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Roles of YehZ, a Putative Osmoprotectant Transporter, in Tempering Growth of *Salmonella enterica* serovar Typhimurium S

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Salmonella, a main cause of foodborne diseases, encounters a variety of environmental stresses and overcomes the stresses by multiple resistance strategies. One of the general responses to hyperosmotic stress is to import or produce compatible solutes so that cells maintain fluid balance and protect proteins and lipids from denaturation. The ProP and ProU systems are the main transport systems for compatible solutes. The OsmU system, recently identified as a third osmoprotectant transport system, debilitates excessive growth as well by reducing production of trehalose. We studied a fourth putative osmoprotectant transport system, YehZYXW, with high sequence similarity with the OsmU system. A *Salmonella* strain lacking YehZ, a predicted substrate-binding protein, did not suffer from hyperosmolarity but rather grew more rapidly than the wild type regardless of glycine betaine, an osmoprotectant, suggesting that the YehZYXW system controls bacterial growth irrespective of transporting glycine betaine. However, the growth advantage of Δ*yehZ* was not attributable to an increase in OtsBA-mediated trehalose production, which is responsible for the outcompetition of the Δ*osmU* strain. Overexpressed YehZ *in trans* was capable of deaccelerating bacterial growth *vice versa*, supporting a role of YehZ in dampening growth. The expression of *yehZ* was increased in response to nutrient starvation, acidic pH, and the presence of glycine betaine under hyperosmotic stress. Identifying substrates for YehZ will help decipher the role of the YehZYXW system in regulating bacterial growth in response to environmental cues.

Keywords: *Salmonella*, YehZ, compatible solute, osmolarity

Introduction

Salmonella enterica serovar Typhimurium (hereafter referred to as *S.* Typhimurium) infects humans through contaminated food and water, and causes gastroenteritis with symptoms of vomiting, abdominal discomfort, and diarrhea. For successful proliferation, *Salmonella* should adapt to deleterious environments encountered during its life cycle. One of these environmental stresses is high osmotic concentrations in the lumen of the host gastrointestinal tract as well as in foods with a high salt content. A general strategy for *Salmonella* to overcome hyperosmolarity is to increase internal concentrations of solutes to maintain cell turgor pressure [20, 32]. Potassium and glutamate are compatible solutes preferred in most bacteria [43, 45]. However, *Salmonella* can increase the uptake or biosynthesis of other osmoprotectants such as glycine betaine, carnitine, ectoine, proline, and trehalose as the principal compatible solutes [10, 15, 24, 36]. Intracellular compatible solutes help cells resist not only high salt pressure but also acidic pH and high temperature [5, 22]. Glycine betaine and proline are two major compatible solutes imported by *S.* Typhimurium, and trehalose is a compatible solute synthesized by the *otsBA* operon in *Salmonella* when glycine betaine cannot be imported [6, 28].

Salmonella utilizes mainly the ProP and ProU transport systems for uptake of glycine betaine under high osmotic stress conditions [11, 17]. The ProP system encoded by the

proP gene is expressed constitutively with 2- to 3-fold induction at high osmolarity and exhibits lower affinity for glycine betaine than the ProU system [4]. The ProU transport system with a high affinity for glycine betaine is a member of the ATP-binding cassette (ABC) transporter using ATP hydrolysis for transport and is composed of three components of ProV, ProW, and ProX functioning as ATPase, membrane pore, and periplasmic substrate-binding protein, respectively [41]. Recently, a third transport system named OsmU was identified to import glycine betaine stimulating bacterial growth under high osmotic stress conditions [15]. The OsmU system consists of ATPase OsmV, two membrane permeases of OsmW and OsmY, and substrate-binding protein OsmX, and each component shows high sequence similarity to its counterpart in the ProU system except for OsmX. Interestingly, in other study of the OsmU system, the absence of the *osmU* operon accelerated trehalose accumulation in cells and thereby rendered *Salmonella* more resistant to hyperosmolarity, acidic pH, hydrogen peroxide, and killing by macrophages [38], suggesting a role of the OsmU system in balancing intracellular solute contents to limit bacterial outgrowth under deleterious environments.

