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Identification of a Novel Dye-Decolorizing Peroxidase, EfeB, Translocated by a Twin-Arginine Translocation System in *Streptococcus thermophilus* CGMCC 7.179

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***Streptococcus thermophilus* is a facultative anaerobic bacterium that has the ability to grow and survive in aerobic environments, but the mechanism for this remains unclear. In this study, the *efeB* gene, encoding a dye-decolorizing peroxidase, was identified in the genome of *Streptococcus thermophilus* CGMCC 7.179, and purified EfeB was able to decolorize reactive blue 5. Strikingly, genes encoding two components (TatA and TatC) of the twin-arginine translocation (TAT) system were also found in the same operon with the *efeB* gene. Knocking out *efeB* or *tatC* resulted in decreased growth of the strain under aerobic conditions, and complementation of the *efeB*-deficient strains with the *efeB* gene enhanced the biomass of the hosts only in the presence of the *tatC* gene. Moreover, it was proved for both *S. thermophilus* CGMCC 7.179 and *Escherichia coli* DE3 that EfeB could be translocated by the TAT system of *S. thermophilus*. In addition, the transcriptional levels of *efeB* and *tatC* increased when the strain was cultured under aerobic conditions. Overall, these results provide the first evidence that EfeB plays a role in protecting cells of *S. thermophilus* from oxidative stress, with the assistance of the TAT system.**

Streptococcus thermophilus is a Gram-positive bacterium of the genus *Streptococcus*, which comprises several harmful pathogenic species, such as *Streptococcus pyogenes* and *Streptococcus pneumoniae*. With long-term usage in fermented dairy products, *S. thermophilus* has lost its virulence-related genes and is given a "generally recognized as safe" (GRAS) status. This organism is commonly used with *Lactobacillus delbrueckii* subsp. *bulgaricus* or other lactobacilli for yogurt making as well as for the production of mozzarella, Swiss, and cheddar cheeses (1, 2).

However, *S. thermophilus* encounters various stress conditions during the fermentation and storage processes (3). Among these environmental stresses, the presence of toxic reactive oxygen species (ROS) is the most important survival challenge, as it affects the organism's growth, fermentative capabilities, and viability and, consequently, the texture and flavor of the final fermented products (4). Though *S. thermophilus* cannot eliminate oxygen by respiration and lacks catalase activity (5), it can grow in the presence of oxygen and has an inducible capacity to survive in the presence of low concentrations of superoxide and hydroxyl radicals (6, 7), suggesting that this bacterium has evolved a specific inducible defensive system against ROS damage.

In *S. thermophilus*, a single H_2O-forming NADH oxidase is found, which could reduce the amount of intracellular O_2 (8). A well-characterized antioxidant enzyme in *S. thermophilus* is the manganese-containing superoxide dismutase (SodA), which converts superoxide anions to molecular oxygen and hydrogen peroxide, and the activity of SodA is not regulated by O_2 (9). Recently, a functional thioredoxin system composed of NADPH, a thioredoxin reductase, and thioredoxin was identified in *S. thermophilus* (10). This system provided protection against oxidative stress through its disulfide reductase activity regulating the protein dithiol/disulfide balance (11). Also, a bifunctional gamma-glutamate-cysteine ligase/glutathione synthetase (GshF) that deals with oxidative damage has been reported for *S. thermophilus* (12). However, how *S. thermophilus* metabolizes H_2O_2 remains unclear, and none of these proteins have been verified to be involved in the inducible defensive system against ROS damage.

To identify the genes involved in oxidative stress resistance, insertional mutagenesis was carried out experimentally with *S. thermophilus* CNRZ368, and the mutants were screened by menadione sensitivity and resistance. Among the mutant genes, the *ossH* gene showed 55% identity to the gene for a potential membrane-spanning permease of an Fe^{3+} ABC transporter (3), and the *osrD* gene showed significant identity to the genes for predicted iron-dependent peroxidases belonging to the family of dye-decolorizing peroxidases (13). Unfortunately, the genetic organization and physiological functions of these two genes were not further characterized.

Dye-decolorizing peroxidases were classified as a novel peroxidase family because of their broad substrate specificity, low pH optima, lack of a conserved active site distal histidine, and structural divergence from classical plant and animal peroxidases (14). They can decolorize a broad spectrum of dyes by utilizing H_2O_2 as an electron acceptor. Large amounts of putative dye-decolorizing peroxidases have been registered in the PeroxiBase database (http://peroxibase.toulouse.inra.fr/), but few of them have been characterized (15). Interestingly, the reported bacterial dye-decolorizing peroxidases from *Escherichia coli* O157:H7 (YcdB/EfeB), *Bacillus*

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TABLE 1 Strains and plasmids used in this study

Bacterial strain or plasmid	Genotype or characteristics	Reference or source
Strains		
S. thermophilus strains		
CGMCC 7.179	Wild-type strain	
ST16814	CGMCC 7.179 Δ*tatC*	This study
ST1314	CGMCC 7.179 Δ*efeB*	This study
ST16503	CGMCC 7.179 Δ*tatC* Δ*efeB*	This study
CGMCC 7.179/SP-CAT	CGMCC 7.179 with pSec-SP-CAT	This study
ST16814/SP-CAT	ST16814 with pSec-SP-CAT	This study
ST1314/Sec	ST1314 with pSec:Leiss:Nuc	This study
ST1314/efeB	ST1314 with pSec-efeB	This study
ST16503/Sec	ST16503 with pSec:Leiss:Nuc	This study
ST16503/efeB	ST16503 with pSec-efeB	This study
E. coli strains		
DH5α	F^- ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	
DE3	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	
DE3/pET	DE3 with pET22b	This study
dE	DE3 Δ*tatE*	This study
dEAD	DE3 Δ*tatE* Δ*tatABCD*	This study
dEAD/efeB	dEAD with pET-efeB	This study
dEAD/efeB-TatCA	dEAD with pET-efeB and pSec-tatCA	This study
Plasmids		
pG$^+$ host4	Erm^r; temperature-sensitive vector	27
pG$^+$ efeUp-Down	pG$^+$ host4 derivative with upstream and downstream sequences of *efeB* gene	This study
pG$^+$ tatUp-Down	pG$^+$ host4 derivative with upstream and downstream sequences of *tatC* gene	This study
pET22b	Ap^r; carries T7 promoter and *lac* operator	Novagen
pET-efeB	Ap^r; expresses EfeB under T7 promoter control	This study
pSec:Leiss:Nuc	pWV01 replicon; expresses Nuc under PnisA control; Cm^r	28
pSec-efeB	pSec:Leiss:Nuc derivative; expresses EfeB	This study
pSec-SP-CAT	pSec:Leiss:Nuc derivative; expresses SP-CAT	This study
pSec-tatCA	pSec:Leiss:Nuc derivative; expresses TatCA	This study
pKD46	Ap^r; λ Red recombinase expression	29
pKD4	Ap^r Kan^r; *kan* cassette template	29
pCP20	Ap^r Cm^r; FLP recombinase expression	30

subtilis 168 (YwbN/EfeB), and *Staphylococcus aureus* subsp. *aureus* N315 (FepB) were verified to be substrates of the twin-arginine translocation (TAT) system (16–18).

