Lactic acid bacteria have a variety of mechanisms for tolerance to oxygen and reactive oxygen species, and these mechanisms differ among species. Lactobacillus casei strain Shirota grows well under aerobic conditions, indicating that the various systems involved in oxidative stress resistance function in this strain. To elucidate the mechanism of oxidative stress resistance in L. casei strain Shirota, we examined the transcriptome response to oxygen or hydrogen peroxide exposure. We then focused on an uncharacterized gene that was found to be up-regulated by both oxygen and hydrogen peroxide stress; we named the gene hprA1 (hydrogen peroxide resistance gene). This gene is widely distributed among lactobacilli. We investigated the involvement of this gene in oxidative stress resistance, as well as the mechanism of tolerance to hydrogen peroxide. Growth of L. casei MS105, an hprA1-disrupted mutant, was not affected by oxygen stress, whereas the survival rate of MS105 after hydrogen peroxide treatment was markedly reduced compared to that of the wild-type. However, the activity of MS105 in eliminating hydrogen peroxide was similar to that of the wild-type. We cloned hprA1 from L. casei Shirota and purified recombinant HprA1 protein from Escherichia coli. We demonstrated that the recombinant HprA1 protein bound to iron and prevented the formation of a hydroxyl radical in vitro. Thus, HprA1 protein probably contributes to hydrogen peroxide tolerance in L. casei strain Shirota by binding to iron in the cells and preventing the formation of a hydroxyl radical.

**INTRODUCTION**

Lactic acid bacteria, which are facultative anaerobes, can grow under aerobic conditions. Many studies have focused on the mechanisms of oxidative stress resistance in lactic acid bacteria, and several enzymes associated with oxidation reactions, including NADH oxidase (Higuchi et al., 1993, 1999), NADH peroxidase (Miyoshi et al., 2003), superoxide dismutase (Chang & Hassan, 1997; Sanders et al., 1995) and manganese catalase (Kono & Fridovich, 1983), are known to be directly involved in this resistance. The actions of superoxide dismutase and manganese catalase have been determined as being specific to particular species or strains of lactic acid bacteria.

Complete genome sequencing and comparative genome analyses have been performed on some Lactobacillus casei/Lactobacillus paracasei group strains (Ai et al., 2011; Makarova et al., 2007; Matsumoto et al., 2012; Matsumoto et al., 2013; Makarova et al., 2006; Mazé et al., 2010; Wang et al., 2014; Zhang et al., 2010), revealing that several strains have a number of oxidative stress resistance genes in common. Annotation information on these oxidative stress resistance genes can be found in the GenBank/EMBL/DDBJ database. However, because we have only a limited number of biochemical reports, the functions of these genes among the L. casei/L. paracasei group of bacteria are still undefined.

L. casei strain Shirota (YIT 9029), one of the most intensively studied probiotics (Asahara et al., 2001; Kaga et al., 2004; Shida et al., 2006), can grow well under aerobic conditions, where it exhibits good viability. This implies that several systems involved in oxidative stress resistance function in this strain. In an analysis of the redox system in L. casei strain Shirota, we have demonstrated that the thioredoxin–thioredoxin reductase system is essential for aerobic growth of this strain (Serata et al., 2012).

To elucidate the other systems involved in oxidative stress resistance, we performed a micro-array analysis treated with oxygen or hydrogen peroxide. We observed the up-regulation of several genes, one of which was the hprA1 gene.
regulation of several genes under both oxygen and hydrogen peroxide stress conditions. Among these up-regulated genes, we focused on an uncharacterized gene, which, because of its involvement in hydrogen peroxide resistance (hpr), we named hprA1. We verified the involvement of this gene in oxidative stress resistance and investigated the mechanism of tolerance to hydrogen peroxide.

