Phenotype Enhancement Screen of a Regulatory spx Mutant Unveils a Role for the ytpQ Gene in the Control of Iron Homeostasis

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Abstract

Spx is a global regulator of genes that are induced by disulfide stress in Bacillus subtilis. The regulon that it governs is comprised of over 120 genes based on microarray analysis, although it is not known how many of these are under direct Spx control. Most of the Spx-regulated genes (SRGs) are of unknown function, but many encode products that are conserved in low %GC Gram-positive bacteria. Using a gene-disruption library of B. subtilis genomic mutations, the SRGs were screened for phenotypes related to Spx-controlled activities, such as poor growth in minimal medium and sensitivity to methyglyoxal, but nearly all of the SRG mutations showed little if any phenotype. To uncover SRG function, the mutations were rescanned in an spx mutant background to determine which mutant SRG allele would enhance the spx mutant phenotype. One of the SRGs, ytpQ was the site of a mutation that, when combined with an spx null mutation, elevated the severity of the Spx mutant phenotype, as shown by reduced growth in a minimal medium and by hypersensitivity to methyglyoxal. The ytpQ mutant showed elevated oxidative protein damage when exposed to methyglyoxal, and reduced growth rate in liquid culture. Proteomic and transcriptomic data indicated that the ytpQ mutation caused the derepression of the Fur and PerR regulons of B. subtilis. Our study suggests that the ytpQ gene, encoding a conserved DUF1444 protein, functions directly or indirectly in iron homeostasis. The ytpQ mutant phenotype mimics that of a fur mutation, suggesting a condition of low cellular iron. In vitro transcription analysis indicated that Spx stimulates transcription from the ytpPQR operon within which the ytpQ gene resides. The work uncovers a link between Spx and control of iron homeostasis.

Introduction

Transcriptome profiling can place genes into regulons or stimulons by providing evidence for coordinate control, governed by a transcriptional regulator and responsive to a specific metabolic or environmental stimulus [1]. In Gram-positive bacteria, some regulons, such as those controlled by alternative RNA polymerase sigma subunits [2] and global regulators [3] are large and complex. For example, the general stress SigmaB regulon, transcription of which requires the alternative RNA polymerase sigma subunit, is estimated to include over 200 genes in L. monocytogenes [4] and over 120 genes in B. subtilis [5,6,7,8]. Many of the genes within complex regulons are of unknown function and the sites of mutations having no detectable phenotype. Hence our view of the roles global regulators play in bacterial physiology remains incomplete. We can imagine that the genes within these complex regulons reside in functionally redundant or genetically buffered subgroups required for alleviating stress by detoxifying or removing harmful agents, or repairing the damage such agents inflict upon macromolecules and supramolecular structure.

The Spx protein is a global regulator of the Gram-positive bacterium’s stress response [9]. It is highly conserved in low G+C Gram-positive bacteria [10], and in B. subtilis it interacts with RNA polymerase to exert positive and negative transcriptional control over a genome-wide scale [11,12,13]. The products of genes having known function that are induced by Spx include thioredoxin, thioredoxin reductase, and products that function in cysteine biosynthesis as well as synthesis of the low molecular weight redox buffer, bacillithiol [12,14,15]. Spx activates the transcription of its regulon in response to disulfide stress and in cells treated with various toxic agents including paraquat, nitric oxide, cell wall-acting agents, toxic electrophiles and hypochloric acid [12,16,17,18,19,20,21]. Spx is under tight regulation that involves positive and negative transcriptional control [22,23,24] and proteolytic control by a substrate-binding factor, YjbH, together with the ATP-dependent protease, ClpXP [25,26,27,28]. Additionally, its activity is controlled by a disulfide redox switch involving a CXXC motif at the N-terminal end of Spx that affects the protein’s productive interaction with RNA polymerase [11]. Spx governs a large regulon with about 120 of its members designated as “y” genes of unknown function (Table S1). Because

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the Spx regulon is induced under a variety of stress conditions, uncovering the function of the Spx-regulated genes (SRGs) would further define the role of Spx in the cell’s response to encounters with harmful agents.

In recent years, methods of genetic analysis have been developed to exploit the vast collections of genomic data generated from whole genome sequencing projects. Large gene knock-out libraries have been created and utilized to uncover functional genetic modules consisting of genes that influence essential cellular processes. One way this has been accomplished is by the systematic and automated screening of strains with paired mutations (double mutants) to search for synthetic phenotypes indicative of genetic interaction [29,30,31,32,33,34,35]. The rationale for uncovering modules of interacting genes has its origins in concepts of functional redundancy and genetic buffering [36]. Elegant studies using classic genetic systems, and screens for unlinked non-complementation and synthetic lethality, uncovered genes that reside within functional modules that affect, for example, morphogenesis and the dynamics of cytoskeletal components [37,38,39,40,41]. More recent studies of genomewide synthetic genetic arrays uncovered new factors involved in iron metabolism and in the activity of the transcription complex [29]. Thus genetic screens for synthetic interaction and phenotype enhancement can shed light on the functions of genes for which no known function has been assigned. Hence, we undertook a phenotype enhancement screen of ytpQ mutants bearing knock-outs of each of the SRGs of unknown function. Strains with the ysp mutation paired with each srg mutation that have defects in growth or elevated sensitivity to methylglyoxal, to which spx mutants are sensitive, were detected. One such srg mutation, in the ytpQ gene, was studied using proteomic and transcriptomic analyses, which uncovered a role for ytpQ in iron metabolism/homeostasis.

