleotides in the FabR binding box in the promoter of trhAEC (trhAEC mut) increased trhAEC expression, while the trhAEC mut expression no longer increased in the ΔfabR mutant (Fig. 1B). Oleate, which promotes FabR repression, strongly repressed trhAEC expression, but not the trhAEC mut expression (Fig. 1C). This effect, dependent on the presence of FabR, was also observed by monitoring levels of TrhAEC-TAP protein (Fig. 1D). Taken together, these data confirm that trhAEC is regulated by FabR, suggesting that it may influence fatty acid membrane composition.

The ΔtrhAEC mutant derivative of E. coli has subtle changes in its fatty acid composition compared to E. coli WT. The eukaryotic PAQRs characterized to date are implicated in FA metabolism or modification of phospholipids to adjust membrane fluidity (27–29, 41–45). Thus, we analyzed the FA composition of E. coli membranes from WT and strains lacking the TrhA homolog (ΔtrhAEC) grown at physiological (37°C) and low (16°C) temperatures to challenge cells with membrane rigidity stress. When grown at 37°C, the ΔtrhAEC strain produced fewer long, unsaturated C18:1w7c FAs and fewer C13:0 and C13:0 hydroxy FAs than the WT (Fig. 2A). It also produced more saturated C14:0 FAs and C17:0 cyclopropanes than the WT (Fig. 2A). At 16°C, the ΔtrhAEC strain only increased its abundance of C17:0 cyclopropanes compared to the WT (Fig. 2B). Cells lacking TrhAEC thus have subtle changes in membrane FA composition compared

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**FIG 1** The gene coding for TrhA in E. coli is coregulated with genes of unsaturated fatty acid synthesis. (A) Sequence of the trhAEC promoter. The transcription start site corresponds to the one identified in reference 38. The FabR box was identified in reference 36. The mutations introduced in the FabR box are indicated below the sequence. (B) Strains ΔfadR and ΔfadRΔfabR were transformed by the plasmids containing the indicated transcriptional fusions. Relative fluorescence intensities were measured after overnight growth at 30°C in LB supplemented with kanamycin (see Materials and Methods). (C) Strains ΔfadR and ΔfadRΔfabR were transformed by the plasmids containing the indicated transcriptional fusions. Relative fluorescence intensities were measured after overnight growth at 30°C in M9 minimal medium with 0.2% glucose or 0.2% oleate as the carbon source. (D) Strains ΔfadR and ΔfadRΔfabR were transformed by the pUA-trhA-TAP plasmid. Cultures were performed at 37°C in M9 minimal medium with 0.2% glucose or 0.2% oleate as the carbon source. Total lysates were then analyzed by Western blotting using anti-TAP antibodies.
to the WT, but there were no significant differences in the proportions of phospholipid headgroups produced by either strain (Fig. 2C).

The ΔthrAEC mutant made adjustments to its FA profile similar to those of the WT (see Fig. S1A in the supplemental material). Both strains decreased their production of short, saturated FA and C17:0 cyclopropanes at 16°C compared to 37°C, and both increased production of long, unsaturated C16:1 w7c at 16°C (Fig. S1B). These findings indicate that ThrAEC has small effects on FA composition, but it is not specifically implicated in a temperature-mediated membrane rigidity stress response.

Membrane fluidity control is not the primary function of ThrAEC. Next, we used 1,6-diphenyl 1,3,5-hexatriene (DPH) to compare the membrane fluidity of E. coli WT and the ΔthrAEC strain grown at 37°C and 16°C. The fluorescence anisotropy of DPH increases in ordered membranes and decreases in fluid membranes (46). For WT, the net membrane fluidity remained constant from 37°C to 16°C (Fig. 2D), consistent with E. coli maintaining fluidity across a range of growth temperatures (47). The ΔthrAEC mutant membrane was more fluid at 37°C than at 16°C and was comparable to that of E. coli WT at 16°C (Fig. 2D). These data are consistent with the subtle effects of a ΔthrAEC mutation on membrane FA composition at this temperature (Fig. 2B) and highlight an incongruence between the membrane fluidity and FA compositions of the ΔthrAEC strain at 37°C. The ΔthrAEC strain’s membrane is expected to be more rigid than that of the WT at 37°C, given the membrane FA profiles of the mutant (i.e., fewer long,
unsaturated FAs and more saturated FAs than *E. coli* WT), but we found fluidity was increased (Fig. 2D). The primary function of TrhAEC is thus unlikely to be membrane fluidity regulation.

**Membrane proteomics support a negligible effect on membrane fluidity.** Next, we compared the membrane proteomes of *E. coli* WT and the ΔtrhAEC strain, grown at 37°C and 16°C to identify membrane-specific function(s) associated with TrhAEC. The analysis captured soluble proteins that are overly abundant and/or which interact at the membrane. At both temperatures, ΔtrhAEC had a membrane proteome with pleiotropic changes compared to the WT, suggesting a major role for TrhAEC in cell physiology (Fig. 3). A total of 301 proteins were differentially abundant in the ΔtrhAEC strain compared to *E. coli* WT and belonged to distinct functional groups based on Gene Ontology (GO-term) categories (Fig. 3 and Tables S1 and S2) (48).