**METHODS**

**Strains, plasmid, media and growth conditions.** The strains and plasmids used are listed in Table 1. *L. casei* strain Shirota (YIT 9029) was used as the wild-type. *Escherichia coli* JM109 was purchased from Toyobo as competent cells for DNA transformation. *E. coli* BL21 (DE3) was purchased from Merck. Plasmid pYSSE3 (Yasuda et al., 2008) was used as a cloning vector for insertion mutagenesis, and pET-28b (+) (Merck) was used as an expression vector for recombinant protein in *E. coli*. Lactic acid bacteria were grown at 37°C in MRS medium (Becton Dickinson). *E. coli* was grown at 37°C in LB medium (Sambrook & Russell, 2001). Erythromycin (Abbott Japan) was added to a final concentration of 20 μg ml⁻¹ for lactic acid bacteria and 500 μg ml⁻¹ for *E. coli* if needed. Chloramphenicol (Sigma-Aldrich) was added to a final concentration of 5 μg ml⁻¹ for lactic acid bacteria and 10 μg ml⁻¹ for *E. coli* if needed. The turbidity of the cultures was measured with a Klett–Summerson photometric colorimeter (Klett MFG) or a DU 800 spectrophotometer (Beckman Coulter). The culture conditions used for anaerobic, aerobic and static aerobic conditions have been described previously (Serata et al., 2012). Briefly, to create anaerobic conditions, oxygen in the test tube was replaced with nitrogen gas. To generate aerobic conditions, cells were cultured in either thick test tubes (10 ml medium in 250 mm test tube) or flasks (30 ml medium in 300 ml flask) capped with a breathable silicone plug and vigorously shaken at 160 r.p.m. For pre-culture or exposure to hydrogen peroxide, we used static aerobic conditions (culture without shaking).

**Construction of insertion mutants.** Insertion mutants were constructed as reported previously (Serata et al., 2012). The primer pairs used to amplify the truncated fragment of the target genes are listed in Table S1 (available in the online Supplementary Material). Erythromycin-resistant clones were selected, and plasmid integration was confirmed by using PCR with the appropriate primers (Table S1). One primer was designed upstream of the multi-cloning site of pYSSE3, and the other was downstream of the target gene on the chromosome.

**Complementation of gene disruption mutants.** The complementation vector pYAP310 was constructed using pYAP300 (Yasuda et al., 2008), which was able to integrate into the *L. casei* chromosome at the attB site for phage phiSW and to express a cloned gene in *L. casei*. The chloramphenicol acetyltransferase gene fragment was amplified with a pair of primers (Table S1) and pC194 (Horinouchi & Weisblum, 1982) as a template. This fragment was digested by BgII and FspI and cloned into pYAP300, which was pre-digested by the same restriction enzymes. For complementation of MS105, the wild-type hprA1 gene was amplified with appropriate primers (Table S1) and cloned into pYAP310, and pYAP310–hprA1 was introduced into MS105 in a similar manner as for gene disruption. Chloramphenicol-resistant clones were then selected.

**RNA isolation for microarray and quantitative PCR analyses.** RNA was isolated as described previously (Serata et al., 2012). An oxidative stress response was induced by cell exposure to oxygen or hydrogen peroxide. Exposure to oxygen was performed as follows. A single 100 ml culture of *L. casei* strain Shirota, which was inoculated at 1% (v/v) with an overnight culture, was grown in MRS for 7 h under anaerobic conditions and then divided into two 50 ml aliquots. One aliquot was grown for 30 min under anaerobic conditions as a control, and the other was grown for 30 min under aerobic conditions with vigorous shaking at 160 r.p.m. in a water bath shaker. Exposure to hydrogen peroxide was performed as follows. A single 100 ml culture of *L. casei* strain Shirota, which was inoculated at 1% (v/v) with an overnight culture, was grown in MRS for 7 h under static aerobic conditions and then divided into two 50 ml aliquots. One aliquot was grown for 30 min under static aerobic conditions as a control; 0.5 mM hydrogen peroxide was added to the other aliquot, and this culture was also grown for 30 min under static aerobic conditions. Two millilitres of each culture were added to 4 ml of RNase protect Bacteria Reagent (QIAGEN). The mixture was then incubated at room temperature for 5 min. Cells were harvested by centrifugation at 10 min at 5000 g. The cells were suspended in 200 μl of TE buffer [10 mM Tris/HCl and 1 mM EDTA (pH 8.0)] containing 15 mg ml⁻¹ lysozyme (Sigma-Aldrich) and 0.05 mg ml⁻¹ N-acetyl-muramidase SG (Seikagaku) and incubated at room temperature for 10 min with occasional vortexing. Then, total RNA was purified using an RNasey Mini Kit (QIAGEN) in accordance with the protocol provided by the supplier. Contaminating DNA was digested using an RNase-Free DNase Set (QIAGEN) in the purification step. RNA integrity for all samples was confirmed with an Agilent 2100 Bioanalyzer (Agilent Technologies).

**Micro-array analysis.** A 60-mer oligonucleotide spotted microarray based on the genome sequence of *L. casei* strain Shirota was designed, produced and validated by Takara Bio. RNAs were labelled with Cy3-dUTP or Cy5-dUTP using a CyScribe First-Strand cDNA Labelling Kit (GE Healthcare). The labelled cDNAs were hybridized and the microarrays were washed in accordance with GE Healthcare protocol. Microarrays were scanned with a DNA microarray scanner G2505B (Agilent). The acquired images were analysed by DNASIS Array (Hitachi Software Engineering). Experiments were done in two technical replications by switching the dyes. The fold-change values are expressed as a mean of two replicates. Genes with fold-change values >2 or <0.5 in both replicates were listed as up- or down-regulated, respectively (Tables S2 to S5).

