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YehZYXW of *Escherichia coli* Is a Low-Affinity, Non-Osmoregulatory Betaine-Specific ABC Transporter

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Supporting Information

ABSTRACT: Transporter-mediated osmolyte accumulation stimulates the growth of *Escherichia coli* in high-osmolality environments. YehZYXW was predicted to be an osmoregulatory transporter because (1) osmotic and stationary phase induction of *yehZYXW* is mediated by RpoS, (2) the Yeh proteins are homologous to the components of known osmoregulatory ABC transporters (e.g., ProU of *E. coli*), and (3) YehZ models based on the structures of periplasmic betaine-binding proteins suggested that YehZ retains key betaine-binding residues. The betaines choline-O-sulfate, glycine betaine, and dimethylsulfoniopropionate bound YehZ and ProX with millimolar and micromolar affinities, respectively, as determined by equilibrium dialysis and isothermal titration calorimetry. The crystal structure of the YehZ apoprotein, determined at 1.5 Å resolution (PDB ID: 4WEP), confirmed its similarity to other betaine-binding proteins. Small and nonpolar residues in the hinge region of YehZ (e.g., Gly223) pack more closely than the corresponding residues in ProX, stabilizing the apoprotein. Betaines bound YehZ-Gly223Ser an order of magnitude more tightly than YehZ, suggesting that weak substrate binding in YehZ is at least partially due to apo state stabilization. Neither ProX nor YehZ bound proline. Assays based on osmoprotection or proline auxotrophy failed to detect YehZYXW-mediated uptake of proline, betaines, or other osmolytes. However, transport assays revealed low-affinity glycine betaine uptake, mediated by YehZYXW, that was inhibited at high salinity. Thus, YehZYXW is a betaine transporter that shares substrate specificity, but not an osmoregulatory function, with homologues like *E. coli* ProU. Other work suggests that *yehZYXW* may be an antivirulence locus whose expression promotes persistent, asymptomatic bacterial infection.

Osmolytes are compounds that promote cell survival and growth by accumulating in the cytoplasm of cells under osmotic and other abiotic stresses.[1] Four osmolyte transporters are known to confer osmotic and urea stress tolerance on *Escherichia coli*: ProP, ProVWX (also known as ProU), BetT, and BetU.[2,3] ProP is a H^+-osmolyte symporter and Major Facilitator Superfamily (MFS) member, whereas ProU is an ATP-Binding Cassette (ABC) transporter. ProP and ProU mediate osmotic stress protection by transporting overlapping arrays of zwitterionic osmolytes, including proline, glycine betaine (GB), dimethylsulfoniopropionate (DMSP), and choline-O-sulfate (COS).[2,4] In contrast, BetT and BetU are choline- and betaine-specific members of the betaine/carnitine/choline transporter (BCCT) family, respectively.[2,4] Structural and functional homologues of these systems are present in other bacteria, both Gram positive and Gram negative, and osmoregulatory systems with no ortholog in *E. coli* also exist.[5] Thus, the osmoregulatory transporters constitute an array of structurally diverse systems with a variety of energy coupling mechanisms and overlapping substrate specificities.

This diversity, and evidence that additional osmoregulatory systems may be present in uropathogenic *E. coli*,[3,6] suggested that further osmoregulatory transporters may await discovery in this organism. Ly et al. predicted that three additional putative transporters may contribute to the osmotic stress response of *E. coli*: MFS member YhjE, ABC transporter YehZYXW, and Tripartite ATP-independent Periplasmic (TRAP) transporter YiaMNO.[3] This prediction was based on sequence similarity to known osmoregulatory systems and either osmoregulated gene expression (*yhjE, yehZYXW*) or enhanced bacterial sensitivity to osmotic stress upon disruption of the encoding gene (*yiaMNO*). Here, we report efforts to identify substrates and demonstrate osmoregulatory functions for transporter YehZYXW. This system did not mediate osmoprotection despite the provision of diverse solutes. However, substrates were identified and transport activity was detected for system YehZYXW.

In other organisms, ABC transporters related to ProU and YehZYXW of *E. coli* mediate the uptake of quaternary ammonium compounds as osmoprotectants and/or for catabolism (ref 7 and references cited therein). The membrane-integral and ATP-binding subunits of YehZYXW

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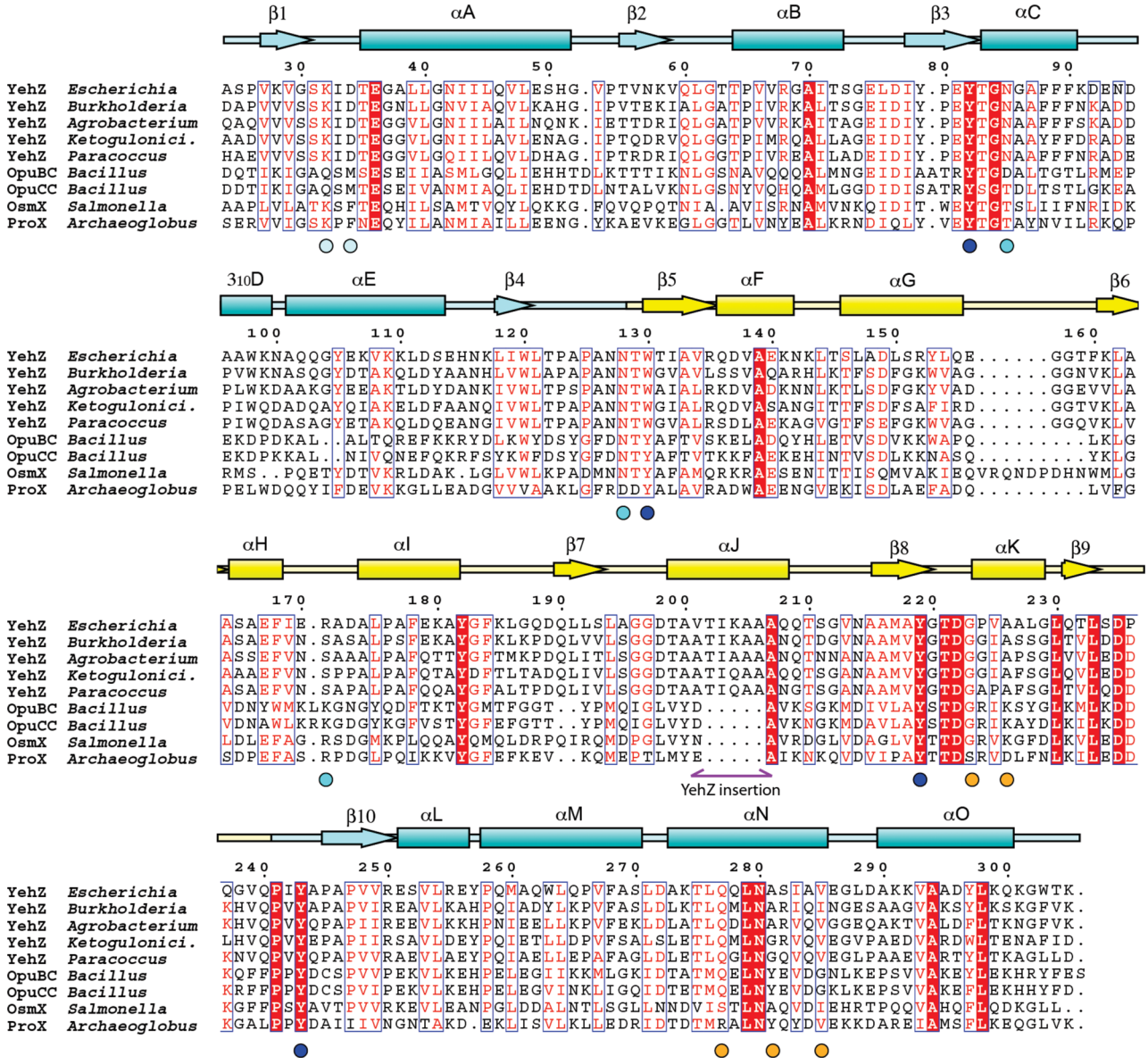

Figure 1. Multiple sequence alignment of YehZ and related proteins. Sequence of *E. coli* YehZ (GenBank: gil405862; shown from the site of cleavage of the N-terminal secretion signal) is aligned with those of related proteins, including OpuBC homologues *Burkholderia multivorans*, GenBank: gil 493440876; *Agrobacterium tumefaciens*, PDB ID: 4NE4, GenBank: gil499273328; *Ketogulonicigenium vulgare*, GenBank: gil503133803; and *Paracoccus* sp. N5, GenBank: gil516626458, as well as *Bacillus subtilis* OpuBC, GenBank: gil16080424; *Bacillus subtilis* OpuCC, GenBank: gil16080434; *Salmonella enterica* OsmX, GenBank: gil16764838; and *Archaeoglobus fulgidis* ProX, GenBank: gil11498587. Known functions of these proteins include betaine binding by those from *E. coli* (this work), *B. subtilis*,[45,46] and *A. fulgidus*[44] and osmoprotection by those from *B. subtilis*[61] and *S. enterica*.[55] The ATP-binding subunits associated with the proteins from *B. subtilis*, *S. enterica*, and *A. fulgidus* include C-terminal CBS domains, whereas the other proteins do not. Secondary structures, numbering, and colors are shown as in Figure 4. Aromatic residues that form the betaine box are marked with a blue circle; other substrate binding residues are indicated with a cyan circle. Residues that mediate key interactions between αN and αK are marked with orange circles. The YehZ-specific insertion in αJ, which packs against and buttresses αK, is marked by the purple symbol.