Bioinformatic analysis identified another putative glycine betaine transport system, YehZYXW, which shares high sequence similarity with the OsmU and ProU systems except for the substrate-binding component of YehZ. YehY and YehW show 54% sequence similarity to ProW membrane permease, respectively, and YehX matches ProV ATPase with 55% similarity. However, there is no significant homology between YehZ and ProX as low sequence similarity between OsmX and ProX [15], suggesting that the YehZYXW system is a member of the ABC-type transport system for compatible solutes but imports the substrates with different affinities from those of the ProU system. Although the possibility of osmoprotectants transport *via* the YehZYXW system has to be tested, we hypothesized the presence of multiple transport systems for compatible solutes such as glycine betaine in *Salmonella*. Each transport system may have distinct roles in bacterial proliferation besides glycine betaine transport in response to environmental conditions. For example, the OsmU system limits *Salmonella* stress resistance by reducing trehalose production [38]. Trehalose is a more effective compatible solute, giving *Salmonella* resistance to acidic pH, hydrogen peroxide, and hyperosmorality [20, 38]. However, excessive growth of *Salmonella* may cause severe inflammation responses threatening bacterial persistence in host tissues, and thereby *Salmonella* may utilize the OsmU system to titrate trehalose production for fitness inside the host. In phylogenetic analysis on the OsmU system, the substrate-binding protein OsmX, was closely related to YehZ, showing sequence similarity of 50% with an expected value [E] of 6e-37 [15], suggesting functional redundancy between OsmX and YehZ although the substrate specificity for YehZ is not known. Therefore, we examined the possibility of glycine betaine transport *via* the YehZYXW system and characterized the function of YehZYXW with respect to *Salmonella* growth.

Materials and Methods

Bacterial Strains and Plasmids

All *Salmonella* strains used in this study are *Salmonella enterica* serovar Typhimurium 14028s [14] or its derivatives. For Δ*yehZ* or Δ*otsBA* strain construction, nonpolar in-frame gene deletion was carried out using the phage λ Red recombination system [12]. The kanamycin resistance (*kan*) cassette of pKD13 was amplified by PCR using primers with 40-nucleotide flanking sequences homologous to target genes, and the subsequent PCR products were introduced into recipient cells harboring pKD46 to replace the target genes with a *kan* cassette. Primer sequences used in deletions of *yehZ* and *otsBA* are as follows. YehZ-RF1 (5′-TCT CTG AAA AAG GCC GTA AAA GGA TGA GGA AAG CAT CAT G GTG TAG GCT GGA GCT GCT TC-3′) and YehZ-RR1 (5′- CAG CAT CAC TCA CAG ATT ACT TCA CCC ACC CTT TTT GTC GTT CCG GGG ATC CGT CGA CCT-3′) are for *yehZ* deletion, and OtsB-RF1 (5′-TTG TGA GTC TCA ATA TGA TGA TAA GGA GGA GAC CAG GGT GGT GTA GGC TGG AGC TGC TTC-3′) and OtsA-RR1 (5′-CGC CGC TCG CGA TAT TTC AGG CCA GCT TAG GGA ACG TCG CTT CCG GGG ATC CGT CGA CCT-3′) are for *otsBA* deletion. The *kan* cassette was removed by flip recombinase produced from pCP20 in order to result in in-frame deletion that was presumably nonpolar [12]. For the construction of pYehZ producing YehZ *in trans*, a DNA fragment containing *yehZ*-coding sequences with its putative ribosome binding site (RBS) was PCR-amplified using YehZ-CF1 (5′-AAA AAG AAT TCT GAA AAA GGC CGT AAA AGG ATG AGG AAA G-3′) and YehZ-CR1 (5′-ATA TAG TCG ACC GTG ATT TTT TTG GCA GCA TCA CTC A-3′) and cloned into pBAD18 [18] under an arabinose-inducible promoter through *Eco*RI and *Sal*I sites.

Growth Conditions

In order to cultivate *Salmonella* under diverse stressful conditions tested in this study, bacteria were grown in Luria-Bertani (LB) medium overnight beforehand and diluted into magnesium minimal medium (MgM) [13, 47] at a 1:20 ratio after washing with MgM, unless otherwise noted. The formula for MgM is composed of 100 mM Tris-Cl, 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 0.2% glycerol, 0.1% casamino acids, and 8 mM $MgCl_2$. The pH was adjusted to 7.0 in all experiments but lowered to 5.0 using HCl at acidic stress conditions. For high osmotic stress conditions, 0.6 M NaCl was added in MgM and 1 mM glycine

betaine was supplied when required. L-Arabinose (0.2%) was used to induce *yehZ* on pYehZ. Antibiotics were added as indicated: kanamycin, 50 μg/ml; ampicillin, 50 μg/ml.

