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The YjbH Adaptor Protein Enhances Proteolysis of the Transcriptional Regulator Spx in *Staphylococcus aureus*

Jakob Engman,[a] Annika Rogstam,[a,b] Dorte Frees,[b] Hanne Ingmer,[b] and Claes von Wachenfeldt[a]

Department of Biology, Lund University, Lund, Sweden,[a] and Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg C, Denmark[b]

Spx is a global regulator that is widespread among the low-G+C-content Gram-positive bacteria. Spx has been extensively studied in *Bacillus subtilis*, where it acts as an activator and a repressor of transcription in response to disulfide stress. Under nonstress conditions, Spx is rapidly degraded by the ClpXP protease. This degradation is enhanced by the YjbH adaptor protein. Upon disulfide stress, the amount of Spx rapidly increases due to a decrease in degradation. In the opportunistic pathogen *Staphylococcus aureus*, Spx is a global regulator influencing growth, biofilm formation, and general stress protection, and cells lacking the *spx* gene exhibit poor growth also under nonstress conditions. To investigate the mechanism by which the activity of Spx is regulated, we identified a homolog in *S. aureus* of the *B. subtilis yjbH* gene. The gene encodes a protein that shows approximately 30% sequence identity to YjbH of *B. subtilis*. Heterologous expression of *S. aureus yjbH* in a *B. subtilis yjbH* mutant restored Spx to wild-type levels both under nonstress conditions and under conditions of disulfide stress. From these studies, we conclude that the two YjbH homologues have a conserved physiological function. Accordingly, inactivation of *yjbH* in *S. aureus* increased the level of Spx protein and transcription of the Spx-regulated gene *trxB*. Notably, the *yjbH* mutant exhibited reduced growth and increased pigmentation, and both phenotypes were reversed by complementation of the *yjbH* gene.

All aerobic organisms encounter oxidative stress and have evolved different ways to reduce stress-induced damage. Oxidative stress can lead to the formation of unwanted disulfide bonds, a phenomenon known as disulfide stress (2). Spx is a transcriptional regulator that has been thoroughly studied in *Bacillus subtilis*, where it acts both as an activator and a repressor of transcription in response to disulfide stress by interacting with the C-terminal domain of the RNA polymerase α subunit (31, 42). Among the genes that are repressed by Spx are many that are involved in cellular metabolism during normal growth, such as biosynthesis of amino acids, vitamins, purines, and pyrimidines, while some of the induced genes are involved in maintenance of the cellular thiol-redox homeostasis (29). By inducing the Spx regulon, the cell can repair damage caused by disulfide stress and return the cytoplasm to its normal reducing state, while not spending energy on the biosynthesis of cellular components.

The nature of the Spx regulon, where several genes needed for vegetative growth are repressed, calls for a tight regulation of Spx. Under nonstress conditions, Spx is kept at a very low concentration by the ClpXP protease, and upon disulfide stress, there is a decrease in degradation, thereby increasing the amount of Spx in the cell (29, 42). ClpXP is a multisubunit protease complex, where ClpP acts as a protease that works together with the AAA+ ClpX unfoldase to degrade misfolded and truncated proteins (8, 13). Substrate specificity is provided by ClpX (3, 6, 13), which utilizes ATP for unfolding and translocation of the substrate into the ClpP proteolytic chamber (13). Mutations in *B. subtilis clpP* and *clpX* give pleiotropic phenotypes with respect to stress tolerance, competence for DNA uptake, high-temperature tolerance, sporulation, morphology, and motility, and the *clpP* and *clpX* mutants display an extended lag phase (10, 19). Originally, the *spx* gene was discovered as the site for mutations that could suppress the pleiotropic phenotype of a *clpXP* mutation in *B. subtilis* (suppressor of *clpP* and *clpX*) (27). It was demonstrated that the accumulation of Spx in the *clpXP* mutants is what causes the observed pleiotropic phenotype (30). A similar phenotype is also seen in a strain lacking the *yjbH* gene, which encodes a 34-kDa cytosolic protein that acts as an adaptor protein to enhance Spx degradation by binding to Spx, thereby making it more available for ClpX recognition (9, 21). YjbH has not been structurally characterized; however, bioinformatics analysis predicts that it is a member of the thioredoxin-like superfamily. It is not fully understood how the adaptor activity of YjbH is regulated. However, recently is has been reported that in *B. subtilis* the 54-amino-acid protein YirB may function as an antiadaptor protein (18). It is suggested that YirB functions by interacting with YjbH, causing a subsequent release of Spx from YjbH (18). However, it is not known under which conditions YirB might be functional. Moreover, YirB is not conserved in *Firmicutes* and it is, for example, not present in *Staphylococcus*, suggesting that alternative mechanisms are used to control the activity of YjbH. In addition to the proteolytic control, the activity of Spx is regulated by a disulfide redox switch involving a CXXC present in Spx that affects the interaction between Spx and the RNA polymerase (28).

Homologs of Spx are widespread among the low-G+C-content Gram-positive bacteria (17, 39, 42). In the opportunistic pathogen *Staphylococcus aureus*, Spx has been identified as a global regulator influencing growth, biofilm formation, and general stress protection (34). A proteomic analysis comparing *S. aureus* wild-type and *spx* mutant cells indicated that Spx acts both as a

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Address correspondence to Claes von Wachenfeldt, claes.von_wachenfeldt@biol.lu.se.
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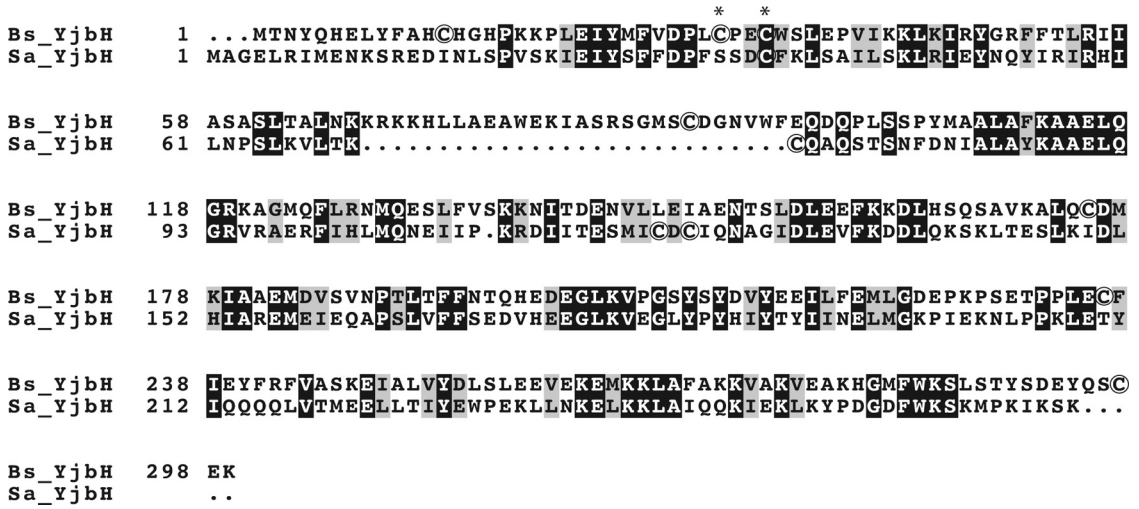

