RESEARCH ARTICLE

*Bacillus licheniformis* Contains Two More PerR-Like Proteins in Addition to PerR, Fur, and Zur Orthologues

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Abstract

The ferric uptake regulator (Fur) family proteins include sensors of Fe (Fur), Zn (Zur), and peroxide (PerR). Among Fur family proteins, Fur and Zur are ubiquitous in most prokaryotic organisms, whereas PerR exists mainly in Gram positive bacteria as a functional homologue of OxyR. Gram positive bacteria such as *Bacillus subtilis*, *Listeria monocytogenes* and *Staphylococcus aureus* encode three Fur family proteins: Fur, Zur, and PerR. In this study, we identified five Fur family proteins from *B. licheniformis*: two novel PerR-like proteins (BL00690 and BL00950) in addition to Fur (BL05249), Zur (BL03703), and PerR (BL00075) homologues. Our data indicate that all of the five *B. licheniformis* Fur homologues contain a structural Zn^{2+} site composed of four cysteine residues like many other Fur family proteins. Furthermore, we provide evidence that the PerR-like proteins (BL00690 and BL00950) as well as PerRBL (BL00075), but not FurBL (BL05249) and ZurBL (BL03703), can sense H_{2}O_{2} by histidine oxidation with different sensitivity. We also show that PerR2 (BL00690) has a PerR-like repressor activity for PerR-regulated genes *in vivo*. Taken together, our results suggest that *B. licheniformis* contains three PerR subfamily proteins which can sense H_{2}O_{2} by histidine oxidation not by cysteine oxidation, in addition to Fur and Zur.

Introduction

The ferric uptake regulator (Fur) protein is an iron-sensing transcriptional regulator which controls the expression of genes involved in intracellular iron homeostasis [1]. Under iron-replete conditions, Fur mediates the repression of genes involved in intracellular iron increase to prevent iron overload. Since its first recognition in *Escherichia coli*, Fur family proteins have been found and characterized in a variety of organisms ranging from bacteria to archaea [1, 2]. Fur family proteins are not only responsible for the acquisition and storage of iron, but also involved in the oxidative stress response as well as in the acquisition and storage of other metal ions. Now it is appreciated that there are various subgroups of Fur family proteins, which
include zinc uptake regulator (Zur), manganese uptake regulator (Mur), nickel uptake regulator (Nur), heme-dependent iron response regulator (Irr) and metal-dependent peroxide repressor (PerR), in addition to Fur itself [1, 2].

Fur family proteins are homo dimeric DNA binding proteins, and each monomer is composed of two domains, a winged-helix DNA-binding domain at the N-terminus and a dimerization domain at the C-terminus, connected by a loop. The available structures of Fur family proteins indicate that many, but not all, of these proteins have at least two metal binding sites in each monomer: a structural Zn2+-binding site and a regulatory metal-binding site [3–9]. The structural Zn2+ is usually coordinated by four Cys residues arranged in two CXXC motifs and stabilizes the dimerization domain. The regulatory metal binding site, located in the hinge region between the DNA binding domain and the dimerization domain, engages amino acids from both domains. In Fur and PerR proteins, the regulatory metal binding site is penta- or hexa-coordinated by three His residues and two Asp/Glu residues [4, 6, 8, 9], whereas this site is tetra-coordinated by two His residues, one Asp/Glu residue, and one Cys residue in Zur proteins [3, 5, 7].

While Fur and Zur are widely distributed in both Gram positive and Gram negative bacteria, PerR is mainly found in Gram positive bacteria as a functional homologue of OxyR [10]. PerR regulates genes involved in oxidative stress response like OxyR. However, unlike cysteine-thiol based peroxide sensor OxyR, PerR senses \( \text{H}_2\text{O}_2 \) by Fe-mediated histidine oxidation [11]. Reaction of \( \text{H}_2\text{O}_2 \) with \( \text{Fe}^{2+} \) leads to a rapid oxidation of either one of the two His residues involved in \( \text{Fe}^{2+} \)-coordination (His37 and His91 in \( \text{B. subtilis} \) PerR), resulting in the loss of repressor activity. However, Fur does not react with \( \text{H}_2\text{O}_2 \), despite the strong similarity of regulatory metal binding site [11, 12].

\( \text{B. licheniformis} \) is a Gram-positive, spore-forming soil bacterium which is closely related to the well-studied model organism \( \text{B. subtilis} \) [13]. \( \text{B. licheniformis} \) is an industrially important organism widely used for the manufacture of enzymes, peptide antibiotics and specialty chemicals. Despite the importance of stress physiology during the fermentation process, little is known about the physiology and stress response of \( \text{B. licheniformis} \) compared with its close relative \( \text{B. subtilis} \) [14].

Here we investigated the Fur family proteins from \( \text{B. licheniformis} \). \( \text{B. licheniformis} \) genome encodes five Fur family proteins, with three of them clustering in the PerR group. We have unequivocally identified BL00075, BL03703, and BL05249 as PerR, Zur, and Fur, respectively, using \( \text{B. subtilis} \) promoter fusion reporter systems. In addition, we provide several lines of evidence that two novel PerR-like proteins, BL00690 and BL00950, are also able to sense \( \text{H}_2\text{O}_2 \) by histidine oxidation, and that BL00690 has a transcriptional repressor activity for PerR-regulated genes.

**Materials and Methods**

**Bacterial strains and culture conditions**

The bacterial strains used in this study are described in Table 1. \( \text{E. coli} \) and \( \text{B. subtilis} \) strains were routinely grown in Luria-Bertani (LB) media at 37°C with appropriate antibiotics. MOPS buffered minimal medium was used for the metal-limited minimal media (MLMM) as described previously [15]. Ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) were used for the selection of \( \text{E. coli} \) strains. Spectinomycin (100 μg/ml), erythromycin (1 or 5 μg/ml), neomycin (25 μg/ml), kanamycin (20 μg/ml), chloramphenicol (10 μg/ml), tetracyclin (10 μg/ml), and lincomycin (8 μg/ml) were used for the selection of \( \text{B. subtilis} \) strains. For the induction of \( \text{xylA} \) promoter, 1% xylose (w/v) was used.
Table 1. Bacterial strains used in this study.

