YcjW: a transcription factor that controls the emergency generator of H₂S in *E. coli*.

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Abstract

Hydrogen sulfide (H₂S) is a ubiquitous gaseous molecule that is endogenously produced in both eukaryotes and prokaryotes. Its role as a pleiotropic signaling molecule has been well characterized in mammals^{1,2}. In contrast, the physiological role of H₂S in bacteria only recently became apparent; H₂S acts as a cytoprotectant against antibiotics-induced stress and affect the cell's ability to maintain redox homeostasis³⁻⁵. In *E. coli*, endogenous H₂S production is primarily dependent on 3-mercaptopyruvate sulfurtransferase (3MST), encoded by *mstA*, previously known as *sseA*^{3,4}. Here, we show that cells lacking 3MST acquired a unique phenotypic suppressor mutation resulting in compensatory H₂S production and tolerance to antibiotics and oxidative stress. Using whole genome sequencing, we mapped a non-synonymous single nucleotide polymorphism (SNP) to uncharacterized LacI-type transcription factor, YcjW. We identified transcriptional regulatory targets of YcjW and discovered a major target, thiosulfate sulfurtransferase PspE, as an alternative mechanism for H₂S biosynthesis. Deletion of *pspE* was sufficient to antagonize phenotypic suppression. Our results reveal a complex interaction between cell metabolism and H₂S production and the role, a hitherto uncharacterized transcription factor, YcjW, plays in linking the two.

Main

H₂S can be generated in numerous pathways-both enzymatically and non-enzymatically and from various substrates beyond cysteine⁶. The main pathway by which *E. coli* generate H₂S when grown aerobically in nutrient rich LB is via 3MST, encoded by *mstA*^{3,4}. Phenotypic consequences of decreased H₂S production include greater susceptibility to multiple classes of antibiotics and H₂O₂^{3,4,7}. However, we discovered that antibiotics-sensitive strain $\Delta mstA$ reverted to the resistant phenotype of the isogenic parent when challenged with different antibiotics. The $\Delta mstA$ variant, referred to as $\Delta mstA$ -sup in this study, was indistinguishable from wild type in time-kill assay analysis and growth curves of cells exposed to gentamicin, nalidixic acid and carbenicillin (Fig. 1a and Supplementary Fig.1). Furthermore, this strain also had increased tolerance to hydrogen peroxide (H₂O₂), compared to its still sensitive parent $\Delta mstA$ strain (Fig. 1b). Using both the classic lead acetate reactivity test for H₂S detection and a fluorescent-based probe, WSP5⁸, we confirmed that this phenotypic reversion was concurrent with increased H₂S production, comparable to wild type (Fig. 1c). In contrast, significant levels of H₂S remained undetectable in $\Delta mstA$ till OD₆₀₀ 1.5.

We utilized whole genome sequencing to identify possible SNPs coding regions that could be responsible for the observed phenotypic suppression. We mapped and validated by PCR a single missense mutation unique to $\Delta mstA$ -sup to uncharacterized transcription factor, *ycjW*. The nucleotide substitution, G to A on the coding strand, results in an amino acid change from serine to asparagine at residue 258 (Fig. 2a).

YcjW is annotated as a putative member of the LacI/GalR family of repressors that are largely responsible for carbohydrate metabolism. Common features of the family include an N-terminal helix-turn-helix DNA-binding domain, a linker domain, and a C-terminal ligand-binding domain⁹. To investigate SNP functionality, we constructed two strains, bearing a plasmid expressing either wild type *YcjW* (pLLY1) or S258N *YcjW* (pLLSN1), in the background of $\Delta mstA/\Delta ycjW$. Figure 2b shows that only plasmid-expressed mutated *YcjW* is able to restore H₂S production, quantitated by utilizing the WSP5 probe and qualitatively shown by lead acetate assay. Furthermore, only $\Delta mstA/\Delta ycjW$;P_{LL}-*ycjW* (S258N) have an increased survival rate when challenged with gentamicin, H₂O₂ and nalidixic acid (Fig. 2c and 2d and Supplementary Fig. 2). Thus, we

confirm that S258N YcjW in $\Delta mstA$ -sup is responsible for the increased hydrogen sulfide production and antibiotics and oxidative stress tolerance relative to $\Delta mstA$.