**Quantitative PCR assay.** cDNA was synthesized using a PrimeScript First-Strand cDNA Synthesis Kit (Takara Bio) in accordance with the protocol provided by the supplier. Total RNA (1 μg) was used as a template. Quantitative real-time PCR was performed with an ABI 7500 Real-Time PCR System (Life Technologies) with SYBR Premix Ex Taq (Takara Bio). The primers are listed in Table S1. The reaction mixture contained 12.5 μl of 2 × SYBR Premix Ex Taq, 0.5 μl of 10 μM forward primer, 0.5 μl of 10 μM reverse primer, 0.5 μl of ROX reference Dye II, 9 μl of dH₂O and 2 μl of diluted cDNA template. Three independent samples were run in duplicate. Gene expression values were normalized against those of the 16S rRNA gene as an internal standard. A melting curve analysis was performed after each run to confirm the amplification specificity. Data were analysed using Sequence Detection Software (Life Technologies) and the standard-curve quantification method. Standard curves for both internal standard and target genes were generated by amplifying 10-fold serial dilutions of cDNA. Gene expression data from quantitative real-time PCR were analyzed using a two-tailed Student’s t-test. P<0.05 was considered significant.

**Database search.** To search for the sequence identities of HprA1 to other proteins, we performed a homology search with blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To predict the physiological function of HprA1, we used an InterProScan search (http://www.ebi.ac.uk/Tools/ pfam/scan/).

**Phylogenetic analysis.** CLUSTALW was used to align protein sequences. A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987).
Survival after hydrogen peroxide treatment. Overnight cultures were harvested, washed with 50 mM potassium phosphate buffer (pH 6.8) and suspended in a 10-fold volume of the same solution. After the addition of hydrogen peroxide at 0.5 mM, cells were incubated at 37°C for 3 h. The numbers of viable cells at 0 and 3 h were counted by plating dilutions onto MRS agar, and survival rates were then calculated.

Measurement of hydrogen peroxide concentration. Overnight cultures were inoculated at 1% (v/v) into 4 ml of MRS medium. Strains were then grown at 37°C under static aerobic conditions. Hydrogen peroxide at a concentration of 0.5 mM was added to the cultures after 7 h incubation. Strains were then grown further for 1 h. Cells were harvested from 1 ml cell cultures of OD600 cultures after 7 h incubation. Strains were then grown at 37°C for 3 h. The numbers of viable cells at 0 and 3 h were counted by plating dilutions onto MRS agar, and survival rates were then calculated.

Cloning of hprA1 and HprA1 protein preparation. The hprA1 locus was amplified from L. casei strain Shirota genomic DNA by PCR, using KOD Plus DNA polymerase (Toyobo). Primers were designed to contain the Ndel site or the EcoRI site (Table S1). The fragment of PCR product was digested with Ndel/EcoRI and cloned into pET-28b(+), which had previously been digested with the same restriction enzymes. This plasmid [pET-28b(+)-hprA1] was amplified in E. coli JM109 and purified using a Wizard Plus SV Minipreps DNA purification system (Promega) and transferred into E. coli BL21(DE3).

His-tagged HprA1 was overproduced in the E. coli cells in accordance with directions in the pET system manual (Merck), with some modifications. E. coli BL21(DE3) harbouring pET-28b(+)-hprA1 was grown at 37°C with shaking at 160 r.p.m. in LB medium containing 30 µg ml−1 kanamycin (Sigma-Aldrich) until OD600=0.6. Then, 3 ml of the culture was inoculated into 200 ml of fresh medium containing 30 µg ml−1 kanamycin. After incubation of the culture at 37°C (with shaking at 160 r.p.m.) to OD600=0.6, IPTG (1 mM) was added. Incubation was then continued for 3 h to induce the production of HprA1 protein. The E. coli cells were harvested and resuspended in 10 ml of BugBuster reagent (Merck). Cell lysis was prepared in accordance with the protocol for the BugBuster reagent. His-tagged HprA1 protein was purified with an Ni-NTA Fast Start Kit (QIAGEN). The protein concentration was determined with a Bio-Rad Protein assay (Bio-Rad). The purity of purified HprA1 protein was confirmed to be greater than 95% by SDS-PAGE with NuPAGE 4–12 % Bis-Tris Gels (Life Technologies) (Fig. S1).