are particularly similar to the corresponding subunits of osmoregulatory transporter OpuC from *Bacillus subtilis*. Periplasmic substrate-binding protein YehZ shows only weak sequence identities with the corresponding subunits of osmoregulatory systems but still conserves key substrate recognition motifs (Figure 1). Homologue OpuA from *Lactococcus lactis* is the best characterized osmosensing ABC transporter; it activates with increasing osmotic pressure in both intact cells and, after purification, in proteoliposomes.[8] The osmotic activation of OpuA has been associated with dual cystathionine-β-synthase (CBS) domains that terminate the cytoplasmic C-terminus of ATP-binding protein OpuAA.[9] Chen and Beattie suggest that such CBS domains may be predictors of osmoregulatory activity for this protein family.[7] Unlike the ATP-binding subunits of the *B. subtilis* OpuC, *L. lactis* OpuA, and *E. coli* ProU systems, YehX does not include C-terminal CBS domains.[7] We therefore tested the alternative hypothesis that YehZYXW transports quaternary ammonium compounds and is inactive at high osmotic pressure.

Here, we report that YehZYXW is a low-affinity betaine transporter that differs in structure and mechanism from its osmoregulatory homologues found in *E. coli* (ProU) and other

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organisms.[10] The structure and characteristics of a gain-of-function variant suggest that the weak ligand affinity of YehZ results from a particularly stable apo state.

EXPERIMENTAL PROCEDURES

Materials. Glycine betaine (GB) and proline were obtained from Sigma-Aldrich, and dimethylsulfoniopropionate (DMSP, also known as (2-carboxyethyl) (dimethyl)sulfonium chloride was purchased from TCI America (Portland, OR). The identity and purity of these compounds were verified by nuclear magnetic resonance (NMR) spectroscopy as previously reported.[2] Choline-O-sulfate (COS) was synthesized as previously described,[11] and its structure was verified by acquiring a proton NMR spectrum using a Bruker 400 MHz spectrometer at the University of Guelph NMR Centre. ^{3}H-labeled glycine betaine (known as betaine [glycine-2-^{3}H], 1.48 × 10^{15} Bq/mol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO).

Bacterial Strains, Growth Media, and Genetic Manipulations. The *E. coli* strains and plasmids used for this study are listed in Table 1. Genetic and molecular biological manipulations were performed as described by Miller[12] or Sambrook and Russell,[13] unless otherwise indicated. Polymerase chain reaction (PCR) was performed as described by Brown and Wood.[14] Oligonucleotides were purchased from Operon Technologies (Eurofins MWG Operon, Huntsville, AL) and are listed in Table S1. Plasmid sequences were verified by DNA sequencing at the Genomics Facility, Advanced Analysis Centre, University of Guelph.

Table 1. *Escherichia coli* Strains and Plasmids[a]

strain	genotype	ref
MG1655	wild type	60
M15 pREP4	not available	Qiagen Inc.
WG1232	MG1655 Δ*proP837::FRT* Δ*betT856::FRT* Δ*otsA847::FRT*	2
WG1246	MG1655 Δ*proP837::FRT* Δ*proW859::FRT* Δ*betT856::FRT* Δ*otsA847::FRT*	2
WG1289	WG1246 Δ*yehW854::FRT*	this work
WG1314	WG1289 pDC306	this work
WG1319	WG1289 pBAD24	this work
WG389	F$^-$ *trp lacZ rpsL thi* Δ(*putPA*)*101* Δ(*proU*)*600* Δ(*proP-melAB*)*212* Δ(*brnQ phoA proC*)	33
WG1406	M15 pREP4 pMC3	this work
WG1407	M15 pREP4 pMC4	this work
WG1466	M15 pREP4 pDC311	this work

[a]Plasmid pDC306 encodes putative transporter YehZYXW. Plasmids pMC3, pMC4, and pDC311 encode proteins ProX, YehZ, and YehZ-G223S, respectively.

Unless otherwise indicated, bacteria were cultivated, at 37 °C with shaking at 200 rpm, in LB medium,[12] modified minimal medium A (MMA)[2] supplemented with $(NH_4)_2SO_4$ (7.6 mM, as nitrogen source) and glycerol (0.4% (v/v)) or glucose (0.5% (w/v)) as carbon source), or MOPS medium[15] supplemented with NH_4Cl (9.5 mM) as nitrogen source and glycerol (0.4% (v/v)) as carbon source, with thiamine (0.0001% (w/v)) and tryptophan (0.005% (w/v)) as required to meet auxotrophic requirements. Ampicillin (100 μg/mL) was added as required to maintain plasmids.

Gene *yehW* was deleted from *E. coli* WG1246 as described by Datsenko and Wanner.[16] Keio collection isolate JW2116-2 was obtained for this purpose from Dr. E.D. Brown (McMaster University). It served as donor of Δ*yehW754::kan*,[17] yielding strain WG1289 (Δ*yehW854::FRT*). To create plasmid pDC306, *yehZYXW* was PCR amplified with chromosomal DNA from *E. coli* MG1655 as template using primers yehWXYZ-us and yehWXYZ-ds (Table S1), thereby adding restriction sites (underlined). The amplicon was restricted with *Nco*I and *Hind*III, and the resulting oligonucleotide was inserted into similarly digested vector pBAD24.[18]

The genotypes and phenotypes of the *E. coli* strains were routinely verified by streaking them on the following media: LB, LB with kanamycin (50 μg/mL), tetracycline (25 μg/mL), streptomycin (100 μg/mL), chloramphenicol (30 μg/mL), or ampicillin (50 μg/mL), MacConkey agar (Difco), and proline-supplemented TTC medium[19] (TTC is 2,3,5-triphenyl tetrazolium chloride, and proline was provided at 0.2% (w/v)). Wild-type bacteria (*otsA*$^+$) grew on MOPS (3-(*N*-morpholino)propanesulfonic acid-buffered) medium supplemented with 0.5 M NaCl ,whereas *otsA*$^-$ bacteria were unable to synthesize trehalose in response to osmotic stress and did not. Radial streak tests were performed to verify other osmoregulatory phenotypes.[2] Choline or GB (20 μmol) was applied to a filter disc on MOPS medium[15] supplemented with 0.5 M NaCl, and zones of growth stimulation were scored. GB stimulated the growth of bacteria that were ProP$^+$ or ProU$^+$; choline stimulated the growth of bacteria that were BetT$^+$, ProP$^+$, or ProU$^+$. Restriction analysis was employed to confirm plasmid content, and PCR reactions with chromosomal DNA as template were performed to confirm chromosomal deletions.

Protein Overexpression and Purification. To create plasmids pMC3 and pMC4, *proX* and *yehZ* were PCR amplified with *E. coli* MG1655 DNA as template using primer pairs proXNterm with proXCterm2 and yehZNterm with yehZC-term2, respectively (Table S1), thereby adding restriction sites (underlined). Each amplicon was restricted with *Bam*HI and *Bgl*II, and the resulting oligonucleotides were inserted into similarly restricted vector pQE60 (Qiagen, Inc.). The resulting plasmids were each transformed into *E. coli* M15 pREP4 (Qiagen, Inc.), creating strains WG1406 and WG1407 (Table 1). Plasmid pDC311 was created by site-directed mutagenesis of plasmid pMC4 using primers yehZ-G223S and yehZ-G223S-r as previously described.[20]

For batch purification of YehZ-His$_6$ or YehZ-G223S-His$_6$, LB (20 mL) supplemented with ampicillin (100 μg/mL) and kanamycin (25 μg/mL) was inoculated with *E. coli* WG1407 (M15 pREP4 pMC4) or WG1466 (M15 pRep4 pDC311), respectively, and incubated overnight at 37 °C with vigorous shaking (200 rpm). The overnight culture was diluted (1:50) into LB (1 L) supplemented with ampicillin (100 μg/mL) and kanamycin (25 μg/mL), and the subculture was incubated until the optical density at 600 nm reached 0.5−0.6 (Pharmacia Novaspec spectrometer). YehZ-His$_6$ expression was then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM), and the culture was incubated overnight at 15 °C with vigorous shaking (200 rpm). The cells were harvested by centrifugation (Beckman Coulter JLA 8.100 rotor, 8000 rpm at 4 °C for 20 min). The resulting pellet was weighed before proceeding with the purification.