To identify transcriptional targets of YcjW, we performed ChIP-seq using an antibody against chromosomal 3xFLAG-tagged YcjW from wild type, and $\Delta mstA$ cells, and 3xFLAG-tagged YcjW S258N from $\Delta mstA$ -sup cells. Figure 3a shows representative peaks identified by MACS2¹⁰ from aligned sequence reads. The most enriched regions, for all three strains, are at two sites near *ycjW*; the first site is before the translation start site of *ycjM* but after a predicted transcription start site and the second lies between *ycjT* and *ycjU*. The binding motifs for LacI type family transcription factors are typically palindromes with a conserved central CG pair¹¹. Recently, Zuo and Stormo experimentally tested the predicted binding motif for YcjW¹². Combined with our analysis of peak summits, we found the same sequence in our data. Using the putative binding sequence, we further restricted peaks to ones containing the conserved 14bp motif, allowing up to three mismatches and with a fold enrichment greater than 5. With those criteria, we identified two additional peaks specifically in $\Delta mstA$, that are not enriched in wild type or $\Delta mstA$ -sup. The two sites are near the promoter of *narP*, encoding a two-component nitrate/nitrite response system and the other is located within the coding sequence of *cyaA*, encoding adenylate cyclase (Supplementary Fig. 3).

We then validated transcription factor binding through electrophoretic mobility shift assay (EMSA). We designed 50bp DNA probes containing the predicted binding sequence in the center. The YcjW protein reduced the mobility of the upstream *ycjM* DNA probe at about a 1:0.5 DNA:protein ratio. Increasing amounts of protein corresponded to an increase in YcjW-DNA complex (Fig. 3b). YcjW (S258N) also reduced DNA probe mobility at the same DNA:protein ratio, using *narP* probe (Fig. 3c). Titration of the normal protein and S258N YcjW showed that they both bound DNA probe starting at a DNA:protein ratio of 1:0.5. At a ratio of 1:2, no free DNA probe could be detected.

The region downstream of *ycjM* contains a predicted operon consisting of 10 genes-*ycjMNOPQRSTUV* and *ompG*. To test functionality of transcription factor-DNA binding to gene expression, we determined the amount of relative mRNA fold change using qRT-PCR. In $\Delta ycjW$, representative genes, *ycjM*, *ycjT*, *ycjU*, and *ompG* are

significantly upregulated, confirming that YcjW is a repressor (Fig. 3d). The absence of YcjW results in constitutive derepression of its regulatory transcriptional targets. The same genes exhibit a similar pattern, increased expression, in $\Delta ycjW$;P_{LL}-*ycjW* S258N relative to wild type MG1655 but not to the extent of its isogenic parent, $\Delta ycjW$. Consistent with $\Delta ycjW$;P_{LL}-*ycjW* S258N, those genes are also upregulated in $\Delta mstA$ -sup, but not $\Delta mstA$, suggesting that S258N YcjW affects DNA occupancy *in vivo* but not necessarily *in vitro*. Mutational analyses of other LacI-TFs have demonstrated that a single amino acid change in the C-terminal can alter effector or co-repressor binding and therefore DNA affinity at target sites^{13,14}. The SNP is located in the C-terminal effector pocket of the protein, thus raising the possibility that it broadens specificity of inducer recognition, co-repressor binding affinity or oligomerization.

qRT-PCR of *narP* and *cyaA* showed no significant change in $\Delta ycjW$. A small subset of genes regulated by NarP was tested as downstream targets (Supplementary Fig. 4). Two genes, *nrfA* and *ydhU* did have a modest increase while two others didn't. NarP regulation however is complex and involves multiple regulators. Therefore, it is difficult to assess if YcjW-binding upstream of *narP* and *cyaA* is functional.

Because many LacI-type repressors act locally in response to some specific effector, we sought to identify the inducer for YcjW by considering its targets. YcjT is homologous to kojibiose phosphorylase from *Thermoanaerobacter brockii* and *Pyrococcus* sp. Strain ST04^{15,16}. Kojibiose phosphorylase can reversibly catabolize kojibiose to D-glucose and beta-D-glucose 1 phosphate. The downstream gene, *ycjU*, has experimentally been shown to encode a beta-phosphoglucomutase¹⁷. Again, utilizing EMSA, we tested to see if kojibiose is the effector molecule for YcjW. The addition of kojibiose at 1mM disrupts the YcjW-DNA complex (Fig. 3e). Other disaccharides tested in excess of up to ten times, trehalose and sucrose, did not affect binding. However, attempts to grow *E. coli* K-12 MG1655 on minimal media with kojibiose as the sole carbon source were unsuccessful (data not shown and¹⁸). Growth on EZ Rich Defined media supplemented with kojibiose as the carbon source did grow but had a rather pronounced defect. Deletion of *ycjW* did not improve growth rates either (Supplementary Fig. 5). However, the concentration of kojibiose added to media is limited by its low solubility. It is possible that a higher concentration of kojibiose supplied would support enhanced growth. Taken together, our results indicate that kojibiose might