Analysis of the ΔtrhAEC mutant membrane proteome did not provide evidence for a prominent role in membrane fluidity. Compared to *E. coli* WT, the ΔtrhAEC mutant produced lower abundances of FabA and FabF at 37°C (Table S1A) and slightly higher abundances of FabG at 37°C and FabH at 16°C (Table S1B). TesB, which is implicated in FA turnover, was more abundant in the ΔtrhAEC mutant at 37°C compared to *E. coli* WT (Table S1B). These changes do not correlate with the net membrane fluidity increase of the mutant at 37°C; the increase in FA elongation enzyme FabG and decrease in unsaturated FA synthesis protein FabA are expected to result in a more rigid membrane for the mutant. Altered abundances of these proteins in the ΔtrhAEC mutant were not common across the two temperatures, suggesting that other confounding factors affect FA composition and membrane fluidity in the mutant. No other proteins with known functions in FA metabolism that would suggest a role in membrane fluidity were differentially expressed. Outer membrane proteins (OMPs) and lipoproteins decreased in abundance in the ΔtrhAEC proteome compared to the WT (Fig. 3C and D and Table S1A). Relative to the WT, proteins involved in cell surface structure and membrane remodeling increased and decreased in abundance, respectively, in the ΔtrhAEC strain (Fig. 3C and D and Table S1A and B). These findings are consistent with TrhAEC exerting pleiotropic effects on homeostasis of the membrane.

**E. coli strains lacking trhAEC are impaired in motility and membrane energetics.** The most profound change in the ΔtrhAEC membrane proteome relative to *E. coli* WT was the reduction in abundance of flagellar and chemotaxis proteins (Fig. 3B and Table S1B). Compared to *E. coli* WT we also observed by light microscopy that the ΔtrhAEC mutant cells either did not swim or swam slowly in liquid media, suggesting that some flagella are present, and it did not chemotax in semisolid agar plates, consistent with impaired motility (Fig. 4A).

Membrane proteomics indicated that transporters, proton symporters, and antiporter proteins, most of which transport positively charged amino acids, divalent cations, and positively charged metals, were lower in abundance in the ΔtrhAEC strain than in the WT (Fig. 3B to D and Table S1A). For example, substrate(s) denoted FeoB (Fe2+), HisQ (lysine, arginine, ornithine, and histidine), NiK (Ni2+), ZntA (Zn2+/Cd2+/Pb2+), and ZntB (Zn2+: H+) were reduced in abundance in the ΔtrhAEC mutant relative to *E. coli* WT (Tables S1B and S2). Proteins involved in aerobic and anaerobic respiratory redox chains which pump protons, including dehydrogenases SdhD, NuoBJ, and HybA, and terminal reductases and oxidases NarGH, FrdD, and CyoA were less abundant in the mutant than in the WT (Fig. 3C and D and Table S1A). At 37°C, the mutant also reduced the abundance of three components of the ATP synthase complex (AtpDHG) (Table S1A). Consistent with these changes, the mutant produces less ATP than the WT (Fig. 4B). Proteomics thus suggest that the abundance of proteins that regulate or utilize gradients of charged molecules across the membrane, including protons, charged amino acids, metals such as iron, zinc, nickel (Table S1A), and ATP synthesis, are perturbed by the lack of TrhA. These changes are expected to affect membrane energetics.

**TrhAEC in E. coli is required for optimal (WT-like) membrane potential.** To test the role of TrhA in membrane energetics, we used two fluorescent, hydrophobic, cationic reporters, DiOC3(3) and ThT, to compare the membrane potential (Δψ) of *E. coli*
FIG 3 Membrane proteome of the ΔtrhAEC mutant reveals large changes in protein abundance, cellular physiology, and metabolism. (A) Venn diagram representing numbers of the differentially abundant proteins in the membrane proteome of ΔtrhAEC relative to E. coli WT. (B) Heat map of proteins that are found in decreased or increased abundance at both 37°C and 16°C in the membrane proteome of ΔtrhAEC relative to E. coli WT. The average log2 fold change of six biological replicates is represented. (C and D) Representation of proteins that are found in decreased abundance in the membrane proteome of ΔtrhAEC relative to WT E. coli at (C) 37°C and (D) 16°C. Proteins are functionally classified into GO-terms. (E and F) Representation of proteins that are found in increased abundance in the membrane proteome of ΔtrhAEC relative to WT E. coli at (E) 37°C and (F) 16°C.
WT and the ΔtrhAEC strain. DiOC(3) and ThT fluoresce once accumulated within polarized cells (49), but their fluorescence decreases upon membrane depolarization (49). Depolarization using carbonyl cyanide m-chlorophenyl hydrazone (CCCP) caused DiOC(3) and ThT fluorescence to decrease relative to the dimethyl sulfoxide (DMSO) controls in both WT and ΔtrhAEC strains (Fig. 5A and B). However, the resting membrane potential (Δψ) of the ΔtrhAEC mutant was significantly depolarized compared to that of the WT (Fig. 5A and B). While similar dyes have been used by others (e.g., 13–16), we note that fluorescent reporters for membrane potential are active within specific ranges (50). We expect a technical limitation in the ability to capture the absolute dynamic range of membrane potential.
Expressing parental trhAEC from a low-copy-number plasmid (pRH005) in the ΔtrhAEC strain (ΔtrhAEC[pRH005 trhAEC]) restored the mutant $\Delta \psi$ to near-WT levels (Fig. 6A). This complemented ΔtrhAEC mutant strain had a higher resting $\Delta \psi$ than the ΔtrhAEC (pRH005) mutant that was not significantly different from that of the E. coli WT (pRH005) (Fig. 6A). The motility defect of the ΔtrhAEC mutant was also partially complemented in this strain (Fig. 6B and Fig. S2A). Our attempts to perform functional complementation using inducers or different-copy-number plasmids (pTrc99a, pBAD33) failed to produce complementation (Fig. S2B and C). Poor transport of the inducers could be implicated here, given the altered membrane proteome and membrane energetics of ΔtrhAEC. The inability to transport these inducers could contribute to the minimal expression of TrhAEC from any plasmid or promoter. An alternative or additional

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**FIG 5** Cells lacking TrhAEC have a depolarized membrane. (A and B) The membrane potential of E. coli WT and the ΔtrhAEC strain was measured using (A) ThT and (B) DiOC2(3) fluorescent reporters. DMSO treatments represent the resting membrane potential of cells. The membrane potential of each strain was collapsed when treated with CCCP, as indicated by decreased reporter fluorescence. The data represent fluorescence from three biological replicates with three technical replicates. Error bars represent the standard deviation.