Macrophage Infection

Murine macrophage-like cells (ATCC RAW264.7) were seeded at 2×10^5 cells per well in 24-well tissue culture plates and incubated in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS overnight at 37°C with 5% CO_2. *Salmonella* cells grown overnight in LB were resuspended in DMEM and added to macrophage monolayers at an input multiplicity of infection (MOI) of 100. Infections were synchronized by centrifuging at 1,000 ×*g* for 5 min and the plates were then incubated at 37°C with 5% CO_2 for 30 min. Extracellular bacteria were subsequently removed by washing the cells with phosphate-buffered saline (PBS) and incubating them in DMEM containing gentamicin (Gibco) at 100 μg/ml for 1 h. After treatment with gentamicin (100 μg/ml), the cells were washed with PBS three times and overlaid with DMEM containing 20 μg/ml gentamicin for the remainder of the experiments. Macrophages were lysed with 1% Triton X-100 in PBS at the indicated times, and serially diluted lysates were spread on agar plates.

Competitive Index Assay

To compare the fitness between wild-type and Δ*yehZ* strains inside macrophages, the reference wild-type strain MA6054 [21] and the Δ*yehZ* strain were grown separately in LB broth overnight and mixed at a 1:1 ratio in PBS as an inoculum at macrophages infection. *S.* Typhimurium MA6054 carries a gene that encodes an arabinose-inducible β-galactosidase on the chromosome. The bacterial mix was used to infect macrophage cells as described above. To enumerate intracellular bacteria, macrophages were lysed at 2 and 12 h post-infection and the lysates were plated on LB agar plates containing 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, Sigma) and 1 mM arabinose (Sigma). The competitive index (CI) was calculated as follows [21, 46]: CI = (% of Δ*yehZ* recovered/% of MA6054 recovered)/(% of Δ*yehZ* inoculated/% of MA6054 inoculated).

Resistance Test Against Hydrogen Peroxide and Paraquat

Salmonella strains were grown in LB broth overnight and resuspended in the same volume of MgM (pH 7.0) after washing with MgM (pH 7.0). Bacteria in MgM were diluted serially from 10^0- to 10^{-9}-fold, and 10 μl of each dilution was dotted and dried on MgM agar plates containing 100 μM H_2O_2 or 100 μM paraquat.

RT-PCR

Total RNA was isolated using the RNAprotect Bacteria Reagent (Qiagen) and RNeasy mini kit (Qiagen) at the indicated time points. Residual chromosomal DNA was removed by a TURBO DNA-free kit (Ambion) according to the manufacturer's recommendations. cDNA was synthesized using RNA to cDNA EcoDry Premix (Clontech), and cDNA corresponding to 10 ng of input RNA was used as template in each real-time PCR using SYBR green reagent to detect the duplex DNA product (iQ SYBR Green Supermix, Bio-Rad). The primers used in RT-PCR are listed in Table S1. RT-PCR was performed in an iCycler iQ real-time detection system (Bio-Rad) by 40 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 20 sec, following 95°C for 10 min. The expression ratio of each gene was the average from at least three independent RNA samples and was normalized to the level of *gyrB* [37, 47].

Results and Discussion

Δ*yehZ* Mutants are Resistant to Hyperosmotic Stress

The high sequence similarity of the YehZYXW system to the ProU and OsmU systems suggests the possibility that YehZ, the putative substrate-binding protein, may transport glycine betaine as the counterparts of ProX and OsmX and render *Salmonella* more resistant to high osmotic pressure. In order to test this possibility, we constructed a mutant

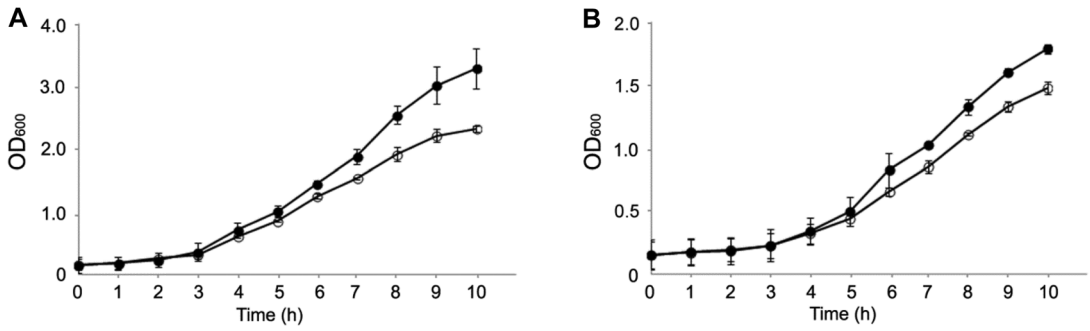

Fig. 1. Growth curves of wild type and Δ*yehZ* in high salt medium.
The wild-type (open circle) and Δ*yehZ* (closed circle) strains were cultivated in minimal medium with 0.6 M NaCl in the presence of 1 mM glycine betaine (**A**) or in the absence of glycine betaine (**B**). Optical density from each culture was measured at 600 nm every hour and averaged from three independent cell cultures.