To purify YehZ-His$_6$ or YehZ-G223S-His$_6$, cell pellets were resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride, 2 mM β-mercaptoethanol) supplemented with magnesium sulfate (5 mM), deoxyribonuclease (30 μg/mL), and phenylmethylsulfonyl fluoride (0.5 mM) at 5

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mL per gram of wet weight. Cells were disrupted by two or three passes through a French pressure cell at an internal pressure of 1000 psi. The lysate was centrifuged in the Beckman Coulter JA25.5 rotor at 8000 rpm at 4 °C for 20 min. The supernatant derived from six 1 L cell cultures was incubated for 1 h at 4 °C with 2.5 mL of HisPur Ni-NTA superflow chromatography beads per liter of culture (Pierce Biotechnology Inc.) that had been washed with lysis buffer supplemented with 10 mM imidazole. The chromatography medium was loaded into four Econo-Pac chromatography columns (Bio-Rad 1.5 × 12 cm), and the flow through was collected. The columns were washed with at least 10 bed volumes of wash buffer 1 (lysis buffer containing 10 mM imidazole) and 10 bed volumes of wash buffer 2 (lysis buffer supplemented with 20 mM imidazole). YehZ-His_6 or YehZ-G223S-His_6 was eluted with 20 mL of lysis buffer supplemented with 200 mM imidazole. Approximately ten 2 mL fractions were collected per column. Protein-containing fractions (detected by Bradford assay[21]) were pooled and stored at 4 °C. The purified protein was concentrated with an Amicon Ultra-15 10 K centrifugal filtration device and dialyzed against 10 mM K phosphate, pH 7.4. Glycerol was added (20% (w/v)), and the protein was stored at −80 °C.

ProX-His_6 was overexpressed and purified in the same way except that the final subculture was incubated for 4 h at 37 °C.

Protein concentrations were determined with the bicinchoninic acid (BCA) assay[22] using bovine serum albumin (BSA) as standard (absorbance values measured at 595 nm using a Titertek Multispek Plus spectrophotometer) or by measuring the absorbance at 280 nm with a Cary 100 Bio UV−visible spectrophotometer and applying extinction coefficients calculated by ProtParam:[23] 40 910 M^{-1} cm^{-1} for YehZ-His_6 and 64 860 M^{-1} cm^{-1} for ProX-His_6. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli[24] using gels prepared with 12% (w/v) acrylamide and 1.1% (w/v) bis-acrylamide and stained with GelCode Blue (Thermo Scientific) reagent. Intact protein molecular weights were determined using an Agilent UHD 6530 Q-ToF electrospray mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph. The instrument was configured with the standard ESI (electrospray ionization) source and operated in positive-ion mode. Data analysis was performed using the MassHunter Qualitative Analysis, version B.06.00 (Agilent), software. Deconvolution of the m/z spectrum was achieved using the Maximum Entropy algorithm within BioConfirm software (Agilent).

Structure Determination and Refinement. Crystals of apo YehZ were grown in a sitting drop configuration using 20 mg/mL YehZ in a solution of 25% (w/v) PEG 3350 with 0.1 M citric acid, pH 3.5, as the well solution. Crystals grew as clusters of thin plates, with individual plates up to 300 μm in diameter. Crystals were frozen after immersion in paratone N oil to remove surface water. Data was collected at the Canadian Light Source 08 ID. Crystals diffracted to 1.5 Å and proved to be of the orthorhombic space group $P2_12_12_1$. Data was processed in XDS and scaled in XSCALE.[25] Molecular replacement was undertaken in Phaser,[26] using a single monomer of the unpublished structure of *A. tumefaciens* protein Atu0199 (PDB ID: 4NE4). Model rebuilding was undertaken in Coot,[27] and refinement, in Phenix.[28] The structure proved to have two copies in the asymmetric unit, with chain A modeled from residue 24 to 304 (i.e., with the first two residues of the construct disordered; numbering includes the first 22 residues, which form the secretion signal) and chain B modeled from 24 to 305 with the first residue of the C-terminal hexahistidine tag (Arg305) also modeled. The two chains in the asymmetric unit can be superimposed with an rmsd of 0.446 Å. The structure of the YehZ glycine betaine complex was modeled in Swiss-Model[29] using the structure of the ProX glycine betaine complex (PDB ID: 1SW2) as a template. Structure figures were prepared in PyMOL.

Binding Assays. Ligand binding to purified ProX-His_6, YehZ-His_6, and YehZ-G223S-His_6 was examined by equilibrium dialysis, fluorescence spectroscopy, and isothermal titration calorimetry.

Intrinsic protein fluorescence spectra were recorded at wavelengths from 300 to 500 nm using a Photon Technology International (PTI) spectrofluorimeter with 2 nm excitation and emission slit widths. Excitation wavelengths of 275 and 290 nm were used to favor tyrosine and tryptophan fluorescence, respectively. A quartz cuvette containing 50 μg/mL protein in 50 mM K phosphate, 0.5 mM EDTA, pH 7.4, was incubated with or without a putative ligand (2 mM) for 10 min at 22 °C before each spectrum was acquired. Photobleaching precluded sequential ligand addition to the same cuvette. The PTI FeliX32 computer software program was used to collect all fluorescence data. The buffer and buffer plus substrate controls were subtracted from corresponding fluorescence data.

Isothermal titration calorimetry (ITC) was performed with a Microcal VP-ITC calorimeter. Experiments were performed in duplicate under the following instrumental parameters: temperature, 30 °C; reference power, 15 μcal/s; spacing between injections, 180 s; injection volume, 3 μL first injection, 10 μL for the remaining injections. Proteins were dialyzed into 10 mM K_2HPO_4, pH 7.4, and ligands were prepared in the corresponding dialysate. Control titrations of buffer−buffer and ligand−buffer were collected, all using the same parameters, after all protein titrations were completed. Each interaction was examined at least twice, yielding consistent parameters, and in some cases, the protein concentration was adjusted to optimize the second measurement. Data were analyzed by first subtracting the appropriate control titration and then fitting to a one set of sites model in Origin 7.0. Data were also analyzed with a fixed binding stoichiometry (n) of 1, but the resulting fits were not meaningful.

Equilibrium dialysis experiments were performed with 96-well equilibrium dialyzers (Harvard Apparatus, Holliston, MA). Eight sample compartments were separated from eight assay compartments by a membrane with a molecular weight cutoff of 10 000 Da. Samples in 0.1 M potassium phosphate, pH 7.4, were equilibrated at room temperature for 24 h. For ProX, the sample compartments were filled with ProX-His_6 (200 μL, 1.92 μM) and the assay compartments were filled with 200 μL of ^{3}H-glycine betaine (740 GBq/mol) at the specified concentrations. Samples (3 × 50 μL) from each compartment were collected and transferred to vials containing 5 mL of Filtron X scintillation fluid (National Diagnostics, Altlanta, GA) for counting on a Beckman LS6500 scintillation counter. For DMSP inhibition studies, the sample compartments were filled with ProX-His_6 (200 μL, 1.92 μM) and the assay compartments were filled with 200 μL of a solution containing ^{3}H-glycine betaine (4 μM) and unlabeled DMSP at the specified concentrations. For proline inhibition studies, the sample compartments were filled with ProX-His_6 (250 μL, 1.92 μM) and the assay compartments were filled with 250 μL of a solution containing ^{3}H-glycine betaine (4 μM) and unlabeled

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proline at the following concentrations (mM): 0, 10, 20, 50, 100.

For YehZ, the sample compartments were filled with YehZ-His_6 (100 μL, 55 mg/mL (1.8 mM)) and the assay compartments were filled with 100 μL ^{3}H-glycine betaine (1.85 GBq/mol) at the specified concentrations. The radioactivity in samples (3 × 25 μL) from each compartment was measured as described above. For these assays, which required high concentrations of both protein and ligand, a significant fraction of assay mixture volume was expected to be occupied by YehZ-His_6 and hence excluded from occupancy by GB.[30] This volume exclusion was expected to offset the evidence of GB binding to YehZ-His_6. Bovine serum albumin (BSA) does not bind GB.[31] With the assumption that the specific volumes of YehZ-His_6 and BSA would be similar, BSA was used at equivalent mass concentration to estimate the contribution of volume occupancy to the GB binding assay. Sample compartments were filled with BSA (Sigma, purified as described by Courtenay et al.;[32] 200 μL, 55 mg/mL), and the assay compartments were filled with 200 μL of ^{3}H-glycine betaine (1.85 GBq/mol) at the specified concentrations. Samples (3 × 25 μL) were taken from each compartment, and radioactivity was measured as described above for YehZ-His_6. The regression line representing the linear dependence of GB exclusion on GB concentration was used as a standard curve to compute the quantity of GB that would have been present in each YehZ-His_6 assay sample if volume exclusion had not occurred.

Functional Assays. Tests for YehZYXW function included an osmoprotection assay, a proline auxotrophy assay, and a radioisotope-based glycine betaine uptake assay.