Results and Discussion

Results of previously published microarray hybridization data [12], uncovered about 125 genes of the “y” designation that were more than 3-fold upregulated by high Spx concentrations (Table S1). The majority of the genes are of unknown function, although many encode products that are highly conserved in other, mostly Gram-positive, bacterial species. While we do not know at this point if all of the genes are under the direct transcriptional control of Spx, we decided to designate the genes as SRGs, or Spx-regulated genes.

The Spx regulon is induced by a number of toxic agents and stress conditions; [10,12,16,17,20,22,42]. To understand further the role of Spx in the cell’s response to these diverse stress conditions, we sought to gain information on the function of the individual SRGs. A B. subtilis ORF knock-out library, obtained from K. Kobayashi and N. Ogasawara (NAIST, Japan), contains gene disruptions in over 2,000 ORFs that are assigned the “y” genomic designation [43]. Each disruption was created by an integrated DNA fragment, derived from plasmid pMUTIN [43], that was inserted within the target gene’s coding sequence. The fragment contains a promoter-less lacZ gene at its 5’ end, followed by the E. coli lacI gene, the product of which controls an IPTG-inducible P Jas promoter [44] residing at the 3’ end of the fragment and oriented in the 3’ direction. The P Jas promoter in this position can drive expression of genes located downstream of the insertion [43], thus alleviating potential insertion-dependent polar effects. The use of pMUTIN-mediated gene disruption was used in previous genome-wide mutational screens and in gene interaction studies [45,46]. Gene disruptions in the SRGs listed in Table S1 were tested by screening for growth on nutrient broth sporation medium (DSM) and minimal glucose (TSS) medium plates. Some slight defect in growth on TSS agar plates was detected for some of the SRG mutants, but the majority showed no obvious defect in growth based on colony size. We next tested the SRG mutant strains for defects in growth on DSM agar plates containing methylglyoxal (MG); a toxic alpha-oxoaldehyde to which spx mutants are sensitive (data not shown). Again, we observed only minor growth defects compared with the wild-type parent on DSM agar medium containing concentrations of methylglyoxal to which spx mutants are sensitive.

Phenotype enhancement screen of srg mutations in the srx mutant background

Possible reasons for the absence of SRG mutant phenotype related to stress resistance include genetic interactions built into the srx regulon that contribute to functional redundancy and genetic buffering. We reasoned that uncovering phenotype would require overcoming regulon genetic interactions that mask defects conferred by the SRG mutations. Hence, we undertook a screen of the SRG mutations within the srx deletion mutant background. The reasoning is illustrated in Figure 1, where the srx mutation is shown to cause a reduction in overall SRG expression, thereby reducing the contributions of genetic interactions among SRGs. Introduction of the srg mutation into the srx mutant background potentially confers hypersensitivity to the agents to which the srx mutant is sensitive, but the srg srx mutant would now be predicted to exhibit further reduction in growth on minimal medium, and hypersensitivity to lower toxic agent concentrations that have little to no effect on the parent srx mutant.

Each plate was inoculated with 10 μl of a dilution series of a log phase culture. The size of the isolated colonies was then measured using the Plixiellus application (Material and Methods). An example of a plate and data collected is shown in Figure S1, where four strains, the wild type JH642, the srx mutant, the SRG mutant, yitV, and the ysp yitV double mutant plated on DSM and DSM plus MG is shown. The average isolated colony size as determined by Plixiellus indicated that the double mutant has a slight growth defect on the DSM-MG plate compared with the srx mutant parent. Most of the double mutants screened showed a result similar to that uncovered in the yitV/srx mutant.

Disruption of ytpQ, an Spx-regulated gene of unknown function, enhances srx mutant phenotype when combined with an srx null mutation

Other double mutants showed more dramatic phenotype enhancement on TSS agar plates than was observed in the case of the yitV mutant. The linked genes ytoQ and ytpQ (Figure 2A) were sites of two gene disruptions that showed growth defects in the srx mutant background. The ytpQ srx mutant showed a severe growth defect on TSS minimal medium (Figure 2B and 2C). The phenotypes of the ytoQ srx and ytpQ srx mutants were examined in growth curves of cultures in liquid TSS medium (Figure 2D and E). Both ytpQ and ytoQ mutants and the srx null mutant showed defects in growth as evident in the slope of the log phase portion of the growth curves or final OD600 (doubling times: JH642: 42.8 min, srx null mutant; 93.8 min, ytoQ 60.3 min, ytpQ 73.3 min). The double mutants showed a further reduction in growth rate ytoQ srx: 103.8 min, ytpQ srx: 164.9 min) and had a lower growth yield. While the introduction of the ytpQ mutation into the srx null mutant resulted in a slower growth rate than the srx null, the doubling time of the ytoQ srx strain was not significantly longer than that of the srx mutant. Hence, we did not further analyze the ytoQ mutant.
The ytpQ and ytpR genes are linked and divergently oriented in the B. subtilis chromosome (Figure 2A). The ytpQ gene product is a member of the DUF1444 family of bacterial proteins with no known function. The ytpQ gene is part of a tricistronic operon that also contains ytpP and ytpR. Disruption of ytpR showed no noticeable phenotype on DSM or TSS medium with or without MG, and confers no phenotype enhancement in the ytpQ mutant background (data not shown). The disruption of the ytpP gene, encoding a thioredoxin-like protein [47], conferred phenotype enhancement in the spx background (Figure 2B), but this was reversed by addition of IPTG, showing that the defect was due to a polar effect of the ytpP insertion (data not shown), most likely causing reduction in ytpQ gene expression.