strain lacking the *yehZ* gene and examined the growth of the mutant in minimal medium containing 0.6 M NaCl and 1 mM glycine betaine (Fig. 1A). To our surprise, *Salmonella* lacking YehZ did not suffer from growth defects under high osmotic pressure but rather showed an improved growth rate to wild-type *Salmonella*. The improved growth of Δ*yehZ* in the growth study measuring the optical density of bacterial culture was verified by enumerating viable bacteria from each culture under the same condition (data not shown). Frossard *et al*. [15] speculated that there would be no additional efficient uptake system for glycine betaine besides the ProP, ProU, and OsmU systems since the accumulation of glycine betaine in a triple mutant lacking ProP, ProU, and OsmU was less than 1% of the level seen in wild-type *Salmonella*. In accordance with their inference, our observation of no growth defect of Δ*yehZ* strains in glycine betaine-containing high salt medium suggests that the YehZYXW system may not be effective in importing glycine betaine, although the specificity of YehZ for other compatible solutes such as choline and proline should be tested further to define a role of the YehZYXW system in osmoprotectants transport. Pilonieta *et al*. [38] found that the absence of the OsmU system increased *Salmonella* resistance to high salt stress by producing more trehalose in the cytoplasm. The growth increase of Δ*yehZ* strains might be attributable to more trehalose production as observed in Δ*osmU* strains, independently of importing glycine betaine. Indeed, *Salmonella* lacking YehZ still showed a steeper slope in growth curve than the wild type in glycine betaine-depleted minimal medium (Fig. 1B), which is reminiscent of the growth advantage by increased trehalose in Δ*osmU* strains [38]. Likewise, Δ*yehZ* strains also survived better than wild-type bacteria after phagocytosis by macrophages as demonstrated in Δ*osmU* strains with the increased resistance against macrophages killing [38]. When the wild-type and Δ*yehZ* strains were mixed equivalently and used in macrophages infection, the intracellular number of the mutant was greater than that of the wild-type strain at 12 h post-infection, showing a CI of 1.35 (Fig. 2).

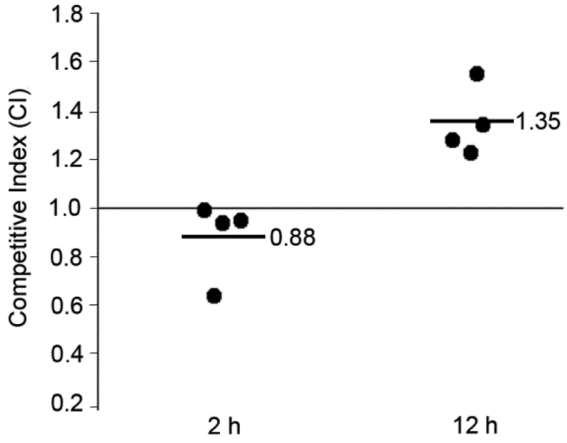

Fig. 2. Competitive index of Δ*yehZ* in murine macrophages.
A wild-type reference strain, MA6054, harboring arabinose-inducible β-galactosidase activity and a Δ*yehZ* strain were mixed at 1:1 ratio and the mix was used in infection of RAW264.7 macrophages at MOI 100. At 2 and 12 h post-infection, macrophage cells were lysed and the lysates were spread on agar plates containing arabinose and X-gal after serial dilutions. Colonies of wild type (blue) and Δ*yehZ* (white) were counted and the competitive index (CI) was calculated as described in Materials and Methods. The averaged CI from four infections (black dots) is shown as a solid line with the value.