| Strains          | Relevant genotype or purpose | Reference or source |
|------------------|-----------------------------|---------------------|
| B. subtilis      |                             |                     |
| HB9700           | CU1065 zur::tet             | [26]                |
| HB9703           | CU1065 perR::tet            | [15]                |
| HBL100           | CU1065 fur::kan             | This study          |
| LB1066           | CU1065 fur::kan, zur::tet, perR::cat | This study |
| LB1532           | HB9703 amyE::spc, SPβΔ2::Tn917::Φ(mrgA-cat-lacZ) | This study |
| LB9738           | HB9703 amyE::perRBS-FLAG, SPβΔ2::Tn917::Φ(mrgA-cat-lacZ) | This study |
| LB1023           | HB9703 amyE::Δ::bi00075-FLAG, SPβΔ2::Tn917::Φ(mrgA-cat-lacZ) | This study |
| LB1034           | HB9700 amyE::spc, SPβΔ2::Tn917::Φ(yciC-cat-lacZ) | This study |
| LB1035           | HB9700 amyE::zurBS-FLAG, SPβΔ2::Tn917::Φ(yciC-cat-lacZ) | This study |
| LB1036           | HB9700 amyE::bl03703-FLAG, SPβΔ2::Tn917::Φ(yciC-cat-lacZ) | This study |
| LB1040           | HBL100 amyE::spc, SPβΔ2::Tn917::Φ(keuA-cat-lacZ) | This study |
| LB1041           | HBL100 amyE::furBS-FLAG, SPβΔ2::Tn917::Φ(keuA-cat-lacZ) | This study |
| LB1042           | HBL100 amyE::bl05249-FLAG, SPβΔ2::Tn917::Φ(keuA-cat-lacZ) | This study |
| LB1227           | LB1066 thrC::spc            | This study          |
| LB1287           | LB1066 thrC::P_perR::bi00950-FLAG | This study |
| LB1288           | LB1066 thrC::P_perR::bi00690-FLAG | This study |
| LB1490           | LB1066 thrC::P_perR::perRBS-FLAG | This study |
| LB1491           | LB1066 thrC::P_perR::furBS-FLAG | This study |
| LB1493           | LB1066 thrC::P_perR::zurBS-FLAG | This study |
| LB1233           | LB1227 SPβΔ2::Tn917::Φ(mrgA-cat-lacZ) | This study |
| LB1234           | LB1227 SPβΔ2::Tn917::Φ(keuA-cat-lacZ) | This study |
| LB1235           | LB1227 SPβΔ2::Tn917::Φ(yciC-cat-lacZ) | This study |
| LB1297           | LB1287 SPβΔ2::Tn917::Φ(mrgA-cat-lacZ) | This study |
| LB1288           | LB1287 SPβΔ2::Tn917::Φ(keuA-cat-lacZ) | This study |
| LB1299           | LB1287 SPβΔ2::Tn917::Φ(yciC-cat-lacZ) | This study |
| LB1300           | LB1288 SPβΔ2::Tn917::Φ(mrgA-cat-lacZ) | This study |
| LB1301           | LB1288 SPβΔ2::Tn917::Φ(keuA-cat-lacZ) | This study |
| LB1302           | LB1288 SPβΔ2::Tn917::Φ(yciC-cat-lacZ) | This study |
| LB4031           | LB1490 SPβΔ2::Tn917::Φ(mrgA-cat-lacZ) | This study |
| LB4065           | LB1491 SPβΔ2::Tn917::Φ(keuA-cat-lacZ) | This study |
| LB4066           | LB1493 SPβΔ2::Tn917::Φ(yciC-cat-lacZ) | This study |
| LB1010           | HB9703 amyE::spc            | This study          |
| LB2128           | HB9703 amyE::P_perR::NdeI-perRBS-FLAG | This study |
| LB4034           | HB9703 amyE::P_perR::NdeI-bi00690-FLAG | This study |
| LB4106           | HB9703 amyE::P_perR::NdeI-bi00950-FLAG | This study |
| E. coli          |                             |                     |
| LE0001           | BL21(DE3)pLysS pET-11a::bi00950 | This study |
| LE0002           | BL21(DE3)pLysS pET-16b::bi00690 | This study |
| LE0008           | BL21(DE3)pLysS pET-11a::bi00075 | This study |
| LE0009           | BL21(DE3)pLysS pET-11a::bi05249 | This study |
| LE0010           | BL21(DE3)pLysS pET-11a::bi03703 | This study |
| LE1374           | BL21(DE3)pLysS pET-15b::His6-bi00950 | This study |

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Construction of *E. coli* strains overexpressing Fur family proteins

The open reading frames (ORFs) of *bl05249*, *bl03703*, *bl00075*, *bl00950* and *bl00690* were PCR-amplified with *B. licheniformis* ATCC14580 chromosomal DNA as template. The PCR fragments of *bl05249*, *bl03703*, *bl00075*, and *bl00950* were individually cloned into the *Nde*I and *Bam*HI sites of expression vector pET-11a (Novagen) resulting in plasmids named pJL303, pJL304, pJL302, and pJL201, respectively. The PCR fragments of *bl00690* were cloned into the *Nco*I and *Bam*HI sites of expression vector pET-16b (Novagen) resulting in plasmid named pJL202. For the purification of N-terminally His-tagged BL00950, the PCR-fragments of *bl00950* were cloned into *Nde*I and *Bam*HI sites of pET-15H-oxyR [16] resulting in plasmids named pJL853. The plasmids were introduced into *E. coli* BL21 (DE3) pLysS cells for the over-expression of encoded proteins.