FIG 1 Alignment of YjbH from *B. subtilis* and *S. aureus*. White letters on black background represent identical residues. The asterisks indicate the cysteine residues of the CXXC motif in *B. subtilis* YjbH. Cysteine residues are encircled.

negative and a positive regulator of genes encoding proteins involved in DNA metabolism, protein synthesis, cell division, and thiol homeostasis. An *spx* mutation causes severely impaired growth in *S. aureus*, and it has been shown that the transcription of the essential *trxB* gene, encoding thioredoxin reductase, is virtually undetectable in an *spx* mutant, possibly causing the growth defect of the mutant. The *spx* mutant is hypersensitive to a variety of stresses, including high and low temperatures as well as oxidative and disulfide stress (34). The *B. subtilis* and *S. aureus* Spx proteins share 79% sequence identity (see Fig. S1 in the supplemental material), and Spx was suggested to be a substrate of ClpXP in *S. aureus*, because Spx accumulates strongly in *clpP* and *clpX* mutants despite that transcription of the gene is reduced (34).

In *B. subtilis*, the efficient degradation of Spx by ClpXP under nonstress conditions requires the Spx adaptor protein YjbH (21). In *S. aureus*, a gene encoding a protein that exhibits some similarity (~30% identity) to *B. subtilis* YjbH (hypothetical protein SAOUHSC_00938) is present, here referred to as *S. aureus* YjbH (Fig. 1). In *B. subtilis*, *yjbH* is located in an operon together with the *yjbI* gene, encoding a truncated hemoglobin (35). The *S. aureus yjbH* gene is located downstream of a gene encoding a protein with a high sequence similarity (~47% identity) to *B. subtilis* YjbI, which has been experimentally shown to encode a truncated hemoglobin (P. Kjelgaard and C. von Wachenfeldt, unpublished data). This chromosomal arrangement suggests that *S. aureus* YjbH may have a role similar to YjbH in *B. subtilis*. Interestingly, global mutagenesis screens designed to identify virulence genes in *S. aureus* and *Listeria monocytogenes* have identified transposon insertions in the *yjbH* gene (14, 37, 41). However, the connection to virulence remains to be clarified.

The low overall conservation of YjbH, and the finding that the N-terminal redox-sensitive region of *B. subtilis* YjbH is absent altogether in *S. aureus* YjbH, prompted us to investigate if the function of YjbH is conserved between the two organisms. Heterologous expression of *S. aureus yjbH* in a *B. subtilis yjbH* mutant restored Spx levels to wild-type levels both under nonstress conditions and under conditions of disulfide stress. From these studies, we conclude that the two YjbH homologues have a conserved physiological function. Accordingly, inactivation of *yjbH* in *S. aureus* increased the level of Spx protein and transcription of the Spx-regulated gene *trxB*. Notably, the *yjbH* mutant exhibited reduced growth and increased pigmentation, and both phenotypes were reversed by complementation of the *yjbH* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study are listed in Table 1. The plasmids used in this work are listed in Table 2. *Escherichia coli* strain TOP10 was used for construction and maintenance of plasmids and was grown in LB or on LB agar plates (36). *S. aureus* strains were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA). *B. subtilis* strains were grown on tryptose blood agar base (TBAB) or in nutrient sporulation medium with phosphate supplemented with 0.5% glucose (NSMPG) (40). All bacterial cultures were grown at 37°C with shaking at 200 rpm. The following antibiotics were added to the growth medium when required: kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), spectinomycin (150 μg ml^{-1}), and erythromycin (100 μg ml^{-1}) for *E. coli* strains; spectinomycin (150 μg ml^{-1}), erythromycin (5 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), and tetracycline (4 μg ml^{-1}) for *S. aureus* strains; spectinomycin (150 μg ml^{-1}), chloramphenicol (15 μg ml^{-1}), tetracycline (20 μg ml^{-1}), and a mixture of erythromycin (0.5 μg ml^{-1}) and lincomycin (12.5 μg ml^{-1}) for *B. subtilis* strains. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to solid medium (50 μg ml^{-1}) as an indicator of β-galactosidase activity. When appropriate, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 100 μg ml^{-1}.

To alter the cellular thiol redox balance, *S. aureus* or *B. subtilis* cells were treated with diamide [diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)$(CH_3)_2$NCON = NCON$(CH_3)_2$] (5 mM [*S. aureus*] or 1 mM [*B. subtilis*]) at mid-exponential growth phase, and cells were harvested immediately before and 20 min after addition, unless stated otherwise.

Expression from the tetracycline-inducible promoter in pRMC2 and derived plasmids was induced by the addition of between 100 and 500 ng ml^{-1} anhydrotetracycline (Atet).