Microtiter plate-based osmoprotection assays were conducted essentially as described by Murdock et al.[2] Bacteria were cultivated in 2 mL of LB medium at 37 °C for approximately 8 h, subcultured (1/50 dilution) into 2 mL of glycerol-supplemented MMA for approximately 16 h, then subcultured to an optical density (600 nm) of 0.2 (Bausch and Lomb Spectronic 88 spectrometer) or 0.1 (Pharmacia Novaspec spectrometer) in glycerol-supplemented MMA without or with L-arabinose (0.2% (w/v)), and grown for 1−2 h. This subculture (180 μL) was added to the wells of a microtiter plate that had been loaded with NaCl and mixtures of putative osmoprotectants (listed in Table S2) to attain a final volume of 200 μL. NaCl was added to 0.5 M, and the putative osmoprotectants, which included γ-aminobutyric acid (GABA), γ-butyrobetaine, D-carnitine, L-carnitine, choline, COS, *N*,*N*-dimethylglycine, DMSP, ectoine, GB, hydroxyectoine, hypotaurine, myo-inositol, pipecolate, proline, sarcosine, sorbitol, taurine, thiaproline, TMAO, and trigonelline, were added to 1 or 10 mM. As illustrated in Table S3, there were six replicate samples/wells for the positive control strain (WG1232) and the test strain (WG1314) and four replicate samples/wells for the negative control strain (WG1319), and each experiment was performed twice. The plate was covered with a Breathe-Easy sealing membrane (Sigma-Aldrich) and incubated, with shaking, at the indicated temperature. The optical density was measured repeatedly at 600 nm with a PerkinElmer Victor^{3}V multilabel plate reader (99 readings with 10 min of elliptical, 0.1 mm diameter shaking between readings over a period of approximately 19 h) or once, after 19 or 40 h, at 595 nm with a Titertek Multiskan Plus spectrophotometer.

A test for proline uptake activity based on proline auxotrophy was performed as previously described.[33] Briefly, *E. coli* WG389 lacks the known proline uptake systems of *E. coli* (Δ(*putPA*) *101* Δ(*proU*)*600* Δ(*proP-melAB*)212) and is also proline auxotrophic (Δ(*brnQ phoA proC*)). Strain WG389 can grow in media supplemented with proline at a high concentration (25 mM) but not in proline-free media or media supplemented with proline at a low concentration (25 μM). Introduction of a plasmid encoding a proline transporter restores growth at low proline concentration to this strain. Plasmids encoding known or putative transporters and the corresponding vector were introduced separately to WG389, and the ability of the resulting bacteria to grow on MOPS medium supplemented with no proline, 25 μM proline, or 25 mM proline was tested.

For measurements of the uptake of radiolabeled GB, bacteria were cultivated in MOPS medium[15] without or with arabinose at the indicated concentrations. GB uptake by whole cells was measured with a filtration assay as described[34] with the following modifications. Bacteria were harvested, washed twice, and resuspended in MOPS medium[15] without organic supplements (unsupplemented MOPS). Assay mixtures were composed of unsupplemented MOPS medium plus 0.2% (w/v) glucose and 0.8 mg/mL chloramphenicol. Cells introduced to the assay mixture were incubated at 25 °C for 3 min before GB was added. Samples were applied to 0.45 μm pore size HA filters (Millipore, Billerica, MA) at the indicated intervals. The wet filters were dissolved in 5 mL of Filtron X scintillation fluid (National Diagnostics) before counting with a Beckman LS6500 scintillation counter. Uptake was a linear function of time for each assay described below, and uptake rates were determined by linear regression.

To measure the time course of GB uptake, the cell suspension (50 μL) was added to a 910 μL assay mixture. Transport was initiated by adding 40 μL of ^{3}H-labeled GB (37 GBq/mol) to attain a final concentration of 4 mM. Samples (100 μL) were filtered at the indicated times and immediately washed with 5 mL of unsupplemented MOPS medium. To measure rates of GB uptake at various salinities, the cell suspension (25 μL) was added to a 455 μL assay mixture containing no NaCl, 0.25 M NaCl, or 0.50 M NaCl. Transport was initiated by adding 20 μL of ^{3}H-labeled GB (37 GBq/mol) to attain a final concentration of 4 mM. Samples (150 μL) were applied to filters after 3, 6, and 9 min and immediately washed with 5 mL of unsupplemented MOPS medium adjusted to the corresponding salinity. To measure initial rates of GB uptake at various GB concentrations, the cell suspension (25 μL) was added to a 455 μL assay mixture. Transport was initiated by adding 20 μL of ^{3}H-labeled GB (185 GBq/mol) to attain the indicated concentrations. Samples (150 μL) were applied to filters after 30, 60, and 90 s and immediately washed with 5 mL of unsupplemented MOPS.

RESULTS

YehZYXW Did Not Mediate Osmotic Stress Protection. To determine whether YehZYXW could provide osmotic stress protection, the *yehZYXW* operon was placed under the control of the L-arabinose-inducible *araBAD* promoter in expression vector pBAD24.[18] The resulting plasmid was introduced to an *E. coli* strain lacking all known osmolyte accumulation mechanisms (OtsA, BetT, ProP, and ProU) and the membrane-integral component YehW; see Experimental Procedures and Table 1). No YehZYXW-dependent growth stimulation was observed when these bacteria were cultivated without or with arabinose (0.2% (w/v)) at 18 or 37 °C and with the diverse osmolytes listed in Experimental Procedures (each provided at concentrations of 1 and 10 mM).

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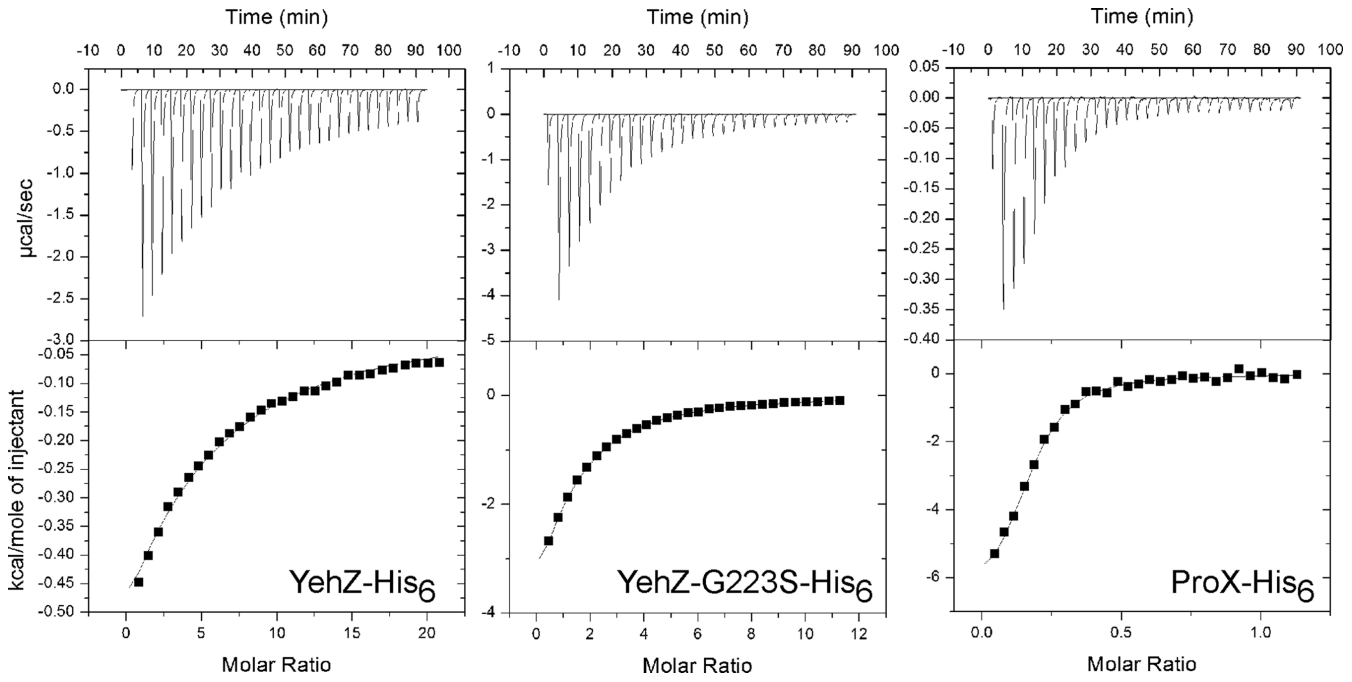

Figure 2. Detection of glycine betaine (GB) binding to YehZ-His_6, YehZ-G223S-His_6, and ProX-His_6: isothermal titration calorimetry. ITC was performed as described in Experimental Procedures. The figure shows representative thermograms for the binding of glycine betaine to YehZ-His_6, YehZ-G223S-His_6, and ProX-His_6. The resulting parameters are summarized in Table 2.