Complementation was conducted using an IPTG-inducible version of the ytpQ gene ectopically expressed from the amyE locus of the B. subtilis genome. For these experiments, a deletion ytpQ mutation was constructed in which part of the ytpQ coding sequence was replaced by a spectinomycin-resistance cassette. An spx ytpQ double mutant (ORB7816) was then constructed by introducing the ytpQ:spx mutation into a spx::tet (tetracycline resistance) mutant. As was observed with the ytpQ:pMUTIN spx double mutant, the ORB7816 strain enhanced the growth-defective phenotype compared with either the ytpQ or spx mutants in the presence or absence of MG (Figure S2). Thus, a strain bearing the new ytpQ:spx allele and the ectopic inducible ytpQ construct was grown in TSS minimal medium in the presence and absence of 0.5 mM IPTG. The results (Figure S3) showed that the reduced growth rate of the ytpQ mutant (doubling time of 79.4 min) was reversed when the ectopically expressed ytpQ gene was induced. The induced complemented strain (ORB8011) had a growth rate similar to the wild type parent (JH642; doubling time of 47.3 min. OR8011; doubling time of 46.5 min).

The ytpPQR operon is under direct Spx control

To validate the previous microarray results, the ytoQ and ytpQ pMUTIN insertions, both generating transcriptional lasZ::lacZ fusions, were used to measure ytoQ- and ytpQ-directed β-galactosidase activity in cells expressing an IPTG-inducible, protease resistant form of Spx (SpxDD) that were grown in liquid DSM medium (Figure S4). In keeping with the microarray results, the fusions showed elevated expression when the spxDD allele was induced. Furthermore, microarray analysis (described below) indicated reduced ytpQ transcript levels in the spx mutant (Table S2). Validation was also accomplished by transcription analysis performed in vitro, which showed that addition of Spx in a reaction with the ytpPQR promoter region DNA and RNA polymerase resulted in the synthesis of a transcript of the predicted size (Figure 3). Synthesis of the transcript is stimulated by the addition of Spx and initiates near a σA-recognized promoter sequence (Figure 3A and B). The reaction utilized His-tagged RNA polymerase from an ytoQ::tetracycline mutant from which RNA polymerase lacking σA was obtained. Purified σA was required for each reaction in order to observe a transcript from the ytpP promoter template (data not shown). This result is consistent with the presence of a σA-utilized promoter upstream of the ytpP coding sequence.

The ytpQ mutant has increased levels of protein damage after MG treatment

We further examined the phenotype of the ytpQ mutant in order to gain more information about its possible role in the B. subtilis stress response. Previous microarray hybridization studies indicated that ytpQ was derepressed in a perR [peroxide regulator [48]] mutant background [49]. That ytpQ is derepressed in a perR mutant and activated by Spx suggests that its product might function in the oxidative/electrophile stress response. We determined if the ytpQ mutant shows elevated levels of protein carboxylation damage by performing an oxyblot [50] on cell extracts from cultures of JH642, spx mutant, and ytpQ::spc mutant cells that were untreated or treated with MG. The untreated wild-type cells showed some evidence of oxidative protein damage (Figure 4), which was increased upon MG treatment. The untreated spx mutant cells showed more damage than the wild type parent, with some increase in damage following MG treatment. The untreated ytpQ mutant cells resembled the untreated wild-type parent in the level of oxidatively damaged protein, but the mutant underwent a dramatic increase in the level of protein damage upon MG treatment that was much higher than the treated wild-type parent or spx mutant. The result suggests that the ytpQ product plays a role in preventing protein damage resulting from an encounter with a toxic electrophile (MG).

Transcriptomic and proteomic analyses indicate a role of ytpQ in iron homeostasis

To gain more insight into the function of ytpQ, the transcriptome of the ytpQ mutant was analyzed, and compared with that of the wild type and the spx mutant. Similarities in the transcriptomic changes conferred by the spx and ytpQ mutations would provide clues to the role played by YtpQ within the Spx regulon. The wild-type parent, ytpQ, and spx mutants were grown in a glucose minimal medium, with and without 2.8 mM MG, to mid-log phase. Cells were harvested and RNA was extracted for
microarray hybridization analysis to determine if any change could be detected in the composition of the transcriptome that were attributable to the ytpQ mutation (data in Tables S2 and S3). Previous microarray hybridization analysis identified putative Spx-controlled genes by detecting elevations in transcript levels when protease resistant forms of Spx were produced [12]. The transcriptome was reexamined, this time, by conducting microarray analysis with an spx null mutant. As predicted from the previous work, the spx mutation causes extreme changes to the cell transcriptome [12,14,27,51]. There is a dramatic increase in the