not be the natural inducer of YcjW but perhaps some derivative of kojibiose. Recently, the substrate for YcjM was identified as glucosylglycerate, alongside kojibiose for YcjT¹⁸. Glucosylglycerate is an osmoprotectant in bacteria and archaea and accumulates under salt stress and limited nitrogen availability^{19,20}. However, most of our experiments were conducted in LB with amino acids constituting the main carbon source. We find it unlikely that synthesis of either the glycoside or disaccharide could occur without the appropriate substrate, and therefore is not likely involved in $\Delta mstA$ phenotypic suppression.

While it is not directly evident how a cluster of carbohydrate catabolic genes regulated by YcjW can lead to an alternative pathway for H₂S production, *pspE* encoding a thiosulfate sulfurtransferase, lies immediately upstream of *ycjM*. PspE has mercaptopyruvate sulfurtransferase activity, albeit low compared to thiosulfate²¹. It is part of a cluster of genes known as the phage shock operon, consisting of *pspABCDE*. Although it can be co-regulated with the other *psp* genes, it can also be transcribed independently from its own promoter²². PspF is the transcriptional activator and phage shock protein A, encoded by *pspA*, negatively regulates PspF²³.

pspE mRNA expression is significantly increased in $\Delta mstA$ -sup and $\Delta ycjW$ relative to wild type cells. In contrast, there are no significant differences in relative expression of the other two thiosulfurtransferase genes (Fig. 4a). Furthermore H₂S production is undetectable during early exponential and mid-log phase in $\Delta mstA$ -sup/ $\Delta pspE$ (Fig. 4b). However, at late logarithmic phase, H₂S levels are now detectable to the same degree as MG1655 and $\Delta mstA$ -sup. In addition, overnight incubation with lead acetate strips shows no discernable difference in H₂S extracellular production between the three strains (Supplementary Fig. 6). We conclude from the significant delay of H₂S generation in $\Delta mstA$ -sup/ $\Delta pspE$ that PspE is capable of generating H₂S in early growth phases as observed in $\Delta mstA$ -sup. However, at later growth stages, another pathway for H₂S production is activated and/or PspE is no longer sufficient. Moreover, $\Delta mstA$ -sup/ $\Delta pspE$ also has increased sensitivity to gentamicin treatment compared to $\Delta mstA$ -sup and *E. coli* MG1655 but not quite as sensitive as $\Delta mstA$. Overexpression of PspE in $\Delta mstA$ increases survival rate but only to the extent of $\Delta mstA$ -sup/ $\Delta pspE$, not $\Delta mstA$ -sup or wild type (Fig. 4c). Altogether, we conclude that the SNP in *ycjW* resulted in increased expression of *pspE* in $\Delta mstA$ -sup. This is sufficient but not

wholly responsible for increased H₂S biosynthesis and in turn, the phenotypic suppression observed in $\Delta mstA$ -sup. We propose a model wherein, *E. coli* cells lacking MstA acquires a SNP in transcription factor YcjW. The SNP imparts moderate constitutive expression of YcjW targets, one of which is *pspE*. Thiosulfate sulfurtransferase PspE is then able to increase H₂S production in $\Delta mstA$, and subsequently protect the cells from antibiotics and H₂O₂ induced stress (Fig. 4d).

The region upstream of *pspE* does not contain a strong binding motif for YcjW, nor do the regions flanking regulators PspA and PspF. This is not entirely unexpected since none of the other genes tested in the *psp* operon were upregulated in $\Delta mstA$ -sup or $\Delta ycjW$ (Supplementary Fig. 6). Additionally, the moderate but significant increase of *pspE* mRNA in $\Delta ycjW$, in comparison to *ycjMNOPQRSTUVWXYZ-ompG*, suggests a more complicated interaction than direct DNA-binding. It may be indicative of “leaky” expression driven by the proximity of the 3’ end of *pspE* to predicted transcription start sites for *ycjM* and the binding site of YcjW. Perhaps, YcjW acts via a co-repressor. Another possibility is that the metabolic state of $\Delta mstA$ -sup with mutated *ycjW* induces *pspE* as a consequence. And finally, the enzyme(s) that is induced in $\Delta mstA$ -sup; $\Delta pspE$, after exponential growth, has not been identified and remains a target for future studies.