Iron-binding analysis. Apo-HprA1 was prepared by incubating HprA1 (200 µM) with 10 mM EDTA and 2 mM diethytothreitol at 37°C for 60 min; the samples were then passed through a PD-10 column (GE Healthcare). Apo-HprA1 (50 µM) was incubated with Fe(NH4)2(SO4)2 (10, 100 or 1000 µM) in the presence of 2 mM diethytothreitol at room temperature for 5 min. The samples were purified by passage through the PD-10 column. The amounts of iron in the protein samples were measured by ICP-OES with Vista-PRO (Agilent Technologies).

Iron-dependent hydroxyl radical formation analysis. Iron-dependent hydroxyl radical formation was analysed using the methods of Hallwell & Gutteridge (1981) and Yamamoto et al. (2002). The hydroxyl...
radical was generated non-enzymatically by iron salt in solution. The hydroxyl radical degrades deoxyribose to a malondialdehyde-like compound, which forms a chromogen with thiobarbituric acid. Fluorescence of the chromogen was used as an indicator of the amount of hydroxyl radical formed.

A basal reaction mixture containing 10 mM potassium phosphate buffer (pH 7.4), 63 mM NaCl and 4 mM deoxyribose was prepared. Appropriate amounts of apo-HprA1, BSA (Sigma-Aldrich), ferritin (apoferritin from equine spleen; Sigma-Aldrich) and deferoxamine (Sigma-Aldrich), as well as ferrous ammonium sulfate at a final concentration of 10 µM, were added to 0.3 ml of the basal reaction mixture. The reaction mixture (total volume 2 ml) was incubated at 37°C for 15 min. After incubation, 0.25 ml of 1% (w/v) thiobarbituric acid and 0.25 ml of 2.8% (w/v) trichloroacetic acid were added to the reaction mixture. After the mixture had been boiled for 10 min and rapidly cooled on ice, fluorescence of the chromogen formed in the sample was measured with a fluorescence spectrophotometer (RF-5300PC; Shimadzu Corporation) (λex=532 nm, λem=553 nm).

RESULTS

Transcriptome response of *L. casei* strain Shirota to oxygen or hydrogen peroxide exposure

On microarray analysis, 11 genes were up-regulated and one was down-regulated in response to oxygen stress (Fig. 1a, Tables S2 and S4). Among the up-regulated genes, that encoding NADH oxidase was the most strongly up-regulated, followed by the genes encoding NADH peroxidase and hypothetical proteins. Fifty-seven genes were up-regulated and 59 were down-regulated in response to hydrogen peroxide stress (Fig. 1a, Tables S3 and S5). Among the up-regulated genes were those encoding l-lactate oxidase, heat shock proteins, NADH peroxidase, hypothetical proteins and cysteine biosynthesis proteins such as CysE and CysK.

Expression of five genes, encoding NADH peroxidase (*npx*), protein-methionine-S-oxide reductase (*msrB*), heat shock protein (*hsp*) and two hypothetical proteins, was induced under both oxygen and hydrogen peroxide stress (Fig. 1a). We considered that these genes play an important role in oxidative stress resistance.

To confirm the up-regulation of these five genes, we conducted quantitative real-time PCR analysis. The expression of four genes (*npx*, *msrB*, CDS1839 and CDS2657) was significantly increased by both oxygen and hydrogen peroxide stress (Fig. 1b). CDS2657 expression was especially dramatically induced (44-fold) by hydrogen peroxide stress. We named this gene *hprA1*. In contrast, although *hsp* expression was significantly up-regulated by hydrogen peroxide stress, it was not significantly up-regulated by oxygen stress. Therefore, we decided to exclude *hsp* from the subsequent analysis.

Involvement of up-regulated genes in oxygen and hydrogen peroxide stress

To elucidate the involvement of the four up-regulated genes (*npx*, *msrB*, CDS1839 and *hprA1*) in resistance to oxidative stress, we constructed four gene disruption mutants and investigated the effects of oxygen or hydrogen peroxide stress on the growth of these mutants. Growth of none of the mutants was altered under anaerobic conditions.