DNA manipulations and transformations. All molecular biology techniques, including *E. coli* transformations, were performed as described by Sambrook and Russell (36). Preparations of *B. subtilis* chromosomal DNA and *B. subtilis* transformations with plasmid or chromosomal DNA were performed as described by Hoch (16). All oligonucleotides

TABLE 1 Bacterial strains used in this study

Strain	Relevant characteristic(s)[a]	Source or reference
E. coli TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD*139 Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
S. aureus		
NCTC 8325-4	Wild type, derivative of NCTC 8325 cured of all known prophages	33
RN4220	Restriction-deficient derivative of NCTC 8325-4	33
8325-4 Δ*spx* mutant	Δ*spx*	34
8325-4 Δ*clpX* mutant	Δ*clpX*	7
8325-4 Δ*clpP* mutant	Δ*clpP*	7
Newman	Human clinical isolate	5
LUSA1	NCTC 8325-4 Δ*yjbH*::*spc*, Sp^r	This study
LUSA2	Newman Δ*yjbH*::*spc*, Sp^r	This study
LUSA3	RN4220 pCL25, Tc^r	This study
LUSA4	RN4220 pCL25_*yjbIH*, Tc^r	This study
LUSA7	Newman pCL25, Tc^r	This study
LUSA8	Newman pCL25_*yjbIH*, Tc^r	This study
LUSA9	Newman pCL25 Δ*yjbH*::*spc*, Sp^r Tc^r	This study
LUSA10	Newman pCL25_*yjbIH* Δ*yjbH*::*spc*, Sp^r Tc^r	This study
B. subtilis		
1A1	*trpC2*	BGSC[b]
LUW272	Δ*yjbH*::*spc*, Sp^r	35
LUW297	spxΩpMUTIN2, Em^r	21
LUW362	spxΩpMUTIN2 *amyE*::spx_{Bs} *spc*, Em^r Sp^r	This study
LUW400	Δ*yjbH*::*spc amyE*::*cat*, Cm^r Sp^r	This study
LUW428	spxΩpMUTIN2 *amyE*::spx_{SA} *cat*, Em^r Cm^r	This study
LUW448	Δ*yjbH*::*spc amyE*::$yjbH_{SA}$ *cat*, Sp^r Cm^r	This study
LUW421	Δ*yjbH*::*spc amyE*::$yjbH_{BS}$ *cat*, Sp^r Cm^r	This study
LUW442	Δ*yjbH*::*spc* spxΩpMUTIN2 *amyE*::spx_{SA} *tet*/pCW7_ $yjbH_{SA}$, Sp^r Cm^r Tc^r Em^r	This study
LUW458	Δ*yjbH*::*spc amyE*::$yjbH_{BS\text{-}Cys}$ *cat*, Sp^r Cm^r	This study
LUW454	Δ*yjbH*::*spc amyE*::pCW101_ $yjbH_{SA}$, Cys-free, Sp^r Cm^r	This study

[a] All *B. subtilis* LUW strains are derivatives of 1A1 and thus carry the *trpC*2 auxotrophic marker. Ap^r, ampicillin resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance.
[b] BGSC, Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus, OH.

used in this work are described in Table 3. All cloned PCR-generated fragments were verified by sequencing.

Construction of plasmids. To create pCW101, a derivative of the $P_{hyperspank}$ plasmid pDR111, plasmid pDG1661, was digested with SphI and treated with exonuclease Bal-31 to remove the SphI and SalI restriction sites, and finally the plasmid was recircularized with T4 DNA ligase. After propagation in *E. coli*, the resulting plasmid was isolated, and a fragment containing the chloramphenicol resistance gene and *amyE* was excised from the plasmid using the restriction enzymes EcoRI and PstI and ligated into pDR111 digested with the same enzymes. In the resulting plasmid, pCW101, the spectinomycin resistance gene of pDR111 was replaced by the chloramphenicol resistance gene of pDG1661.

The *spxA* gene was amplified by PCR from chromosomal DNA from *S. aureus* 8325-4, using primers SASPX1 and SASPX2. The PCR product was cloned into pCR-BLUNT II-TOPO (Invitrogen). The sequence of the cloned fragment was verified by sequencing and confirmed to have 100% sequence identity to the expected sequence. The insert was excised using XbaI and SalI and cloned into pCW7. The resulting plasmid was named pCW7_spx_{SA}.

To create pCW101_spx_{SA}, the spx_{SA} fragment including a *B. subtilis* ribosome-binding site from pCW7_spx_{SA} was excised using HindIII and SalI and cloned into pCW101. Plasmid pCW7_$yjbH_{SA}$ was created in the same manner as pCW7_spx_{SA} except that the primers SAYJBH3 and SAYJBH4 were used for the PCR amplification. Plasmid pCW101_$YjbH_{BS}$ was created by excising the *yjbH* fragment from pCW7_$YjbH_{BS}$ with XbaI and HindIII and ligating it into pCW101 cut with the same enzymes.

Genes encoding cysteine-free variants of *yjbH* ($yjbH_{SA\text{-}Cys}$ and $yjbH_{BS\text{-}Cys}$) were synthesized by Eurofins MWG Operon, and the fragments were cloned into pCW101 using HindIII, creating pCW101_$yjbH_{SA\text{-}Cys}$ and pCW101_$yjbH_{BS\text{-}Cys}$, respectively. For complementation in *S. aureus*, the *S. aureus yjbH* gene (SAOUHSC_00938) was amplified by PCR from *S. aureus* 8325-4 chromosomal DNA using primers SAYJBH10 and SAYJBH11. The resulting fragment containing the *yjbH* gene and a ribosome binding site was isolated, cut (KpnI-EcoRI), and cloned into the KpnI-EcoRI sites of the expression vector pRMC2. This plasmid allows tetracycline-inducible expression of genes that are cloned downstream of a TetR-controlled promoter (4). The plasmid (pRMC2_$yjbH_{SA}$) was amplified and purified from *E. coli*. The plasmid was then transformed into the restriction-negative strain *S. aureus* RN4220, isolated, and subsequently transformed into *S. aureus* Newman by electroporation.

Construction of *B. subtilis* strains. Strain LUW428 was generated by transformation of LUW297 (spxΩery) with pCW101_spx_{SA} and selection of transformants with chloramphenicol. The successful integration of the *S. aureus spx* gene into the *amyE* locus of the transformants was confirmed by the lack of halo formation upon growth on plates containing 1% starch with subsequent exposure of the plates to iodine.

Strain LUW421 was generated by transforming the wild-type strain 1A1 first with pCW101_$yjbH_{BS}$ and by subsequent transformation of the resulting strain with chromosomal DNA of strain LUW272 (Δ*yjbH*::spc). Confirmation of an AmyE-negative phenotype was done as described above. It should be noted that the deletion of *yjbH* severely affects competence development (21). Therefore, the introduction of the *yjbH* mutation was the final step in the strain constructions.