Each *E. coli* BL21 (DE3) pLysS strain carrying pJL303, pJL304, pJL302, pJL853, or pJL202 was grown in 1 L of LB medium containing 0.4% (w/v) glucose, chloramphenicol, and ampicillin. At OD<sub>600</sub> of ~0.4, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM (with additional final 50 μM ZnSO<sub>4</sub> for cells expressing BL03703), and the cells were allowed to grow for an additional 2 h. The cells were harvested by centrifugation, and lysed by sonication for protein purification. BL00075, BL05249, and BL03703 were purified by heparin-Sepharose and MonoQ chromatography using buffer A (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 5% glycerol (v/v)) containing 10 mM EDTA for BL00075 and BL05249, or 2 mM EDTA for BL03703 with the application of a linear gradient of 0.1–1 M NaCl as described previously [15]. BL00950 was purified by heparin-Sepharose and SP-Sepharose chromatography using buffer A containing 10 mM EDTA with the application of a linear gradient of 0.1–1 M NaCl. Since BL00950 did not bind to heparin-Sepharose resin unlike other Fur family proteins, we used His-tagged BL00950 for this study. His-tagged BL00950 was first purified by Ni-NTA chromatography, and subsequently by SP-Sepharose chromatography using buffer A containing 10 mM EDTA with the application of a linear gradient of 0.1–1 M NaCl. All the proteins were further purified using a Superdex 200 HiLoad 16/60 column (GE Healthcare) equilibrated with Chelex-100-treated buffer A. Note that BL00950 was purified as monomer whereas all the other proteins were purified as dimers as judged by elution profiles from Superdex 200 HiLoad gel filtration chromatography. The purities of all of the purified proteins were checked by SDS-PAGE, and their concentrations were determined by measuring A<sub>280</sub> nm using the calculated values of molar extinction coefficient of each protein (BL05249: 11,460 M<sup>-1</sup>cm<sup>-1</sup>, BL03703: 10,430 M<sup>-1</sup>cm<sup>-1</sup>, BL00075: 8,940 M<sup>-1</sup>cm<sup>-1</sup>, BL00690: 10,430 M<sup>-1</sup>cm<sup>-1</sup>, BL00950: 8,940 M<sup>-1</sup>cm<sup>-1</sup>.

**Electrophoretic mobility shift assay**

The 431 bp DNA fragment containing *B. subtilis* mrgA promoter region was generated by PCR, and subsequently digested with *Eco*RI, resulting in a 273 bp fragment containing PerR box and a 154 bp fragment used for a non-specific control. The DNA fragments were end labelled with [γ-<sup>32</sup>P] ATP using T4 polynucleotide kinase (NEB) and unincorporated labels were removed using nucleotide removal kit (Qiagen). Protein (BL00690 or BL00950) and a labelled probe were mixed in binding buffer (20 mM Tris-HCl pH 8.0, 50 mM KCl, and 5% glycerol (v/v), 50 μg/ml BSA and 100 μM MnCl<sub>2</sub>), and separated by 6% PAGE with a 45 mM Tris-borate buffer containing 100 μM MnCl<sub>2</sub>. After 2 h at 120 V, the gel was dried and exposed to X-ray film with an intensifying screen (Kodak) at -80°C.

**Measurement of Zn<sup>2+</sup> release by H<sub>2</sub>O<sub>2</sub> using PAR**

Measurement of Zn<sup>2+</sup> release by H<sub>2</sub>O<sub>2</sub> was performed as described previously [15, 17]. 5 μM protein in buffer A was treated with 0, 1, 10, or 100 mM H<sub>2</sub>O<sub>2</sub> in the presence of 100 μM 4-
(2-pyridylazo)resorcinol (PAR), and Zn²⁺-release was measured by monitoring the Zn²⁺-PAR complex at 494 nm every 1 s for 30 min. The Zn²⁺ content of purified proteins by PAR assay was determined using a molar extinction coefficient of 85,000 M⁻¹cm⁻¹ at 494 nm for Zn²⁺-PAR complex.

MALDI-TOF MS and LC-ESI MS/MS analysis

The analysis of protein oxidation after overexpression in *E. coli* was performed as previously described [17, 18]. Briefly, aliquots of *E. coli* cells (1.8 ml of culture of LE0001, LE0002, LE0008, LE0009, or LE0010) were either treated with 1 mM H₂O₂ (final concentrations) for 1 min or not. Cells harvested by centrifugation after the addition of 200 μM of trichloroacetic acid (TCA) were sonicated in 500 μl of 10% TCA. The pellets obtained by centrifugation were resuspended with 20 μl IA buffer (50 mM iodoacetamide, 0.5 M Tris pH 8.0, 5% glycerol, 100 mM NaCl, 1 mM EDTA, 2% SDS) and incubated for 1 h in the dark to alkylate free thiols. After separation on 13.3% Tris-Tricine SDS-PAGE and staining with Coomassie Brilliant Blue R-250, protein bands were cut and analyzed by MALDI-TOF MS using a Voyager-DE STR instrument (Applied Biosystems) after in-gel tryptic digestion. The sites of oxidation were identified by LC-MS/MS analyses using an Agilent nanoflow-1200 series HPLC system connected to a linear ion trap mass spectrometer (Thermo Scientific).

Construction of deletion mutant, complementation, and reporter fusion strains

The *B. subtilis fur* deletion mutant strain (HBL100) was constructed using long-flanking homology PCR as described previously [19]. The *fur zur* double mutant strain (HBL112) was generated by transformation of HBL100 with zur:tet cassette, and the *perR fur zur* triple mutant strain (LB1066) was generated by transformation of HBL112 with perR:cat cassette.

For the expression of FLAG fusion proteins from their own promoter in *B. subtilis*, the PCR fragments containing ORF and about 200 bp upstream region (bl00075, bl05249, bl03703, bl00690, bl00950, furBS, zurBS) were individually cloned into BamHI and EagI sites of pJL070. For the expression of FLAG fusion proteins from xylA promoter in *B. subtilis*, the pXT plasmid which can fuse a xylose-inducible promoter to the gene of interest was used. The PCR fragments containing ribosome binding sequence and *perR* ORF from pJL070 were cloned into BamHI and EcoRI sites of pXT, generating pJL240. Then, the PCR fragments containing consensus ribosome binding sequence and ORF (perRₐₕ, furₐₕ, zurₐₕ, bl00690, and bl00950) were each cloned into BamHI and EagI sites of pJL240. For the expression of PerRₐₕ-FLAG, BL00690-FLAG, and BL00950-FLAG from *B. subtilis perR* promoter in *B. subtilis* (for the construction of LB2128, LB4034, and LB4106 strains), NdeI site was introduced at the beginning of perR ORF in pJL070 by QuikChange site-directed mutagenesis (Stratagene) generating pJL448. Then, the PCR amplified bl00690 and bl00950 ORFs were each cloned into NdeI and EagI sites of pJL448. The ScaI digest of each plasmid was introduced to the corresponding *B. subtilis* strain to generate a transformant containing FLAG-fused gene in the *amyE* (pJL070-derived plasmids) or *thrC* (pJL240-derived plasmids) locus. The reporter fusion strains were constructed by transduction with SPβ phages, and β-galactosidase assays were performed, as described previously [15].