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**FIG 6** Functional complementation of the ΔtrhAEC strain by expressing WT trhAEC using the pRH005 plasmid. (A) Membrane potentials of E. coli WT (pRH005), ΔtrhAEC (pRH005), and ΔtrhAEC (pRH005) were measured using ThT. Data represent fluorescence from five biological replicates with three technical replicates. Error bars represent the standard deviation. a, significantly different from E. coli WT (pRH005) DMSO ($P < 0.05$); b, significantly different from ΔtrhAEC (pRH005) DMSO ($P < 0.05$); c, significantly different from ΔtrhAEC (pRH005 trhAEC) DMSO ($P < 0.05$). (B) E. coli WT (pRH005), ΔtrhAEC (pRH005), and ΔtrhAEC (pRH005 trhAEC) cells were plated on the center of a soft agar (0.3%) plate. Plates were imaged after 18 h of incubation at 28°C.
possibility is that the cellular stoichiometry of TrhAEC could also be a determining factor in its yet unknown function. Consistent with this notion, expressing pRH005 with trhAEC in the parental *E. coli* WT strain, thereby increasing the abundance of TrhAEC, produces a dominant negative phenotype (Fig. S3A to C).

The effect of TrhAEC on the mutant membrane potential compared to its parent but the lack of a measurable growth defect (Table S3) prompted us to analyze the sequenced genomes of the *E. coli* WT and ΔtrhAEC strains. None of the few genotypic changes detected in the genome of the ΔtrhAEC strain relative to the *E. coli* WT strain explained the effect of TrhAEC on the membrane proteomes or energetics (Table S4). These mutations are either commonly found in laboratory strains of *E. coli* (insB-5 and insA-5) (52), are in a pseudogene (stfe), or have a predicted function in DNA recombination (mutC). The only exception is a mutation in pgk, which encodes the glycolytic enzyme phosphoglycerate kinase (52). However, complementation of the ΔtrhAEC strain using WT pgk in pRH005 did not restore motility to the mutant (Fig. S4A) or its membrane potential compared to the WT (Fig. S4B). These results are in agreement with functional (albeit partial) complementation of the trhA mutation and the dominant negative phenotype upon overexpression of trhA described above.

**Modified metabolism and multiple stresses are associated with dysregulated membrane energetics.** Consistent with altered membrane energetics, metabolic changes were evidenced in the mutant proteome relative to the WT (Fig. 3B to F and Tables S1 and S2). The ΔtrhAEC mutant increased abundances of proteins which synthesize cofactors (cofactor annotated)—CoaA (coenzyme A), PanC (pantothenate), IspAE (isoprenoids), LipA (fatty acid anion lipoate), UbiC (ubiquinone), NadE (NAD⁺), HemHL (heme), MoeA (molybdenum), RibC (riboflavin), Thil (thiamine), and EntC (enterobactin) (Fig. 3B, E, and F and Table S1B). Enzymes involved in glycolysis, the glyoxylate shunt pathway, and the production of glyoxylate, increased in the mutant relative to the WT. Changes in abundance of proteins were implicated in amino acid and nucleotide metabolism (Fig. 3B, E, and F and Tables S1B and S2). DNA replication and modification (Fig. 3C to F and Tables S1B and S2), transcription, and translation (Fig. 3E and F and Tables S1B and S2) were affected in the mutant relative to the wild type. These changes suggest that the mutant’s metabolism is dedicated to conservation of carbon, replenishment of metabolic intermediates, turnover pool of cofactors, and remodeling of information transfer processes.

Stress resistance responses were increased in the mutant compared to *E. coli* WT. The abundance of proteins with function in DNA damage repair and iron-sulfur cluster repair as well as glutaredoxin systems which help maintain oxidative states of small redox-sensitive thiol sensor proteins (53) increased in the ΔtrhAEC mutant proteome (Fig. 3E and F and Table S1B), suggestive of upregulation of oxidative stress pathways. Reduction in the abundance of Cyo and Nuo proton-pumping complexes in the ΔtrhAEC mutant compared to the WT could suggest mild alkaline stress (Fig. 3B to D and Table S1A), as *E. coli* reduces these complexes when grown at alkaline pH (54). The abundance of several transcriptional regulators important for acid and osmotic stress responses increased in the ΔtrhAEC strain compared to *E. coli* WT (Fig. 3E and F and Table S1B). The mutant had increased abundances of EvgA, which mediates acid resistance, osmotic adaptation, and drug resistance and represses motility (55), as well as OmpR, which plays a central role in acid stress response and is involved in osmoregulation (56) (Table S1B). Additional evidence of the mutant’s increased acid stress responses included increased abundance of glutamic acid decarboxylase regulator GadW and hydrolase PxpA, which consumes ATP to form L-glutamate (52) (Table S2). The abundance of enzymes that break down amino acids to release ammonium ions, which would acidify the cytoplasm, decreased in the ΔtrhAEC mutant compared to the WT (Fig. 3C and D and Table S1A). Growth of the ΔtrhAEC mutant, despite a depolarized membrane potential relative to the WT, is thus likely at the expense of reduced metabolism, motility, and expression of acid, alkaline, osmotic, and/or oxidative stress responses. These data also support that changes in the membrane potential, as reported here with fluorescent dyes, are relevant readouts to further probe the role of TrhA.