TABLE 2 Plasmids used in this study

Plasmid	Relevant characteristic(s)[a]	Source or reference
pCRBlunt II TOPO	Cloning vector; Km^r	Invitrogen
pUC18	Cloning vector; Ap^r	32
pUC18_*yjbIH*$_{SA}$	Ap^r	This study
pUC18_*yjbIH*$_{SA}$ spc	Ap^r Sp^r	This study
pDG1727	Integration vector for *B. subtilis*; Sp^r Ap^r	15
pMAD	Temperature-sensitive *S. aureus* shuttle vector with the *bgaB* gene encoding a β-galactosidase; Ap^r Er^r	1
pMAD_*yjbIH*$_{SA}$ spc	Ap^r Er^r Sp^r	This study
pRMC2	Ap^r Cm^r	4
pDG1661	Cm^r	15
pDR111	Insertional vector for IPTG-inducible expression from *B. subtilis amyE* locus; *lacI*; Ap^r Sp^r; $P_{hyperspank}$	D. Rudner
pRMC2_*yjbH*$_{SA}$	Cm^r	This study
pCL25	Tc^r	23
pCL25_*yjbIH*$_{SA}$	Tc^r	This study
pCW7	Cm^r	35
pCW7_*yjbH*$_{SA}$	Cm^r	35
pCW101	Insertional vector for IPTG-inducible expression from *B. subtilis amyE* locus; *lacI*; Ap^r Cm^r; $P_{hyperspank}$	This study
pCW101_*yjbH*$_{BS}$	Cm^r	This study
pCW101_*yjbH*$_{SA}$	Cm^r	This study
pCW101_*yjbH*$_{BS\text{-}Cys}$	Cm^r	This study
pCW101_*yjbH*$_{SA\text{-}Cys}$	Cm^r	This study
pDR111_*spx*$_{BS}$	Cm^r	This study
pCW101_*spx*$_{SA}$	Cm^r	This study
pCm::Tc	Tc^r	38

[a] Ap^r, Cm^r, Em^r, Km^r, Sp^r, and Tc^r indicate resistance to chloramphenicol, erythromycin, kanamycin, spectinomycin, and tetracycline, respectively.

LUW448, LUW454, and LUW458 were created in the same manner as LUW421 with the exception that pCW101_*yjbH*$_{SA}$, pCW101_*yjbH*$_{SA\text{-}Cys}$, or pCW101_*yjbH*$_{BS\text{-}Cys}$ was used instead of pCW101_*yjbH*$_{BS}$.

To create the *B. subtilis* strain LUW442, in which the *yjbH* and *spx* genes are replaced with their *S. aureus* counterparts, the marker for LUW428 was first switched from chloramphenicol to tetracycline by using plasmid pCm::Tc. The resulting strain was then transformed with pCW7_*yjbH*$_{SA}$, selecting for chloramphenicol resistance. Finally, the *yjbH* mutation was introduced by transformation with chromosomal DNA from the Δ*yjbH* strain LUW272.

TABLE 3 Oligonucleotide sequences

Oligonucleotide	Sequence (5′→3′)
SA2	CTGCACTACGCATAAGAGTTAAAG
SA3	CCGGATCCACACTTCTATATGAATTATTATG
SASPX1	CCTCTAGATGGTAACATTATTTACTTCACC
SASPX2	CCGTCGACACATAGTTAAATGGTTATTAG
SAYJBH1	GGGGATCCATTAACGCCAACTAGAATG
SAYJBH2	CGCATGCTTAATCCTCCTCT
SAYJBH3	TCTAGATGGCTGGAGAATTACGAATAATG
SAYJBH4	GTCGACTTATTTTGATTTGATTTTAGGCAT
SAYJBH10	GGTACCAAAGGAGGTAAAGATGTATGGCTGG AGAATTACGAATAATGG
SAYJBH11	TACGTTCTAATAATTTAATGTTGC
SCV1	GCAACACCACATAATGGTTCAC
SCV8	GCACATAATTGCTCACAGCCA
SPC2	CGTATGTATTCAAATATATCCTCC
SPC10	GCCGTATGATTTTAACTATGGACAC
NW-spxA-RT-fw	AAATGACTGAAGACGGTACTGATG
NW-spxA-RT-rev	CGTTGTGCTTCTTGTAATTGG
NW-trxB-RT-fw	AAGACGGCAAAGTGGGTTC
NW-trxB-RT-rev	AACATCTCCTGCTGCAAAAATAC
NW-yjbI-RT-fw	GCAGAAACAAGTCGTAAACAAAAAC
NW-yjbI-RT-rev	CTCCAACACCTTGTGGAAAC
NW-yjbH-RT-fw	AAGCCCCTTCTCTCGTTTTC
NW-yjbH-RT-rev	TTTAAAAGTTTTTCTGGCCATTC

Diamide sensitivity assay. Disc diffusion sensitivity assays were conducted by streaking a TBAB plate with a solution containing 1×10^8 cells/ml and then placing a 6-mm filter disc (AA discs; Whatman) containing 10 μl of 25 mM diamide onto it. Plates were incubated at 37°C for 18 h, and the zone of clearing around the discs was measured.

Pigment isolation. Isolation and quantification of *S. aureus* carotenoid pigments were done according to Morikawa et al. (26).

Construction of an *S. aureus yjbH* deletion mutant. The *yjbIH* region was amplified by PCR from *S. aureus* 8325-4 chromosomal DNA using primers SA2 and SA3 (Table 3). The fragment was cloned into pCR-BLUNT II-TOPO (Invitrogen). Transformants were selected for on kanamycin-containing plates. The resulting plasmid was cut with SalI and BamHI, and the 2,373-bp fragment containing the *yjbIH* region was ligated into pUC18 cut with the same enzymes and transformed into *E. coli* selecting for ampicillin resistance. The resulting plasmid was named pUC18_*yjbIH*$_{SA}$ (see Fig. S2 in the supplemental material). The spectinomycin resistance gene of pDG1727 (see Fig. S2 in the supplemental material) was excised with PvuII and EcoRV and was ligated into pUC18_*yjbIH*$_{SA}$ cut with MscI and EcoRV and transformed into *E. coli* selecting for spectinomycin and ampicillin resistance. The resulting plasmid, pUC18_*yjbH*$_{SA}$_spc, was cut with SalI and BamHI, and the 3,117-bp fragment containing the *S. aureus yjbIH* region where the *yjbH* gene is partially replaced by the spectinomycin resistance gene was ligated into pMAD, which contains a temperature-sensitive *S. aureus* origin of replication, cut with SalI and BamHI (see Fig. S2 in the supplemental material). The ligate was used to transform *E. coli*, selecting for ampicillin resistance. The resulting plasmid was named pMAD_*yjbIH*$_{SA}$_spc and was used to transform the restriction-deficient *S. aureus* strain RN4220 grown at 30°C to erythromycin resistance, selecting for blue colonies on plates containing X-Gal. The plasmid was then extracted and used to transform *S. aureus* NCTC 8325-4 grown at 30°C to erythromycin resistance, again selecting for blue colonies on plates containing X-Gal. Transformants were grown in TSB supplemented with erythromycin at 30°C for 2 h, after which they were harvested and resuspended in TSB without