Results

Identification of five Fur family proteins in *B. licheniformis*

Many Gram positive bacteria such as *B. subtilis*, *L. monocytogenes* and *S. aureus* encode three Fur family proteins: Fur, Zur, and PerR [20–22]. Interestingly, the BLAST homology searches
of the *B. licheniformis* ATCC14580 genome sequence [13] with each one of the *B. subtilis* Fur family proteins revealed the presence of five putative genes encoding Fur family proteins. BL00075, BL03703, and BL05249 of *B. licheniformis* show the highest similarity to PerR<sub>BS</sub>, Zur<sub>BS</sub>, and Fur<sub>BS</sub> from *B. subtilis*, respectively, and all these proteins cluster with their homologues from *L. monocytogenes* and *S. aureus* as well as *B. subtilis* (Fig 1A). Although the sequence identity between BL00690 and BL00950 is not high (33%), both proteins cluster with PerR proteins with sequence identities ranging between 41 and 44% for BL00690 and between 41 and 46% for BL00950 (Fig 1B). In comparison, BL00690 and BL00950 exhibit sequence identities of ~25% to Fur and Zur proteins (Fig 1B), which are comparable to those between PerR and Fur or between PerR and Zur [1, 2, 22, 23].

As shown in Fig 1C, all the five Fur family proteins from *B. licheniformis* retain four highly conserved Cys residues corresponding to Cys96, Cys99, Cys136, and Cys139 of *B. subtilis* PerR. These four Cys residues arranged in two CXXC motifs are involved in high affinity structural Zn<sup>2+</sup>-binding in most Fur family proteins including PerR<sub>BS</sub> [9, 15]. In addition to this structural Zn<sup>2+</sup>-binding site, Fur family proteins also have a regulatory metal binding site. For PerR<sub>BS</sub>, this site is composed of His37, Asp85, His91, His93 and Asp104 [9, 11]. These five residues are conserved in BL00690 as well as PerR proteins including BL00075. Although Fur proteins and BL00950 also have conserved N-donor ligands (corresponding to His37, His91, and His93 of PerR<sub>BS</sub>), these proteins have a Glu residue in place of Asp104 (for Fur proteins) or Asp85 (for BL00950) as an O-donor ligand. Zur proteins are known to use S-donor ligand corresponding to Cys84 of Zur<sub>BS</sub> instead of O-donor ligand corresponding to Asp85 of PerR<sub>BS</sub> for regulatory Zn<sup>2+</sup>-binding, and do not have a conserved N-donor ligand corresponding to His37 of PerR<sub>BS</sub> [3, 5, 7]. Based on their repressor activities as described below as well as their sequence similarity and conserved amino acid residues involved in putative structural and regulatory metal binding, we functionally annotate BL00075, BL03703, and BL05249 as PerR<sub>BL</sub>, Zur<sub>BL</sub>, and Fur<sub>BL</sub>, respectively. And, the new Fur homologues, BL00690 and BL00950, were annotated as PerR2 and PerR3, respectively, based on their sequence similarity to the PerR proteins and their ability to sense peroxide by histidine oxidation as described below.

**All the five Fur family proteins from *B. licheniformis* contain structural Zn<sup>2+</sup>**

The sequence analysis indicates that all the Fur family proteins from *B. licheniformis* have conserved cysteine residues putatively involved in structural Zn<sup>2+</sup>-binding. To investigate the involvement of cysteine residues in Zn<sup>2+</sup> coordination, we purified all the five Fur family proteins after overexpression in *E. coli* (Fig 2A), and measured Zn<sup>2+</sup>-release from each protein upon H<sub>2</sub>O<sub>2</sub> treatment by monitoring the formation of PAR-Zn<sup>2+</sup> complex (Fig 2B) as described previously [15, 17]. Interestingly, unlike other Fur family proteins PerR3 did not bind to heparin-Sepharose (which is widely used for the purification of DNA-binding proteins). Furthermore, PerR3 was purified as monomeric protein by a gel filtration chromatography, whereas the other four Fur family proteins were purified as dimeric proteins (see Materials and Methods).

PAR-Zn<sup>2+</sup> complex formation was not detected for 30 min without H<sub>2</sub>O<sub>2</sub> treatment, and the rate of Zn<sup>2+</sup>-release was dependent on added H<sub>2</sub>O<sub>2</sub> concentrations (Fig 2B). These results indicate that all the Fur family proteins from *B. licheniformis*, including PerR3 purified as monomers, have stably bound Zn<sup>2+</sup> which cannot easily be removed by high affinity Zn<sup>2+</sup>-chelator PAR ([<sup>K<sub>app</sub></sup> ~ 10<sup>13</sup> M<sup>-1</sup> for PAR<sub>2</sub>-Zn<sup>2+</sup> complex, [24])] in the absence of H<sub>2</sub>O<sub>2</sub>. Furthermore, the dependence of Zn<sup>2+</sup>-release on H<sub>2</sub>O<sub>2</sub> strongly suggests that Zn<sup>2+</sup> is coordinated by conserved cysteine residues as observed with PerR proteins [15, 17]. The second-order rate
constants of Zn\(^{2+}\) release by H\(_2\)O\(_2\) were determined to be ~0.03 M\(^{-1}\)s\(^{-1}\) for PerR\(_2\), PerR\(_3\), and FurBL, ~0.04 M\(^{-1}\)s\(^{-1}\) for PerRBL, and ~0.01 M\(^{-1}\)s\(^{-1}\) for ZurBL. The slow rates of H\(_2\)O\(_2\)-mediated Zn\(^{2+}\) release for Fur family proteins from *B. licheniformis*, which are comparable to those observed with *B. subtilis* and *S. aureus* PerR proteins (~0.05 M\(^{-1}\)s\(^{-1}\))[15, 17], suggest that the Zn\(^{2+}\) sites play a structural rather than a H\(_2\)O\(_2\) sensing role. The Zn\(^{2+}\) contents of the purified proteins per monomer were determined to be ~0.8 for PerR\(_2\), ~0.9 for PerR\(_3\), ~0.5 for FurBL, ~0.7 for PerRBL, and ~0.5 for ZurBL. The retention of ~0.5–0.9 Zn\(^{2+}\) per monomer, despite the use of strong metal chelator EDTA during protein purification (see Materials and Methods), also supports the notion that all the Fur family proteins from *B. licheniformis* have a structural Zn\(^{2+}\) site. Altogether, these data indicate that all the five Fur family proteins from *B. licheniformis* contain a structural Zn\(^{2+}\) presumably coordinated by conserved cysteine residues like many other Fur proteins.