Resistance of Δ*yehZ* Mutants to Hyperosmolarity is Independent of OtsBA-Mediated Trehalose Production

Similar growth phenotypes between Δ*yehZ* and Δ*osmU* strains prompted us to examine the effect of the YehZYXW system on the metabolism of trehalose. *Salmonella* possesses two trehalose biosynthesis pathways mediated by OtsBA (utilizing UDP-glucose) and TreZY (utilizing glucan), but the *otsBA* operon responds more strongly to environmental stresses such as hyperosmolarity, nutrient depletion, and dehydration [16, 19, 30, 42]. Pilonieta *et al*. [38] also showed that OtsBA is the dominant trehalose biosynthesis system over TreZY in *Salmonella* showing insignificant trehalose accumulation in Δ*otsBA* strains. OtsA (trehalose-6-phosphate

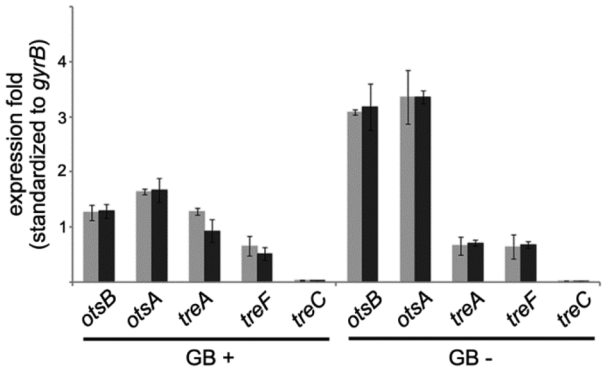

Fig. 3. RT-PCR of genes involved in trehalose metabolism.
The wild-type (gray bar) and Δ*yehZ* (black bar) strains were cultivated in high salt minimal medium with or without glycine betaine (GB). RNAs were isolated when cells were in mid-log phase and subjected to RT-PCR with primers specific to trehalose metabolism genes. mRNA expression levels were normalized with *gyrB* mRNA and the expression ratios relative to that of *gyrB* were averaged from three independently isolated RNA samples.

synthase) converts UDP-glucose to trehalose-6-phosphate, and OtsB (trehalose-6-phosphate phosphatase) removes a phosphate residue to produce trehalose [42]. Surplus trehalose is then degraded by TreC (trehalose-6-phosphate hydrolase) and TreA (periplasmic trehalase)/TreF (cytoplasmic trehalase) [23, 34, 39]. In order to examine the effect of YehZ on trehalose accumulation, wild-type and Δ*yehZ Salmonella* strains were cultivated in minimal medium containing a high salt concentration (0.6 M NaCl) with or without glycine betaine, and mRNAs were isolated and subjected to RT-PCR to measure the expression levels of *otsB*, *otsA*, *treA*, *treF*, and *treC* (Fig. 3). There was no significant difference in the expression of trehalose metabolism-relevant genes between wild-type and Δ*yehZ* strains regardless of the presence of glycine betaine in the medium, although the *otsBA* operon was transcribed more than 2-fold when glycine betaine could not be used as an osmoprotectant under high salt pressure in the wild-type and Δ*yehZ* strains. These results suggest that the growth advantage of Δ*yehZ* strains under high osmotic conditions was not caused by an increase in trehalose accumulation in the cytoplasm, albeit it cannot be ruled out that OtsB and OtsA proteins may be produced more in Δ*yehZ* strains at the post-transcriptional level [26]. Pilonieta *et al*. [38] showed that accumulation of trehalose in the absence of the OsmU system resulted in *Salmonella* being more resistant not only to high osmolarity but also to oxidative stress, a major hostile stress inside macrophages [38]. However, *Salmonella* deprived of *yehZ* exhibited comparable viability to the wild-type strain on minimal medium agar plates containing 100 μM hydrogen peroxide or 100 μM paraquat (Fig. S1). This phenotypic discrepancy in oxidative stress resistance between Δ*yehZ* and Δ*osmU* suggests that the resistance of Δ*yehZ* strains under stress conditions was accomplished in a manner distinct from the trehalose-mediated resistance of Δ*osmU* strains.

To verify that the growth advantage of Δ*yehZ* strains was independent of OtsBA-mediated trehalose accumulation, deletion of the *otsBA* operon was introduced in the wild-type and Δ*yehZ* strains and their growth was compared under high osmotic stress conditions (Fig. 4A). As noted, the absence of *otsBA* in wild-type *Salmonella* exhibited severe reduction in the growth rate in high salt medium lacking compatible solutes [24]. However, the deletion of *yehZ* rather increased the growth regardless of the absence of OtsBA (compare between filled and open symbols in circle or triangle symbols, respectively, in Fig. 4A). This result indicates that the increased growth of Δ*yehZ* strains was not resulted from OtsBA-mediated trehalose accumulation, and the inverse effects of YehZ and OtsBA on the growth are likely to work independently of each other. Furthermore, production of YehZ *in trans* on a plasmid retarded bacterial growth in the wild-type *Salmonella* (Fig. 4B), demonstrating a negative role of YehZ in *Salmonella* growth.