The TAT system is present in the cytoplasmic membranes of most bacteria and archaea and has the highly unusual property of transporting fully folded proteins across the cytoplasmic membrane. The TAT system in *E. coli* includes five components (TatA, TatB, TatC, TatD, and TatE), while it comprises two components (TatA and TatC) in most Gram-positive bacteria. The TAT system has been proved to be essential for viability in a few bacteria and archaea (19). However, the function of the TAT pathway still remains unknown for *S. thermophilus*, since this organism exhibits a striking level of gene decay (10% pseudogenes) (20).

In this study, *S. thermophilus* CGMCC 7.179, isolated from the traditional fermented dairy products of Inner Mongolia, was partially sequenced. A dye-decolorizing peroxidase gene (*efeB*) and two TAT component genes (*tatC* and *tatA*) were identified in the *S. thermophilus* CGMCC 7.179 genome, and they were located in the same operon. The protective role of EfeB against oxidative stress was investigated, and the functionality of the TAT system was analyzed. This is the first demonstration that a dye-decolorizing peroxidase is translocated by the TAT pathway in *S. thermophilus*.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *S. thermophilus* CGMCC 7.179 was isolated from a traditional yogurt of Inner Mongolia, China. *S. thermophilus* strains were propagated in M17 broth (Oxoid, Basingstoke, United Kingdom) supplemented with 1% (wt/vol) lactose (LM17 broth) anaerobically at 42°C. *Escherichia coli* DH5α was used as the host for standard cloning procedures. All *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37°C. Chloramphenicol or erythromycin was added at 5 μg/ml or 2.5 μg/ml, respectively, for *S. thermophilus*. Antibiotics for *E. coli* were used at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and kanamycin, 50 μg/ml.

Multiple-sequence alignment. Multiple-sequence alignment was performed using Clustal X, version 2.0 (21), and ESPript 3.0 (22). The amino acid sequence of EfeB was aligned with those of YcdB, YwbN, FepB, DyP, MsP1, MsP2, and PoDyP (16–18, 23–25).

Construction of plasmids. The primers used in this study are listed in Table 2. All the molecular manipulations in this study were performed as described previously (26). *Taq* polymerase, restriction enzymes, T4 DNA ligase (TaKaRa, Tokyo, Japan), and *Pfu* polymerase (TransGen, Beijing, China) were used according to standard procedures.

To knock out the *efeB* gene in *S. thermophilus* CGMCC 7.179, pG$^+$ efeUp-Down was constructed as follows. Sequences upstream (amplified with primers efUpF and efUpR) and downstream (amplified with primers

TABLE 2 Oligonucleotide primers used in this study

Primer	Sequence (5′-3′)[a]	Restriction site
efUpF	AAACTCGAGTCATTATTAATGATAAAACGACGAT	XhoI
efUpR	TTTTCTTATTTCCCATCTAGGATAA	
efDownF	TTATCCTAGATGGGAAATAAGAAAAACTCCTCGTTTTGCTTGGG	
efDownR	AAAAGATCTTAATTTCACGGTTAGCCCCTG	BglII
tUpF	AGGCTCGAGAAAAACGGCTGCGGCATCTATG	XhoI
tUpR	TCTGTCGACGCTGCTTCACCGTCACAAAGGC	SalI
tDownF	GATGTCGACAAAGGAAATCTCATGGGAATTC	SalI
tDownR	CCTCTGCAGTTAACCAGTGTATGAACCGTAG	PstI
KanF	GTGTAGGCTGGAGCTGCTTC	
KanR	ATGGGAATTAGCCATGGTCC	
EupF	CTGGATACCACCTGTTATATGGTG	
EupR	GAAGCAGCTCCAGCCTACACAGATACCTTCTTGACATATAACCGG	
EdownF	GGACCATGGCTAATTCCCATCGTGGCGAGCAGGACGC	
EdownR	GAAACCAATGAAGAGATTATTGAAG	
AupF	GCGCCAGGGCAAGTATTTAC	
AupR	GAAGCAGCTCCAGCCTACACACATGTTCCTCTGTGGTAGATGATG	
DdownF	GGACCATGGCTAATTCCCATAGTTTGCGGAACTCGGTGTT	
DdownR	GATTCATACCACGACTATCAACGC	
efF	AACATATGACTGATAAAAAATTTTTAGACCAA	NdeI
efR	ATAGTCGACTTAGTGGTGGTGGTGGTGGTGTTCAAAGAGTGCTTGACCAAGG	SalI
SPF	ATGACTGATAAAAAATTTTTAGACC	
SPR	AGCAGATGCACCAGAAAGAC	
PtatF	AAGGATCCGTGAAAAGACAAATCGTGGACTC	BamHI
PtatR	GGTCTAAAAATTTTTTATCAGTCATGAATGTCTCCTATTTTCAAGATAGT	
PcmF	AAAAGATCTCTGCAGTCGACGGCAATAGTTAC	BglII
PcmR	GGTCTAAAAATTTTTTATCAGTCATTTGATATGCCTCCTAAATTTTTATC	
cmF	GTCTTTCTGGTGCATCTGCTATGAACTTTAATAAAATTGATTTAG	
cmR	AAAAGATCTCTGTAATATAAAAACCTTC	BglII
RepF	AAAAGATCTTCAGCAAATAACTCTTTTTCTTTG	BglII
RepR	AAAAGATCTATTGTAAAAGTGTCACTGCTGCTAG	BglII
P1F	AAAAGATCTAGCGCGCCAAAGAAGTAG	BglII
P1R	AAGAATTCATATGTCGAGCCTCCTGAAGTACTG	NdeI
TatCF	AACATATGGCAAGAAGTAGAGATGAGATG	NdeI
TatAR	AAGTCGACCTAGTCTTTAGTTTTGTCTTCTTCG	SalI
RT-16sF	CTAACTACGTGCCAGCAGC	
RT-16sR	GGTTGAGCCACAGCCTTTA	
RT-efF	ATGATGAACAAGTAGCCTTCC	
RT-efR	CCTTACGGTCACCAATAGC	
RT-tatF	GCTGTTCTCTTTGAATTTCCTGTAG	
RT-tatR	GTGTCAGAATCACCGCCAAA	
ST tufF	GGTGCGATCCTTGTAGTTGCA	
ST tufR	ACACCAACCTGACGTGAAAGAA	
RPOST-F	ACTGTCATTGTTGCTTGGAATG	
RPOST-R	AGCTGAGGTTACTGCTGGAGAT	

[a] The restriction sites in the primer sequences are underlined.

efDownF and efDownR) of the *efeB* gene were PCR amplified from the genomic DNA of *S. thermophilus* CGMCC 7.179 and spliced by an overlap extension PCR using primers efUpR and efDownF; subsequently, the fused fragment was inserted into the temperature-sensitive vector pG$^+$ host4 (27). Plasmid pG$^+$ tatUp-Down was constructed by the same procedure, using primers tUpF, tUpR, tDownF, and tDownR. To express EfeB, the *efeB* gene from *S. thermophilus* CGMCC 7.179 was amplified by a PCR with primers efF and efR. The PCR product was digested with NdeI and SalI and inserted into the corresponding sites of pET22b, resulting in plasmid pET-efeB. To perform *efeB* complementation, plasmid pSec-efeB was constructed as follows. The *Ptat* promoter was amplified from the *S. thermophilus* CGMCC 7.179 genome by a PCR using primers PtatF and PtatR, and *efeB* was amplified from the *S. thermophilus* CGMCC 7.179 genome by a PCR using primers SPF and efR. *Ptat* and *efeB* were fused, and the fusion fragment was digested with BamHI and SalI and inserted into the *Lactococcus lactis*/*E. coli* shuttle vector pSec:Leiss:Nuc (28) digested with BglII and SalI. To test the interaction of the TAT system and the EfeB signal peptide (SP), the sequence of the putative EfeB SP (amplified with primers SPF and SPR) was fused between the promoter (*Pcm*; amplified with primers PcmF and PcmR) and the chloramphenicol acetyltransferase gene (*CAT*; amplified with primers cmF and cmR), generating *Pcm-SP-CAT*. The fusion fragment was then digested with BglII and ligated with the digested replicon *Rep*AC, amplified by PCR from the vector pSec:Leiss:Nuc, generating plasmid pSec-SP-CAT. To constitutively express TatCA, *P1x* (amplified with primers P1F and P1R), which is the promoter of the *xyl* operon from *Lactobacillus fermentum* 1001, and the *tatCA* sequence were amplified (with TatCF and TatAR) and digested with NdeI. The *P1x-tatCA* fragment was amplified from ligated *P1x* and *tatCA* by a PCR using primers P1F and TatAR, and the PCR product was digested with BglII and SalI and inserted into the corresponding sites of pSec:Leiss:Nuc, resulting in pSec-tatCA.