PerR\(_2\) (BL00690) and PerR\(_3\) (BL00950) as well as PerRBL can sense H\(_2\)O\(_2\) by protein oxidation

Previously we have shown that the oxidation of PerR proteins can be easily and efficiently evaluated using *E. coli* system [17, 18]. To investigate the oxidation of Fur family proteins from *B. licheniformis*, we analyzed protein oxidation by MALDI-TOF MS after overexpression in *E. coli* with or without H\(_2\)O\(_2\) treatment (Fig 3) as described previously [17, 18]. As noted for PerR\(_{BS}\), PerRBL showed H\(_2\)O\(_2\)-dependent oxidation at two tryptic peptides, T\(_5\) (His\(_{25}\) to Lys\(_{45}\), m/z = 2401.19) containing His\(_{37}\) and T\(_{11}\)/C\(_{3}\) (Phe\(_{84}\) to Arg\(_{98}\), m/z = 1910.85) containing His\(_{91}\) (Fig 3A). In contrast, FurBL and ZurBL displayed no detectable changes in tryptic peptide peaks after H\(_2\)O\(_2\) treatment (Fig 3D and 3E). Interestingly, PerR\(_2\) exhibited significant degree of oxidation at T\(_8\) peptide (Asn\(_{38}\) to Arg\(_{50}\), m/z = 1506.80) containing His\(_{39}\) (corresponding to His\(_{37}\) in PerR\(_{BS}\)) after H\(_2\)O\(_2\) treatment (Fig 3D and 3E). PerR\(_3\) also displayed H\(_2\)O\(_2\)-dependent oxidation, although less when compared with PerR\(_{BL}\) and PerR\(_2\), at T\(_7\) peptide (Thr\(_{27}\) to Arg\(_{42}\), m/z = 2170.99) containing His\(_{34}\) (corresponding to His\(_{37}\) in PerR\(_{BS}\)) (Fig 3C). As expected, the sites of oxidation responsible for the 16 Da mass increase were mapped to be His\(_{37}\) and His\(_{91}\) for PerRBL, His\(_{39}\) and His\(_{92}\) for PerR\(_2\), and His\(_{34}\) for PerR\(_3\) (S1–S5 Figs). The site of oxidation for T\(_{11}\)/C\(_{3}\) +16 from PerR\(_3\) could not be exactly mapped partially due to the weak signal intensity. The presence of significantly oxidized T\(_7\) peptide (T\(_7\)+16 from PerR\(_2\)) as compared to that (T\(_{5}\)+16 from PerR\(_{BL}\)) in the absence of H\(_2\)O\(_2\) treatment suggests that PerR\(_2\) is more sensitive than PerR\(_{BL}\) to oxidation by H\(_2\)O\(_2\) encountered during aerobic growth of *E. coli* [17]. In addition, no detectable oxidation without external H\(_2\)O\(_2\) treatment and the inefficient oxidation by H\(_2\)O\(_2\) treatment for PerR\(_3\) suggest that PerR\(_3\) is less sensitive to oxidation by H\(_2\)O\(_2\) than PerR\(_{BL}\) or PerR\(_2\).

All the peptides (T\(_{11}\)’ peptide of PerR\(_{BL}\), T\(_{13}\)’ peptide of PerR\(_2\), and T\(_{13}\)’ peptide of PerR\(_3\)) containing putative Zn\(^{2+}\)-binding motif CXXC motif (corresponding to C\(_{99}\)XXC\(_{99}\) in PerR\(_{BS}\)) were detected in their fully alkylated form (Fig 3, S2 and S4 Figs). Note that the
Fig 2. Zn²⁺ contents of Fur family proteins from *B. licheniformis*. (A) Purified Fur family proteins from *B. licheniformis*. *B. licheniformis* Fur-like proteins were purified after overexpression in *E. coli*, and analyzed by SDS-PAGE after alkylation by iodoacetamide. (B) H₂O₂-dependent Zn²⁺ release. Release of Zn²⁺ from proteins (5 μM) was measured by monitoring Zn²⁺-PAR complex at 494 nm every 1 sec for 30 min after treatment of 0, 1, 10, and 100 mM H₂O₂. Data for experiments with 100 mM H₂O₂ are only shown for clarity. The Zn²⁺ content of proteins was calculated using a molar extinction coefficient of 85,000 M⁻¹ cm⁻¹ at 494 nm for Zn²⁺-PAR complex.

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small amount of T13 peptide of PerR2, which is detected without alkylation even in the absence of H$_2$O$_2$ treatment, underwent no further oxidation after H$_2$O$_2$ treatment (Fig 3B). This observation that the cysteine residues are refractory to oxidation by H$_2$O$_2$ treatment is consistent with the idea that these cysteine residues are involved in structural Zn$^{2+}$-binding. All these data together suggest that PerR2 and PerR3 as well as PerRBL can sense H$_2$O$_2$ with differential sensitivity, by histidine oxidation but not by cysteine oxidation.

Functional annotation of PerRBL (BL00075) as PerR, FurBL (BL05249) as Fur, and ZurBL (BL03703) as Zur

The function of PerR, Zur, and Fur have intensively been studied both structurally and molecular genetically in *B. subtilis* [11, 25–28], a close relative of *B. licheniformis*. Thus, *B. subtilis* provides an excellent model system for the characterization of Fur family proteins from *B. licheniformis*. To investigate whether PerRBL (BL00075) functions as PerR, FurBL (BL05249) as Fur, and ZurBL (BL03703) as Zur, heterologous complementation studies were performed using *B. subtilis*. For this, PerRBL-FLAG, FurBL-FLAG, or ZurBL-FLAG was expressed from its own promoter (with ~200 nucleotide sequence upstream of ORF) in *B. subtilis* strain lacking a functional perR, zur, or fur gene, respectively (Fig 4). Since the FLAG epitope-tagged *B. subtilis* Fur family proteins are fully functional and the epitope tag provides a convenient means of monitoring protein levels *in vivo* [11, 17, 26, 29]. The repressor activity of PerRBL-FLAG was monitored using a *B. subtilis* mrgA promoter-lacZ reporter fusion (P$_{mrgA}$-lacZ) which is under the control of PerRBS. As reported previously [11], the P$_{mrgA}$-lacZ was repressed in cells expressing PerRBS-FLAG but derepressed in the perR null mutant cells. The P$_{mrgA}$-lacZ was also fully repressed by PerRBL-FLAG, and the repression was relieved upon H$_2$O$_2$ treatment as observed with PerRBS-FLAG (Fig 4A). FurBL showed a full repressor activity for FurBS-regulated feuA promoter-lacZ reporter fusion (P$_{feuA}$-lacZ) (Fig 4B). ZurBL-FLAG exhibited a full repressor activity for *B. subtilis* yciC promoter-lacZ reporter fusion (P$_{yciC}$-lacZ) which is under the control of ZurBS, despite the lower levels of expression when compared to ZurBS-FLAG (Fig 4C). We also examined the metal-dependent repressor activities of FurBL and ZurBL using a metal-limited minimal medium (MLMM). As expected, FurBL fully repressed the P$_{feuA}$-lacZ in the presence of Fe like FurBS and ZurBL fully repressed the P$_{yciC}$-lacZ in the presence of Zn like ZurBS (Fig 4G and 4H).