---

Fig. 1. Genes up-regulated under oxidative stress. (a) Venn diagram representing the number of genes that were up-regulated by oxygen and hydrogen peroxide stress on microarray analysis. (b) Quantitative real-time PCR analysis to confirm five genes up-regulated by both stressors. *npx*, NADH peroxidase (CDS0427); *msrB*, protein-methionine-S-oxide reductase (CDS1501); *hsp*, heat shock protein (CDS2656); *hprA1*, hypothetical protein (CDS2657). Black bars represent expression under exposure to oxygen relative to that under anaerobic conditions, and grey bars represent expression under exposure to hydrogen peroxide relative to that under static aerobic conditions. Results are shown as fold changes compared to each control condition. Data are means of three independent experiments. Error bars indicate standard deviations. Significant differences compared to unstressed control are indicated by an asterisk (P<0.05).
conditions (Fig. 2b). In contrast, the growth of MS102 (npx mutant) was impaired under aerobic conditions compared with that of the wild-type (Fig. 2a). We also measured the survival rates after hydrogen peroxide treatment. As in the wild-type, the numbers of viable cells of MS102, MS103 (msrB mutant) and MS104 (CDS1839 mutant) were slightly decreased after treatment with 0.5 mM of hydrogen peroxide (Fig. 3). In contrast, MS105 (hprA1 mutant) was more sensitive than the wild-type to hydrogen peroxide. Furthermore, the survival rate of MS124, which was complemented by hprA1, was fully recovered to the wild-type level. These results showed that hprA1 was involved in hydrogen peroxide stress resistance. Therefore, we then focused on hprA1 in our further experiments to reveal the function of this gene.

**hprA1 is not involved in eliminating hydrogen peroxide**

Whereas the wild-type decomposed half of the added hydrogen peroxide, MS102 was unable to decompose the hydrogen peroxide at all. MS105 was able to decompose hydrogen peroxide almost as well as the wild-type could (Fig. 4). These results indicated that NADH peroxidase, at least, was involved in the decomposition of hydrogen peroxide, but that HprA1 was not.

![Fig. 2. Growth of *L. casei* strain Shirota and gene disruption mutants under (a) aerobic and (b) anaerobic conditions. Overnight MRS cultures were inoculated into fresh MRS cultures. •, wild-type strain; ▲, MS102 (npx mutant); ■, MS103 (msrB mutant); ◆, MS104 (CDS1839 mutant); and ×, MS105 (hprA1 mutant). Data are representative of two independent experiments.](http://mic.microbiologyresearch.org)

![Fig. 3. Survival of *L. casei* strain Shirota and mutants upon addition of hydrogen peroxide. Hydrogen peroxide (0.5 mM) and cells were incubated in potassium phosphate buffer (pH 6.8) at 37 °C for 3 h. The numbers of viable cells were counted at 0 and 3 h, and survival rates were measured. WT, wild-type; MS102, npx mutant; MS103, msrB mutant; MS104, CDS 1839 mutant; MS105, hprA1 mutant; MS124, complement of hprA1 mutant. Data are means of triplicates. Error bars indicate standard deviations.](http://mic.microbiologyresearch.org)
Bioinformatics analysis of the hprA1 gene

We used blastp to search for sequence identities of HprA1 to other proteins. HprA1 was widely distributed among lactobacilli and was highly conserved in strains of *L. casei*. Bacteria such as *Enterococcus* and *Pediococcus* were found to have hprA1 homologues, but other bacteria did not possess these homologues. Lactobacilli had two copies of an HprA1 homologous protein. From the results of phylogenetic tree analysis, these were categorized into two different large clusters (Fig. S2). Among *L. casei* strain Shirota, CDS1839 had a high level of identity to HprA1. Table 2 compares the amino acid sequence of *L. casei* strain Shirota HprA1 to those of other representative lactic acid bacteria. The similar proteins for which we obtained hits in the database analysis were mostly 'hypothetical proteins'. To predict the physiological function of HprA1, we used an InterProScan search (http://www.ebi.ac.uk/Tools/pfa/interproscan/). We found that HprA1 was a member of the Fe–S cluster biogenesis family (IPR000361), which includes IscA and HesB.

**HprA1 binds to iron**

MS105 was able to decompose hydrogen peroxide, as described above, suggesting that MS105 is more sensitive than the wild-type to hydrogen peroxide. We assumed that hprA1 was involved in preventing the toxic effects of the hydroxyl radical formed by the Fenton reaction. We initially examined the iron-binding activity of apo-HprA1 protein expressed by, and purified from, an *E. coli* recombinant strain. We used BSA as a negative control and ferritin (apo-ferritin from equine spleen) as a positive control. BSA did not bind to the iron. In contrast, ferritin bound to the iron less than expected. HprA1 bound about two iron atoms per molecule upon incubation with 100 µM iron (Table 3). Samples that turned red-brown upon incubation with 1000 µM Fe(NO₃)₂·(SO₄)₂ were excluded from the analysis. The spectrum of iron-loaded HprA1 had a major peak at a wavelength of 280 nm and a minor peak at 315 nm. The absorbance of these peaks increased in proportion to the concentration of iron.