Gene deletion. To knock out the *efeB* gene in *S. thermophilus* CGMCC 7.179, pG^+ efeUp-Down was used to perform homologous double-crossover recombination in the *S. thermophilus* CGMCC 7.179 genome as described previously (27), generating the *efeB* deletion mutant ST1314, and the *efeB* gene deletion was verified by PCR amplification with primers efUpF and efDownR. The same procedures were carried out with sequences homologous to the up- and downstream regions of *tatC* (with plasmid pG^+ tatUp-Down) in *S. thermophilus* CGMCC 7.179 and ST1314, generating ST16814 and ST16503, respectively.

For deletion of *tatE* in *E. coli* DE3, the kanamycin resistance cassette of plasmid pKD4 was amplified by a PCR using primers KanF and KanR. Subsequently, it was fused with Eup (amplified with primers EupF and EupR) and Edown (amplified with primers EdownF and EdownR), which had about 300 bp of sequence (each) homologous to the up- and downstream regions of *tatE*. Homologous recombination of the PCR product with the chromosomal *tatE* allele was carried out in *E. coli* DE3 as described previously, using the λ Red recombinase expression plasmid pKD46 (29). The kanamycin resistance cassette was eliminated by using the temperature-sensitive plasmid pCP20 (30), by which the new strain dE was obtained. The same procedures were carried out with sequences homologous to the up- and downstream regions of *tatABCD* in dE, resulting in the dEAD strain, in which *tatE* and *tatABCD* were deleted.

Determination of numbers of CFU. The number of CFU was calculated by a previously described method (31). Cultures were 10-fold serially diluted, and the dilution time (T_{dilution}) of the original sample was 0. For objective dilution, 5-μl samples were pipetted onto a plate containing 1.5% agar medium. The plates were air dried and then incubated until colonies were visible, with an average size of 200 to 500 μm. The colony number of every drop (N_{colony}) was then counted. The CFU concentration (number of CFU per milliliter) was calculated by using the following equation:

$$\text{CFU/ml} = 10^{T_{\text{dilution}}} \times 200 \times N_{\text{colony}}$$

Detection of tolerance of *S. thermophilus* to oxidative stress. After overnight static incubation at 42°C, *S. thermophilus* and its derivatives were diluted 50-fold in 5 ml fresh LM17 broth and incubated at 42°C with shaking (200 rpm) at a ratio of liquid to airspace of approximately 1 to 3, and the optical density at 600 nm (OD_{600}) and number of CFU per milliliter were measured at 2-h intervals. Meanwhile, static cultures were used as controls.

Expression and purification of EfeB. Plasmid pET-efeB was transformed into *E. coli* dEAD, generating dEAD/efeB. EfeB with six histidine residues at the C terminus was overproduced by induction with isopropyl-β-D-thiogalactopyranoside (IPTG; Sangon, China) at a final concentration of 0.5 mM when the OD_{600} of cultures reached 0.8. After a further 14 h of growth at 16°C, the cells were harvested by centrifugation at 8,000 × *g* for 5 min and resuspended in 10 ml phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4). Cells were broken by use of an ultrasonic cell crusher, and cell debris was removed by centrifugation. EfeB was purified by nickel affinity chromatography following the procedure described in the pET system manual (Novagen, Inc.). The purified protein was quantified using the Bradford protein assay. The cell extracts and purified protein were separated by SDS-PAGE (12% acrylamide) (32).

EfeB enzyme assay. Dye-decolorizing peroxidase activity was detected as described previously (33), with modifications. Briefly, reactive blue 5 (RB5; Yongxing Dye, Jinan, China), a representative anthraquinone dye, was selected as the substrate and dissolved in 25 mM citrate buffer (pH 5.5) to a final concentration of 360 μM. Two hundred microliters of enzyme solution (0.4 mg/ml) was mixed with 100 μl substrate solution and 0.2 mM H_2O_2. The reaction mixture was then incubated at 42°C for 30 min. The enzyme activity was calculated by measuring the decrease in absorbance at 600 nm of RB5 ($\varepsilon_{600} = 8{,}000\ M^{-1}\ cm^{-1}$). One unit of enzyme activity was defined as the amount of enzyme required for the decolorization of 1 mmol of RB5 (34). In control experiments, a boiled enzyme solution was used instead of the enzyme solution, or H_2O was used instead of H_2O_2.

Test of genetic complementation of *efeB*. pSec:Leiss:Nuc and pSec-efeB were transformed into strains ST1314 and ST16503. After overnight static cultivation at 42°C, strains ST1314/efeB, ST1314/Sec, ST16503/efeB, and ST16503/Sec were diluted 50-fold in 5 ml fresh LM17 broth and incubated at 42°C with shaking (200 rpm) at a ratio of liquid to airspace of approximately 1 to 3. The OD_{600} was measured at 2-h intervals.

Translocation analysis of the EfeB signal peptide. The function of *tatC* in *S. thermophilus* CGMCC 7.17 was investigated by a previously reported method for positive selection for a loss of TAT function (35). Briefly, the plasmid pSec-SP-CAT was transformed into *S. thermophilus* CGMCC 7.179 and ST16814, generating strains CGMCC 7.179/SP-CAT and ST16814/SP-CAT, respectively. Strains CGMCC 7.179/SP-CAT and ST16814/SP-CAT were grown to stationary phase and 10-fold serially diluted. Five-microliter aliquots of a 10-fold dilution were pipetted onto plates containing 5, 8, 9, 10, 11, and 15 μg/ml chloramphenicol. Five-microliter aliquots of a 10^5-fold dilution were pipetted onto plates without chloramphenicol as a control experiment.

Coexpression of *efeB* and *tatCA* in *E. coli*. Plasmid pSec-tatCA was transformed into dEAD/efeB. After overnight incubation at 37°C, dEAD/efeB-TatCA, dEAD/efeB, and DE3/pET were diluted 50-fold in 5 ml fresh LB broth and further incubated until the OD_{600} reached 0.8. IPTG was added to a final concentration of 0.5 mM. After a further 14 h of growth at 16°C, the cells were removed by centrifugation at 10,000 × *g* for 5 min. The cell extracts and supernatant were separated by SDS-PAGE (12% acrylamide). For EfeB immunoblotting, the supernatant was separated by SDS-PAGE (12% acrylamide) and transferred to a polyvinylidene difluoride (PVDF) membrane (36). Immunoreactive bands were visualized using an anti-His antibody (Novagen).