Figure 2. Phenotype of srg mutations in spx mutant background. A. Chromosomal organization of ytoQ ytpQ locus. Arrows represent location and orientation of coding sequences and lollipop figure denotes location of putative transcriptional terminator. B. Phenotype of ytpP and ytpQ mutations in the wild-type and spx mutant backgrounds. Cultures were grown to late log phase and serially diluted 10-fold. Ten µl were spotted on TSS minimal medium plates with and without MG. C. Minimal TSS plate onto which JH642 (wild-type parent), the spx mutant, the ytpQ::pMUTIN mutant, and the double mutant spx ytpQ::pMUTIN were streaked. D and E. Growth curves of ytoQ and ytpQ mutants in wild-type and spx mutant backgrounds. Open circles: JH642. Open squares: spx mutant. Closed triangles ytoQ mutant (D), and ytpQ mutant (E). Closed squares: ytoQ spx mutant (D) and ytpQ spx mutant (E). doi:10.1371/journal.pone.0025066.g002

Figure 3. Spx-activated transcription from the ytpPQR operon promoter. A. The nucleotide sequence of the ytpPQR promoter region is shown. The bold plain text indicates the oligonucleotide primers used to generate the linear DNA promoter template fragment for the in vitro transcription reaction. Also the region underlined and in italics shows the putative promoter region along (in bold) the –10 region (tagat) with extended TG and –35 region (tggtt) and the Spx cis element (tgcatataa) upstream from the –35 region. B. In vitro transcription from ytpP promoter. The ytpP promoter template (10 nM) was incubated with 25 nM σA-depleted RNAP and 25 nM σS in the absence or presence of 75 nM Spx. Samples were collected from the reactions at indicated times during incubation (2, 4, 8, and 12 min). Transcripts were resolved by gel electrophoresis, visualized and quantified as previously described [77]. Marker transcripts were generated using Spx protein and purified RNA polymerase, along with DNA fragments containing the Spx-controlled trxB promoter [11] and the yrrT promoter [14] in transcription reactions. doi:10.1371/journal.pone.0025066.g003
level of transcripts from CymR-controlled genes, whose products function in organosulfur metabolism and the synthesis of cysteine (Figure 5, Table S2) [14,52,53]. In fact, the impaired growth of the spx mutant on minimal medium can be reversed by addition of cysteine, indicating that the spx mutant is a mild Cys auxotroph. The reduced expression of the trxA gene might account for spx growth phenotype on minimal medium, since thioredoxin is required for sulfate utilization [54]. There is an increase in the level of transcript from genes controlled by PerR and Fur, both Fe-binding proteins that regulate, respectively, the peroxide response and genes that are activated under iron starvation (Figure 5, Table S2). ComK-dependent transcription was also derepressed confirming previously reported results [55,56]. Synthesis of transcripts encoded by early sporulation genes controlled by σH, σE, and σF was also up-regulated in the spx mutant background. ClpX was known to be required for σH-dependent transcription (Figure 6, Table S2) [37,50], which was partially relieved by a mutation in the spx gene [55].

The most striking result from the transcriptome analysis of the ytpQ mutant is the dramatic derepression of the Fur (iron uptake regulator) and PerR (peroxide response regulator) regulons (Figure 5, Table S2 and S3). The ytpQ mutation seems to mimic the reported phenotype of the B. subtilis fur mutant [59]. The expression of genes (dhbABCDEF) encoding the enzyme complex that catalyzes dihydroxybenzoyl-glycine (DHB-Gly, or itoic acid) synthesis was dramatically increased. The B. subtilis strain, JH642, bears a mutation in the ytp gene [60] encoding phosphopantethei-
nyl transferase [61] required for non-ribosomal peptide synthetase activity. Hence, JH642 cells are unable to synthesize the siderophore, bacillibactin (DHB-Gly-Thr) [62] despite the fact that genes required for its biosynthesis (the \textit{dhb} operon) show elevated expression. The genes specifying hydroxamate siderophore utilization (\textit{yxEB}, \textit{fhuB}, \textit{fhuD}) were also upregulated in the \textit{ytpQ} mutant. The expression of the Fur-regulated \textit{ykaNOP} operon was elevated higher than 10-fold in the \textit{ytpQ} mutant. The operon encodes two flavodoxins that serve as reductases for nitric oxide synthase catalysis and for two-electron transfer to cytochrome P450 BioI. This latter function can be fulfilled by the product of the \textit{fer} gene, which is a 4Fe-4S ferridoxin. Under conditions of low iron, the \textit{ykaN} and \textit{ykaP} products provide an iron-free substitute. Another characteristic of the \textit{ytpQ} mutant that mimics the \textit{fur} null phenotype is the derepression of the genes belonging to the cryptic prophage, PBSX (Figure S5) [59]. It is not known why these genes show elevated expression in the \textit{fur} mutant.

Notably, several of the genes derepressed in the \textit{ytpQ} mutant were further up-regulated upon MG treatment (Figure 5, Tables S2, S3). These include genes controlled by PerR and Fur, as well as the \textit{yfx} gene itself. Elevated expression of genes encoding the transporter for elemental iron (\textit{yfmLMN}) was observed in the \textit{ytpQ} mutant cells treated with MG.