![Fig. 4. Decomposition of hydrogen peroxide by *L. casei* strain Shirota and gene disruption mutants. Hydrogen peroxide (100 µM) and cells were incubated at 37 °C for 1 h and the concentrations of hydrogen peroxide remaining were then measured. WT, wild-type; MS102, npx mutant; MS105, hprA1 mutant. Data are means of three independent experiments. Error bars indicate standard deviations.](image-url)

**Table 2.** Comparison of the amino acid sequence of *L. casei* strain Shirota HprA1 to those of other lactic acid bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Accession no.</th>
<th>Product</th>
<th>Score</th>
<th>Coverage (%)</th>
<th>E value</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. casei</em> strain Shirota</td>
<td>(HprA1)</td>
<td>Hydrogen peroxide-resistant protein</td>
<td>270</td>
<td>100</td>
<td>4e–97</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>(CDS1839)</td>
<td>Hypothetical protein</td>
<td>85.5</td>
<td>75</td>
<td>7e–25</td>
<td>42.6</td>
</tr>
<tr>
<td><em>L. casei</em> BL23</td>
<td>YP_001988915</td>
<td>Hypothetical protein</td>
<td>270</td>
<td>100</td>
<td>4e–97</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>YP_001988045</td>
<td>Hypothetical protein</td>
<td>85.5</td>
<td>75</td>
<td>7e–25</td>
<td>42.6</td>
</tr>
<tr>
<td><em>L. casei</em> ATCC334</td>
<td>YP_807957</td>
<td>Hypothetical protein</td>
<td>270</td>
<td>100</td>
<td>4e–97</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>YP_807099</td>
<td>Hypothetical protein</td>
<td>82.8</td>
<td>75</td>
<td>6e–24</td>
<td>41.6</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em></td>
<td>GG</td>
<td>Hypothetical protein</td>
<td>224</td>
<td>100</td>
<td>4e–79</td>
<td>80.6</td>
</tr>
<tr>
<td></td>
<td>YP_003172551</td>
<td>Hypothetical protein</td>
<td>85.5</td>
<td>73</td>
<td>6e–25</td>
<td>43.4</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em></td>
<td>WCFS1</td>
<td>Hypothetical protein</td>
<td>122</td>
<td>100</td>
<td>5e–39</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>NP_785627</td>
<td>Hypothetical protein</td>
<td>71.6</td>
<td>71</td>
<td>9e–20</td>
<td>34.7</td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em></td>
<td>ATCC367</td>
<td>Hypothetical protein</td>
<td>119</td>
<td>100</td>
<td>4e–38</td>
<td>47.0</td>
</tr>
<tr>
<td></td>
<td>YP_795855</td>
<td>Hypothetical protein</td>
<td>82.8</td>
<td>84</td>
<td>6e–24</td>
<td>38.9</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em></td>
<td>NCFM</td>
<td>Hypothetical protein</td>
<td>102</td>
<td>87</td>
<td>2e–31</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>YP_193032</td>
<td>Hypothetical protein</td>
<td>79.3</td>
<td>86</td>
<td>1e–22</td>
<td>37.4</td>
</tr>
<tr>
<td><em>Lactobacillus johnsonii</em></td>
<td>NCC533</td>
<td>Hypothetical protein</td>
<td>94.4</td>
<td>100</td>
<td>3e–28</td>
<td>37.3</td>
</tr>
<tr>
<td></td>
<td>NP_964095</td>
<td>Hypothetical protein</td>
<td>79</td>
<td>87</td>
<td>2e–22</td>
<td>36.1</td>
</tr>
<tr>
<td><em>Pediococcus pentosaceus</em></td>
<td>ATCC 25745</td>
<td>Hypothetical protein</td>
<td>121</td>
<td>100</td>
<td>8e–39</td>
<td>50.7</td>
</tr>
<tr>
<td></td>
<td>ABJ67421</td>
<td>Hypothetical protein</td>
<td>73.9</td>
<td>84</td>
<td>9e–21</td>
<td>34.2</td>
</tr>
</tbody>
</table>
iron concentration (Fig. 5). These results suggested that purified HprA1 bound to iron.