Growth Advantage of Δ*yehZ* Mutants Occurs Independently of Osmolarity

The growth benefit by the absence of YehZ was observed in minimal medium containing high salt concentrations and this growth phenotype was independent of glycine betaine in the medium and trehalose production in the cells, suggesting that YehZ does not bind glycine betaine as the substrate or has a very low affinity for glycine betaine and the resistance mechanism in Δ*yehZ* strains is different

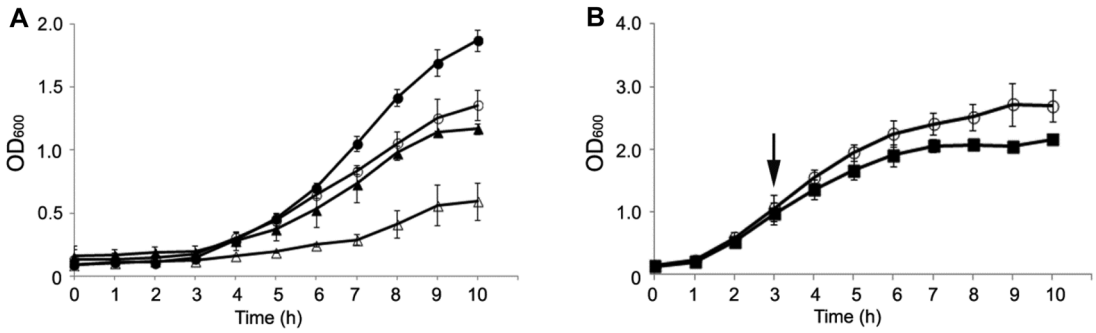

Fig. 4. Growth advantage of Δ*yehZ* independent of OtsBA.

(**A**) Wild-type *Salmonella* (open circle) and its derivatives Δ*yehZ* (closed circle), Δ*otsBA* (open triangle), and Δ*yehZ* Δ*otsBA* (closed triangle) were pre-cultured in LB and diluted in 0.6 M NaCl minimal medium not supplemented with glycine betaine at 1:20 ratio and their growth was measured every hour. Averages of triplicate growth curves were plotted. (**B**) Wild-type *Salmonella* was transformed with pYehZ (closed square) or pBAD18 (open circle) and grown in minimal medium with a 1:20 inoculation ratio. Arabinose (0.2%) was added at 3 h for YehZ expression, as indicated with the arrow.