Table 2. Ligand Binding to YehZ-His_6, YehZ-G223S-His_6, and ProX-His_6[a]

protein (μM)		ligand (mM)		K_a (M^{-1})	K_d (M)	ΔH (kcal/mol)	ΔS (cal/mol °K)	n
YehZ-His_6	217	COS	20	$(3.3 \pm 0.3) \times 10^2$	3.07×10^{-3}	-2.1 ± 0.5	4.44	3.7 ± 0.8
	217	DMSP	20	$(3.3 \pm 0.4) \times 10^2$	3.06×10^{-3}	-1.0 ± 0.2	8.25	5.5 ± 0.9
	217	GB	20	$(5.0 \pm 0.6) \times 10^2$	1.99×10^{-3}	-2.1 ± 0.7	5.54	2.6 ± 0.8
YehZ-G223S-His_6	100	COS	5	$(4.28 \pm 0.06) \times 10^3$	2.34×10^{-4}	-12.3 ± 0.4	−24.1	0.90 ± 0.03
	100	DMSP	5	$(3.04 \pm 0.08) \times 10^3$	3.29×10^{-4}	-8.6 ± 0.7	−12.6	0.99 ± 0.07
	100	GB	5	$(5.56 \pm 0.08) \times 10^3$	1.80×10^{-4}	-8.6 ± 0.2	−11.4	0.99 ± 0.02
ProX-His_6	250	DMSP	5	$(9.2 \pm 0.9) \times 10^3$	1.09×10^{-4}	-0.6 ± 0.1	16.3	0.38 ± 0.04
	20	GB	0.2	$(7.8 \pm 1.1) \times 10^5$	1.3×10^{-6}	-6.7 ± 0.4	4.71	0.20 ± 0.01

[a]Binding was measured by isothermal titration calorimetry, as described in Experimental Procedures. The ligands were choline-O-sulfate (COS), dimethylsulfoniopropionate (DMSP), and glycine betaine (GB). Representative data are shown.

Representative data are shown in Table S3. *E. coli* strain WG1232, which retains system ProU, was employed as a positive control for these experiments. ProU-dependent growth stimulation was as previously reported by Murdock et al.[2]

A proline auxotrophy-based uptake assay[33] also failed to detect proline uptake via system YehZYXW (see Experimental Procedures). As expected, proline uptake-deficient, proline auxotrophic bacteria harboring plasmid pDC79 (encoding ProP) could grow with 25 μM or 25 mM proline.[33] Like those harboring vector pBAD24, bacteria harboring plasmid pDC306 (encoding YehZYXW) could grow when provided with 25 mM proline but not when provided with only 25 μM proline.

It was possible that none of the offered compounds were substrates for YehZYXW. However, some homologues of system YehZYXW transport betaines and are not osmoregulatory.[35−37] We therefore pursued the alternative hypothesis that YehZ is the periplasmic binding protein component of a betaine transporter that is inactivated at high salinity.

YehZ Is a Betaine Binding Protein. To identify potential YehZYXW substrates, ligand binding to YehZ was compared with ligand binding to ProX, the periplasmic binding protein component of the *E. coli* ProU system. ProX served as a positive control for these experiments, having bound GB with a K_d of 0.7 μM when a dialysis assay was applied to the purified protein.[38] C-Terminally histidine-tagged variants of *E. coli* proteins YehZ and ProX were purified. Analysis by Q-ToF (Quadrupole time of flight) electrospray mass spectrometry revealed masses for the purified proteins of 31270.9 and 34,791.1, respectively, indicating that the N-terminal signal sequences were removed and that the C-terminal hexahistidine tags were retained, as expected (the predicted masses were 31270.3 and 34792.9 respectively) (Figure S1). Deamidation of asparagine or glutamine may account for the difference between the expected and observed masses for ProX-His_6.

Neither known nor putative transporter substrates perturbed the steady-state intrinsic tyrosine or tryptophan fluorescence spectra of these proteins (data not shown; see Experimental Procedures). The tested ligands were proline, GB, choline, COS, and trimethylamine-*N*-oxide (TMAO). However, ligand binding to both proteins was detected by isothermal titration calorimetry (ITC) (Figure 2 and Table 2). COS, DMSP, and

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GB bound YehZ-His$_6$ with millimolar affinities. GB bound ProX-His$_6$ with micromolar affinity, as reported before,[38] whereas DMSP bound ProX-His$_6$ with 100-fold lower affinity than that of GB. Proline did not bind to either protein. Thus, we corroborated previous evidence that ProX does not bind proline even though ProU mediates osmoprotection by proline.[2,39,40]

Ligand binding stoichiometries of 1:1 are commonly observed for periplasmic binding proteins. A stoichiometry of 1:1 was assumed for previous work on ProX[38] and corroborated when subsequent crystal structures were obtained for ProX and its homologues bound to a variety of ligands (e.g., Schiefner et al.[41]). Stoichiometries significantly greater than 1 were obtained by ITC for the low-affinity interactions of YehZ-His$_6$ with COS, GB, and DMSP, whereas stoichiometries significantly less than 1 were obtained for interactions of ProX-His$_6$ with GB and DMSP (Table 2). The quality of the ITC data was inevitably limited by protein solubility. For example, a YehZ-His$_6$ concentration in excess of 10 mM (approximately 0.3 g/mL) would be required to optimize characterization of its interaction with GB. An alternative technique was implemented, therefore, to study the interactions of GB with the same protein preparations.

Measurements performed by equilibrium dialysis corroborated the evidence that GB is a ligand for both YehZ-His$_6$ and ProX-His$_6$ and confirmed that the affinities of YehZ-His$_6$ and ProX-His$_6$ differed 1000-fold (Figure 3). Both binding and volume occupancy were expected to contribute to the net interaction of GB with YehZ-His$_6$ in this assay, which required high concentrations of both protein and ligand.[30] BSA is a globular protein that does not bind GB,[31] so it was used as described in Experimental Procedures to estimate the contribution of volume occupancy to these measurements (open circles, Figure 3A). The systematically negative binding values confirmed the significance of volume exclusion in this system.[31] As expected, the BSA data fit a straight line with slope -2.45 ± 0.08 nmol mg^{-1} mM^{-1} and intercept 0.26 ± 0.27 nmol mg^{-1}. They were used to correct the YehZ-His$_6$ data, with the assumption that the specific volumes of BSA and YehZ-His$_6$ are similar. The corrected YehZ-His$_6$ data fit a rectangular hyperbola with asymptote 25 ± 2 nmol GB/mg YehZ-His$_6$, equivalent to a binding stoichiometry of 0.78 GB/YehZ-His$_6$. According to this analysis, the K_d for GB binding to YehZ-His$_6$ was 2.7 ± 0.5 mM, which is in good agreement with the values obtained by ITC.

Equilibrium dialysis measurements also confirmed an earlier report[40] and the ITC data discussed above (Figure 2 and Table 2) by showing that GB and ProX-His$_6$ interact with a K_d of 1.4 ± 0.1 μM (Figure 3B). DMSP weakly inhibited GB binding to ProX-His$_6$, (I_{50} 1.3 ± 0.2 mM; Figure 3B, inset), whereas proline did not (see Experimental Procedures; data not shown). With the measured K_d of ProX-His$_6$ for glycine betaine, these data yielded a K_d for DMSP of 0.3 mM,[42] comparable in magnitude to the interaction detected by ITC (Figure 2 and Table 2).

The Structure of YehZ. The X-ray structure of apo-YehZ-His$_6$ was determined by molecular replacement at a resolution of 1.5 Å and deposited with PDB ID: 4WEP (Table 3). This structure closely resembles that of ProX from *Archaeoglobus fulgidis* (PDB ID: 1SW5), with a type II periplasmic binding protein fold, where two domains are connected by two loops (Figure 4A). Domain A is built from the N- (25−128) and C-terminal (241−305) ends of the polypeptide chain, whereas