**HprA1 protein prevents hydroxyl radical formation**

Because the purified apo-HprA1 protein bound to iron, we examined the suppressive effect of apo-HprA1 protein on hydroxyl radical formation. We measured the amount of hydroxyl radical formed by each sample and present the results as percentage inhibition. We used BSA as a negative control and ferritin or deferoxamine (an iron chelator) as a positive control. Deferoxamine inhibited hydroxyl radical formation in proportion to its concentration; it suppressed 81.2% at 100 µM. In contrast, ferritin was unable to inhibit hydroxyl radical formation under our experimental conditions. Purified apo-HprA1 protein was able to prevent hydroxyl radical formation in proportion to its concentration (Table 4).

**DISCUSSION**

To search for the genes involved in oxidative stress resistance in _L. casei_ strain Shirota, we performed a micro-array analysis under oxygen stress or hydrogen peroxide stress conditions. The number of genes up-regulated under hydrogen peroxide stress was greater than that up-regulated under oxygen stress. The genes up-regulated included oxidative stress resistance genes such as NADH oxidase (CDS0252), NADH peroxidase (CDS0427), molecular chaperone HSP18 (CDS0686, CDS2656), GroEL (CDS2119) and protein-methionine-S-oxide reductase (CDS1501). Activity of the thioredoxin–thioredoxin reductase system is induced by aerobic conditions or hydrogen peroxide (Reott _et al._, 2009; Rocha _et al._, 2007). However, only one of four thioredoxins (CDS0780; _trxA1_) was slightly up-regulated by hydrogen peroxide stress; the other thioredoxin and thioredoxin reductases were not differentially expressed under either oxygen or hydrogen peroxide stress. These results are similar to those of our previous report (Serata _et al._, 2012), suggesting that constitutive expression of the thioredoxin–thioredoxin reductase system plays an important role in oxygen and hydrogen peroxide tolerance in _L. casei_. It was not clear how the genes that were down-regulated were involved in hydrogen peroxide resistance. We hypothesized that those genes that were not essential for escape from hydrogen peroxide stress were down-regulated.

Five genes were up-regulated under both oxygen and hydrogen peroxide stress. From the patterns of gene expression (Fig. 1), culture growth (Fig. 2) and hydrogen peroxide sensitivity (Fig. 3), we concluded that one gene was directly involved in detoxification of hydrogen peroxide. This gene is widely distributed in lactobacilli but its function is still unknown. We named this gene _hprA1_.

It is well known that hydrogen peroxide is decomposed by NADH peroxidase and that some _L. plantarum_ strains produce Mn calatase (Kono & Fridovich, 1983), which also acts to decompose hydrogen peroxide. In this study, the hydrogen peroxide-decomposing activity of the _hprA1_ mutant (MS105) was almost equal to that of the wild-type, whereas the hydrogen peroxide-decomposing activity of the _npx_ mutant (MS102) was lost. Nevertheless, there still appeared to be a difference between MS105 and the wild-type. We believe that this slight difference occurred in the mechanisms of hydrogen peroxide resistance.

<table>
<thead>
<tr>
<th>Protein conc. (µM)</th>
<th>Iron conc. (µM)</th>
<th>Iron conc. of iron-loaded protein solution (mg kg⁻¹)</th>
<th>Iron atoms per molecule or subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>HprA1</td>
<td>50</td>
<td>0.29</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1.70</td>
<td>1.83</td>
</tr>
<tr>
<td>BSA</td>
<td>50</td>
<td>&lt;0.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>&lt;0.1</td>
<td>–</td>
</tr>
<tr>
<td>Ferritin (subunit)</td>
<td>50</td>
<td>&lt;0.1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.27</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Table 3. Iron-binding capacity of HprA1

Data are means of two independent experiments.
because of the difference in the numbers of viable cells between the two. First, pre-treatment was a necessary step because the hydrogen peroxide-decomposing activity of L. casei strain Shirota could not be detected without pre-treatment with hydrogen peroxide. However, this pre-treatment likely decreased the viability of the hprA1 mutant (Fig. 3), suggesting that this process killed some of the mutant cells. In contrast, most of the wild-type cells were viable (Fig. 3). In the experiment, cell quantities were made uniform by using turbidity, not the number of viable cells. Dead cells were thus likely included in the mutant line before the experimental hydrogen peroxide treatment, and we believe that this led to a slight, but unimportant, decrease in hydrogen peroxide-decomposing activity.