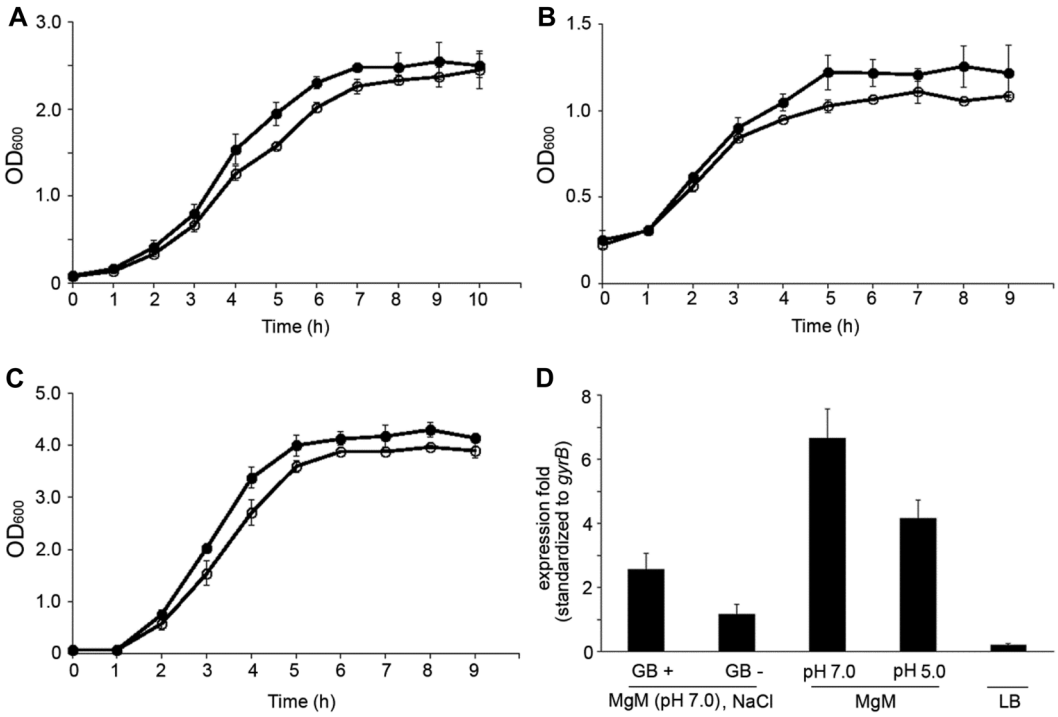

Fig. 5. Growth curves of Δ*yehZ* in diverse conditions and differential *yehZ* mRNA levels depending on growth conditions.
The wild-type (opened circle) and Δ*yehZ* (closed circle) strains were cultivated in minimal medium (pH 7.0) without high salt stress (**A**), acidic minimal medium (**B**), and nutrient-rich LB medium (**C**). Bacteria were added at a 1:20 ratio in minimal media and at a 1:100 ratio in LB broth, respectively. Growth was measured at 600 nm every hour and the averages from triplicate cultures were plotted for each strain. In parallel with growth curves, the expression levels of *yehZ* were compared between growth conditions (**D**). Total RNAs were isolated at mid-log phase from five different conditions; high osmotic minimal medium with glycine betaine (GB) or not, minimal medium of pH 7.0 or pH 5.0 without osmotic stress, and LB medium. *yehZ* expression levels were normalized using *gyrB* in each growth condition and the relative expression folds were averaged.

from the trehalose-mediated resistance in Δ*osmU* strains. These distinct growth phenotypes of Δ*yehZ* from those of Δ*osmU* led us to examine the growth of Δ*yehZ* in other environmental conditions. First, in order to define the role of the YehZYXW system with regard to high osmotic stress, the growth of Δ*yehZ* strains was studied in minimal medium without NaCl addition (Fig. 5A). Both wild-type and Δ*yehZ* bacteria had shortened lag phases in the absence of high osmotic stress, compared with the growth under high salt concentrations. However, the mutant lacking YehZ still grew faster than the wild-type strain even without high salt pressure, indicating that the growth advantage of Δ*yehZ* strains was independent of high osmolarity. This increased growth of Δ*yehZ* strains was also observed in other minimal medium, M9, without high salt concentrations (data not shown). The growth profit by the lack of YehZ under minimal medium was still maintained when the pH was lowered to 5.0 (Fig. 5B), which mimics the intracellular environment after phagocytosis [13, 29, 47]. Next, the deletion of *yehZ* also brought about the growth benefit in nutrient-rich medium, LB (Fig. 5C). The increased growth of Δ*yehZ* strains under various growth conditions suggests that YehZ might dampen *Salmonella* proliferation in response to an unknown environmental stimulus. To get insights into the environmental signals stimulating transcription of the *yehZYXW* operon, we analyzed the expression levels of *yehZ* mRNA under conditions tested in this study (Fig. 5D). The *yehZYXW* operon is known to be induced under osmotic shock conditions and stationary phase by σ^s [7]. The expression of *yehZ* was induced in nutrient-depleted minimal medium (pH 7.0 and 5.0 both), compared with that in LB medium, and this was likely due to the σ^s responding to nutrient starvation in the minimal medium. Under high osmotic pressure, the expression of *yehZ* was doubled by the presence of glycine betaine, indicating that compatible solutes such as glycine betaine stimulate the induction of the *yehZYXW* operon under high osmotic stress conditions. This result suggests that the role of the YehZYXW system may be important when *Salmonella* suffers from nutrient starvation and high osmolarity in the

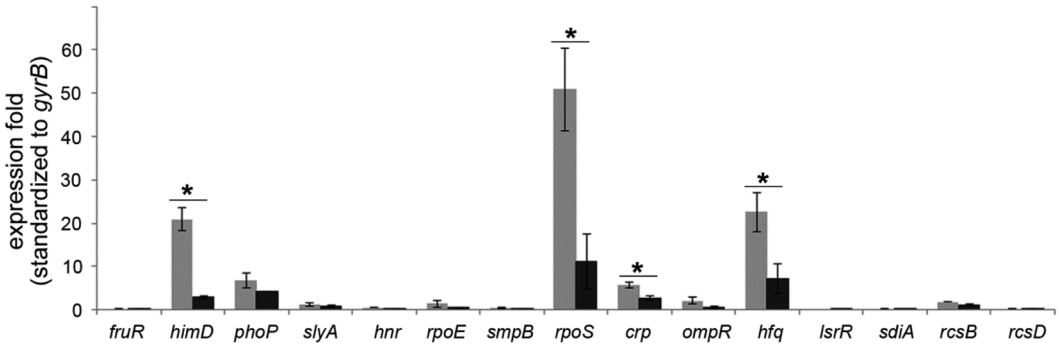

Fig. 6. Expression of regulator genes in Δ*yehZ*.