Altogether, our results reveal an alternative H₂S generator (PspE) that is mobilized as a result of rapid evolutionary adaptation to antibacterial stress. The SNP in YcjW, regulating metabolism to at least two rare sugars, presents an interesting link between sulfur metabolism and carbon availability. Moreover, a SNP in YcjW reflects the striking genetic plasticity employed by bacteria to promptly adapt to environmental changes and stimuli and highlights the survival advantage imparted by endogenous H₂S.

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Methods

General growth conditions

For the general cultivation of *E. coli*, strains were grown in LB broth supplemented with kanamycin (50ug ml⁻¹), or chloramphenicol (30ug ml⁻¹) as appropriate. Growth on solid medium contained 1.5% agar added to LB. Where noted, MOPS EZ Rich Defined Medium Kit (Teknova) was used in place of LB²⁴. Cysteine solutions were prepared immediately before use, as needed.

Construction of strains and plasmids

For a list of all strains used throughout this work, refer to Table 1.1. BW25112 and its derivatives are from the *E. coli* Keio Knockout Collection²⁵ (Thermo Scientific). Introduction of new mutations into *E. coli* MG1655 were achieved through P1 transduction as previously described²⁶. Temperature-sensitive FLP recombinase plasmid pCP20 was used for the excision of selective markers as needed²⁷. All constructs were verified with PCR and sequencing. Primers used throughout this study are listed in Table 1.2.

To generate pLLY1, *ycjW* was PCR amplified from *E. coli* MG1655 using primers LL10 and LL11 and cloned into pACYC184 plasmid (NEB) using the Gibson Assembly Mastermix, according to the manufacturer's protocol (NEB). Plasmid pLLSN3 was generated as above except *ycjW* was PCR amplified from *mstA*-sup. The Q5 Site-Directed Mutagenesis Kit (NEB) was used to generate pLLSN1 from pLLY1, according to manufacturer's protocol.

Transformations were performed using the CaCl₂ competent cell protocol²⁸. All plasmids were sequenced for verification.

Addition of 3xFLAG tag to *ycjW* at its chromosomal locus was achieved as previously described, with slight modifications²⁹. Briefly, primers pLL14 and pLL15 were used to PCR amplify Cm^R cassette from pKD4. PCR product was transformed into appropriate electrocompetent strains.

H₂S detection

End-point detection of H₂S production by lead acetate strips were performed as previously described³. Test strips were purchased from Sigma-Aldrich. Monitoring H₂S generation with the WSP5 fluorescent probe followed a modified protocol from Peng et al.⁸ Briefly, cells were grown in LB at 37°C to desired OD₆₀₀ and aliquots of approximately 4x10⁸ cells were taken. The extinction coefficient used for calculations is OD₆₀₀ of 1.0 is equal to 8x10⁸ cells. A working solution of WSP5 was made immediately before use and added to cells for a final concentration of 10uM. Samples were incubated

at 37°C for 30 minutes and then washed in PBS buffer, pH 7.4, to remove excess probe. Cells were resuspended in PBS buffer and incubated at room temperature for 30 minutes. Cytation3 (Biotek) was used to take fluorescent readings, at excitation 500 nm and emission 533 nm. All experiments were repeated for a total of three times. Background values were subtracted during analysis.

Time-kill assay and growth curves

Overnight cultures of *E. coli* were diluted 1:300 into fresh media and grown to an OD₆₀₀ of ~0.2. A 1ml aliquot was serially diluted and plated onto LB agar plates to determine initial colony forming units per ml (cfu ml⁻¹) after overnight incubation at 37°C. Antibiotics were added to the cultures at indicated concentrations. Aliquots of 1ml were collected at specified time intervals, serially diluted and plated. Results from three independent experiments were plotted in GraphPad version 5.0.

Growth curves were generated from Bioscreen C automated growth analysis system as previously described³. Antibiotics were purchased from Sigma-Aldrich or Gold Biotechnology.