**The ΔtrhAEC mutant was less affected than the WT under stressful growth conditions.** To test the hypotheses generated by the proteomics data described above, we compared the abilities of *E. coli* WT and the ΔtrhAEC mutant to grow under acid, alkaline, and osmotic...
stress. We did not test oxidative stress, since it is associated with acid or osmotic stresses (54). When exposed to acid stress, the ΔtrhAEC mutant resumed logarithmic growth 6 h post-shift at pH 4.5, which was shorter than the 10-h delay for E. coli WT (Fig. 7A). The ΔtrhAEC mutant grew faster than the WT at pH 4.5, and it reached a maximal optical density close to that of both the WT and ΔtrhAEC at pH 7.0 (Fig. 7A). The fast growth of the mutant at pH 4.5 correlated with an increased Δψ relative to its Δψ at pH 7.0 (Fig. 7F). This pattern was opposite to the depolarization of the E. coli WT Δψ at pH 4.5 compared to pH 7.0 (Fig. 7F). When grown under alkaline stress (pH 9.0), the ΔtrhAEC mutant had a shorter lag phase and grew faster and to a higher cell density than the WT (Fig. 7B). The maximal cell density of the mutant at alkaline pH remained low compared to growth at pH 7.0, indicating that the mutant still experienced stress, albeit not as severely as that WT (Fig. 7B). At pH 9.0, the Δψ of WT and ΔtrhAEC strains became hyperpolarized (Fig. 7G), but the increase in Δψ was greater for the ΔtrhAEC mutant strain than for the WT, consistent with the mutant’s faster growth under these conditions (Fig. 7G). The mutant lacking TrhA is not able to adjust its membrane potential to levels similar to those of the WT when challenged with conditions causing proton gradient stress. The observations that the mutant’s membrane potential is hyperpolarized under both acid and alkaline stress compared to the WT under similar conditions further suggest the that mutant is impaired in its ability to adjust membrane potential with changing conditions.

Next, we manipulated charge gradients through osmotic stress. In the presence of 0.75 M NaCl, the ΔtrhAEC strain initiated growth roughly 5.5 h post-exposure to the stress, while E. coli WT began to grow after about 7 h (Fig. 7C). With 1.0 M NaCl, the mutant showed minimal slow growth after approximately 17 h post-stress exposure, while the E. coli WT did not grow (Fig. 7C). These differences are consistent with the mutant being preacclimated to osmotic stress suggested by proteomics. High concentrations of NaCl produced a similar growth defect in the E. coli WT and the ΔtrhAEC mutant, with both strains reaching equivalent low maximum densities under these conditions, consistent with the two strains experiencing similar osmotic stress (Fig. 7C). Similar small growth advantages of the mutant relative to the WT were observed using KCl (Fig. 7D). There was no such preacclimation or growth advantage for the mutant relative to the WT when the uncharged sorbitol was used to provoke osmotic stress (Fig. 7E). These findings suggest the preacclimation of the mutant is specific to changes in the concentrations of protons or salt in the cells’ environment.

**Additional physiological changes of the ΔtrhAEC mutant support a defective Δψ.** Cells with a perturbed membrane potential have pleiotropic defects, consistent with the central role of membrane potential in cellular physiology (57). Susceptibility to some antibiotics is associated with a perturbed membrane potential (57). The ΔtrhAEC mutant was more resistant to antibiotics representing different mechanisms of action—protein (chloramphenicol and tetracycline) or cell wall (ampicillin) synthesis, membrane permeability (gramicidin D and filipin III), and membrane potential (nigericin and valinomycin) (Fig. 4C). One exception was polymyxin B, for which we did not detect any difference between the strains. The greatest differences between E. coli WT and the ΔtrhAEC strain were for membrane pore-forming antibiotics (gramicidin D) and antibiotics targeting transmembrane ion gradients (nigericin and valinomycin). Membrane potential also affects the ability of cells to form and communicate within and between biofilms (13, 14), and we found that the ΔtrhAEC strain produced roughly 1.5 times as much biofilm as E. coli WT (Fig. 4D). The pleiotropic phenotypes are consistent with TrhA’s role in membrane energetics.

**TrhA homologs in other bacteria function in membrane energetics homeostasis linked to FA metabolism.** Given the conservation of TrhA homologs in several other bacteria, we speculated that their functions should also be conserved. Comparison of the Δψ of a different strain of E. coli, UB1005, and its derivative lacking TrhA (ΔtrhAUB) using ThT and DiOC2(3) indicated the ΔtrhAUB mutant had a hyperpolarized Δψ relative to its parent, regardless of the reporter probe used (Fig. SSA and B). The hyperpolarized Δψ of the ΔtrhAUB mutant, opposite to that of the mutant derivative of MG1655, was associated with reduced growth in the presence of acid, alkaline, or salt stresses, while
sorbitol had no apparent effect (Fig. S5C to G). Therefore, the deletion of trhA in UB1005 and MG1655 yields opposite effects on growth with protons or salt stress. This pattern of behavior is consistent with a dysregulated maintenance of membrane energetics in the mutant lacking TrhA relative to their parent strains and an inability of mutant cells to establish a WT-like membrane potential under given growth conditions. We refer to this mutant phenotype as a dysregulation of membrane potential homeostasis because E. coli strains lacking TrhA experience either a membrane de- or hyper-polarization relative to their parent under various growth conditions.