Transcriptional analysis by real-time qPCR. RNA isolation and real-time quantitative PCR (qPCR) were performed as described previously (37). Briefly, exponential-phase cells from 1-ml cultures were collected by centrifugation at 7,000 × *g* for 5 min, and the total RNA was extracted by use of an RNA Simple total RNA kit (Tiangen, Beijing, China) according to the manufacturer's protocols. The quantity and purity of RNA were determined spectrophotometrically at 260 nm and 280 nm. Reverse transcription was performed with random 6-mer primers and an oligo(dT) primer, using a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. Real-time qPCR was performed with SYBR Premix Ex TaqII (TaKaRa, Tokyo, Japan) by applying the protocol for a real-time PCR detection system (Bio-Rad, Hercules, CA). The *efeB* transcript was PCR amplified with the primers RT-efF and RT-efR, and the *tatC* transcript was PCR amplified with the primers RT-tatF and RT-tatR. The 16S rRNA gene, the elongation factor Tu (*tuf*) gene, and the RNA polymerase subunit alpha (*rpoA*) gene, which were used as internal standards, were amplified with the primers RT-16sF and RT-16sR, ST tuff and ST tufR (38), and RPOST-F and RPOST-R (39), respectively.

Nucleotide sequence accession number. The GenBank accession number for the *efeOBU-tatCA* operon is KR106994.

RESULTS

Genetic organization of the *efeB* gene. To investigate why *S. thermophilus* CGMCC 7.179 can grow under aerobic conditions, a genome-wide search of its genome for peroxidase genes revealed an ortholog of dye-decolorizing peroxidase genes which was an allele of the *osrD* gene. This gene was named *efeB* in accordance with the names of the dye-decolorizing peroxidase genes from *E. coli* and *B. subtilis*. The complete DNA sequence of *efeB* is 1,206 bp long. The deduced amino acid sequence of EfeB was aligned with those of three bacterial dye-decolorizing peroxidases (FepB, YwbN, and YcdB) and four fungal dye-decolorizing peroxidases (DyP, MsP1, MsP2, and PoDyP). The heme-binding His^{313} resi-

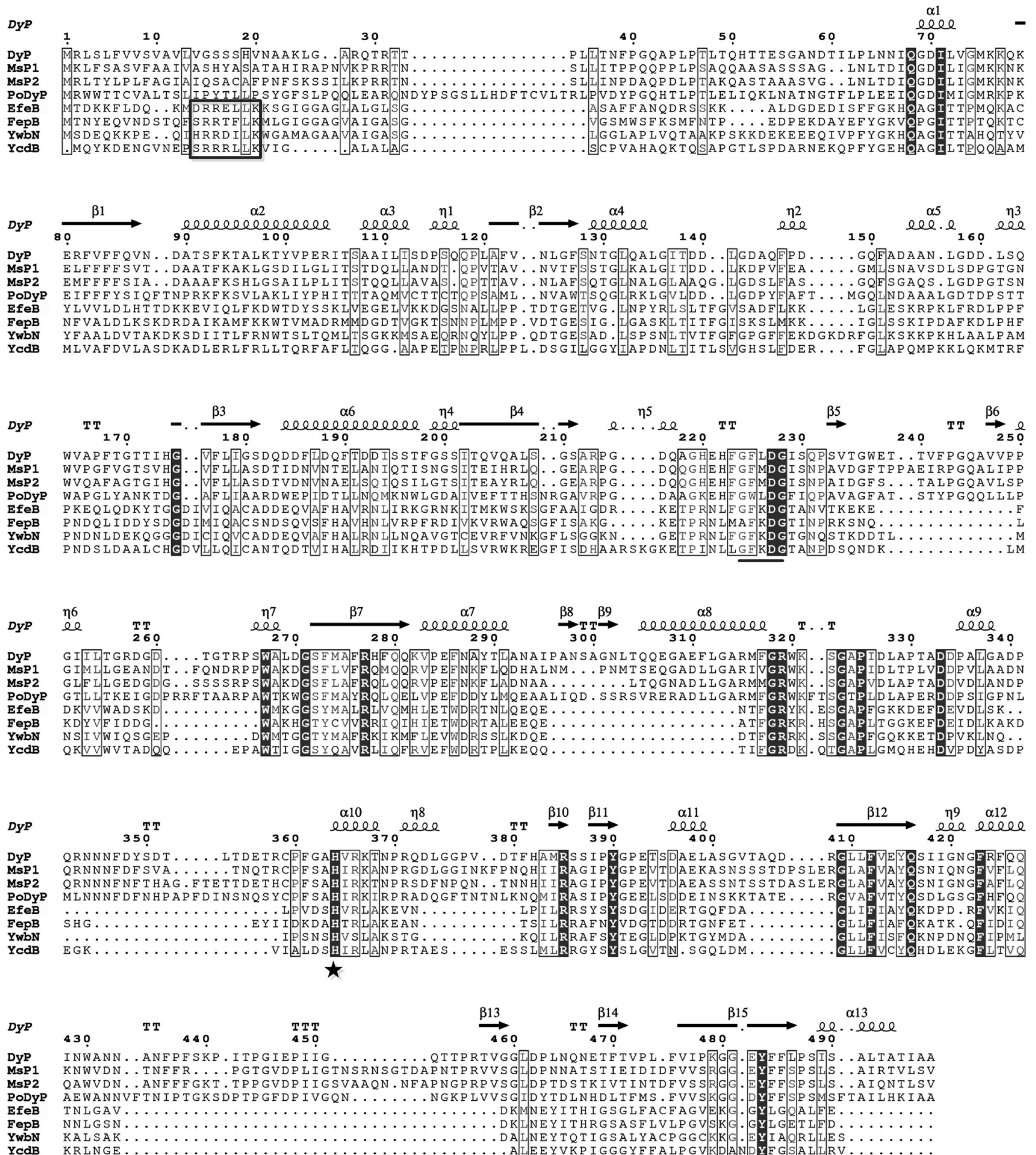

FIG 1 Multiple-sequence alignment of EfeB and dye-decolorizing peroxidases. All sequences are displayed as full-length sequences before processing. The secondary structure of DyP is shown at the top of each set of sequences. Abbreviations: α, α-helix; β, β-sheet; η, 3_{10}-helix; TT, β-turn. Perfectly matched residues are displayed in white on black. Similar amino acid residues are boxed with a thin line. The His[313] residue of EfeB is indicated with a star. The G-X-X-D-G motif is underlined. The S/T-R-R-X-F-L-K motif is boxed with a bold line.