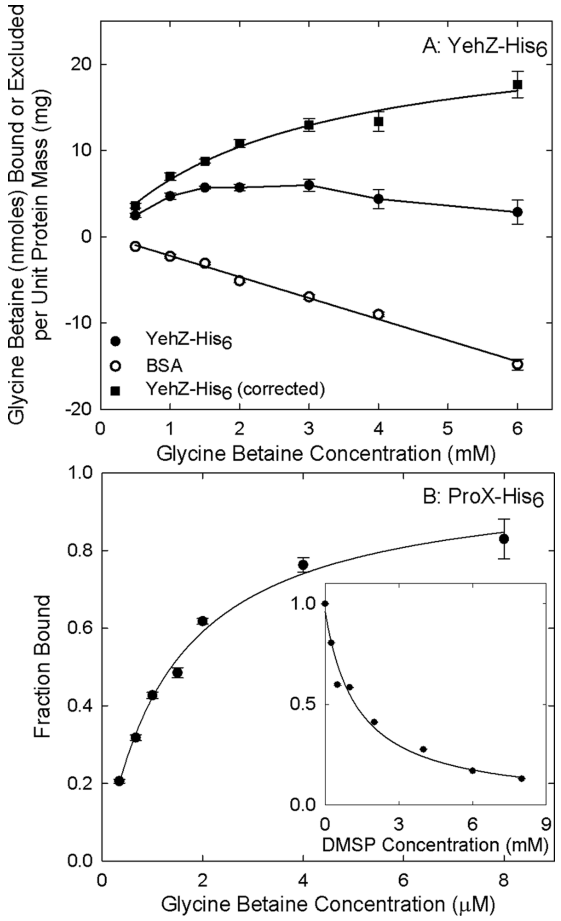

Figure 3. Detection of ^{3}H-glycine betaine (GB) binding to YehZ-His$_6$ and ProX-His$_6$: equilibrium dialysis. Equilibrium dialysis was performed as described in Experimental Procedures. (A) The interactions of glycine betaine with YehZ-His$_6$ and bovine serum albumin (BSA) are compared. The data showing preferential exclusion of GB from BSA fit the illustrated straight line with slope -2.45 ± 0.08 nmol mg^{-1} mM^{-1} and intercept 0.26 ± 0.27 nmol mg^{-1}. Assuming the specific volumes of BSA and YehZ-His$_6$ to be similar, the BSA data were used to correct the binding data for YehZ-His$_6$. The corrected data fit the illustrated rectangular hyperbola with asymptote 25 ± 0.2 nmol GB/mg protein and K_d 2.7 ± 0.5 mM. This asymptote would be 32 nmol GB/mg protein if the binding stoichiometry was 1:1 and all protein bound ligand. (B) GB binding to ProX-His$_6$, plotted as the fraction of ProX-His$_6$ molecules occupied by GB. The data fit the illustrated binding isotherm with a stoichiometry of 0.99 ± 0.03 and a K_d of 1.4 ± 0.1 μM. Inset to (B): DMSP inhibition of GB binding to ProX-His$_6$. The data fit the illustrated inhibition isotherm with a stoichiometry of 0.96 ± 0.04 and a K_d of 1.3 ± 0.2 μM.

domain B contains the central part of the protein (129−240). Each domain is built as a five-stranded mixed β-sheet, with α-helices arrayed on either face. Searching the PDB with DALI lite,[43] the structure of *E. coli* YehZ most closely resembles the unpublished structure of *Agrobacterium tumefaciens* protein Atu0199 (PDB ID: 4NE4; Z-score 44.2, 56% sequence identity). YehZ also resembles the following periplasmic binding proteins from known and putative betaine transporters, all with Z-scores greater than 25: ProX from *Archaeoglobus fulgidis* (PDB ID: 1SW5),[44] OpuCC from *Staphylococcus aureus* (PDB ID: 3O66) and *B. subtilis* (PDB ID: 3PPN),[45] and OpuBC from *B. subtilis* (PDB ID: 3R6U).[46]

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Table 3. Data Collection, Model Refinement, and Final Structure Statistics

crystallographic data collection statistics	
space group	$P2_12_12_1$
wavelength (Å)	0.97949
resolution (Å)	1.50
unique reflections[a]	80 739 (5869)
redundancy[a]	7.0 (5.0)
completeness (last shell)[a]	1.00 (0.98)
$\langle I/\sigma(I)\rangle$ (last shell)[a]	15.7 (1.7)
R_{sym} (last shell)[a]	0.085 (0.938)
X-ray structure refinement statistics	
R_{cryst}	0.1692
R_{free}[b]	0.1926
Asymmetric Unit Contents	
protein chains	2
water molecules	671
Average ADPs (Å^2)	
protein	21.0
water	34.6
rmsd bond lengths (Å)	0.005
rmsd bond angles (deg)	0.954
Ramachandran favored (%)	98.6
Ramachandran outliers (%)	0.17

[a]The last shell includes all reflections between 1.54 and 1.50 Å. [b]R_{free} calculated using 5% of the data which were chosen randomly.

The structure of the YehZ−GB complex was modeled by superposing the experimental structure of the N- and C-terminal domains of YehZ on the structure of the *A. fulgidis* ProX complex with GB (PDB ID: 1SW2).[44] In this model, Lys32 (Lys13), Arg171 (Arg149), and Asn85 (Thr66) all form hydrogen bonds to the carboxylate group (bracketed residues are from ProX). The betaine group interacts with Tyr82 (Tyr63) and Tyr243 (Tyr214) from domain A and Trp130 (Tyr111) and Tyr219 (Tyr190) from domain B. These aromatic residues together form a betaine box, and similar arrangements of residues are seen in most proteins that bind betainyl ligands. ProX Asp109, which provides a countercharge to the betaine group, is replaced by Asn128 in YehZ. Asp143 is also replaced with Ser165, resulting in a less electronegative pocket overall. The YehZ pocket is also somewhat larger, with Phe15 (ProX) being replaced by the less bulky Asp34.

Apo State Stabilization in YehZ Predicts Weaker Ligand Binding. The structure of apo YehZ differs subtly from that of apo ProX in the interaction between domains A and B, especially in the interaction between the helices αN and αK (Figure 4B). Helix αK approaches αN much more closely in YehZ than in ProX: 3.7 vs 4.6 Å. This is facilitated by the fact that in YehZ this region is characterized by small and nonpolar residues (Gly223, Ala226, Ala227, and Ala281) that pack together closely in a way that maximizes buried nonpolar surface area. Analysis in PDBePISA[47] indicates that YehZ buries a larger surface area between the two helices (301.1 Å^2 in apo YehZ vs 273.1 Å^2 in apo ProX (PDB ID: 1SW5)) and that formation of the interface in YehZ is associated with a considerably larger favorable free energy solvation gain (−5.7 vs −3.5 kcal/mol). Interactions in this region were predicted, therefore, to result in a more stable apo conformation in YehZ than in ProX. In addition, none of the relatively short side chains present on αN and αK are in contact in the YehZ glycine betaine bound model, whereas in ProX, the corresponding longer residues (e.g., Tyr251 and Leu198) rearrange and remain in van der Waals contact in the closed structure. If other protein−protein interactions in the apo and substrate-bound states do not change significantly, then YehZ should exhibit a lower affinity for a ligand that makes equi-energetic interactions with each protein, as some of the interaction energy must be diverted to offset the energetic costs of breaking interactions that stabilize the apo state but not the ligand complex.

To test these predictions, Gly223 of YehZ-His$_6$ was replaced with serine, which is the corresponding amino acid in ProX. Gly223 is wholly buried in interactions with αN in the apo structure. If these interactions contribute significantly to apo state stabilization, then the Gly223Ser replacement would be expected to create steric clashes in the apo structure and result in a protein with a higher ligand affinity. YehZ-G223S-His$_6$ was purified, and its ligand affinities were determined by ITC. It displayed significantly higher affinities for GB, COS, and DMSP than YehZ-His$_6$ (Figure 2 and Table 3). The similar improvements in binding exhibited by these chemically diverse ligands are consistent with the destabilization of a state that competes with ligand binding rather than a direct effect on protein−ligand interactions. It is notable that the enthalpy of ligand binding in YehZ-Gly223Ser-His$_6$ actually exceeds that of ProX-His$_6$, indicating that enthalpically unfavorable interactions present in apo YehZ mask strongly favorable interactions made by the ligand.

YehZYXW Is a Glycine Betaine Transporter. Evidence that YehZ is a low-affinity betaine-binding protein led to reassessment of the hypothesis that YehZYXW is a betaine transporter. Indeed, YehZYXW did transport GB at low salinity (Figure 5A). Time-dependent GB uptake was not observed if *yehZYXW* was not present (as in control strain WG1319 (data not shown)) or if expression of *yehZYXW* was not induced (Figure 5A). The GB uptake activity was low, even with arabinose induction of *yehZYXW* expression from the ara_{BAD} promoter of vector pBAD24 (Figure 5A). That activity decreased as the salinity of the transport assay medium increased, explaining the inability of this system to mediate osmoprotection by GB (Figure 5B). The initial rate of GB uptake via YehZYXW was a linear function of GB concentration up to 4 mM (Figure 5C), confirming the expectation that YehZYXW has a low affinity for this substrate. Technical limitations precluded uptake measurements at higher GB concentrations.

DISCUSSION

This research was motivated in part by evidence that transporters other than ProP, ProU, BetT, and BetU may contribute to osmolyte uptake by *E. coli*.[3] Murdock et al. sought evidence that previously unidentified systems would promote osmoprotectant uptake by *E. coli* strains lacking all other known mechanisms.[2] No such evidence was found, but the experimental conditions employed for that work may have failed to promote transporter gene expression or enhance transporter activity. In this work, putative transporter operon *yehZYXW* was placed under the control of the arabinose-inducible ara_{BAD} promoter in vector pBAD24. Again, no evidence of osmoprotection was found.