Whole genome sequencing

Overnight cultures of *E. coli* cells were used for genomic DNA isolation. The MasterPure Complete DNA Purification Kit (Epicentre) was used to purify DNA according to the manufacturer's protocol. DNA samples were quantified using the Quant-IT PicoGreen dsDNA assay kit (Thermo Fisher) according to manufacturer's protocol. DNA was sheared to appropriate size with Covaris, followed by adaptor ligation. Sequencing was performed at New York University School of Medicine's Genome Technology Center.

Quantitative RT-PCR

Cells were grown until appropriate OD₆₀₀ and aliquots were collected and treated with RNAprotect Bacteria Reagent (Qiagen). After 5 minutes, cells were harvested and resuspended in lysis buffer (RNase-free TE buffer, 10 mg ml⁻¹ lysozyme, 100 ug ml⁻¹). Trizol LS (Thermo Scientific) was used according to manufacturer's protocol to extract total RNA. Samples were treated with DNase (Invitrogen) and purified using spin columns (Zymo Research). Superscript III reverse transcriptase (Invitrogen) was used to synthesize cDNA. qPCR reactions were amplified using Power SYBr Green PCR Master Mix (Applied Biosystems) with appropriate primer sets and cDNA template.

ChIP-seq

ChIP was carried out as previously described with the following modifications³⁰. Briefly, cells were grown at 37°C to OD₆₀₀ ~0.4 and a final concentration of 1% formaldehyde was added for in vivo cross-linking of nucleoprotein. A final concentration of 0.5M glycine was added to the culture to quench the reaction after a 20 minute incubation. Cells were collected by centrifugation and washing twice with 1X cold Tris-buffered saline then frozen in liquid nitrogen and stored at -80. Cells were resuspended in lysis buffer (50mM Tris [pH 7.5], 100mM NaCl, 1mM EDTA, protease inhibitor [Roche], 10 mg ml⁻¹ lysozyme). After incubation at 37°C, IP buffer (50mM HEPES-KOH, 150mM NaCl, 1mM EDTA, 1% Triton X100, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitor [Roche]) was added at a 1:3 ratio. DNA was sheared using ultrasonicator Covaris M220 on a 10 seconds on and 10 seconds off cycle for a total of 50 cycles.

The supernatant was incubated with 3xFLAG antibody (Biolegend) and Dynabeads Protein G (Thermo Scientific) overnight at 4°C. Samples were then washed twice with IP buffer, once with IP buffer+500mM NaCl, once with wash buffer (10mM Tris, 250mM LiCl, 1mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate), and a final wash with TE. Immunoprecipitated complexes were eluted in elution buffer (50mM Tris, 10mM EDTA, 1% SDS) at 65°C for 20 minutes. Samples were treated with RNase A (Qiagen), at 42°C and then uncross-linked with elution buffer+pronase for 2 hours at 42°C, followed by 6 hours at 65°C. DNA was purified using ChIP Clean and Concentrate (Zymo Research). Prior to sequencing, DNA was checked on TapeStation 2200 for appropriate size (Agilent). ChIP experiments were repeated for a total of three replicates.

For sequencing, sample libraries were prepared by using the NEBNext ChIP-seq Library (Illumina), according to manufacturer's protocol. Samples were sequenced on NextSeq 500 (Illumina). Bowtie and MACS2 were used for aligning and peak-calling, respectively¹⁰.

Electrophoretic mobility shift assay

Protein purification. YcjW and S258N YcjW were cloned into plasmid pet28-SUMO using the Gibson Assembly Mastermix kit, according to the manufacturer's protocol (NEB). Auto-induction media was used for protein production (152). Cells were harvested and resuspended in lysis buffer (1M NaCl, 5mM Imidazole, 5% Glycerol, protease inhibitor cocktail [Roche]) and sonicated. AKTA Start system was used for chromatography with HisTrapHP columns (GE Healthcare Life Sciences). Columns were

washed in wash buffer (50 mM Tris-Cl [pH 8.0], 10 mM Imidazole, 5% Glycerol, 500 mM NaCl), followed by gradient elution with elution buffer (50 mM Tris-Cl [pH 8.0], 250 mM Imidazole, 5% Glycerol, 250 mM NaCl). The SUMO tag was cleaved with SUMO protease in dialysis buffer (200 mM NaCl, 50 mM Tris-Cl [pH 8.0], 5% Glycerol, 1mM DTT). Samples were applied to a HiTrap HeparinHP column (GE). Columns were washed with buffer (20mM Tris [pH 8.0], 50mM NaCl, 5% glycerol), and eluted in elution buffer (20 mM Tris [pH 8.0], 1.5 M NaCl, 5% glycerol). The sample was concentrated to 5 mL and injected onto a Superdex 200 column with GF buffer (20 mM Tris-Cl [pH 8.0], 50 mM NaCl, 1 mM DTT).