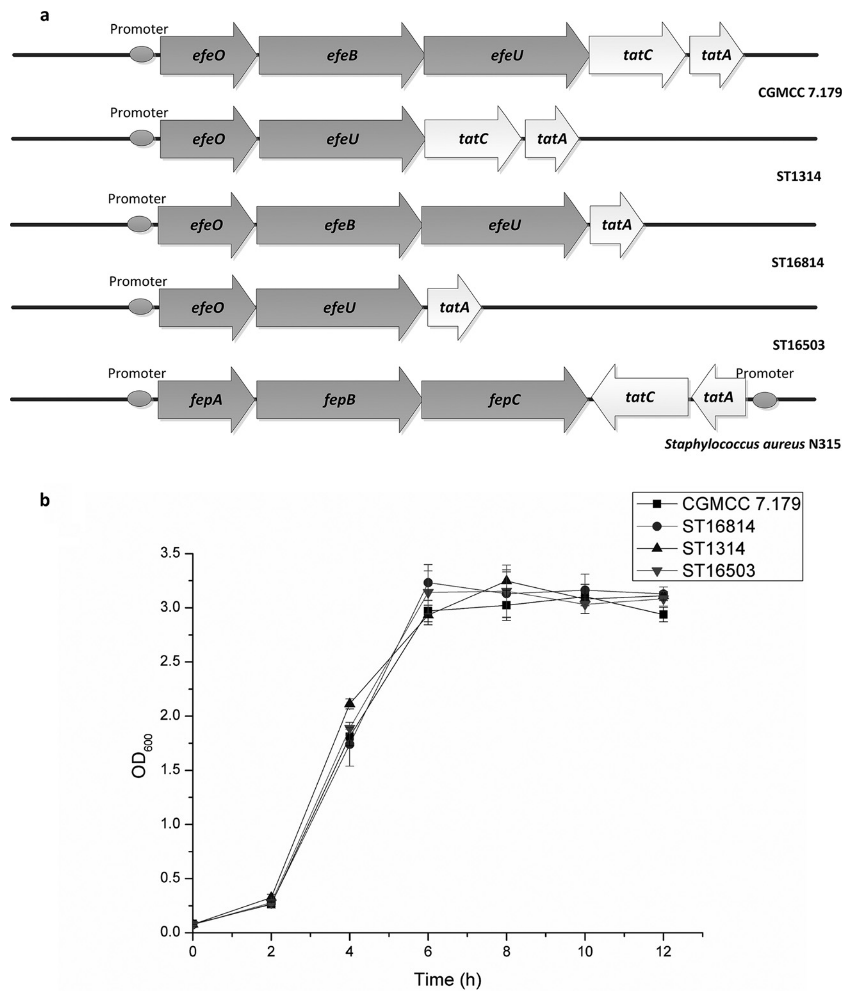

FIG 2 (a) Gene organization of the *efeOBU-tatCA* operon in *S. thermophilus* CGMCC 7.197 (wild type), ST1314 (*efeB* deleted), ST16814 (*tatC* deleted), and ST16503 (*efeB* and *tatC* deleted) and *Staphylococcus aureus* N315. The operon encodes five proteins. Arrows indicate the orientations of the genes. (b) Growth curves for *S. thermophilus* CGMCC 7.197 (■), ST1314 (▲), ST16814 (●), and ST16503 (▼) under anaerobic conditions.

due and the highly conserved G-X-X-D-G box of dye-decolorizing peroxidases were found in EfeB (Fig. 1). In particular, EfeB showed 48.13% similarity to FepB of *Staphylococcus aureus* N315.

When the sequence alignment was focused on the bacterial dye-decolorizing peroxidases, a typical twin-arginine signal peptide containing the consensus motif S/T-R-R-X-F-L-K was revealed at the N terminus of EfeB (Fig. 1). Genetic organization analysis showed that the *efeB* gene was located in a 5-gene cluster containing genes encoding a putative periplasmic iron transport lipoprotein (*efeO*), a putative dye-decolorizing peroxidase (*efeB*), a putative iron permease (*efeU*), and two putative components of a TAT system (*tatC* and *tatA*) (Fig. 2a). In contrast to the genetic organization of the *tatCA* genes in *Staphylococcus aureus* N315, the *tatCA* genes in *S. thermophilus* CGMCC 7.179 had the same orientation as the *efeOBU* genes (Fig. 2a). It was surmised that these 5 genes were probably located in the same operon.

Function of EfeB in defense against oxidative stress. To examine the protective role of EfeB against oxidative stress, the *efeB*-deficient mutant ST1314 was generated in *S. thermophilus* CGMCC 7.179 by homologous double-crossover recombination (Fig. 2a). The deletion of the *efeB* gene was verified by PCR amplification with primers efUpF and efDownR. Strain CGMCC 7.179 and its derivative ST1314 displayed the same growth rate under anaerobic conditions (Fig. 2b). To detect the tolerance of strains CGMCC 7.179 and ST1314 to oxygen, their optical densities and viable counts were measured when they were grown aerobically in LM17 medium. As shown in Fig. 3a, both the OD_{600} and the number of CFU of the *efeB*-deficient strain ST1314 were lower than those of the wild-type strain CGMCC 7.179 throughout the whole growth period. After incubation for 6 h, the viable cell counts of both strains CGMCC 7.179 and ST1314 reached the maximum, with a gap of about 160 million/ml (30.02%), indicating that the deletion of the *efeB* gene resulted in an increase of the sensitivity of *S. thermophilus* cells to oxygen. Therefore, EfeB was able to protect cells against oxidative stress.

EfeB enzyme activity assay. According to sequence alignment, EfeB is distinctly related to the dye-decolorizing peroxidase family. To investigate the possible peroxidase activity of EfeB, this protein was first expressed in *tatABCDE*-deficient *E. coli* dEAD and purified by nickel affinity chromatography. As shown in Fig. 3b, SDS-PAGE analysis of the purified EfeB protein revealed a

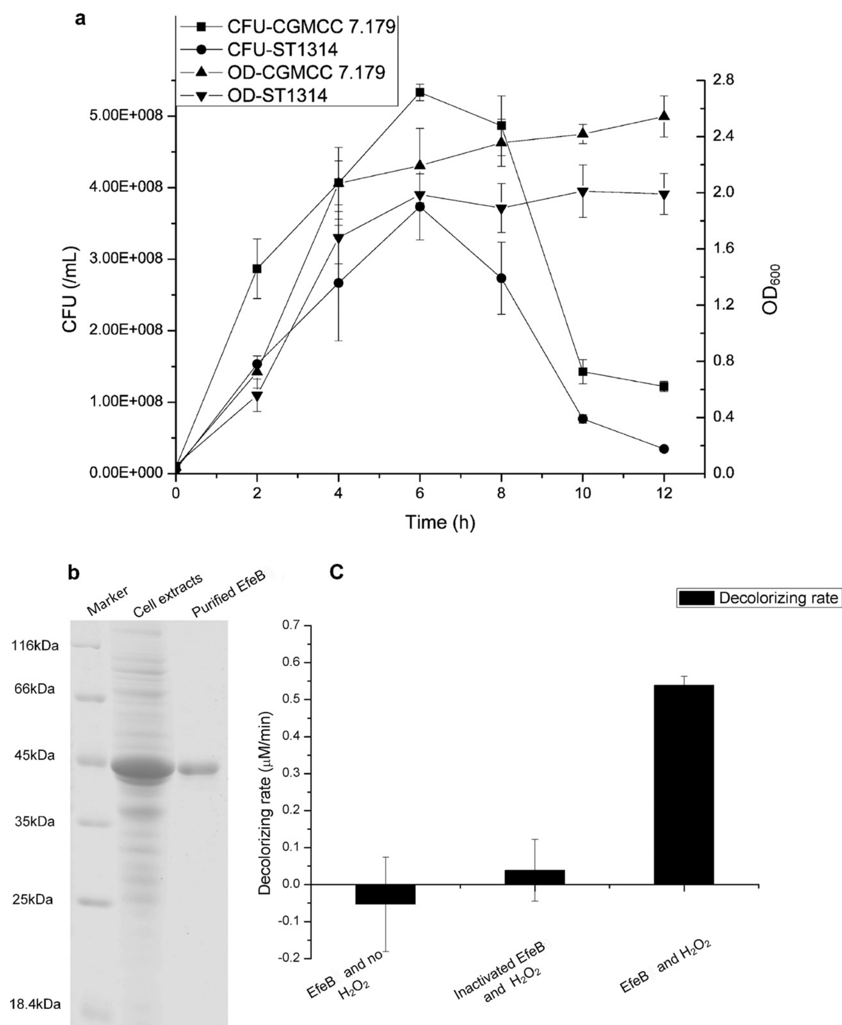

FIG 3 Function, purification, and activity of EfeB. (a) Numbers of CFU and OD_{600} values for *S. thermophilus* CGMCC 7.197 and ST1314 under aerobic conditions. Symbols: ■, number of CFU of CGMCC 7.197; ●, number of CFU of ST1314; ▲, OD_{600} of CGMCC 7.197; ▼, OD_{600} of ST1314. (b) SDS-PAGE analysis of EfeB in cell extract sample or purified sample. (c) RB5 decolorization by EfeB, with controls of inactivated EfeB and supplying no H_2O_2.