Another class of genes showing elevated expression in the \textit{yfx} and \textit{ytpQ} mutant is the \textit{sD} regulon, which includes genes that function in motility and chemotaxis [63,64]. The level of the \textit{hag} gene transcript, encoding flagellin, was increased 80-fold in the \textit{ytpQ} mutant (Figure 6). It is not clear why the expression of the \textit{sD} regulon is elevated in the \textit{ytpQ} mutant. Reduced growth rate of the \textit{ytpQ} mutant could be related to the elevated expression of genes that

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**Figure 6. Hierarchical clustering analysis of gene expression profiles up- and downregulated in \textit{spx} and \textit{ytpQ} mutants.** Gene expression data were clustered based on the induction or repression ratios in the \textit{spx} and \textit{ytpQ} mutants leading to different nodes specific to regulons. Nodes including regulons involved in motility, competence and sporulation are shown (Com, SigmaD, SigmaH, CodY, SinR, Spo0A, SigmaL, AhrC, Rok, SigmaE, F, G, K regulons). Red indicates induction and green repression in the \textit{spx} or \textit{ytpQ} mutants under control conditions, and MG stress. doi:10.1371/journal.pone.0025066.g006

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increased amounts in the transcription uncovered in the transcriptome analysis. The elevated protein phenotype is the elevated expression of function in motility and chemotaxis. Possibly linked to this phenotype is the elevated expression of \( \text{ywaC} \) in the \( \Delta \text{ytpQ} \) mutant (3.5-fold). The \( \text{ywaC} \) gene product is a GTP pyrophosphokinase that can catalyze ppGpp formation at the expense of GTP \([65,66]\). Induction of the stringent response has been observed to heighten expression of the motility/chemotaxis regulons through reduced activity of the CodY repressor \([67]\). Another report has linked iron-dependent control and CodY with the stringent response \([68]\).

2D-gel based cytoplasmic proteome analysis provides validation of the transcriptomic results (Figure 7). Consistent with the microarray data of the \( \text{spx} \) mutant, the most strongly up-regulated proteins in the proteome are controlled by the CymR repressor. These include \( \text{YtmI}, \text{YtmO}, \text{YtmJ}, \text{YrhB}, \text{CysK}, \text{SsuA} \), and \( \text{SsuD} \). Furthermore, the PerR-controlled proteins \( \text{KatA}, \text{AhpC}, \text{AhpF} \) were strongly elevated in the \( \Delta \text{spx} \) mutant proteome and less strongly in the proteome of the \( \Delta \text{ytpQ} \) mutant. The proteins of the Fur-regulated genes \( \text{dhbA}, \text{dhbB}, \text{dhbE} \) were also observed at increased amounts in the \( \Delta \text{ytpQ} \) mutant. Finally, the \( \text{Hag} \) protein amount was also elevated in mutant cells. The elevated protein levels in the \( \Delta \text{ytpQ} \) mutant are likely the result of the enhanced transcription uncovered in the transcriptome analysis.

The phenotype enhancement screen is one way to gain information about SRG function. In the present study, \( \text{Spx} \) phenotype enhancement was tested by examining growth on minimal medium and sensitivity to methylglyoxal. However, the \( \text{spx} \) regulon is induced under a variety of harsh conditions, and an \( \text{spx} \) null mutant is sensitive to other toxic agents including electrophiles, paraquat, selenite, hypochlorite and certain antibiotics (unpublished data). Resistance to each of these agents might require the contributions of one or more specific SRGs. With this in mind, further phenotype enhancement screens can be conducted in the presence of each toxic agent with SRG mutations in the \( \Delta \text{spx} \) mutant background to initiate an effort to uncover function of other \( \text{Spx} \) regulon members.

The \( \Delta \text{spx} \) phenotype enhancement screen of SRGs uncovered the \( \Delta \text{ytpQ} \) gene as being an important member of the \( \text{spx} \) regulon. The double \( \Delta \text{spx} \Delta \text{ytpQ} \) mutant exhibited severely impaired growth in minimal medium. Phenotype analysis of the \( \Delta \text{ytpQ} \) mutant showed that it had an elevated level of oxidative protein damage after methylglyoxal treatment compared to that of the wild-type parent. The increased protein damage upon MG treatment could be a consequence of dysfunctional iron metabolism, which was evident from the transcriptome results showing heightened expression of Fur regulon genes in \( \Delta \text{ytpQ} \) mutant cells. While phenotype of the \( \Delta \text{ytpQ} \) mutant resembled that of a fur mutant, indicating a condition of low cellular iron, this also creates a condition of elevated free, chelatable iron that could mediate the observed oxidative damage \([69,70]\)(Figure 4). These results implicate \( \text{ytpQ} \) gene as a link between \( \text{Spx} \) dependent control and regulation of iron homeostasis. The study herein provides evidence that the influence of \( \text{Spx} \) in oxidative stress management extends to participation in the control of iron metabolism. Hence, the severity of the \( \text{spx} \) phenotype is enhanced by the loss of \( \text{ytpQ} \) function, and the accompanying dysfunction in iron homeostatic mechanisms.

Attempts at gaining more information about YtpQ function will involve a search for interacting proteins, with hopes of finding binding partners with known function. Screening other SRG mutations in the \( \text{ytpQ} \) background to find synthetic effects or phenotype enhancement will uncover other \( \text{spx} \) regulon members that reside in the same functional domain occupied by \( \text{ytpQ} \). Suppressor mutations that relieve the growth impairment of the \( \Delta \text{ytpQ} \Delta \text{spx} \) mutant will also identify genes that are influenced by YtpQ function.