antibiotics. This culture was grown at 42°C for 6 h in order to allow for a double-crossover recombination event in the chromosomal *yjbH* locus. Cells were plated on TSA supplemented with spectinomycin and X-Gal and grown at 42°C, selecting for white colonies. The double-crossover recombination event was confirmed by PCR using primer pairs SPC10/SAYJBH1 and SAYJBH2/SPC2 (see Fig. S3 in the supplemental material). The *yjbH* mutant strain in the strain 8325-4 background, denoted LUSA1, did not show any apparent phenotype separating it from the wild type. One possible reason for this might be that strain 8325-4 lacks a functional copy of *rsbU*, which impairs signaling by the alternative sigma factor SigB that is involved in the general stress response (11). Because of this, the mutation was moved to *S. aureus* strain Newman by transduction as described by McNamara and Iandolo (25). The presence of the *yjbH*::*spc* mutation in strain Newman was confirmed by PCR as described for strain 8325-4 above.

Complementation of the *S. aureus yjbH* deletion mutant. The Δ*yjbH* mutation was complemented with a chromosomally integrated copy of *yjbH* expressed from its own promoter (P_{yjbIH}) using the single-copy integration plasmid pCL25 (24). The *yjbH* gene was excised from pUC18_$yjbIH_{SA}$ and cloned into pCL25 using HindIII and XbaI. The cloned fragment in the resulting plasmid was sequenced to confirm that the sequence was correct. The plasmid was transformed into RN4220 containing the pYL11219 plasmid encoding the integrase from the phage L54a that catalyzes site-specific integration of the plasmid (pCL25_*yjbIH*) into the *attB* site located within the *geh* gene (22). The integrated copy of pCL25_*yjbIH* was then transduced into the Newman strain using the lysogenic phage ϕ11. The Δ*yjbH* mutation in strain LUSA1 was then transduced into the strain containing the integrated copy of pCL25_*yjbIH*. PCR, using the primers SCV1 and SCV8, was used to confirm that the strain contained both the *yjbH* deletion and the intact copy of *yjbH* inserted into the *geh* gene. The strain was named LUSA8. A control strain with pCL25 instead of pCL25_*yjbIH* was created in the same way and denoted LUSA7.

Immunoblot analysis. Soluble extracts were prepared by harvesting 25 ml of a culture of exponentially growing *S. aureus* or *B. subtilis* cells and dissolving the pellet in 100 mM Tris-HCl and 5 mM Na-EDTA (pH 8.0), supplemented with one tablet of Complete EDTA-free protease inhibitor (Roche). To lyse *S. aureus* cells, lysostaphin was added to a final concentration of 25 $\mu g\ ml^{-1}$, followed by incubation at 37°C for 30 min, while *B. subtilis* cells were lysed by sonication. To remove cell debris, the lysates were centrifuged at 20,000 × *g* for 45 min at 4°C. The protein concentration was determined using the bicinchoninic acid method (Thermo Scientific) with bovine serum albumin as the standard. A total of 20 μg of the extracts was then separated by Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18%) and transferred to Hybond P membranes (GE Healthcare). Spx, $YjbH_{BS}$, and $YjbH_{SA}$ were detected using an antiserum from a rabbit that had been immunized with a synthetic peptide ($NHCOCH_3$)CGYNEDEIRRFLPRKVR($CONH_2$), ($NHCOCH_3$)MLGDEPKPSETPPLC($CONH_2$), or ($NHCOCH_3$)CGDFWKSKMPKIKSK($CONH_2$), respectively, conjugated at terminal cysteines to Keyhole limpet hemocyanin (*KLH*). The used peptides corresponded to sequence Gly-104 to Arg-119 of Spx, Met-221 to Leu-234 of $YjbH_{BS}$, and Gly-248 to Lys-261 of $YjbH_{SA}$. Spx antisera were used at a 1,250-fold dilution, and the $YjbH_{BS}$ and $YjbH_{SA}$ antisera were used at a 5,000-fold dilution. The secondary antibodies (ECL anti-rabbit IgG, horseradish peroxidase-linked species-specific whole antibody from donkey [GE Healthcare]) were used at a 5,000-fold dilution. Immunodetection was carried out by chemiluminescence using the Super Signal West Pico system (Thermo Scientific). Quantification was done using a Kodak image station 440CF.

RNA isolation and cDNA synthesis. For isolation of total RNA, 10 ml of an exponentially growing *S. aureus* culture was added to a 50-ml centrifuge tube containing 10 g of crushed ice. The sample was centrifuged immediately at 17,000 × *g* for 5 min at 4°C, and the pellet was used for extraction of total RNA using the Qiagen RNeasy minikit according to the manufacturer's protocol. The integrity of the RNA was checked by electrophoresis on a 2% agarose gel containing ethidium bromide, and the RNA was quantified spectrophotometrically. A total of 2 μg RNA was treated with RNase-free DNase I (Fermentas) and was then used for cDNA synthesis with the RevertAid H Minus First-Strand cDNA synthesis kit (Fermentas) and random hexamer primers according to the manufacturer's protocol. A control without reverse transcriptase (NoRT) was made in parallel to ensure that no chromosomal DNA gave a false-positive signal in downstream applications.