To predict the physiological function of HprA1, we used an InterProScan search (http://www.ebi.ac.uk/Tools/pfa/iprscan/). We found that HprA1 was a member of the Fe–S cluster biogenesis family (IPR000361), which includes IscA and HesB. In cyanobacteria, hesB, the function of which is not fully understood, is expressed only under nitrogen fixation conditions (Huang et al., 1999). iscA belongs to the isc gene cluster, which is involved in the assembly of Fe–S clusters (Nakamura et al., 1999; Takahashi & Nakamura, 1999). Although the physiological function of IscA remains elusive, it has been reported to have iron-binding activity (Ding et al., 2004; Wang et al., 2010). Ferrous iron reacts with hydrogen peroxide, resulting in the generation of an extremely toxic hydroxyl radical by the Fenton reaction. The iron-binding protein, Dpr, which is found in Streptococcus mutans, prevents iron-dependent hydroxyl radical formation in vitro (Yamamoto et al., 2002). These facts led us to hypothesize that the function of hprA1 is to bind ferrous iron, thereby preventing iron-dependent hydroxyl radical formation.

Our results showed that HprA1 binds about two iron atoms per molecule during incubation in 100 µM iron solution (Table 3), and it prevents hydroxyl radical formation in proportion to its concentration (Table 4). The iron-storage protein, ferritin, did not have high iron-binding activity or hydroxyl radical formation-preventing activity (Tables 3 and 4). It has previously been reported that ferritin barely inhibits hydroxyl radical formation (Yamamoto et al., 2002). In a study by Yamamoto et al. (2002), the efficiency of inhibition of hydroxyl radical formation at 1000 nM ferritin was only 12.3 %. Because of possible competition between non-enzymatic oxidation by molecular oxygen and iron chelation by ferritin, the ability of ferritin to incorporate iron may be insufficient to inhibit iron oxidation. Furthermore, human ferritin expressed in yeast contains little iron, and the presence of an iron chaperone increases the amount of iron loaded into ferritin (Shi et al., 2008). For these reasons, we considered it likely that, in our study, ferritin without an iron chaperone was unlikely to have high iron-binding activity.

Iron–sulfur proteins are widely distributed, from bacteria to mammals, and are involved mainly in electron transport. The details of iron–sulfur cluster biogenesis in lactic acid bacteria are still largely unknown. HprA1 may not be involved in iron–sulfur cluster biogenesis because growth of the hprA1 disruption mutant was not affected by oxygen stress. However, we need to study this issue further.

CDS1839, which was up-regulated by both oxygen and hydrogen peroxide stress, had homology with HprA1 (Table 2). However, we could not characterize CDS1839 protein because the recombinant protein was not sufficiently expressed in E. coli cells. Although the properties of MS104 were not altered under oxidative stress (Figs 2 and 3), functional analysis of CDS1839 protein will be required in the future.

In conclusion, our results suggest that HprA1 protein contributes to resistance to the effects of hydrogen peroxide in L. caseiL. paracasei group bacteria by binding to iron in the cell, thus preventing the formation of a hydroxyl radical. hprA1 is commonly distributed in lactobacilli. Here, we added hydrogen peroxide artificially. However, under natural aerobic conditions, hydrogen peroxide forms in the course of oxygen metabolism. A hydroxyl radical then forms from a reaction between hydrogen peroxide and trace iron in the cell. In lactobacilli, which grow well under aerobic conditions, hprA1 expression may be a reasonable and effective mechanism for avoiding high levels of toxicity of the hydroxyl radical.

**Table 4. Effect of HprA1 on hydroxyl radical formation**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. (µM)</th>
<th>Extent of hydroxyl radical formation (arbitrary units)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
<td>310.2±3.7</td>
<td>&lt;0</td>
</tr>
<tr>
<td>HprA1</td>
<td>0.1</td>
<td>329.2±0.4</td>
<td>&lt;0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>246.2±16.2</td>
<td>20.6±5.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>178.8±2.2</td>
<td>42.4±0.7</td>
</tr>
<tr>
<td>BSA</td>
<td>1</td>
<td>326.1±4.9</td>
<td>&lt;0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>863.9±22.2</td>
<td>&lt;0</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.1</td>
<td>321.5±1.6</td>
<td>&lt;0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>306.3±11.1</td>
<td>1.3±3.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>387.8±9.2</td>
<td>&lt;0</td>
</tr>
<tr>
<td>Deferoxamine</td>
<td>1</td>
<td>309.4±7.4</td>
<td>0.3±2.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>237.5±4.4</td>
<td>23.4±1.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>58.3±2.6</td>
<td>81.2±0.8</td>
</tr>
</tbody>
</table>

Data are means±standard deviations of triplicates.
ACKNOWLEDGEMENTS

We thank Tomoyuki Sako of Yakult Europe for his helpful discussions and suggestions. We also thank Kosuke Kondo of Yakult Central Institute for his help with the ICP-OES measurement.

REFERENCES


Edited by: P. W. O’Toole, K. P. Scott and J. Kok