EMSA. dsDNA probes containing the binding sequence were radiolabeled with gamma ³²P rATP using T4 polynucleotide kinase (NEB). Labeled probes were purified by passage through size exclusion columns (Bio-Rad). Binding reactions were done as previously described¹². The gel was then exposed to a phosphor screen and visualized on Storm 820 Phosphorimager (GE Healthcare). Experiments with various disaccharides were done in a similar fashion, except purified protein was incubated with appropriate sugar for 20 minutes at room temperature before addition of radiolabeled probe.

Figure 1 *E. coli* MG1655 lacking 3MSTA acquires phenotypic suppression and has increased H₂S levels and tolerance to Gm and H₂O₂ (a) $\Delta mstA$ -sup has increased survival rate compared to $\Delta mstA$ when treated with 2 μ g ml⁻¹ gentamicin in a time-kill assay (b) $\Delta mstA$ -sup also has increased tolerance after exposure to 5mM H₂O₂ for 30 minutes (c) H₂S production as measured with fluorescent probe, WSP5. Relative fluorescent units are normalized to OD₆₀₀ and minus the background fluorescent of PBS buffer+100 μ M L-cysteine and WSP5. H₂S reacts with lead acetate, leading to staining of strips (Sigma-Aldrich). Values are means $\pm$ SD (n=3). * p<0.05

Figure 2 Unique nonsynonymous SNP in $\Delta mstA-sup$ is mapped to putative transcription factor *ycjW*. (a) PCR validation of whole genome sequencing. Displayed are sequences from *E. coli* MG1655, $\Delta mstA$, and $\Delta mstA-sup$ of (-) strand. The SNP changes amino acid 258 from serine to asparagine. (b) $\Delta mstA/\Delta ycjW$ strains with plasmid expressed $P_{LL}-ycjW$ or $P_{LL}-ycjW$ S258N were measured for H₂S production. Only $\Delta mstA/\Delta ycjW; P_{LL}-ycjW$ S258N had increased H₂S production with no significant differences with WT or $\Delta mstA-sup$. At OD₆₀₀ 1.5 no significant differences exist between strains. (c) Cells exposed to 2ug ml⁻¹ Gm. $\Delta mstA/\Delta ycjW; P_{LL}-ycjW$ S258N had increased survival rate. $\Delta mstA/\Delta ycjW; P_{LL}-ycjW$ was more sensitive to Gm compared to wild type and $\Delta mstA-sup$. (d) $\Delta mstA/\Delta ycjW; P_{LL}-ycjW$ S258N improved tolerance to H₂O₂. Values are means $\pm$ SD (n=3) for all experiments.

Figure 4 Deletion of *pspE* in $\Delta mstA$ -sup decreases H₂S biosynthesis during exponential growth (a) qRT-PCR of three thiosulfate sulfurtransferase genes. RNA was isolated from cells grown to OD₆₀₀ ~ 0.4. Only *pspE* expression was significantly increased in both $\Delta mstA$ -sup and $\Delta ycjW$ (b) H₂S levels in $\Delta mstA$ -sup; $\Delta pspE$ were undetectable till OD₆₀₀ reached 0.8. (c) $\Delta mstA$ -sup/ $\Delta pspE$ had increased sensitivity to gentamicin compared to WT and $\Delta mstA$ -sup. Overexpression of *pspE* in $\Delta mstA$ increased tolerance to gentamicin compared to $\Delta mstA$. Values are means $\pm$ SD (n=3) (d) In cells lacking MstA which is responsible for H₂S production, S258N YcjW upregulates PspE as an alternate route of H₂S biosynthesis.