Methods

Bacterial strains and growth media

The \( B. \text{subtilis} \) knock-out library \([45]\) was constructed using a previously described method \([43]\), and was obtained from the laboratory of N. Ogasawara (NAIST, Nara, Japan). Mutants from the collection were re-checked by PCR using primers that hybridize to the genomic region where the disruptions reside and primers specific to \( \text{pMutIN} \). This was done to ensure that the \( \text{pMutIN} \)
insertion was in the assigned gene. DNA from the SRG members (Table S1) of the mutant collection was used to transform JH642 and ORB6701 (spc::spc) competent cells to create two sets of isogenic SRG mutants. All strains used in this study were derived from JH642 (ytpC2 pheD) and are Tyr- and Phe- auxotrophs.

*B. subtilis* cells were grown on TSS minimal medium [71], 2xYT (yeast extract/tryptone [72]), or DSM nutrient broth sporulation medium [73]. *E. coli* plasmid-bearing strains were propagated in 2xYT. Antibiotic concentrations used in growth media were ampicillin (50 μg ml⁻¹), chloramphenicol (5 μg ml⁻¹), spectinomycin (75 μg ml⁻¹), and erythromycin/lincomycin (1 μg ml⁻¹ and 25 μg ml⁻¹, respectively).

**Construction of ytpQ deletion mutant**

The ytpQ gene was amplified from JH642 chromosomal DNA using a forward primer oMN10-505 (cgCCGCAAAAAAA-GAAGAA; only upper case letters correspond to chromosomal sequence) and a reverse primer oMN10-506 (gCGA-CACCTCTTTTATTATA) by PCR. The amplified DNA was cloned using Topo-cloning kit (Invitrogen) to generate pMMN806. The ytpQ gene isolated from EcoRI-digested pMMN806 was cloned into pUC8 digested with EcoRI to generate pMMN807. The spectinomycin-resistant-cassette was isolated from pDG1727 digested with BamHI and Stul and the resistance gene was used to replace the BglII/EcoRV-digested fragment of ytpQ in pMMN807. The resultant plasmid pMMN808 led to a substitution of a 54-bp DNA in ytpQ with the spectinomycin-resistance gene. The plasmid pMMN808 was transformed into JH642 and the transformant (ORB7815) was selected for the spectinomycin resistance. The disruption of ytpQ in ORB7815 was confirmed by PCR using oMN10-505 and oMN10-506. ORB7816 (spc::tet ytpQ::spc) was constructed by transforming ORB6786 (spc::tet) with chromosomal DNA prepared from ORB7815.

**Preparation of chromosomal DNA and transformation**

Small-scale chromosomal DNA preparation was conducted by growing a culture of 3 ml 2xYT. The cells were harvested at 14,000 rpm in a Sorvall super T21 centrifuge with SL-50T rotor at 4°C for 10 min. The cells were resuspended in EDTA buffer (25 mM NaCl, 50 mM EDTA) and sonicated for 30 min or 1 h. The supernatant containing the chromosomal DNA was then stored at 4°C. The chromosomal DNA from the mutants was used to transform JH642 competent cells and spc mutant (ORB6781) competent cells. The transformation mixture was incubated for 30 min at 37°C and then 1 ml of 2xYT was added in each test tube. The transformation mixture was incubated for 1 h and plated on DSM Erm-Ln (1 μg ml⁻¹) and DSM Spc (75 μg ml⁻¹) plates, respectively. The colonies were then streaked for single cell clones. After genotype testing, strains were stored at −80°C.

**Mutant Screening**

The SRG mutations in the wild-type and spc mutant backgrounds were tested on TSS, TSS+IPTG (0.5 mM), TSS+MG (2 mM), TSS+MG+IPTG plates and the phenotype of each strain was recorded. The mutants in both backgrounds along with JH642 and spc::spc were grown in TSS media with appropriate antibiotics. The cells were diluted to OD₆₀₀ = 0.1 and then serially diluted to 10⁻¹ in TSS medium. Ten μl from each dilution were transferred to TSS plates and TSS and MG (2 mM) plates. To assess the extent of growth impairment on minimal medium or caused by treatment with methylglyoxal, we employed a Matlab graphical user interface to measure colony size on agar medium containing the toxic agent. The application, Pixicillus, utilizes triangulation of coordinates corresponding to the dimensions of the petri plate as a standard to calculate colony size (the design and use of the program is available upon request).

**Construction of ytpQ complementation strains**

The ytpQ ORF as well as 37 bases upstream harboring the ytpQ Shine-Dalgarno sequence was amplified by PCR from JH642 purified genomic DNA using primers oSB1 (TAGGGAGCTTCA-GACCTCTTCTGGTAGAAAGCTTAAGGA, HindIII cut site underlined) and oSB2 (TAGGGTCTAGACTATCCCTTTCG-GACGGCTTTTCGC, Xbal cut site underlined). The HindIII - Xbal digested linear amplicon was cloned into pDR67 [74], which contains an IPTG-inducible spc promoter [44], a chloramphenicol resistance cassette, and flanking *amyE* homologous regions for chromosome integration. pDR67::P::spc::ytpQ (pSB1) was sequenced and introduced by transformation into several competent isogenic strains of *B. subtilis*. i) *spc::tet* (ORB6670), ii) *ytpQ::spc* (ORB7815), iii) *spc::tet ytpQ::spc* (ORB7816), and iv) parental JH642. Strains were tested for the disruption of the *amyE* locus by plating on LB plus 0.5% starch.

**Growth curves**

The preculture of JH642, spc::spc and mutants constructed in the JH642 or spc::spc genetic backgrounds were grown in TSS media with appropriate antibiotics until mid-log phase. The cells were then diluted to OD₆₀₀ = 0.03 and incubation was continued with shaking at 37°C. The OD₆₀₀ was taken at time intervals of 30 min or 1 h.