These results imply that PerRBL (BL00075), FurBL (BL05249), and ZurBL (BL03703) may function as PerR, Fur, and Zur, respectively, in *B. licheniformis*, and that each protein can be expressed from its own promoter located in ~200 nucleotide sequence upstream of each ORF.

PerR2 (BL00690), but not PerR3 (BL00950), has a PerR-like repressor activity

PerR2-FLAG and PerR3-FLAG could not be expressed with ~200 nucleotide sequence upstream of their ORFs, thus it is likely that the genes encoding these proteins do not have their own promoters. To express PerR2-FLAG and PerR3-FLAG and investigate the roles of...
In vivo repressor activities of PerR, Fur, and Zur

Fig 4. (A) Repressor activities of PerR and PerR for P_\text{mrgA}-\text{lacZ} reporter fusion. B. subtilis cells expressing no PerR orthologue (LB1532), PerR-FLAG (HB9738), or PerR-FLAG (LB1023) were treated without or with 100 μM H_2O_2 for 30 min, and β-galactosidase activities were measured using P_\text{mrgA}-\text{lacZ} reporter fusion. (B) Repressor activities of Fur and Fur for P_\text{PfeuA}-\text{lacZ} reporter fusion. B. subtilis cells expressing no Fur orthologue (LB1040), Fur-FLAG (LB1041), or Fur-FLAG (LB1042) were treated without or with 100 μM H_2O_2 for 30 min, and β-galactosidase activities were measured using P_\text{PfeuA}-\text{lacZ} reporter fusion. (C) Repressor activities of Zur and Zur for P_\text{PyciC}-\text{lacZ} reporter fusion. B. subtilis cells expressing no Zur orthologue (LB1034), Zur-FLAG (LB1035), or Zur-FLAG (LB1036) were treated without or with 100 μM H_2O_2 for 30 min, and β-galactosidase activities were measured using P_\text{PyciC}-\text{lacZ} reporter fusion. (D-F) Western blot analyses of FLAG-fused PerR orthologues (D), Fur orthologues (E), and Zur orthologues (F). The FLAG-fused proteins were probed by anti-FLAG antibody. (G) Dependent repressor activities of Fur and Fur for P_\text{PfeuA}-\text{lacZ} reporter fusion. B. subtilis cells expressing no Fur orthologue (LB1040), Fur-FLAG (LB1041), or Fur-FLAG (LB1042) were grown in MLMM supplemented with or without 10 μM FeSO_4, and β-galactosidase activities were measured using P_\text{PfeuA}-\text{lacZ} reporter fusion. (H) Zn-dependent repressor activities of Zur and Zur for P_\text{PyciC}-\text{lacZ} reporter fusion. B. subtilis cells expressing no Zur orthologue (LB1034), Zur-FLAG (LB1035), or Zur-FLAG (LB1036) were grown in MLMM supplemented with or without 10 μM ZnCl_2, and β-galactosidase activities were measured using P_\text{PyciC}-\text{lacZ} reporter fusion.

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these proteins in vivo, we used the pXT system which fuses a xylose-inducible promoter to the gene of interest, and a triple mutant B. subtilis strain which lacks all of the three fur family genes (Fig 5). Despite the use of same xylA promoter along with the consensus ribosome binding site, the expression levels of Fur family proteins were not identical possibly by differences in mRNA and/or protein stability (Fig 5A). However, as observed with single mutant background with their own promoters (Fig 4), PerR-FLAG, Fur-FLAG, and Zur-FLAG expressed from xylA promoter fully repressed the P_\text{PmrgA}-\text{lacZ}, P_\text{PfeuA}-\text{lacZ}, and P_\text{PyciC}-\text{lacZ}, respectively (Fig 5B). Although PerR-FLAG was highly expressed under the control of xylA promoter, PerR-FLAG showed no repressor activity for PerR-regulated reporter fusion as well as for Fur- and Zur-regulated reporter fusions. Interestingly, PerR-FLAG exhibited repressor activity for the PerR-regulated P_\text{PmrgA}-\text{lacZ}, but no repressor activity for the Fur-regulated P_\text{PfeuA}-\text{lacZ} nor the Zur-regulated P_\text{PyciC}-\text{lacZ}. This specific repressor activity of PerR for the known PerR-regulated promoters, along with its H_2O_2-dependent histidine oxidation, suggest that PerR may act as a second PerR in B. licheniformis.

It is known that a B. subtilis perR null mutant strain grows very poorly in nonstressed conditions due to Fe deficiency resulting from elevated levels of FurR and KdA [19]. To examine whether PerR can complement the perR null mutant strain and rescue the small colony phenotype, complementation experiments were performed (Fig 5C). As expected, the perR null mutant strain expressing PerR-FLAG showed a wild-type like colony phenotype. The perR null mutant strain expressing PerR also exhibited significantly increased colony size, indicating that PerR can rescue the Fe-deficiency presumably by reducing the levels of KdA and/or FurR. In contrast, the perR null mutant strain expressing PerR still exhibited the small colony phenotype (Fig 5C) consistent with the lack of repressor activity for the PerR-regulated gene (Fig 5B).