visible protein band with a molecular mass of 45 kDa, which was consistent with the theoretical molecular mass of EfeB plus the His tag. The enzyme activity of EfeB was biochemically detected with the substrate RB5, which is a staple substrate of dye-decolorizing peroxidases. The results showed that the purified EfeB protein had the capability to decolorize RB5 with the aid of H_2O_2, displaying a specific activity of 6.74 U/mg. When EfeB was inactivated by boiling or H_2O instead of H_2O_2 was added to the reaction system, no decolorization was observed, suggesting that EfeB is a member of the dye-decolorizing peroxidase family (Fig. 3c).

Dependence of EfeB on the TAT system. A TAT substrate search was performed in the whole genome of a completely sequenced *S. thermophilus* strain, and the dye-decolorizing peroxidase was the only candidate TAT-exported protein (8). Taken together, the genetic construction of the *efeB* gene and the translocation pathway of other bacterial dye-decolorizing peroxidases suggested that EfeB might be translocated by the TAT system. To clarify the TAT dependence of EfeB, *tatC*-deficient mutants ST16814 and ST16503 were generated in *S. thermophilus* CGMCC 7.179 and ST1314, respectively. Strains CGMCC 7.179, ST16814, ST1314, and ST16503 displayed the same growth rate under anaerobic conditions (Fig. 2b). After the strains were cultured aerobically, the growth curves for strains ST16503, ST16814, and ST1314 displayed the same pattern, and all of them showed obviously slower growth than that of the wild-type strain CGMCC 7.179 (Fig. 4a). Therefore, both EfeB and TatC are necessary for the antioxidant response. In other words, EfeB and TatC are interdependent in defending against oxidative stress.

To further test whether the function of EfeB depended on the presence of TatC, the *efeB* gene was genetically complemented in strains ST1314 and ST16503. Strains ST1314/efeB, ST1314/Sec, ST16503/efeB, and ST16503/Sec were grown aerobically. As shown in Fig. 4b, the OD_{600} values for strain ST1314/efeB and strain ST1314/Sec were 1.89 and 1.68, respectively. The significant growth improvement of the complemented ST1314/efeB strain revealed the positive effect of EfeB on the defense against oxidative stress in the presence of the TAT system. However, the growth of the ST16503/efeB strain was not restored compared to that of ST16503/Sec, indicating that EfeB indeed depends on the TAT system to defend against oxidative stress.

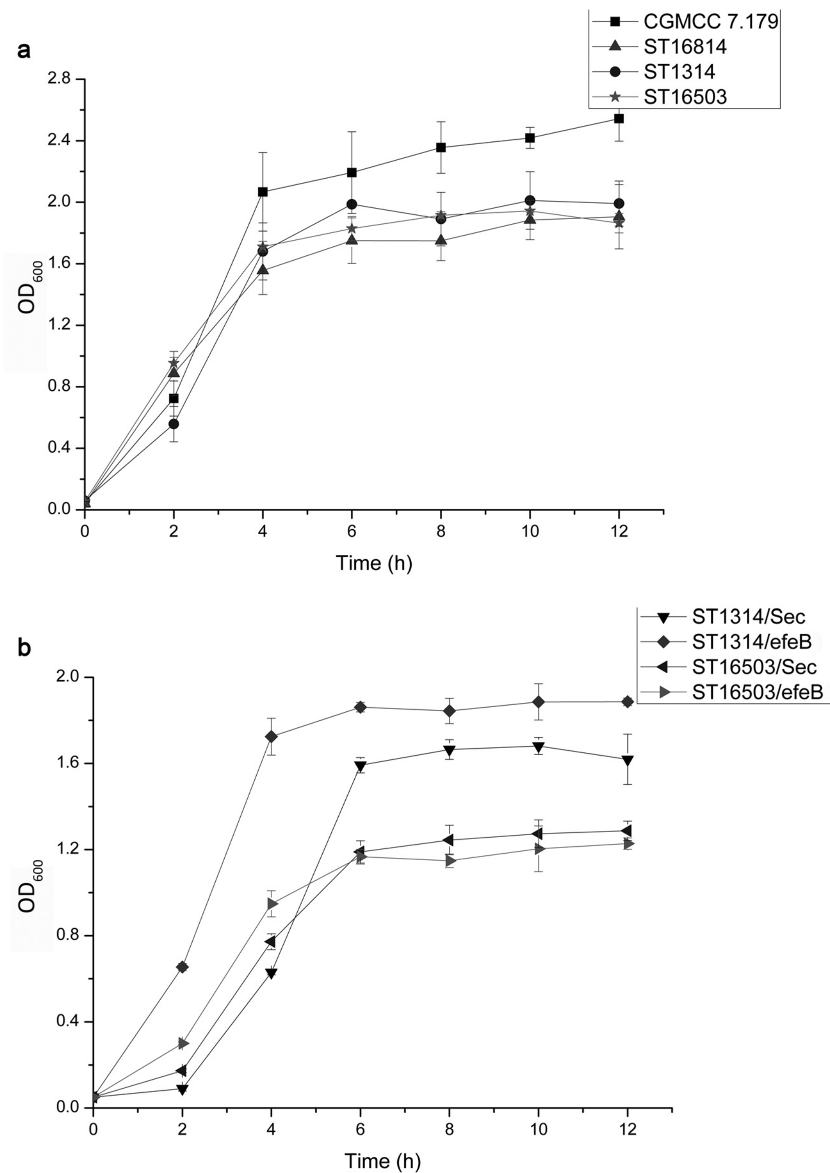

FIG 4 Function of the *tatC* gene. (a) Growth curves for *S. thermophilus* CGMCC 7.197 (■), ST16814 (▲), ST1314 (●), and ST16503 (⋆) under shaking conditions. (b) Growth curves for *S. thermophilus* ST1314/Sec (▼), ST1314/efeB (◆), ST16503/Sec (◀), and ST16503/efeB (▶) under shaking conditions.