**OxyBlot Assay**

The wild-type strain, ytpQ mutant, and spc::spc mutant were grown in 100 ml of 2xYT at 37°C. At OD₆₀₀ = 0.5, the culture was divided equally in baffled flasks for each sample. Methylglyoxal (2.8 mM) was added into one of the two flasks. The cells were grown for 6 h and harvested at 7000 rpm, 4°C for 20 min in a Sorvall Super T21 centrifuge using a 5L-50T rotor. The harvested cells were resuspended in 50 mM NaCl, 25 mM EDTA pH 7.0 and lysed using a French press. The crude cell lysate was centrifuged at 4°C. The supernatant was collected and the protein concentration was determined by the Bradford Assay [75]. Fifteen μg of the crude sample protein was derivatized with 2, 4 dinitrophenyl hydrazine. A control reaction with extract of untreated cells was also assembled. The samples were then applied to a 12% SDS polyacrylamide gel and the protein was electroblotted onto a nitrocellulose membrane. The membrane was treated with primary antibody provided in the OxyBlot kit (Millipore) specific for 2,4 dinitrophenyl hydrazone. After the treatment with antibodies the membrane was treated with chemi-luminescent reagent (Millipore) and labeled protein was visualized on a photographic film (Fuji).

**In vitro transcription**

RNA polymerase and Spx protein was purified as previously described [28,76]. For the study reported herein, RNA polymerase was purified from an *rhoDL2664* mutant (A gift from C. P. Moran, Jr., Emory University), which lacks SigA protein after a three-column purification procedure [76]. The enzyme does not transcribe a consensus σ⁺ promoter DNA fragment (from the *rhoD* gene) unless purified σ⁺ [58] is added to the reaction. In vitro transcription reactions were performed according to previously published work [11,76,77], with further details in Figure legend 3.
Proteome and mass spectrometry analysis

*B. subtilis* wild type (JH642), *ytpQ* and *spx* mutant cells were grown in Belitsky minimal medium [78] to an OD_{600} = 0.4 and harvested before (control) and 20 min after exposure to 2.8 mM methylglyoxal. Preparation of cytoplasmic protein extracts and separation by two-dimensional gel electrophoresis (2D-PAGE) were performed as described [79]. The protein content was determined using the Bradford assay [75]. For two-dimensional gel electrophoresis (2D-PAGE), 200 μg of the protein extracts were separated using the non-linear immobilized pH gradients (IPG) in the pH range 4–7 for cytoplasmic proteins (Amersham Biosciences) and a Multiphor II apparatus (Amersham Pharmacia Biotech) as described previously [80]. The resulting 2D gels were fixed in 40% (v/v) ethanol, 10% (v/v) acidic acid and stained with Colloidal Coomassie Brilliant Blue (Amersham Biosciences). The image analysis was performed from the Coomassie-stained 2D gels using the DECODON Delta 2D software (http://www.decodon.com).

For protein identification from 2D gels, spot-cropping, tryptic digestion of the proteins, and spotting of the resulting peptides onto the MALDI-targets (Voyager DE-STR, PerSeptive Biosystems) were performed using the Ettan Spot Handling Workstation (Amersham-Biosciences, Uppsala, Sweden) as described previously [81]. The MALDI-TOF-TOF measurement of spotted peptide masses was carried out on a Proteome-Analyzer 4800 (Applied Biosystems, Foster City, CA, USA) as described previously [81].

Transcriptome analysis

For microarray analysis, *B. subtilis* wild type, *ytpQ* and *spx* mutant cells were grown in Belitsky minimal medium to OD_{600} = 0.4 and harvested before and after exposure to 2.8 mM methylglyoxal. Total RNA was isolated by the acid phenol method as described [82]. For transcriptome analysis, 35 μg of RNA were DNase-treated using the RNase-Free DNase Set (Qiagen) and purified using the RNA Clean-Up and Concentration Micro Kit (Norgen). The quality of the RNA preparations was assessed by means of the Agilent 2100 Bioanalyzer according to the manufacturer’s instructions.

Synthesis and purification of fluorescently labeled cDNA were carried out according to Charbonnier et al. [83] with minor modifications. In detail, 10 μg of total RNA were mixed with random primers (Promega) and spike-ins (Two-Color RNA Spike-In Kit, Agilent Technologies). The RNA/primer mixture was incubated at 70°C for 10 min followed by 5 min incubation on ice. Then, the following reagents were added: 10 μl of 5× First Strand Buffer (Invitrogen), 5 μl of 0.1 M DTT (Invitrogen), 0.5 μl of a dNTP mix (10 mM dATP, dGTP, and dTTP, 2.5 mM dCTP), 1.25 μl of Cy3-dCTP or Cy5-dCTP (GE Healthcare) and 2 μl of SuperScript II reverse transcriptase (Invitrogen). The reaction mixture was incubated at 42°C for 10 min and then heated to 70°C for 10 min. After 5 min on ice, the RNA was degraded by incubation with 2 units of RNaseH (Invitrogen) at room temperature for 30 min. Labeled cDNA was then purified using the CyScribe GFX Purification Kit (GE Healthcare). Five hundred ng of Cy3-labeled cDNA and 500 ng of Cy5-labeled cDNA were hybridized together to the microarray following Agilent’s hybridization, washing and scanning protocol (Two-Color Microarray-based Gene Expression Analysis, version 5.5).