FIG 7 The ΔtrhAEC mutant is preadapted to external stressors that perturb charge or ion gradients across the membrane. (A to E) E. coli WT and the ΔtrhAEC mutant cells were grown to the logarithmic phase at 37°C in LB medium at pH 7.0 and then shifted to (A) acid stress at pH 4.5, (B) alkaline stress at pH 9.0, (C) NaCl stress at 0.75 M or 1 M NaCl, (D) KCl stress at 0.5 M or 0.75 M KCl, or (E) sorbitol stress at 1 M or 1.5 M sorbitol. All growth curves are representative of the average and standard deviation of three biological replicates with three technical replicates. (F and G) The membrane potential of E. coli WT and the ΔtrhAEC mutant was measured using (F) ThT during acid stress at pH 4.5 or (G) alkaline stress at pH 9.0. All measurements represent the membrane potential measurements from three biological and three technical replicates. Error bars represent the standard deviation. a, significantly different from E. coli WT control (P < 0.05); b, significantly different from ΔtrhAEC control (P < 0.05); c, significantly different from E. coli WT stress treatment (pH 4.5 or pH 9.0) (P < 0.05).
conditions. We do not yet know what provokes this opposite effect, but it is possible that the genetic background of UB1005 (harboring additional mutations in relA and spoT genes [58]) contributes to this difference.

We also characterized the role of the TrhA homolog (encoded by the yplQ gene) in the distantly related *Bacillus subtilis* strain 168 and a mutant derivative lacking the TrhA homolog (ΔtrhA<sub>168</sub>). Using two different reporter dyes, previously used to measure Δψ of *B. subtilis* (59), we showed that the resting Δψ of *B. subtilis* WT was higher (at least 50% more) than that of the ΔtrhA<sub>168</sub> mutant (Fig. 8A and B). The findings indicate that TrhA<sub>168</sub> supports its role in membrane potential homeostasis in *B. subtilis*.

To determine if the lack of TrhA<sub>168</sub> also provoked changes in membrane FA composition, we compared the FA composition of the *B. subtilis* WT and ΔtrhA<sub>168</sub> mutant membranes and found subtle differences at 37°C versus 16°C. At 37°C, the only differences were that the ΔtrhA<sub>168</sub> mutant produced more C14:0 than *B. subtilis* WT (Fig. 8C). At 16°C, the ΔtrhA<sub>168</sub> strain produced more C14:0 and fewer C17:1 anteiso than *B. subtilis* WT (Fig. 8D). Despite these changes, the ΔtrhA<sub>168</sub> mutant made the same adjustments that *B. subtilis* WT made to its FA profile when acclimating from 37°C to 16°C (Fig. S6A and B), and net membrane fluidity was unaffected (Fig. 8E). Thus, mutants lacking trhA in *B. subtilis* and *E. coli* have similar subtle changes in their FA profiles. These changes are small yet consistent across the strains analyzed, suggesting that TrhA has some unknown, small effects on FA metabolism in both *E. coli* and *B. subtilis*.

**DISCUSSION**

Here, we present evidence that TrhA homologs, which are distantly related to eukaryotic PAQRs, are required for homeostasis of membrane energetics in two *E. coli* strains and in *B. subtilis* using defined mutants, physiological assays, and evaluation of membrane potential using fluorescent dye reporters as a read-out of membrane energetics status. The following lines of evidence support this conclusion: the bacterial mutant strains lacking TrhA homologs (i) possess a dysregulated membrane potential relative to the WT in *E. coli* and *B. subtilis* under various growth conditions, (ii) display either depolarized or hyperpolarized membrane potential relative to WT, (iii) show pleiotropic physiological changes consistent with dysregulated membrane energetics, and (iv) experience further depolarization or hyperpolarization in response to changes in proton or salt gradients in *E. coli*. Given the phylogenetic distance of *B. subtilis* and *E. coli*, control of membrane energetics homeostasis is the likely shared function of bacterial TrhA homologs.

The membrane proteomes of a ΔtrhA<sub>EC</sub> strain revealed concurrent physiological stresses and altered cellular metabolism, which suggested altered membrane energetics with dysregulated membrane potential homeostasis in the mutant relative to the WT, confirming these observations. Perturbation of the bacterial membrane potential is associated with drug potency (57), motility defects (60, 61), and an inability to achieve pH homeostasis (20). We observed each of these phenotypes for *E. coli* lacking TrhA<sub>EC</sub>. Depolarized cells have destabilized membranes, protein mistranslation, and misfolding that is not severe enough to cause cell death (17), consistent with the ΔtrhA<sub>EC</sub> mutant’s ability to grow similarly to the WT. Proteomics changes in the ΔtrhA<sub>EC</sub> mutant compared to the WT indicated that the mutant adjusts its metabolism and physiology to grow under laboratory conditions by reorganizing its metabolism to maintain essential transport and metabolic processes, minimizing transmembrane ions and metal fluxes and elevating cellular stress responses to charged molecules.