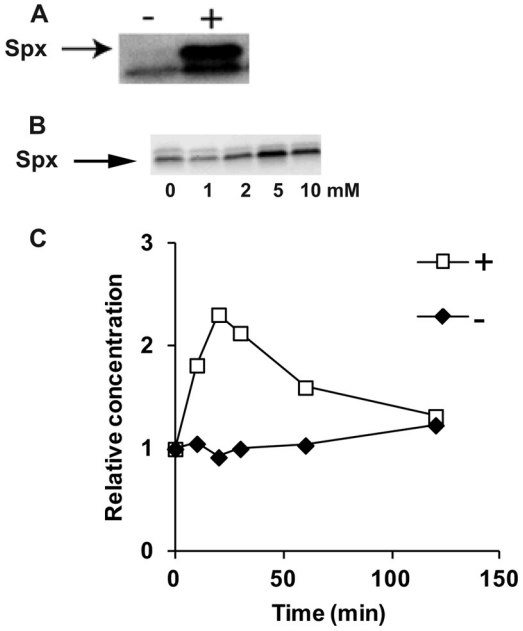

FIG 2 Immunoblot analysis of Spx content before and after the addition of 1 mM diamide to *B. subtilis* 1A1 (A) and 1 to 10 mM diamide to *S. aureus* Newman (B). The relative Spx content as a function of time after no addition (−) or addition (+) of 5 mM diamide to *S. aureus* Newman is shown in panel C.

qRT-PCR. Quantitative real-time PCR (qRT-PCR) was performed in an Mx3000P qPCR system (Stratagene). Reaction mixtures (25 μl) were made using the Maxima SYBR green/ROX qPCR Master Mix (2×) (Fermentas) according to the manufacturer's protocol. Primers used for qRT-PCR are listed in Table 3. The cDNA and NoRT controls were diluted 5-fold in 10 mM Tris-HCl (pH 8.0), and 2 μl of these dilutions was used as templates in qRT-PCR. A total of 2 μl 10 mM Tris-HCl (pH 8.0) was added to the no-template controls. At least three biological replicates were made for each strain and condition, and each biological replicate was run in triplicate in the qRT-PCR. For each primer pair, a control sample containing the gene of interest at an appropriate concentration was included in all runs and used as the calibrator. Data were analyzed using the comparative quantitation feature of the MxPro software (Stratagene). A two-way Student *t* test assuming equal variances was used to compare data.

RESULTS AND DISCUSSION

Influence of diamide on cell growth and Spx concentration. The azo compound diamide is a thiol-specific oxidant used to induce disulfide stress. In *B. subtilis*, the addition of 1 mM diamide to exponentially growing cells leads to a rapid increase in the intracellular level of Spx (Fig. 2A), thereby inducing the disulfide stress response. To investigate the effect of diamide on *S. aureus*, a series of different diamide concentrations were added to exponentially growing wild-type *S. aureus* cells in a broth medium, and growth was monitored for 60 min. The addition of ≥5 mM diamide led to

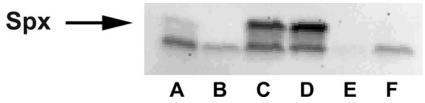

FIG 3 Immunoblot analysis of the Spx content in unstressed cells of different *B. subtilis* strains. Lane A, WT (1A1); lane B, spxΩpMUTIN2 (LUW297); lane C, Δ*yjbH*::*spc* (LUW272); lane D, Δ*yjbH*::*spc* (LUW400); lane E, Δ*yjbH*::*spc amyE*::$yjbH_{BS}$ (LUW421); lane F, Δ*yjbH*::*spc amyE*::$yjbH_{SA}$ (LUW448).

growth inhibition, and the addition of ≥10 mM diamide completely inhibited growth (data not shown).

In *S. aureus*, the basal level of Spx under nonstress conditions appears to be higher than that of *B. subtilis*. Using the same antibodies, raised against an Spx peptide, and comparable growth conditions, very low Spx-specific signal can be detected in cell extracts from nonstressed *B. subtilis* cells (Fig. 2A), while a clear band corresponding to Spx can be seen in immunoblots with cell extracts from nonstressed *S. aureus* cells (Fig. 2B).

The Spx content in extracts from cultures of *S. aureus* to which different concentrations of diamide had been added was analyzed. Concentrations up to 2 mM diamide could be added without any detected increase in the Spx level. However, increasing the added diamide concentration to 5 mM or more gave a significant increase in Spx concentration (Fig. 2B). Interestingly, previous studies have shown that the expression of the *trxB* gene is induced by as little as 0.5 mM diamide in *S. aureus* NCTC 8325-4 cells grown in the same growth medium as the one used in the present study (34). This induction of *trxB* is not seen in an *spx* mutant (34). This suggests that no increase above the basal concentration of Spx observed in nonstressed cells is required to induce *trxB* expression. It seems that the concentration of diamide where the level of Spx increases corresponds to the concentration where growth defects are seen, suggesting that this is a response to severe stress.

Heterologous complementation of a *B. subtilis yjbH*-null mutation. To test whether *S. aureus yjbH* could complement the *B. subtilis yjbH* deletion, we constructed a strain with an IPTG-inducible *S. aureus yjbH* gene integrated in a single copy at the *amyE* locus on the chromosome of a *B. subtilis yjbH*-null mutant. Immunoblot analysis confirmed that the product of the exogenous *yjbH* gene was produced in an IPTG-dependent manner (see Fig. S7 in the supplemental material). *B. subtilis yjbH* mutant strains grow poorly and accumulate high levels of Spx compared with the wild-type strain (21). Expression of *S. aureus yjbH* alleviated the growth phenotype present in the mutant strain (data not shown), suggesting functional complementation by *S. aureus yjbH*. Moreover, the complementation with *S. aureus yjbH* restored the Spx level to wild-type levels as determined by immunoblotting (Fig. 3). The concentration of Spx was inversely dependent on the concentration of YjbH, which was shown by varying the amount of the inducer IPTG (see Fig. S4 in the supplemental material).

The strain complemented with *S. aureus yjbH* responded to the addition of 1 mM diamide by increasing the level of Spx in the same fashion as the strain complemented with *B. subtilis yjbH* and wild-type cells (data not shown). This shows that the ability of $YjbH_{SA}$ to facilitate the proteolysis of Spx is inhibited by diamide.