The wild-type (gray bar) and Δ*yehZ* (black bar) strains were cultivated in minimal medium (pH 7.0) without osmotic stress. RNAs were isolated when cells were in mid-log phase and subjected to RT-PCR with primers specific to 15 regulator genes. mRNA expression levels were normalized with *gyrB* mRNA and the expression ratios relative to that of *gyrB* were averaged from three independently isolated RNA samples. Regulators that showed significant differences between wild-type and Δ*yehZ* bacteria (P-value < 0.05) are depicted with an asterisk.

presence of osmoprotectants, although YehZ is not likely to import glycine betaine.

Growth Control by YehZ is Irrespective of Global Regulators Responding to Environmental Stresses

Summarizing these results suggests that the YehZYXW system may not be responsible for glycine betaine transport but restrains bacterial growth sensing unidentified environmental cues. Controlling bacterial growth is highly complicated and coordinated work by multiple gene products and generally requires global regulators that govern the expression of a myriad of genes for adaptation to unfavorable environments [2, 35, 47]. Although the binding substrate for YehZ is not determined yet, YehZ may recognize an environmental stimulus and link the stress to a global regulator for controlling growth. This assumption led us to search for a global regulator coordinating with YehZ. We chose 15 regulators that are known to respond to extracellular stimuli and regulate bacterial growth in studies elsewhere and measured the expression levels in the presence or absence of YehZ (Fig. 6). Minimal medium not supplemented with NaCl and glycine betaine was used to isolate total RNA, since this condition induced *yehZ* to higher levels than the other conditions did (Fig. 5D). However, there was no regulator whose expression was increased by the lack of YehZ and probably capable of stimulating bacterial growth under nutrient-depleted conditions. Instead, four out of fifteen regulator genes tested were significantly reduced by the absence of YehZ and these four regulators included IHF-β, CRP, RpoS, and Hfq. IHF is a DNA-binding protein with a role in changing DNA structure and thereby controlling transcription of many genes, including classical stationary-phase genes [33]. The CRP-cAMP complex is involved in multiple regulatory networks, including glucose-mediated catabolite repression, and regulates hundreds of genes [27]. In regard to osmoregulation, CRP expression is negatively regulated by osmolarity, and fine-tuning of CRP activity is essential for bacterial viability under low osmotic stress conditions in *Escherichia coli* [3]. RpoS is a sigma factor required for bacterial survival under nutrient-depleted and stress conditions [25] and is found to positively regulate the *yehZYXW* operon [7]. Hfq is a RNA chaperons that mediates the binding of small RNAs to target mRNAs and assists in post-transcriptional gene regulation in bacteria. The importance of Hfq for resistance against harsh environments, including osmolarity, heat, and nutrient starvation, is well demonstrated in a variety of bacteria [8, 31, 40, 44]. Deletion of each regulator did not affect *Salmonella* growth under minimal medium condition, whereas Δ*crp* and Δ*hfq* were rather attenuated in growth under the same condition (data not shown). There may be other regulatory circuits linking extracellular signal transport by YehZ to growth modulation by other regulators not tested here. However, bacterial fitness in changing environments is accomplished by the coordinated regulation network among multiple regulators integrating environmental stimuli [1, 35, 47]. Regarding their roles of IHF, CRP, RpoS, and Hfq as regulators modulating the expression of multiple genes in a coordinated manner, the optimal activity of each regulator may be critical for bacterial viability and adaptation to stressful environments, otherwise leading to abnormal increase or decrease in growth due to imbalance in the regulatory network by suboptimal activities of regulators. Overgrowth is not always beneficial

to bacterial infection into hosts. For example, Δ*ydgT* strains show enhanced replication inside macrophages, due to the increased SPI-2 activity at early time points, but ultimately get attenuated in virulence in a long time infection in a mouse model [9]. A mutant outcompeting intact wild-type *Salmonella* would break the balance between host and pathogen required for bacterial long-term persistence and provoke intensive host immune responses by host tissue damage, leading to early elimination from the site of infection. Growth phenotype study of Δ*yehZ* under other environments, including animal models, will further decipher the roles of YehZ in controlling *Salmonella* growth. In summary, our study demonstrates that the YehZYXW system has a role in tempering bacterial growth and the working mechanism is distinct from that of the OsmU system restraining trehalose production.

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