Translocation analysis of the EfeB signal peptide, with CAT as a reporter protein. In *E. coli*, chloramphenicol acetyltransferase (CAT) is equipped with a typical TAT signal peptide to positively select for a loss of Tat function (35). In the present study, the SP sequence from the *efeB* gene was fused with the *CAT* gene. The difference in resistance to chloramphenicol between *S. thermophilus* CGMCC 7.179/SP-CAT and ST16814/SP-CAT was used to reflect the interaction between the TAT system and the signal peptide of EfeB. Strains CGMCC 7.179/SP-CAT and ST16814/SP-CAT were cultured to stationary phase in LM17 broth. To confirm that these two strains had no growth difference in the absence of chloramphenicol, 5-μl samples of 10^5-fold dilutions of their cultures were first pipetted onto LM17 plates. Subsequently, their sensitivity to chloramphenicol was measured by pipetting 5-μl samples of 10-fold dilutions on plates containing chloramphenicol at various concentrations (5, 8, 9, 10, 11, and 15 μg/ml). As shown in Fig. 5, the recombinant strains CGMCC 7.179/SP-CAT and ST16814/SP-CAT showed almost the same survival in the absence of chloramphenicol. With an increase of the chloramphenicol concentration, strain CGMCC 7.179/SP-CAT became more sensitive to chloramphenicol than strain ST16814/SP-CAT. When the concentration of chloramphenicol reached 9 μg/ml, the growth of CGMCC 7.179/SP-CAT was completely inhibited, while the lawn of ST16814/SP-CAT could easily be observed, suggesting that ST16814/SP-CAT displayed a stronger capability to tolerate chloramphenicol than that of CGMCC 7.179/SP-CAT. Therefore, the TAT system could translocate EfeB SP-fused CAT in CGMCC 7.179.

Translocation of EfeB by the TAT system *in vitro*. To further verify that EfeB was translocated by the Tat system, the subcellular location of EfeB was analyzed in the *tatABCDE*-deficient mutant *E. coli* dEAD/efeB and its *tatCA* complementation strain, dEAD/

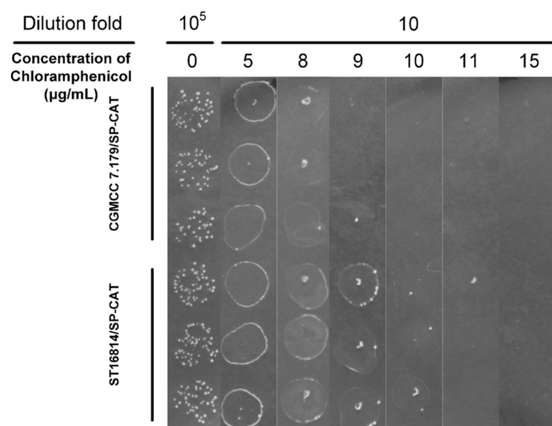

FIG 5 Chloramphenicol resistance of *S. thermophilus* CGMCC 7.197/SP-CAT and ST16814/SP-CAT. Samples of 10^5-fold dilutions were pipetted onto plates without chloramphenicol, and 10-fold dilutions were pipetted onto plates containing chloramphenicol at various concentrations (5, 8, 9, 10, 11, and 15 μg/ml). Each sample was used 3 times.

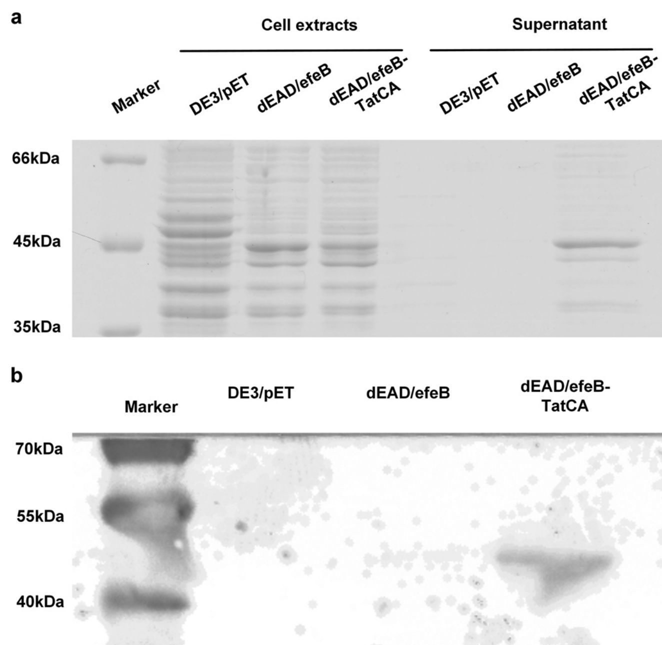

FIG 6 Coexpression of EfeB and TatCA in *E. coli*. (a) SDS-PAGE analysis of EfeB in cell extracts or supernatants of DE3/pET, dEAD/efeB, and dEAD/efeB-TatCA. (b) Western blot of EfeB in supernatants of DE3/pET, dEAD/efeB, and dEAD/efeB-TatCA.

efeB-TatCA. After induction by IPTG and culture for 14 h at 16°C, cell extracts and supernatants of *E. coli* dEAD/efeB and dEAD/efeB-TatCA cultures were separated by SDS-PAGE. The results showed a visible protein band with a molecular mass of 45 kDa for the cell extracts of dEAD/efeB and dEAD/efeB-TatCA as well as the supernatant of the dEAD/efeB-TatCA cultures, but not for the supernatant of the strain dEAD/efeB culture (Fig. 6a). Therefore, EfeB was successfully expressed in *E. coli* dEAD/efeB, but it was not translocated across the cytoplasmic membrane into the medium. When TatCA was expressed simultaneously in *E. coli* dEAD/efeB-TatCA, TatCA served as the translocon to drive EfeB across the cytoplasmic membrane. This result was also confirmed by Western blot analysis (Fig. 6b), in which a single corresponding band was visible only for the supernatants of *E. coli* dEAD/efeB-TatCA cultures. Together, the data showed that EfeB could be translocated by the *S. thermophilus* CGMCC 7.179 TAT system *in vitro*.

Transcriptional analysis of the *efeOBU-tatCA* operon. To investigate whether the *efeOBU-tatCA* operon was induced by O_2, the transcriptional levels of the *efeB* and *tatC* genes under anaerobic and aerobic conditions were quantified by real-time qPCR. Total RNA was extracted from cells whose OD_{600} had reached 1.0. As shown in Fig. 7, the transcriptional level of the *efeB* gene under anaerobic conditions was defined as 1, and it reached 3.11 ± 0.01 under aerobic conditions. Interestingly, corresponding with *efeB*, the same transcriptional pattern was observed for *tatC*, with levels of 1.06 ± 0.01 and 2.91 ± 0.03 under anaerobic and aerobic conditions, respectively. Therefore, it was concluded that transcription of the *efeB* and *tatC* genes was induced by aerobic conditions. These data also further strengthened our hypothesis that the *efeB* and *tatC* genes are organized in the same operon in *S. thermophilus*. Excitingly, this *efeOBU-tatCA* operon might be the first component found to be involved in the inducible defensive system against ROS damage of *S. thermophilus*.

DISCUSSION

S. thermophilus is one of the most widely used bacteria in the dairy industry. Owing to its extensive industrial application and survival challenges, many studies have been performed on the stress resistance (especially ROS resistance) of *S. thermophilus*. Although several ROS resistance enzymes are well characterized, very little information regarding the response to peroxide stress was revealed. In the present study, the *efeB* gene in the *S. thermophilus* CGMCC 7.179 genome was identified as a gene encoding a dye-decolorizing peroxidase. Functional analysis showed that EfeB can defend against oxidative toxicity. Strikingly, the protective role of EfeB depended on the genomically adjacent TAT system, as EfeB was a substrate of the TAT system.