To further establish the functional conservation of the Spx/YjbH system, a complementation of the *B. subtilis spx* mutant with the *spx* gene from *S. aureus* was made. *B. subtilis spx* mutant strains are hypersensitive to diamide (29). Therefore, sensitivity to diamide was used to establish functional production of *S. aureus* Spx in *B. subtilis*. The complementation alleviated the sensitivity of the mutant to diamide as effectively as a complementation with *spx* from *B. subtilis* (Fig. 4). The concentration of *S. aureus* Spx was also shown to be regulated in a similar manner to *B. subtilis* Spx in response to diamide exposure, with a low level in the unexposed cells and a high level in exposed cells (Fig. 5). A *B. subtilis* strain where both *yjbH* and *spx* were deleted and complemented with their *S. aureus* counterparts was created (LUW442). This strain exhibited the same diamide-induced pattern of Spx regulation as the other complemented strains and showed similar resistance to diamide (Fig. 4 and 5). In conclusion, the experiments in this section show that despite the low sequence conservation between the *B. subtilis* and *S. aureus* YjbH proteins, they are similar in all functional aspects tested.

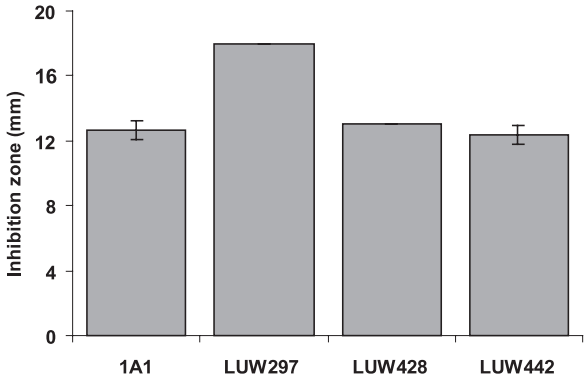

FIG 4 The diagram shows the diameter of the clearing zone of *B. subtilis* 1A1 (WT), LUW297 (spxΩpMUTIN2), LUW428 (spxΩpMUTIN2 *amyE*::spx_{SA}), and LUW442 (spxΩpMUTIN2 Δ*yjbH amyE*::spx_{SA}/pCW7_$yjbH_{SA}$) in the presence of diamide. The error bars represent the standard deviations of the diameters of three separate clearing zones.

Role of cysteine residues in YjbH. Cysteines have the ability to be reversibly oxidized and covalently modified and are often involved in redox sensing. *B. subtilis* YjbH contains an N-terminal CXXC motif (two cysteines separated by two other residues) at residues 31 to 34 and five additional cysteines (Fig. 1). In thioredoxins and related proteins, the CXXC motif is directly involved in redox reactions, suggesting that this motif could have a similar role in YjbH. However, in *S. aureus* YjbH, the cysteine corresponding to C31 is replaced by a serine. Moreover, it has been suggested that YjbH binds zink via a His-Cys-rich domain and that redox-induced inactivation of Zn-binding leads to Zn^{2+} release and liberation of Spx from YjbH (9). In order to investigate the role of the cysteine residues in $YjbH_{BS}$, a mutant where all seven cysteine residues (C13, C31, C34, C89, C175, C236, C297) were exchanged for serines was constructed. Similarly, a mutant of

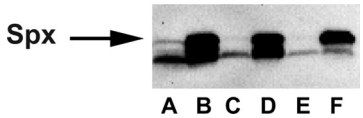

FIG 5 Immunoblot analysis of the heterologous *S. aureus* Spx content in *B. subtilis*. Lanes A and B, LUW362 (spxΩpMUTIN2 *amyE*::spx_{BS}); lanes C and D, LUW428 (spxΩpMUTIN2 *amyE*::spx_{SA}); lanes E and F, LUW442 (spxΩpMUTIN2 Δ*yjbH amyE*::spx_{SA}/pCW7_$yjbH_{SA}$) before (lanes A, C, E) and after (lanes B, D, F) diamide treatment.

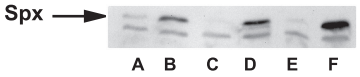

FIG 6 Immunoblot analysis of the Spx content before (lanes A, C, and E) and after (lanes B, D, and F) the addition of diamide to *B. subtilis* WT (lanes A and B) and Δ*yjbH* mutant cells complemented with $YjbH_{BS}$ without cysteines (lanes C and D) or $YjbH_{SA}$ without cysteines (lanes E and F).

FIG 8 Immunoblot analysis of the Spx content of different *S. aureus* strains. Lane A, Newman (wild type); lane B, LUSA2 (Newman Δ*yjbH*); lane C, 8325-4 Δ*spx* mutant; lane D, LUSA2/pRMC2; lane E, LUSA2/pRMC2-$yjbH_{SA}$.

$YjbH_{SA}$ where all four cysteines (C37, C71, C121, C123) were exchanged for serines was also constructed. These cysteine-free YjbH variants were used to complement the *B. subtilis yjbH*-null mutant in the same way as described in the previous section. Immunoblot analysis showed that both of the cysteine-free YjbH variants were produced at similar levels as the wild-type protein and that they were able to facilitate the efficient proteolysis of Spx (data not shown and Fig. 6). It is concluded that the cysteine residues are dispensable for the function of the protein and that the activity of the adaptor is not activated or deactivated via redox-active cysteines. When our investigation was completed, Göhring et al. (12) reported a study on the role of the cysteine residues in *S. aureus* YjbH using a complementary approach. The cysteine residues were replaced with glycine residues, and plasmid-encoded cysteine replacement mutants of YjbH were tested for their ability to complement an *S. aureus yjbH* mutant strain. In contrast to our results, Göhring et al. (12) suggest that the cysteine residues indeed are important for the role of YjbH in disulfide stress management. A possible difference underlying the obtained results is the stability of the mutated YjbH proteins. The difference in helix propensities between cysteine (intermediate), serine (intermediate), and glycine (very low) might affect the stability and folding of YjbH.