Data were extracted and processed using the Feature Extraction software (version 10.5, Agilent Technologies). For each gene on a microarray, the error-weighted average of the log ratio values of the individual probes was calculated using the Rosetta Resolver software (version 7.2.1, Rosetta BioSoftware). Genes showing induction or repression ratios of at least three-fold in three independent experiments were considered as significantly induced. The averages ratios and standard deviations for all induced or repressed genes in the *ytpQ* and *spx* mutants compared to the wild type were calculated from three independent transcriptome experiments each and listed in Table S3. All microarray datasets and accompanying descriptions are MIAME compliant and the datasets are available in the GEO database under accession numbers GSE28872.

Hierarchical clustering analysis

Clustering of gene expression profiles up- and downregulated in *spx* and *ytpQ* mutants compared to the wild type under control conditions and in the *ytpQ* mutant under methylglyoxal stress were performed using Cluster 3.0 [84]. The transcriptome datasets included log2-fold expression changes in the *spx* and *ytpQ* mutant strains versus wild type. After hierarchical clustering, the output was visualized using TreeView [85]. For clustering, genes were used that are induced or repressed in the *ytpQ* and/or *spx* mutants (e.g. CymR, Spx, PerR, Fur, HslR, CatR, Com, SigmaD, SigmaH, CodY, SinR, SpoA0, SigmaE, AhIC, Rok, SigmaE, F, G, K regulons and SPβ-related genes).

Supporting Information

**Figure S1** Colonies of serially diluted, spotted cultures on TSS plates with and without 3 mM methylglyoxal. Strains are JH642 (wild type parent), *spx* mutant, *yitV*, and *yitV spx* mutant. Values are average colony size as determined by Pixicillus. (TIF)

**Figure S2** TSS minimal medium agar containing 0, 0.5, 1, and 2 mM MG. From top to bottom rows: JH642 (Wild type parent), *spx*:tet mutant, *ytpQ* deletion mutant, *spx*:tet *ytpQ* deletion double mutant. Cells were grown in TSS medium to mid-log phase, then serially diluted 10-fold. Ten μl of each dilution was then spotted onto the agar surface. Plates were incubated at 37°C. (TIF)

**Figure S3** Complementation of the Δ*ytpQ::spe* mutation by an IPTG-inducible allele of *ytpQ* expressed from the amyE locus (Strain ORB8011). Growth curves are shown in which cultures of JH642 cells and ORB8011 cells are grown in TSS medium containing Trp and Phe auxotrophic requirements. (TIF)

**Figure S4** Assay of *ytpQ*- and *ytoQ*-directed β-galactosidase activity in wild type cells and cells bearing the IPTG inducible *spx* allele. A. Open circles: *ytpQ::pMUTIN Physpansk*-spe^DD^ without IPTG. Closed circles: with IPTG. B. Open triangles: *ytoQ::pMUTIN Physpansk*-spe^DD^ without IPTG. Closed triangles: with IPTG. C. The expression of the *ytpQ* and *ytoQ*-lacζ fusions was measured in *ytpQ::pMUTIN* and *ytoQ::pMUTIN* cells in the absence of the IPTG-inducible *spx*^DD^ construct. Open circles: *ytpQ::pMUTIN* without IPTG. Closed circles: *ytpQ::pMUTIN* with IPTG. Open triangles: *ytoQ::pMUTIN* without IPTG. Closed triangles: *ytoQ::pMUTIN* with IPTG. (TIF)

**Figure S5** Hierarchical clustering analysis of gene expression profiles up- and downregulated in *spx* and *ytpQ* mutants. Gene expression data were clustered based on the induction or repression ratios in the *spx* and *ytpQ* mutants. Nodes enriched for phase-related genes of *B. subtilis* are shown. (TIF)
Table S1 Expression levels of spx-controlled genes in cells bearing an IPTG-inducible allele of spx (spxDD) encoding a protease resistant form of Spx protein. The values are log2 of transcript level ratio between cells grown in presence and absence of IPTG [12].

Table S2 Induction and repression of genes in the spx and ytpQ mutants under control conditions and in response to 2.8 mM methylglyoxal stress as revealed by transcriptome analyses. The averages ratios and standard deviations as well as log2-fold changes for all induced and repressed genes are calculated from three transcriptome replicate experiments at control conditions and after 10 min of exposure to 2.8 mM methylglyoxal. All genes with induction ratios of at least three-fold were listed and classified according to previously described regulons according to http://dbtbs.hgc.jp and SubtiWiki database [http://subtiwiki.uni-goettingen.de/wiki/index.php/Main_Page].

Table S3 Induction and repression of genes under control conditions and in response to methylglyoxal in the spx and ytpQ mutants as revealed by transcriptome analyses. The transcriptome datasets included log2-fold expression changes of average values of three transcriptome replicates according to Table S2. All induced and repressed genes under control and methylglyoxal stress were listed and classified into regulons according to http://dbtbs.hgc.jp and SubtiWiki database.

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Author Contributions
Conceived and designed the experiments: PZ HA. Performed the experiments: SC PP MMN SG BKC HA UM AAL SBM. Analyzed the data: PZ HA UM. Contributed reagents/materials/analysis tools: PZ MMN HA UM. Wrote the paper: PZ HA.
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