TABLE 1

STRAIN NAME	Description	SOURCE
<i>E. coli</i> K12 MG1655	F- lambda- <i>ilvG</i> - <i>rfb</i> -50 <i>rph</i> -1	31 32
$\Delta mstA$		3
$\Delta mstA$ -sup	$\Delta mstA$; <i>ycjW</i> ^{S258N}	This study
$\Delta mstA$; $\Delta ycjW$		This study
$\Delta mstA$; $\Delta ycjW$; P _{LL} - <i>ycjW</i>		This study
$\Delta mstA$; $\Delta ycjW$; P _{LL} <i>ycjW</i> ^{S258N}		This study
$\Delta ycjW$; P _{LL} <i>ycjW</i> ^{S258N}		This study
WT-3xFlag	<i>E. coli</i> K12 MG1655 (<i>ycjW</i> fused to 3X FLAG)	This study
$\Delta mstA$ -3XFlag	$\Delta mstA$ (<i>ycjW</i> fused to 3X FLAG)	This study
$\Delta mstA$ -sup-3XFlag	$\Delta mstA$; <i>ycjW</i> ^{S258N} (<i>ycjW</i> ^{S258N} fused to 3X FLAG)	This study
$\Delta pspE$		This study
$\Delta mstA$ -sup; $\Delta pspE$	$\Delta mstA$; <i>ycjW</i> ^{S258N} ; ; $\Delta pspE$	This study
$\Delta mstA$; P _{LL} - <i>pspE</i>	$\Delta mstA$; overexpression PspE	This study

TABLE 2

Name	Sequence	target
LL01	TTATACGCAAGGCGACAAGG	pKD3 CmR ²⁷
LL02	GATCTTCCGTCACAGGTAGG	pKD3 CmR ²⁷
LL03	CGGTGCCCTGAATGAACTGC	pKD4 KanR ²⁷
LL04	CAGTCATAGCCGAATAGCCT	pKD4 KanR ²⁷
LL05	CGGCCACAGTCGATGAATCC	pKD4 KanR ²⁷
LL06	CACAATGGCCCGTTAGCAAC	mstA
LL07	TTTGCTGTGAAGCACCTGCG	mstA
LL08	AGTCGCTTCATTGCTGTCCT	ycjW up
LL09	CTTTCCGTTTCGCTGGAATA	ycjW down
LL10	gcataaggagagcgAGGCGCAGATGTCTGTCTCG	pAC-ycjW
LL11	gtagccagcgcgctcTTATTTTCAGCGTCATATGCG	pAC-ycjW
LL12	GAAATTAATGaacATTTATCGTGCGGCAGC	pAC-ycjW SN
LL13	AGGCTGTCAGTGGCAAAC	pAC-ycjW SN
LL14	CGGCGAACTCCATAAGATGT	3xFlag ycjW
LL15	CTGGCATTTCAGGCAAGGTAG	3xFlag ycjW
LL16	CGACGTAACTGTGCGGTAA	glpE
LL17	CGCCATGTAATCAACAAACG	glpE
LL18	GCA AAT GGG TTG AAG GAA AA	cysB
LL19	GCC GAA AAT AAC GCA AGA AA	cysB
LL20	TCGGGCTGCTAATAAACTGG	pspE
LL21	ATGTTGCCTTTCCGTGGTAG	pspE
LL22	AATGGATGGCAATTTTCATCCATAGAAGGAC GCTTACATGGTGTAGGCTGGAGCTGCTTC	pspE-pKD3
LL23	ACCTTTGACCTTCGGCATTGCGATGTCTTTC AGGCCACCGCATATGAATATCCTCCTTAG	pspE-pKD3
LL24	GCCTTTAAAAAAGGCTTACTTGC	pspE-AskA
LL25	CCACCTTTGACCTTCGGCATTGC	pspE-AskA
LL26	CTAATGCCGGGTACCTTGA	cyaA-qRT
LL27	GGCGGATCCTGTACTGACAT	cyaA-qRT
LL28	CGTGAACGTGAAATGTTTGG	narP-qRT
LL29	ATTCAACACCGAGGCAATCT	narP-qRT
LL30	CCGCAATAACGGTGAGTTTT	ompG-qRT
LL31	AAATCATGGCCTTCACGTTC	ompG-qRT
LL44	GATCAACTGGTTCACGCTCA	nrfA-qRT
LL45	CGTCCTGAATTGGCTTCATT	nrfA-qRT
LL46	TCGATGTTTCGTGTTCCAGAG	pspE-qRT
LL47	ATCTCTTTTGCTTGCCCTGA	pspE-qRT
LL48	GCAGGCTTTCCATTAAACCA	glpE-qRT