The cytoplasm of *E. coli* becomes acidified during acid and osmotic stress, and *E. coli* uses similar mechanisms to cope with these stressors (62). Altered acid, alkaline, and salt stress responses compared to parent strains were observed in both ΔtrhA<sub>EC</sub> and ΔtrhA<sub>168</sub>. Exposure to acidic pH decreases the PMF of *E. coli* and depolarizes the Δψ (63). Exposure to external high pH artificially increases the PMF of *E. coli* WT and hyperpolarizes the Δψ (64). Here, we observed similar Δψ changes for the *E. coli* MG1655 strain. However, the Δψ of ΔtrhA<sub>EC</sub> became hyperpolarized under both high
FIG 8 The TrhA homolog in *Bacillus subtilis* affects membrane potential. (A) Membrane potential measurements of *B. subtilis* WT and the ΔtrhA<sub>BS</sub> strain using ThT reporter. DMSO treatments represent the resting membrane potential of cells. The membrane potential of each strain was collapsed when treated with CCCP, as indicated by a decrease in fluorescence of ThT relative to that of the DMSO control. Data are representative of a single experiment, with one biological replicate and three technical replicates. (B) Membrane potential measurements of *B. subtilis* WT and the ΔtrhA<sub>BS</sub> mutant using DiSC<sub>3</sub>(5) reporter. Fluorescence of the polar, hydrophobic DiSC<sub>3</sub>(5) dye is quenched as it enters polarized cells. Because CCCP is incompatible for use with DiSC<sub>3</sub>(5) in this assay (58), we used gramicidin D to depolarize Δψ. Upon membrane depolarization using gramicidin, DiSC<sub>3</sub>(5) is released back into the free medium, and fluorescence is dequenched. The data are representative of a single experiment, with one biological replicate and three technical replicates. (C) Total fatty acid composition of *B. subtilis* WT and the ΔtrhA<sub>BS</sub> strain grown at 37°C. (D) Total fatty acid composition of *B. subtilis* WT and the ΔtrhA<sub>BS</sub> strain grown at 16°C. Error bars represent the standard deviation of two biological and two technical replicates. (E) Membrane fluidities of *B. subtilis* WT and the ΔtrhA<sub>BS</sub> strain grown at 37°C and 16°C. Membrane fluidity of whole cells was measured using DPH anisotropy, where higher values in anisotropy indicate a more rigid membrane. Data represent six biological replicates, where each point is the average of three technical replicates. Error bars represent the standard deviation.
and low external pH conditions. The mutant response is consistent with altered membrane potential homeostasis (i.e., WT-like) in ΔtrhAEC. Changes in external pH or salt concentrations led to reduced growth for both WT and mutant pairs. However, under these conditions, growth of ΔtrhAEC was greater than its parent, while growth of ΔtrhAUB was further reduced compared to its parent. These responses tracked the depolarized (ΔtrhAEC) or hyperpolarized (ΔtrhAUB) Δψ of these mutants compared to their parents. We surmise that low or high external pH and high salts conditions artificially further increase (ΔtrhAEC) or decrease (ΔtrhAUB) the ΔpH and/or Δψ of the mutants compared to parent strains. While we do not know how these opposite effects are produced, these responses are consistent with our hypothesis that the mutants have an altered ability to maintain membrane potential homeostasis.

We do not yet know the mechanism(s) by which TrhA modulates membrane potential homeostasis in bacteria. However, the data obtained here, combined with information available from distant eukaryotic homologs, hint at a possible mechanism(s) to be investigated in the future. Eukaryotic class I PAQRs from humans and C. elegans control the abundances of unsaturated FAs in the membrane in response to conditions that decrease membrane fluidity, such as low temperature or dietary fatty acids (27–30, 41–43, 45). Our study demonstrates that the bacterial TrhA (PAQR class III) homologs do not primarily control membrane fluidity. However, our data suggest a role for TrhA in FA metabolism. TrhAEC is part of the FabR regulon and is expressed similarly to fabAB, implying that TrhAEC is expressed under conditions where long unsaturated FAs are produced. The FA profiles of mutants lacking TrhA in both E. coli and B. subtilis have small but consistent changes relative to parent strains—reduction in abundance of long, unsaturated FAs and accumulation of short, saturated FAs. Furthermore, PAQR family proteins are found in Bacteria phyla in which membranes include ester-linked fatty acids, but not in Archaea (24) with isoprenoid lipids (65). Membrane lipids are essential to membrane protein folding and function (4). In the absence of a molecular mechanism, we cannot dissociate whether the dysregulated membrane potential which occurs with a lack of TrhA is through changes in membrane protein abundances, suggested by proteomics data for the MG1655 and mutant pair, or whether it is associated with an effect of TrhA on membrane FA composition, which was observed for both E. coli strains and B. subtilis WT and mutant pairs analyzed here. Based on the evidence here, together with the role of distantly related eukaryotic PAQRs known to date and the phylogenetic distribution of TrhA bacterial homologs, we favor the hypothesis that TrhA homologs mediate their effect on membrane potential homeostasis through their effect on unsaturated FA metabolism.

The protein sequence of bacterial TrhA homologs and of the most closely related class III eukaryotic PAQRs are significantly shorter than other eukaryotic PAQR homologs from classes I and II studied to date (24). The longer eukaryotic homologs may thus have evolved divergent functions relative to the ancestral class III homologs, such as binding ligands such as adiponectin or modulating membrane fluidity. The findings here lead us to propose that a common function of PAQR family proteins is homeostasis of the membrane (membrane fluidity or energetics) linked to fatty acid metabolism.

MATERIALS AND METHODS

Strains and media. The bacterial strains used in this study are listed in Table 1. E. coli and B. subtilis strains were grown at 37°C in Luria broth (LB), minimal M9 medium containing glucose or oleate, or on LB agar plates supplemented with antibiotics (100 µg/mL ampicillin, 34 µg/mL chloramphenicol, or 50 µg/mL kanamycin). Inducers were used at 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) (pRH005 and pTrc99a) or 1% L-arabinose (pBAD33). The ΔtrhA strain derivatives of E. coli MG1655 and UB1005 were generated by Lambda-red recombination (66). Bacillus subtilis 168 and the ΔtrhA strain were obtained from the Bacillus Genetic Stock Center (BGSC).