To investigate the interaction of PerR with DNA, we performed electrophoretic mobility shift assays using the B. subtilis mrgA promoter regions as probe. As shown in Fig 5D, PerR specifically shifted the DNA fragment containing PerR box but not the DNA fragment lacking PerR box. This result indicates that the repressor activity of PerR observed with the PerR-regulated promoter fusion is due to direct interaction of PerR with PerR box. However, it should be noted that the apparent K_d value of PerR for DNA binding was measured to be ~ 70 nM. This rather weak DNA binding activity of PerR, as compared to that of PerR (K_d ~ 10 nM) [29], is likely to reflect the higher oxidation (inactivation) levels of PerR as shown in Fig 3B. In contrast, consistent with the lack of repressor activity for the PerR regulated promoter fusion, PerR showed no DNA binding activity (Fig 5D).
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A

![Marker blank PerR PerR PerR PerR PerR Fur PerZ Fur](image)

B

![Bar chart for P_{mrqA-lacZ} P_{feuA-lacZ} P_{ycic-lacZ}](image)

C

![Images showing ΔperR + blank ΔperR + PerR ΔperR + PerR ΔperR + PerR](image)

D

![Graphs showing PerR3 (nM) PerR2 (nM)](image)
Discussions

Proteins with Fur-like domain architecture are widespread in prokaryotes with ~20,000 homologues in EMBL-EBI InterPro database (IPR002481). Depending on signals they respond, Fur family proteins are classified as Fur (Fe), Zur (Zn), Mur (Mn), Nur (Ni), PerR (peroxide), and Irr (heme) [1, 2]. Among these, Fur is the most ubiquitous, and Zur, albeit not as ubiquitous as Fur, is also widespread in Gram negative and Gram positive bacteria. In contrast, PerR is mainly found in Gram positive bacteria as a functional substitute for OxyR, although it is also found in some Gram negative bacteria, and, in some cases, coexists with OxyR [1, 10]. Mur and Irr have been found in some α-proteobacteria including Rhizobiales and Rhodobacterales [30], and Nur has been only found in Streptomyces genus [31]. Although four Fur paralogues (Fur orthologue FurA, PerR orthologue CatR, Zur, and Nur) have been found and characterized in S. coelicolor [3, 31–33], many bacteria contains up to three Fur family proteins, usually two or three. For example, Gram negative bacteria E. coli and V. cholerae contain two (Fur and Zur) [4, 7], and Gram positive bacteria B. subtilis and S. aureus contain three (Fur, Zur, and PerR) [20, 22]. In this study, we found that B. licheniformis, a close relative of B. subtilis, contains five Fur family proteins. Like many other Fur family proteins, all these proteins retain a tightly bound Zn\(^{2+}\) presumably coordinated by highly conserved cysteine residues. Three of them were identified as Fur, Zur, and PerR orthologues of B. subtilis based on their repressor activity. The other two were identified as PerR-like proteins based on their sequence similarity to PerR proteins and their \(\text{H}_2\text{O}_2\)-dependent oxidation of histidine residues.

The \(\text{H}_2\text{O}_2\)-sensing mechanism of PerR has only been extensively studied in B. subtilis and S. aureus, despite its wide distribution in most Gram positive bacteria and in some Gram negative bacteria [10, 11, 17, 25]. Unlike OxyR which utilizes the oxidation of cysteine thiol, PerR uses a distinct Fe-dependent histidine oxidation mechanism for \(\text{H}_2\text{O}_2\) sensing, where \(\text{H}_2\text{O}_2\) oxidizes the histidine ligands of the Fe\(^{2+}\) at the regulatory site to 2-oxo-histidine. Our results indicate that PerR\(_{BL}\) also uses a histidine oxidation mechanism for \(\text{H}_2\text{O}_2\) sensing. Furthermore, we found that \(\text{H}_2\text{O}_2\) can also oxidize the two other PerR-like proteins, PerR2 and PerR3, but not Fur\(_{BL}\) and Zur\(_{BL}\). MALDI-TOF MS and ESI-MS/MS analyses of the tryptic peptides, along with sequence analyses, of PerR2 and PerR3 indicate that the oxidation events occur at histidine residues rather than cysteine residues. Despite the high similarity between the regulatory metal binding sites of PerR and Fur, PerR does not react with \(\text{H}_2\text{O}_2\) under conditions where PerR does [11]. Recently, it has been suggested that O-donor ligand corresponding to Asp104 of PerR\(_{BS}\) or Glu108 of Fur\(_{BS}\) is the key residue which determines the accessibility of \(\text{H}_2\text{O}_2\) to Fe\(^{2+}\)-coordination site [12]. It is noteworthy that PerR2 and PerR3, as well as other PerR proteins, also contain a conserved Asp at this position, whereas Fur proteins have a Glu (Fig 1C).

Despite the presence of bona fide PerR\(_{BL}\), PerR2 also showed specific repressor activity on the representative PerR-regulated gene but not on Fur- or Zur-regulated gene, and the perR null mutant small colony phenotype could be rescued by PerR2 (Fig 5). Thus, it is reasonable to speculate that PerR regulon in B. licheniformis is under the control of both PerR\(_{BL}\) and...
PerR2. In the simplest scenario, the two proteins would exert influence on the PerR regulon genes simultaneously. Alternatively, each protein may regulate genes under different conditions. The higher sensitivity of PerR2 than PerRBL seems to suggest the differential role of these proteins under different oxidation conditions.

Unlike PerR2, no repressor activity of PerR3 was observed for any genes under the control of PerR, Fur, and Zur using *B. subtilis* reporter fusion assays. And, PerR3 was purified as monomer after overexpression in *E. coli*, whereas all the other four Fur family proteins from *B. licheniformis* were purified as dimer. Considering that all the biochemically characterized Fur family proteins are dimeric DNA binding proteins, PerR3 may not be a canonical Fur family protein. However, the oxidation of PerR3 by H$_2$O$_2$, albeit less sensitive as compared to PerRBL or PerR2, suggests that PerR3 may play a role as a H$_2$O$_2$ sensor in *B. licheniformis*. It has been previously reported that the transcription of perR3 (bl00950, bli04114) is massively induced after H$_2$O$_2$ treatment [34]. Interestingly, the genes encoding for PerR3 and BL00949 (Bli04115, putative ferrochelatase) are located directly downstream of katA gene (bl00951, bli04113). Furthermore, in contrast to *B. subtilis* katA gene which is monocistronically transcribed under the control of PerRBS, *B. licheniformis* katA gene is cotranscribed with perR3 and bl00949 after H$_2$O$_2$ treatment [34]. These imply that PerR3 may have some role especially under conditions of H$_2$O$_2$ stress.

In summary, we have shown that *B. licheniformis* contains a total of five Fur family proteins: two novel PerR-like proteins in addition to the canonical PerR, Fur, and Zur. The presence of two additional Fur family proteins in *B. licheniformis*, in contrast to its close relative *B. subtilis*, may indicate that the metal ion regulation and peroxide stress response under the control of Fur family proteins are far more complex than previously reported for *B. subtilis*. Further study is required to identify distinct roles of PerR2 and PerR3 along with their relevance to other Fur family proteins in *B. licheniformis*.