Here, we report that YehZYXW is, as predicted, a betaine transporter. Purified periplasmic protein YehZ bound GB, COS, and DMSP with millimolar affinities (Figures 2 and 3 and Table 2), and a filtration assay based on ^{3}H-GB detected a corresponding GB uptake activity (Figure 5). Most bacterial

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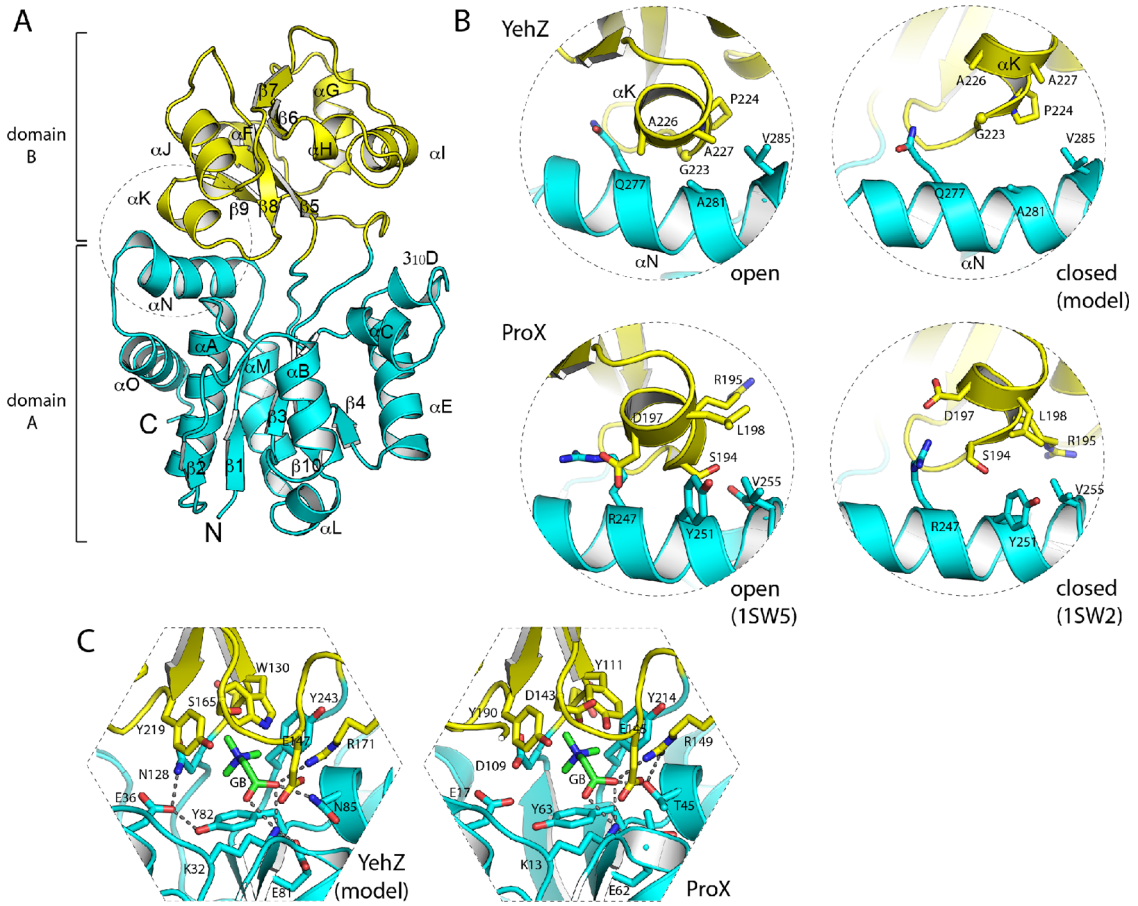

Figure 4. Crystal structure of apo YehZ-His_6. (A) Overall structure of YehZ, with secondary structure elements as indicated. (B) Detail of the interaction between domains A and B in the region of αK and αN in YehZ and ProX. In YehZ, this region is characterized by a series of small residues on αK (Gly223, Ala226, and Ala227) that fits closely with a region of αN in the region of Ala281. Lower inset: The equivalent region in the apo conformation of *Archaeoglobus fulgidis* ProX (PDB ID: 1SW5). Note that the helices in ProX do not approach each other as closely as in YehZ. Right: When YehZ is modeled in the substrate-bound closed state, the short side chains in αN and αK do not interact. In contrast, in ProX, the longer side chains can reorganize and remain in contact. (C) Organization of the ligand binding site in YehZ in comparison to ProX. Left: A homology model of YehZ glycine betaine (GB) complex (modeled on the *Archaeoglobus fulgidis* ProX GB costructure (PDB ID: 1SW2)). Right: The ProX GB complex (PDB ID: 1SW2). Note that while there are some conservative changes in the binding pocket, there are few major differences in the potential interactions between GB and the respective binding proteins other than the fact that the ProX binding site seems to be significantly more acidic. This view is in approximately the same orientation as that in panel A.

amino acid and osmolyte transporters display high (micromolar) substrate affinities. Thus, the low binding affinities of YehZ initially suggested that a true, physiologically significant ligand had not been found. However, several arguments indicate that this is unlikely.

First, homology modeling of the YehZ GB complex on the ProX GB complex indicates that most protein−ligand interactions are conserved, implying similarly favorable binding energies (Figure 4C). In YehZ, it is difficult to rationalize a 3 orders of magnitude loss of binding affinity for a wide variety of substrates on the basis of the very minor differences observed versus ProX. For example, in analogous work on the distantly related GlnPQ transporter, relatively subtle changes at the domain interface were shown to control whether glutamine or asparagine is preferred as a substrate.[48] However, here, small changes in the orientation of the transporter domains compensate for changes in the spacing of key binding motifs (due to glutamine's additional methylene group), but the nature of the chemical groups specified by the binding site remains unaltered.

Along the same line, the betaine-binding box is conserved in YehZ (Tyr82, Trp130, Tyr219, and Tyr243) (Figures 1 and 4C). In experiments using ProX, tryptophan and tyrosine residues could substitute for one another without altering affinities for betainyl ligands.[41] The presence of the betaine box severely restricts the set of potential ligands under consideration, as the strength of the interactions mediated by this motif arise from unusual soft hydrogen bonds between the aromatic rings and the polarized methylene hydrogen atoms. This motif is effective, therefore, only in binding ligands with cationic methylated groups; betaines and methylated thiols. In the absence of the electron-withdrawing nitrogen or sulfur atom, these interactions are far weaker.[44] There are very few naturally occurring small molecules that combine such a motif with a nearby anionic group that could favorably interact with residues Lys32 and Arg171 and that are small enough to fit in the restricted binding pocket. Analysis of the model YehZ−GB complex indicates very few additional interactions that YehZ could make with a ligand to increase binding energy, meaning that it is difficult to envision a substrate that would bind orders of magnitude more tightly than GB.

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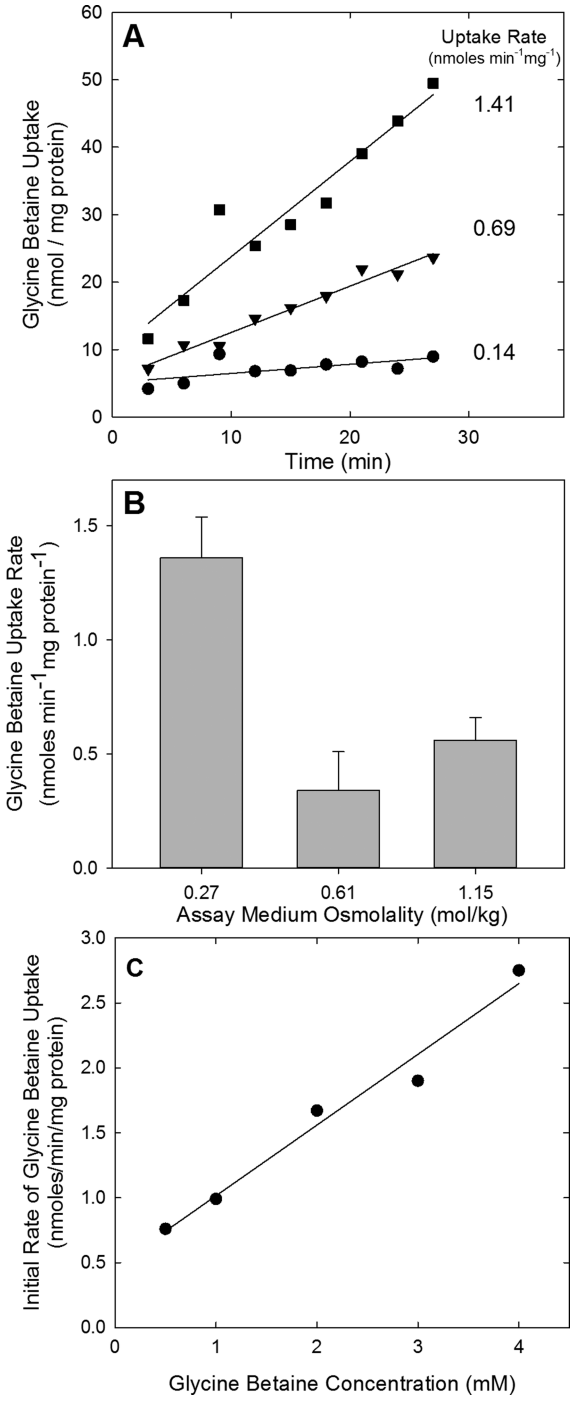

Figure 5. YehZYXW is a glycine betaine (GB) transporter. GB uptake activities of *E. coli* strain WG1314 (harboring pDC306, which encodes YehZYXW) were measured as described in Experimental Procedures using ^{3}H-GB as substrate. (A) The bacteria were cultivated with 0.2% (w/v) L-arabinose (squares), 0.02% L-arabinose (inverted triangles), or no L-arabinose (circles), ^{3}H-GB was provided at 4 mM, and aliquots of the reaction mixture were filtered at the indicated times (representative data for one of three replicate experiments). (B) The bacteria were cultivated with 0.2% (w/v) L-arabinose, and initial rates of GB uptake were determined with ^{3}H-GB at 4 mM in assay media adjusted with NaCl to the indicated osmolalities (representative data (mean ± standard error) for three replicates in one of two replicate experiments). (C) The bacteria were cultivated with 0.2% (w/v) L-arabinose, and initial rates of GB uptake were determined with ^{3}H-GB at the indicated concentrations and an assay medium osmolality of 0.26 mol/kg (representative data from two experiments).