LL49	GACCACATCGTAGCCCTGTT	glpE-qRT
LL50	ATCGTGAATGCCAACATCAA	pspA-qRT
LL51	TGTTCAATACGGCGAGTCAG	pspA-qRT
LL52	CGCGCTATTTCTGGCTATTC	pspB-qRT
LL53	TGCTCACTTTGCGACAATTC	pspB-qRT
LL54	AGCAGGCAAGCTGGTACTTC	pspC-qRT
LL55	CGGCGAGCAACTGATTTTAT	pspC-qRT
LL56	CGTGTTGTGAAATGTTGGGTAA	16S
LL57	CCGCTGGCAACAAAGGATA	16S
LL58	GAGTAGTCTCTCGTTTCATGGGACCGCTACC ACGGAAAGGCCAACatgAAAC	ycjM-EMSA
LL59	GTTTcatGTTGCCTTTCCGTGGTAGCGGTCCC ATGAAACGAGAGACTACTC	ycjM-EMSA
LL60	CTAATGCCGGGTACCTTGATGGTAACGTTC CCAAAGGCATTTGCCTTTACACG	cyaA-EMSA
LL61	CGTGTAAGGCAAATGCCTTTGGGAACGTTA CCATCAAGGTAACCCGGCATTAG	cyaA-EMSA
LL62	GGTAACATTCACGCGCCTGGTAGCGTTACC AACGCTACGCTCAAACATAATGATTC	narP-EMSA
LL63	GAATCATTATGTTTGAGCGTAGCGTTGGTAA CGCTACCAGGCGCGTGAATGTTACC	narP-EMSA
LL64	CCTGCCAATCAGGATCACGATACGCCGCTA TTACAGCAATATTTTTTCGtga	nonspecific
LL65	tcaCGAAAAATATTGCTGTAATAGCGGCGTAT CGTGATCCTGATTGGCAGG	nonspecific
LL66	GTCGGCAAAAAGTGAATGGT	ycjM-qRT
LL67	GCATCTGGAATGGCGTTAAT	ycjM-qRt
LL68	CAACAGAAAACCTGGCGTCAA	ycjT-qRT
LL69	CGCAGATGATAAAGCGCATA	ycjT-qRT
LL70	ACTGGCGTATCGCAAATC	ycjU-qRT
LL71	GCATTCAGGGAGACAGAAGC	ycjU-qRT
LL72	CCGATGATCTGATGCTGGCT	rpoD-qRT
LL73	GTGCGCCCGATTTCAGATTC	rpoD-qRT

SUPPLEMENTARY FIGURES

+2ug ml⁻¹ nalidixic acid

+1ug ml⁻¹ carbenicillin

Fig. S1 Δ mstA-sup has increased tolerance to carbenicillin and nalidixic acid **(a)** Cells were grown in the presence of 2ug ml⁻¹ nalidixic acid and monitored for growth by OD₆₀₀. Δ mstA-sup grows as well as wild type **(b)** Cells were grown in the presence of 1ug ml⁻¹ carbenicillin and monitored for growth by OD₆₀₀. Δ mstA-sup grows as well as wild type.

E. coli MG1655 (WT)
mstA-sup
mstA
Annotations

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Fig. S4 YcjW does not affect *narP* expression or *cyaA*. NarP is a two-component nitrate/nitrite response regulator and can either repress or activate transcription. Of the genes measured for changes in mRNA levels, only *nrfA* and *ydhU* are significantly increased in $\Delta ycjW$. Upregulation however, is modest.

Fig S5 E. coli cells were grown in EZ rich defined media supplemented with either 0.5% glucose or 0.5% kojibiose as the carbon source. WT has a pronounced growth reduction with kojibiose. Knockout of *ycjW*, which results in significant upregulation of *ycjT*, capable of catalyzing kojibiose, did not improve growth.

Fig S6 After overnight incubation with lead acetate strips, $\Delta mstA$ -sup/ $\Delta pspE$ is able to overcome the early deficit in H_2S production seen during exponential growth. This is in contrast to $\Delta mstA$, which does not accumulate extracellular H_2S to the extent of WT after overnight growth.

Fig S7 *YcjW* does not affect other *psp* genes. *pspABC* is upstream of *pspE* which is upregulated in $\Delta ycjW$ indicating that the regulation of *pspE* in this case is distinct and separate from the phage shock operon in general.

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