### Supporting Information

**S1 Fig. Identification of His37 as an oxidation site in T5 peptide (His25 to Lys45) from PerRBL by ESI MS/MS analysis.** (A) Predicted m/z values of b- and y-ions of unmodified T5 peptide of PerRBL. His37 is shown in red. (B) Tandem MS spectrum of T5 peptide. Triple charged precursor ion ([T5+3H]$^{3+}$ = 801.85, shown in green) was analyzed by tandem MS. The b- and y-ions are shown in purple and blue, respectively. (C) Tandem MS spectrum of T5+16 peptide. Triple charged precursor ion ([T5+16+3H]$^{3+}$ = 806.82, shown in green) was analyzed by tandem MS. The b- and y-ions are shown in purple and blue, respectively. The y-ions not containing His37 (y4-y8) appear at the predicted m/z values, whereas the subsequent y-ions containing His37 (y9-y20) have a +16 Da mass shift. Note that almost all the y9- and y10-ions (containing His37 but not Met35) have a +16 Da mass shift. The b-ions not containing His37 (b5-b12) appear at the predicted m/z values, whereas the subsequent b-ions containing His37 (b13-b20) have a +16 Da mass shift. Note that almost all the b10- and b11-ions (containing Met35 but not His37) appear at the predicted m/z values. Taken together, these data indicate that most of the oxidation in T5+16 peptide occurred at His37 rather than Met35.

(TIF)

**S2 Fig. Identification of His91 as an oxidation site in T11$^+$/C3 peptide (Phe84 to Arg98) from PerRBL by ESI MS/MS analysis.** (A) Predicted m/z values of b- and y-ions of unmodified T11$^+$ peptide (containing carboxymidomethylated Cys96 residue) of PerRBL. His91 is shown in red. (B) Tandem MS spectrum of T11$^+$ peptide. Double charged precursor ion ([T11$^++2H$]$^{2+}$ = 956.06, shown in green) was analyzed by tandem MS. The b- and y-ions are shown in purple and blue, respectively. (C) Tandem MS spectrum of T11$^+$+16 peptide. Double charged precursor ion ([T11$^++16+2H$]$^{2+}$ = 964.42, shown in green) was analyzed by tandem MS. The b- and y-ions
are shown in purple and blue, respectively. The y-ions not containing His91 (y3-y7) appear at
the predicted m/z values, whereas the subsequent y-ions containing His91 (y8-y14) have a +16
Da mass shift. The b-ions not containing His91 (b3-b7) appear at the predicted m/z values,
whereas the subsequent b-ions containing His91 (b8-b14) have a +16 Da mass shift. These
data indicate that the oxidation in T11+16 peptide occurred at His91.

S3 Fig. Identification of His39 as an oxidation site in T8 peptide (Asn38 to Arg50) from
PerR2 by ESI MS/MS analysis. (A) Predicted m/z values of b- and y-ions of T8 peptide of
PerR2. His39 is shown in red. (B) Tandem MS spectrum of T8 peptide. Triple charged precur-
sor ion ([T8+3H]3+ = 503.20, shown in green) was analyzed by tandem MS. The b- and y-ions
are shown in purple and blue, respectively. (C) Tandem MS spectrum of T8+16 peptide. Triple
charged precursor ion ([T8+16+3H]3+ = 509.16, shown in green) was analyzed by tandem MS.
The b- and y-ions are shown in purple and blue, respectively. The y-ions not containing His39
(y1-y11) appear at the predicted m/z values, whereas the subsequent y-ions containing His39
(y12-y13) have a +16 Da mass shift. The b-ions containing His39 (b2-b13) have a +16 Da mass
shift. These data indicate that the oxidation in T8+16 peptide occurred at His39.

S4 Fig. Identification of His92 as an oxidation site in T13+ peptide (Phe85 to Lys102) from
PerR2 by ESI MS/MS analysis. (A) Predicted m/z values of b- and y-ions of T13+ peptide
(containing carboxyamidomethylated Cys97 and Cys100 residues) of PerR2. His92 is shown in
red. (B) Tandem MS spectrum of T13+ peptide. Triple charged precursor ion ([T13+3H]3+ =
724.93, shown in green) was analyzed by tandem MS. The b- and y-ions are shown in purple
and blue, respectively. (C) Tandem MS spectrum of T13+16 peptide. Triple charged precursor
ion ([T13+16+3H]3+ = 730.05, shown in green) was analyzed by tandem MS. The b- and y-
ions are shown in purple and blue, respectively. The y-ions not containing His92 (y3-y10)
appear at the predicted m/z values, whereas the subsequent y-ions containing His92 (y11-y17)
has a +16 Da mass shift. The b-ions not containing His92 (b3-b7) appear at the predicted m/z
values, whereas the subsequent b-ions containing His92 (b8-b16) has a +16 Da mass shift.
These data indicate that the oxidation in T8+16 peptide occurred at His92.

S5 Fig. Identification of His34 as an oxidation site in T7 peptide (Thr27 to Arg42) from
PerR3 by ESI MS/MS analysis. (A) Predicted m/z values of b- and y-ions of T7 peptide of
PerR3. His34 is shown in red. (B) Tandem MS spectrum of T7 peptide. Double charged precur-
sor ion ([T7+2H]2+ = 831.40, shown in green) was analyzed by tandem MS. The b- and y-ions
are shown in purple and blue, respectively. (C) Tandem MS spectrum of T7+16 peptide. Double
charged precursor ion ([T7+16+2H]2+ = 839.29, shown in green) was analyzed by tandem
MS. The b- and y-ions are shown in purple and blue, respectively. The y-ions not containing
His34 (y3-y8) appear at the predicted m/z values, whereas the subsequent y-ions containing
His34 (y9-y15) has a +16 Da mass shift. The b-ions not containing His34 (b5-b7) appear at the
predicted m/z values, whereas the subsequent b-ions containing His34 (b8-b16) has a +16 Da
mass shift. These data indicate that the oxidation in T7+16 peptide occurred at His34.

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Author Contributions
Conceived and designed the experiments: JHK CJJ JWL. Performed the experiments: JHK CJJ SYJ YMY SHR YK YBW YEL HY JWL. Analyzed the data: JHK CJJ SYJ YMY SHR YK YBW YEL HY JWL. Wrote the paper: JHK CJJ HY JWL.

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