Finally, both the structure (Figure 4) and experiments with variant YehZ-Gly223Ser-His$_6$ (Figure 2 and Table 2) argue that low substrate affinity arises (at least in part) because ligand binding energy is diverted to dissociate the exceptionally stable apo complex, not because the ligands tested bind suboptimally. The difference in interface stability between apo-YehZ and apo-ProX estimated by PDBePISA predicts a 40-fold weaker binding affinity for YehZ. Additional features of this interface, not accounted for in this calculation, may further exacerbate this difference. For example, in ProX, this region is characterized by larger, more flexible residues that should incur an appreciable loss in conformational free energy when fixed by interactions in the apo conformation (resulting in a further destabilization of the apo state in this protein). Our ITC experiments show that amino acid replacement Gly223Ser causes an order of magnitude decrease in K_d for all tested ligands, confirming that apo state stabilization is an important contributor to weak binding. The residues associated with weak ligand binding appear to be both conserved and actively selected for in YehZ (Figure 1) and are presumably important for YehZ function. Removal of favorable interactions in the binding pocket would be a more direct way to attain weak binding. However, by retaining the requirement that the ligand makes as many favorable interactions as required for strong binding to ProX, YehZ retains a similar degree of substrate specificity. While interactions of the αN and αK helices influenced by replacement Gly223Ser are clearly important, other aspects of the interface beyond the αN-αK helices also likely contribute to the observed 3 orders of magnitude difference in affinity; this may include weaker ligand interactions, weaker interdomain interactions in the closed state, and other interactions that stabilize the YehZ apo state.

Despite the above arguments, it is possible that YehYXW mediates binding protein-independent transport and that the ligand specificity of YehZ does not fully encompass the substrate specificity of YehZYXW. Our analysis of ligand binding to YehZ included a comparison with ProX, the periplasmic binding protein component of the *E. coli* ProU system. The high-affinity interaction of GB with ProX was confirmed (K_d of 1.4 μM), as was the failure of both proteins to bind proline. The inability of ProX to bind proline is surprising because proline is clearly a (low affinity) ProU substrate.[2,39,40] A variant of the MalEFGK system of *E. coli* can transport lactose, even though periplasmic binding protein MalE does not bind lactose.[49] However, we know of no other instance in which an ABC system mediates transport of a substrate that does not bind to the periplasmic component. Osmotic activation may alter the conformation of ProU in a manner that exposes a membrane-integral substrate binding site within subunit ProW.[2] However, YehYXW would not be expected to share such behavior because it was inhibited, not activated, with increasing osmolality (see further discussion below).

Analysis of YehZ homologues suggests that YehZYXW is present in diverse bacteria. YehZ homologues are generally 50% or more identical to *E. coli* YehZ, have all putative GB binding residues conserved, and also have a conserved pattern of small residues in αK and αN that stabilize the apo conformation. All members also have a conserved insertion in αJ that appears to position and buttress the apo state stabilizing helix αK (Figures 1 (magenta symbol) and 4). By these criteria, YehZ appears to be very common in all branches of *Proteobacteria*, but some examples are also found in *Thermotogae*, *Spirochaetes*, *Synergistetes*, *Deferribacteres*, and *Chloroflexi* and members of

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the *Deinococcus-Thermus* grouping. *A. fulgidus* ProX was used as the primary comparator for YehZ in this work. Glycine betaine provides heat stress rather than osmotic stress protection to *A. fulgidus*, which is a hyperthermophilic *Archaeon*.[50]

It is also interesting that, in contrast to those of its osmoregulatory homologues (e.g., ProU (*E. coli*), OpuC (*B. subtilis*), OpuA (*L. lactis*), and OsmU (*S. typhimurium*)), ATP binding subunit YehX does not terminate in CBS domains. Such domains have been implicated in osmosensing by *L. lactis* OpuA,[51] and their presence on the ATP-binding subunit was proposed to predict osmoregulatory function for transporters in this family.[7] As for *yehZYXW*, expression of the operon encoding transporter OpuB of *Listeria monocytogenes* depends on a general stress sigma factor (σ^B) and is induced as the bacteria enter stationary phase or during growth at high salinity.[52,53] However, the ATP binding subunit of this transporter does not include CBS domains, and it did not contribute to osmoprotection by betaines.[54]

Transporter OsmU is an intermediate case. It contributes weakly to osmoprotection of *Salmonella enterica* serovar typhimurium by GB.[55] However, many of the small residues confirmed (Gly223) or predicted (Ala281, Ala227, Ile285) to contribute to apo state stabilization of YehZ are conserved or conservatively replaced in periplasmic ligand binding protein component OsmX of the OsmU system (Figure 4), supporting the prediction that OsmU transports GB with low affinity.[55] Thus, at least one YehZ homologue does contribute to osmoprotection, and the occurrence of CBS domains is a stronger predictor of osmoregulatory function than the occurrence of a ProX-like (and not a YehZ-like) periplasmic binding protein.

The physiological function of YehZYXW remains unclear. The osmotic induction of *yehZYXW* may ensue from the osmotic induction of *rpoS* and reflect stress cross-resistance, not participation of YehZYXW in the osmotic stress response. Betaines stabilize and solubilize proteins,[56] so YehZYXW may contribute to cytoplasmic homeostasis by mediating GB uptake at low osmolality for bacteria entering stationary phase. Some YehZYXW homologues participate in the catabolism of exogenous solutes (e.g., CbcXWV participates in choline catabolism by *Pseudomonas aeruginosa*[37]). YehZYXW is encoded by the core *E. coli* genome,[3] so it is available in *E. coli* strains with diverse genomes and (presumably) metabolic capacities. However, it is unlikely to facilitate catabolism of GB or DMSP by *E. coli*, as tested *E. coli* strains do not degrade either compound. In fact, deletion of *yehZ*[57] or the *osmU* operon[58] enhanced the growth of *S. typhimurium* in laboratory culture (with or without betaine) and conferred a competitive disadvantage during colonization of mice and murine macrophage-like cells, respectively, by *S. typhiumurium*. Thus, *yehZYXW* and *osmU* may be antivirulence loci whose expression limits bacterial growth within host macrophages, thereby promoting asymptomatic, persistent infection.[57,58] Some periplasmic binding proteins contribute to signal transduction by interacting with the periplasmic surfaces of chemotactic receptors, sensor kinases, or other membrane-integral receptors (ref 59 and references cited therein). Those functions may be complementary to or independent of their roles in solute transport. Thus, betaine transport may be a vestigial function for a YehZYXW system that has evolved to play an alternative role in *E. coli* and other bacteria.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00274.

Primer sequences (Table S1), osmoprotection data (Tables S2 and S3), and mass spectra of YehZ-His$_6$ and ProX-His$_6$ (Figure S1) (PDF).

Accession Codes

Coordinates and structure factors for apo-YehZ have been deposited in the Protein Data Bank with accession no. 4WEP.

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Notes

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■ ABBREVIATIONS

ABC, ATP-binding cassette; BCCT, betaine/carnitine/choline transporter; BCA, bicinchoninic acid; BSA, bovine serum albumin; CBS, cystathionine-β-synthase; COS, choline-O-sulfate; DMSP, dimethylsulfoniopropionate; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; GABA, γ-aminobutyric acid; GB, glycine betaine; ITC, isothermal titration calorimetry; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB, Luria broth; MFS, Major Facilitator Superfamily; MMA, modified minimal medium A; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; Q-ToF, Quadrupole time of flight; rmsd, root-mean-square deviation; TMAO, trimethylamine-*N*-oxide; TRAP, tripartite ATP-dependent; TTC, 2,3,5-triphenyl tetrazolium chloride

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