Plasmid construction and genome sequencing. The pUA66, pUA-trhAEC, pUA-fabA, and pUA-fabB plasmids were obtained from the library of E. coli promoters fused to GFP coding sequence (39) or constructed previously (66). PCR site-directed mutagenesis using primers trhAEC mut FabR site forward (Fwd) and trhAEC mut FabR site reverse (Rev) (Table 1) was used to mutate the FabR binding box in the trhAEC promoter. Plasmid pUA66 was modified for translational fusion by introducing the TAP tag sequence from plasmid pLJ72 (67). The sequence of trhAEC (natural promoter and coding sequence) was introduced

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TABLE 1 List of bacterial strains, plasmids, and primers used in this study

| Strain, plasmid, or primer | Relevant properties | Reference or source |
|----------------------------|--------------------|---------------------|
| **Strains**                |                    |                     |
| Escherichia coli MG1655    | F-, λ-, ilvG, rfb-50, rph-1 | 76                  |
| ΔtrhAEC                    | F-, λ-, ilvG, rfb-50, rph-1, ΔtrhAEC::Km | This work          |
| ΔfadR                      | F-, λ-, ilvG, rfb-50, rph-1, ΔfadR | 67                  |
| ΔfadRΔfadR                 | P1 transduction of ΔfadR::Km (Keio collection) in ΔfadR. Km cassette removed using pCP20 plasmid. | This work          |
| MG1655 (pRH005)            | MG1655 containing empty (pRH005) (Km, Cm) | This work          |
| ΔtrhAEC (pRH005)           | ΔtrhAEC containing empty (pRH005) (Km, Cm) | This work          |
| ΔtrhAEC (pRH005 ΔtrhAEC)   | ΔtrhAEC containing (pRH005 ΔtrhAEC) (Km, Cm) | This work          |
| MG1655 (pBAD33)            | MG1655 containing empty (pBAD33) (Cm) | This work          |
| ΔtrhAEC (pBAD33)           | ΔtrhAEC containing empty (pBAD33) (Cm) | This work          |
| ΔtrhAEC (pBAD33 ΔtrhAEC)   | ΔtrhAEC containing (pBAD33 ΔtrhAEC) (Cm) | This work          |
| MG1655 (pTrc99a)           | MG1655 containing empty (pTrc99a) (Ap) | This work          |
| ΔtrhAEC (pTrc99a)          | ΔtrhAEC containing empty (pTrc99a) (Ap) | This work          |
| ΔtrhAEC (pTrc99a ΔtrhAEC)  | ΔtrhAEC containing (pTrc99a ΔtrhAEC) (Ap) | This work          |
| Escherichia coli Top10     | General cloning strain | Invitrogen         |
| Escherichia coli UB1005    | F-, λ-, gyrA37(NalR), relA1, spoT1, metB1, λ8 | 77                  |
| Bacillus subtilis 168      | trpC2              | 78; BGSC           |
| ΔtrhAEC                    | trpC2, ΔylQ::erm   | 78; BGSC           |
| **Plasmids**               |                    |                     |
| pUA66                      | ori sc101, GFPmut2 (Km) | 40                  |
| pUA-fabB                   | fabB promoter in pUA66 | 67                  |
| pUA-fabA                   | fabA promoter in pUA66 | 67                  |
| pUA-trhAEC                 | trhAEC promoter in pUA66 | 40                  |
| pUA-trhAEC::mut            | Site-directed PCR mutagenesis in the FabR binding site on the pUA-trhAEC plasmid | This work          |
| pJL2                       | TAP tag sequence   | 68                  |
| pUA-TAP                    | TAP tag sequence in pJL72 (BamHI/EcoRV) in pUA66 (BamHI/HincII) | This work          |
| pUA-TrhAEC-TAP             | trhAEC gene (promoter and ORF) cloned in phase with TAP sequence in pEB1588(Xhol/BamHI) | This work          |
| pDONR221                   | Gateway-based cloning vector (Km) | Invitrogen         |
| pRH005                     | Gateway-based destination vector expressing proteins fused with YFP at the C terminus; ori RK2orN, (Km, Cm) | 51                  |
| pRH005 ΔtrhAEC             | pRH005 containing WT ΔtrhAEC (Km, Cm) | This work          |
| pTrc99a                    | Expression vector with inducible LacI promoter; ori ColE1/pMB1/pBR322, (Ap) | 79                  |
| pBAD33                     | Expression vector with arabinose-inducible promoter; ori pACYC184/p15A (Cm) | 80                  |
| pBAD33 ΔtrhAEC             | pBAD33 containing ΔtrhAEC (Cm) | This work          |
| **Primers**                |                    |                     |
| trhAEC Fwd                 | CACCATGTTCAAGACGCCCTC |                     |
| trhAEC Rev                 | TTACCCTGCTGCAATAATA |                     |
| UP trhAEC Xhol             | ttgtctgagAAAAATATAGCCTGACTGAAGCT |                     |
| trhAEC BamHI Rev           | cggagatccCGGCTGCCAAATATACAAATAGATC |                     |
| trhAEC::mut Fab site Fwd   | CAGAATTTCATTACCTGAAGCTTTGCTTCTCCAGAC |                     |
| trhAEC:mut Fab site Rev    | GTTCCAGTTCAACACCCCACTAGCTAAAAATAATTCTG |                     |
| GW trhAEC Fwd              | GGGGACAAAGTTTGTGACAAAAGACCGCTATGGTTGAGACGCCCTCATT |                     |
| GW trhAEC Rev              | GGGGACAAATTACCTGAAGACGCCCTCATT |                     |
| GW pgk Fwd                 | GGGGACAAATTACCTGAAGACGCCCTCATT |                     |
| GW pgk Rev                 | GGGGACAAATTACCTGAAGACGCCCTCATT |                     |
| prRH005 ccdB mut Fwd       | ATGGTTCTAAGAAGCAGGTAATGTCGAGC |                     |
| prRH005 ccdB mut Rev       | GCCCTGACATTTAAGGCTGCATTGAGACCAT |                     |
| Xbal trhAEC Fwd            | TAAGCATTAGATCTCAGAGGTTAGTATGGTT |                     |
| HindIII trhAEC Rev         | TGGTTAAGGTTTACGCGCTGCGCCAAATATA |                     |

*KM, kanamycin; Cm, chloramphenicol; Ap, ampicillin; Erm, erythromycin; BGSC, Bacillus Genetic Stock Center. The restriction enzymes used are underlined as part of the primer name, and the location of these restriction sites within the primer is underlined.

using the Xhol and BamHI sites of pUA66. Gateway technology, as per the manufacturer’s protocol (Invitrogen), was used to clone into pRH005 (S1). Cloning in the pTrc99a and pBAD33 plasmids (Table 1) was completed by restriction digestions and ligation using primers Xbal trhAEC Fwd and HindIII trhAEC Rev (Table 1). All plasmids were sequence-verified before being introduced into E. coli by transformation. The genomes of the E. coli MG1655 and ΔtrhAEC strains were sequenced at the Microbial Genome Sequencing Center (MiGS; Pittsburgh, PA, USA).
Transcriptional fusion analysis. Transcriptional GFP fusions in the ΔfadR and ΔfadRΔfabR E. coli strains were done as described in reference 67.