Pleiotropic properties of an *S. aureus yjbH* mutant. To investigate the possible role of YjbH in the regulation of Spx in *S. aureus*, an allelic replacement was used to exchange *yjbH* with a spectinomycin resistance cassette (for details, see Materials and Methods). We compared the growth of the *yjbH* deletion strain with that of the wild type (Newman) and found that the mutation caused a decreased growth rate in rich medium. This is similar to a *B. subtilis yjbH* mutant, which displays growth defects both on plates and in broth medium (21). Loss of YjbH also resulted in decreased carotenoid pigment production by *S. aureus* (Fig. 7). The pigment protects *S. aureus* against oxidative stress due to its ability to absorb excess energy from reactive oxygen species and is linked to virulence (23). We do not know if the reduced pigmentation is due to lack of YjbH or increased levels of Spx. However, it has recently been shown that pigment production is linked to several different metabolic processes in the cell (20). To confirm that the phenotype was caused by the deletion of *yjbH*, we performed complementation analysis by inserting the *yjbH* gene in the pRMC2 plasmid (4) to generate the plasmid pRMC2_$yjbH_{SA}$. The complementation restored the pigment production; however, the growth rate phenotype was not restored in the strain complemented with pRMC2_$yjbH_{SA}$. This observation suggests that timing or level of *yjbH* expression is important. To better mimic the wild-type situation, an additional complementation construct, pCL25_$yjbIH_{SA}$, containing the *yjbIH* operon under the control of its native promoter integrated into the chromosome, was constructed. The resulting strain, LUSA10, alleviated the growth phenotype seen in the mutant and also complemented the pigmentation phenotype (see Fig. S5 in the supplemental material). In *B. subtilis*, the disruption of *yjbH* results in an increased resistance to diamide due to increased levels of thioredoxin (21). In contrast to this, the *S. aureus* Δ*yjbH* mutant showed decreased resistance to diamide compared to that of the wild type and the complemented strain (see Fig. S6 in the supplemental material).

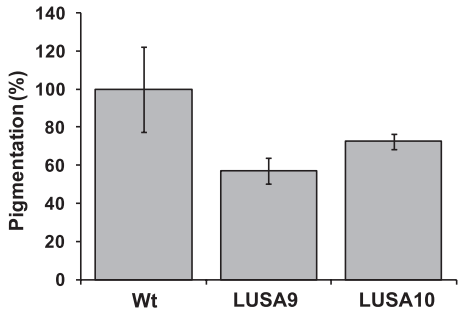

FIG 7 Pigment formation in *S. aureus*. The bars represent the relative amounts isolated from 24-h culture by methanol extraction. The error bars represent the standard deviations from three experiments. Wt, Strain Newman; LUSA9, Δ*yjbH* strain; LUSA10, Δ*yjbH* + *yjbIH* strain.

YjbH influences the level of Spx in *S. aureus*. In *B. subtilis*, a *yjbH*-null mutation leads to the accumulation of Spx (21), which in turn is responsible for the observed pleiotropic phenotype. To investigate if YjbH is required for efficient proteolytic degradation of Spx also in *S. aureus*, the amounts of Spx in the wild type and the *yjbH* mutant strain were analyzed by immunoblotting. A band corresponding to the expected size of Spx was detected at elevated levels in the *yjbH* mutant strain (Fig. 8). In both the complemented strains, the level of Spx returned to a low level similar to that of the wild-type strain. For the pRMC2_$yjbH_{SA}$ construct, the complementation was seen without adding the inducer (Atet) for $YjbH_{SA}$ production. This implies that residual expression yielding very small quantities of $YjbH_{SA}$ are enough to control the level of Spx. Larger amounts of $YjbH_{SA}$ were achieved by adding increasing concentrations of the inducer (see Fig. S8 in the supplemental material). This resulted in Spx concentrations below wild-type levels and a decreased growth rate. It was previously shown (34) that the *spx* mutant has a severe growth defect, so the depletion of Spx seen under $YjbH_{SA}$ overexpression is most likely the cause of the observed growth defect.

Transcriptional analysis. To determine whether the effect on Spx seen at the protein level was due to regulation at the transcriptional or the posttranscriptional level, qRT-PCR was used to quantify the amount of *spx* mRNA (Fig. 9A). No significant difference in the expression of *spx* before or after the addition of 5 mM diamide was found, nor was any significant difference seen between the expression of *spx* in the wild type and the *yjbH* mutant. This suggests that the increased level of Spx is due to changes at the posttranscriptional level that lead to a decreased degradation of Spx.

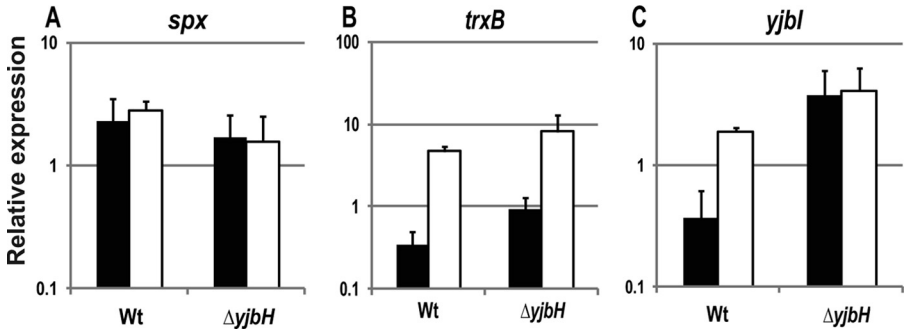

FIG 9 Transcript levels of *spx* (A), *trxB* (B), and *yjbI* (C) and in wild-type (Newman) and Δ*yjbH* (LUSA2) strains before (black columns) and after (white columns) addition of diamide (5 mM). The error bars represent the standard deviations from three experiments.

Previous studies identified *trxB* expression in *S. aureus* as an important target of Spx regulation (34). As expected, the expression of *trxB* was increased after the addition of diamide in the wild-type strain (Fig. 9B). The *yjbH* mutant showed a significantly higher expression of *trxB* in the unstressed cells but also an increase after the addition of diamide, indicating that the amount of Spx and the oxidation state of the cell effect *trxB* expression.

In *B. subtilis*, YjbH has a negative effect on its own expression (21), which is likely mediated through Spx. This ensures that there will be YjbH present in the cell to mediate the breakdown of Spx once the stress is gone. The expression of the *S. aureus yjbIH* operon was investigated. In the wild type, the addition of diamide leads to an induction of the expression of both *yjbI* and *yjbH*. In the *yjbH* mutant, only the level of *yjbI* could be measured and was assumed to be representative of *yjbH*. In the mutant, the level of *yjbI* was elevated compared to that of the unstressed wild type both before and after the addition of diamide (Fig. 9C). The transcriptional data presented here suggest that, when the level of Spx is high in the cell (stressed cells and Δ*yjbH* mutant) the level of expression from the *yjbIH* operon is elevated.

In conclusion, we here demonstrate that despite the low sequence similarity (approximately 30% identity) between YjbH in *B. subtilis* and *S. aureus*, the *S. aureus* protein can functionally complement its counterpart in *B. subtilis*.

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