In the genome of *S. thermophilus* CGMCC 7.179, the *efeB* gene was found to be located in a putative operon with four other genes: *efeU*, *efeO*, *tatC*, and *tatA*. Interestingly, the *efeO* gene is an allele of the *ossH* gene, which was found by insertional mutagenesis (3). Therefore, there was ample reason to believe that this operon was related to the capability of *S. thermophilus* CGMCC 7.179 to defend against oxidative stress. The amino acid sequence of EfeB was aligned with those of seven dye-decolorizing peroxidases. The heme-binding His^{313} residue (40) and the highly conserved G-X-X-D-G box (15) of dye-decolorizing peroxidases were found in EfeB. Specifically, EfeB showed a high level of similarity to the dye-decolorizing peroxidase FepB. Therefore, EfeB might be a member of the dye-decolorizing peroxidase family, by which *S. thermophilus* CGMCC 7.179 may eliminate H_2O_2.

To investigate the protective role of EfeB against oxidative stress, the *efeB*-deficient mutant ST1314 was generated by homologous double-crossover recombination. Strains CGMCC 7.179 and ST1314 displayed the same growth rate and OD_{600} under static conditions. However, the growth and maximum number of CFU of strain ST1314 were obviously lower than those of the wild-type strain CGMCC 7.179 when the strains were exposed to air. The distinct decrease in the number of living bacteria indicated that the *efeB* gene actually worked to defend against oxida-

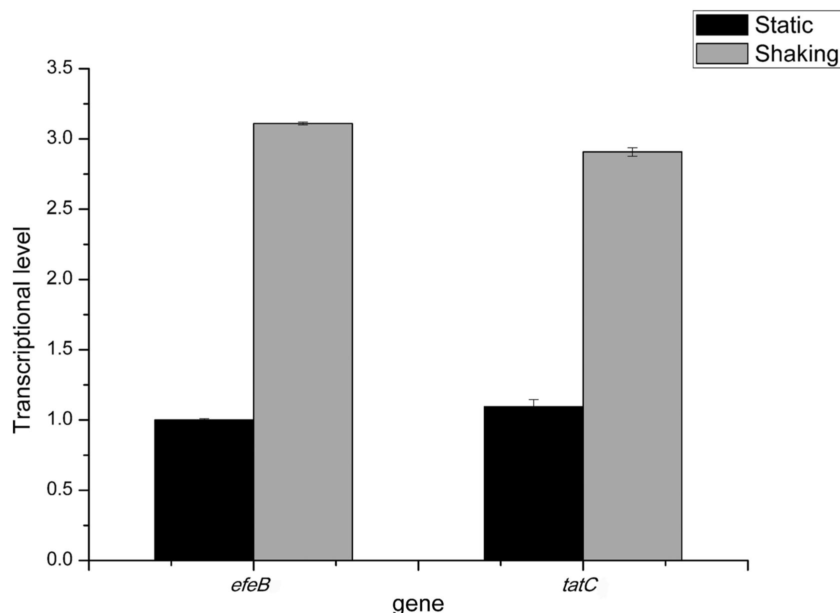

FIG 7 Transcriptional levels of *efeB* and *tatC* under static (black columns) and shaking (gray columns) conditions. The transcriptional level of the *efeB* gene under static conditions was defined as 1.

tive stress. Sequence alignment showed that EfeB contains a conserved His^{313} residue to link heme molecules, but *S. thermophilus* cannot synthesize heme; therefore, whether the peroxidase activation of EfeB depends on heme from the environment needs to be studied further.

Although *in silico* analysis of the *efeB* gene showed that it is a putative dye-decolorizing peroxidase, its function of eliminating H_2O_2 needed to be confirmed by biochemical testing. Reactive blue 5 is the most commonly used substrate to test the activity of dye-decolorizing peroxidases (33, 34, 41, 42). The EfeB protein purified from dEAD/efeB displayed a specific activity toward RB5 of 6.74 U/mg, which is 1/3 that of the DyP protein from *Geotrichum candidum* Dec 1 (34). Although the specific activity of EfeB was somewhat low, it still significantly indicated that EfeB is a member of the dye-decolorizing peroxidase family. However, the natural substrate of EfeB is unknown, and its specific activity may be higher with other substrates *in vivo*.

It has been reported that the bacterial dye-decolorizing peroxidases FepB, YcdB, and YwbN are secreted by the Tat pathway (16–18). In *S. thermophilus* CGMCC 7.179, genes encoding two components of the TAT system were located in the same operon as the *efeB* gene. Furthermore, a typical twin-arginine signal peptide exists at the N terminus of EfeB. Since TatC is essential in all TAT systems (19), the *tatC* gene was deleted to investigate the relationship between EfeB and the TAT system. The experimental data on the deletion of the *tatC* gene and the complementation of the *efeB* gene revealed the interdependence of EfeB and the TAT system in defending against oxidative stress.

However, direct evidence to verify the translocation of EfeB by the TAT system remained poor. We found that overexpression of EfeB in *S. thermophilus* CGMCC 7.179 is lethal (data not shown), so a method used for positive selection of Tat function in *E. coli* was performed to test the translocation of the EfeB signal peptide. CAT inactivates the antibiotic chloramphenicol by acetylation using acetyl-coenzyme A, which is present only in the cytoplasm (35). Thus, when CAT is fused to the SP of EfeB, cells that export the SP-CAT fusion protein should be more sensitive to chloramphenicol than cells that fail to export the fusion protein. The experimental results showed that ST16814/SP-CAT possessed a stronger capability to tolerate chloramphenicol than that of CGMCC 7.179/SP-CAT, suggesting that the Tat system of *S. thermophilus* CGMCC 7.179 can export small quantities of SP-CAT across the cytoplasmic membrane. However, the resistance difference was quite slight, which may have resulted from the expression of the TAT system being too low to produce enough transport capability.

The difference in chloramphenicol resistance between strains CGMCC 7.179/SP-CAT and ST16814/SP-CAT was so small that the deduction appeared not to be very persuasive. Considering that components from other bacterial TAT systems can work efficiently in *E. coli* (43, 44), EfeB and TatCA were coexpressed in *E. coli* dEAD. EfeB could be observed in the supernatant of dEAD/efeB-TatCA by SDS-PAGE, which suggested that a large amount of EfeB was exported. While EfeB was expressed in dEAD/efeB, no EfeB existed in the supernatant. Obviously, TatCA was able to export EfeB efficiently. Thus, it was credible that TatC protected the cells by translocating EfeB across the cytoplasmic membrane.

To our knowledge, none of the characterized antioxidant enzymes are involved in the inducible defensive system against ROS damage in *S. thermophilus*. To test whether the *efeOBU-tatCA* operon was inducible, the transcriptional levels of the *efeB* and *tatC* genes were quantified under anaerobic and aerobic conditions. Compared with the levels in cells cultured anaerobically, the *efeB* and *tatC* transcriptional levels were tripled after aerobic culture, suggesting that the *efeOBU-tatCA* operon could be involved in the inducible defensive system against ROS damage. The transcriptional levels of the *efeB* and *tatC* genes were almost the same under the same conditions, which further indicated that *efeB* and *tatC* are located in the same operon.

In conclusion, EfeB is the first peroxidase identified in *S. ther-*

mophilus, and it provides a novel defense against oxidative stress. This oxidative stress-inducible peroxidase could use an anthraquinone dye as an electron donor, which protected cells from the toxicity of both H_2O_2 and anthraquinone. Moreover, experimental evidence was provided to indicate that EfeB is translocated by the TAT system in *S. thermophilus*. The *S. thermophilus* TAT system identified in this study is unique among lactic acid bacteria (LAB), so it is a promising translocon for the construction of an LAB extracellular protein expression system for the translocation of proteins that are difficult to secrete.

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