SDS-PAGE and Western blotting. Total cell extracts were prepared by resuspending cell pellets in Laemmli buffer 1× at a concentration of 0.3 optical density at 600 nm (OD600) in 10 μL (we did not heat, as we observed that this prevents correct migration/detection of the integral TrhA protein in SDS-PAGE), followed by electrotransfer onto nitrocellulose membranes. TrhA-TAP-tagged protein was detected with peroxidase-antiperoxidase antibodies (Sigma) and colorimetric detection with diaminobenzidine.

Fatty acid methyl ester (FAME) analysis. Whole-cell FAME analysis of E. coli and B. subtilis was done by gas chromatography-mass spectrometry (GC-MS) through Microbial ID (Newark, DE) using cultures grown at 37°C or 16°C to early stationary phases (OD600 of 1.5 to 2.0).

DPH anisotropy. The fluorescent dye DPH (1,6-diphenyl-1,3,5-hexatriene) was used to measure anisotropy, as described previously (46).

Membrane Proteomics. Membrane protein fractions were prepared as described in reference 68. Bacterial cell membrane proteins were denatured, reduced, alkylated, and enzymatically digested into peptides using previously established methods (69, 70). All samples were analyzed on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) coupled with a Proxeon EASY-nLC 1200 liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were separated on a 75-μm inner diameter microcapillary column packed with 30 cm of Kinetex C18 resin (1.7 μm, 100 Å; Phenomenex) that was heated to 60°C in a Phoenix S&T NanocL column heater. For each sample, 2-μg aliquots of peptides were loaded in buffer A (0.1% formic acid, 2% acetonitrile) and eluted with a linear 210-min organic gradient followed by a wash and column reequilibration as follows: 0% to 2% solvent B over 27 min, 2% to 25% solvent B over 148 min, 25% to 50% solvent B over 10 min, 50% to 0% solvent B over 10 min, hold at 0% solvent B for 15 min. The flow rate was kept at 200 nL/min. MS data were acquired with Thermo Xcalibur software v4.27.19 using the topN method, where N can be up to 10.

Data were processed in Proteome Discoverer v2.2.3 (Thermo Scientific) using M5 Amanda v2.0 (71) and Percolator (72). Spectral data were searched against the E. coli reference proteome database, and common laboratory contaminants and identifications were controlled at a false-discovery rate (FDR) of <1% at the peptide level. Proteins were quantified according to the sum of their peptide chromatographic areas under the curve and normalized in Proteome Discoverer by the total peptide amount. To identify differential protein abundances, hypothesis testing was performed by t tests, and adjusted P values were calculated based on the background population of proteins. Log, protein abundance differences were determined to assess the fold change. Proteins were assigned to appropriate Gene Ontology (GO) term categories using EcoCyc. These data are available in the ProteomeXchange Consortium via the MASSIVE repository (https://massive.ucsd.edu/; username, MSV000088025Reviewer; password, ecoli_membrane).

Measurement of Δψ using DiOC6(3), TnT, and Disc6(5). Colonies of E. coli and B. subtilis were grown overnight at 37°C in LB broth, reinoculated (250 μL into 3 mL of fresh LB), and shaken for 2.5 h at 37°C. Membrane potential was measured as described (59, 73).

Physiological assays. Growth of E. coli was determined in 200 μL of LB in a 96-well plate with shaking using an ELx808 absorbance microplate reader (Biotek). The specific growth rate, μ, was calculated as described (74). The ATP concentration was quantified using the BacTiter-Glo kit luminescence assay as per the manufacturer’s protocol (Promega). Biofilms were quantified as described (75).

For soft agar assays, five μL of culture was inoculated into the center of plates containing terrific broth (TB) (1.2 g/L tryptone, 10 g/L yeast extract, 0.4% glycerol [vol/vol]), 10% 1× phosphate-buffered saline (PBS) with 0.3% agar (wt/vol). Plates were incubated for 15 h at 28°C. For acid, alkaline, and osmotic stress tests, the pH of Luria Broth (LB) was adjusted to 4.5, 7.0, or 9.0 using HCl or NaOH or supplemented with 0.75 and 1 M for NaCl, 1.0 M and 1.5 M Sorbitol, and 0.5 M and 0.75 M KCl. Growth in stress conditions was determined as described above.

For MICs, cells were grown in LB to the log phase, diluted in 200 μL of fresh LB containing antibiotics at concentrations denoted in Fig. 4C, and shaken overnight using an ELx808 absorbance microplate reader (Biotek). Concentration (MIC) values were determined by the lowest antibiotic concentration added to the medium at which the growth rate was inhibited.

Statistical analyses. Statistical analyses were performed in GraphPad Prism 9. Two-tailed t tests were applied for normally distributed data with one degree of freedom. Exact n values are provided in each figure. Representative images or results are shown for soft agar motility assays and Disc6(5), where experiments were repeated at least three times.

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 6.8 MB.

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