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Full Paper

NADH peroxidase plays a crucial role in consuming H_2O_2 in *Lactobacillus casei* IGM394

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The facultative anaerobic bacterium *Lactobacillus casei* IGM394 is used as a host for drug delivery systems, and it exhibits the same growth rate under aerobic and anaerobic conditions. *L. casei* strains carry several genes that facilitate oxygen and reactive oxygen species (ROS) tolerance in their genomes, but their complete functions have not been uncovered. To clarify the oxygen and ROS tolerance mechanisms of *L. casei* IGM394, we constructed 23 deficient mutants targeting genes that confer oxidative stress resistance. Significantly decreased growth and high H_2O_2 accumulation were observed in the NADH peroxidase gene-mutated strain (Δ*npr*) compared with the findings in the wild type. The H_2O_2 degradation capacity of Δ*npr* revealed that NADH peroxidase is a major H_2O_2-degrading enzyme in *L. casei* IGM394. Interestingly, Δ*ohrR*, a mutant deficient in the organic hydroperoxide (OhrA) repressor, exhibited higher H_2O_2 resistance than the wild-type strain. Increased Npr expression and H_2O_2 degradation ability were observed in Δ*ohrR*, further supporting the importance of OhrA to ROS tolerance mechanisms. The other mutants did not exhibit altered growth rates, although some mutants had higher growth in the presence of oxygen. From these results, it is presumed that *L. casei* IGM394 has multiple oxygen tolerance mechanisms and that the loss of a single gene does not alter the growth rate because of the presence of complementary mechanisms. Contrarily, the H_2O_2 tolerance mechanism is solely dependent on NADH peroxidase in *L. casei* IGM394.

Key words: NADH peroxidase, oxidative stress, deficient mutants, *Lactobacillus casei*, H_2O_2

INTRODUCTION

Lactic acid bacteria are facultative anaerobic bacteria that do not require oxygen for growth, and they do not have a respiratory chain and catalase; thus, they rely on anaerobic fermentation to produce energy. There is a wide range of variations in tolerance to oxygen and reactive oxygen species (ROS) stress among lactic acid bacteria, even in the same species. This means that stress tolerance in bacteria depends on the genes present in their genomes. ROS are produced via the conversion of oxygen to the superoxide anion radical, which is further converted to hydrogen peroxide (H_2O_2), and Fe^{2+} in cells induces production of the more toxic hydroxyl radical via the Fenton reaction. ROS damage intracellular proteins and DNA and cause cell death [1]. Many studies have examined the tolerance mechanisms of lactic acid bacteria to oxygen and ROS. Enzymes such as NADH oxidase and pyruvate oxidase, which degrade molecular oxygen [2–7], superoxide dismutase (SOD), which targets superoxide as a substrate [8, 9], and NADH peroxidase, which degrades H_2O_2 [10], are involved in the tolerance mechanisms. Further, lactic acid bacteria in the *Lactobacillus casei* group possess multiple types of peroxidase, including NADH peroxidase, glutathione peroxidase, thiol peroxidase and iron-dependent peroxidase. Therefore, several antioxidant enzymes are involved in oxidative stress tolerance in lactic acid bacteria. In addition to enzymes that confer direct resistance to oxygen and ROS, some enzymes contribute to oxidative tolerance. Thioredoxin reductase (TrxB2), which maintains the intracellular redox state balance, has been reported to be involved in oxygen tolerance in *Lactococcus lactis*, *Lactobacillus plantarum* WCFS1, and *Lactobacillus casei* Shirota [11–13], and similar findings have been reported for *Escherichia coli* [14]. *Streptococcus mutans* carries an iron-binding protein (Dpr) to avoid the Fenton reaction, in addition to antioxidant enzymes such as NADH oxidase [15, 16]. Furthermore, *L. casei* Shirota expresses the iron-binding protein HprA1, which is involved in H_2O_2 resistance via a different mechanism than Dpr [17]. HprA1 is involved in H_2O_2 resistance, but it does not exhibit H_2O_2-decomposing activity. It has also been reported that the disruption of the NADH peroxidase gene (*npx*) of *L. casei* Shirota results in a decreased growth rate under shaking and the

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loss of H_2O_2-decomposing activity. On the contrary, in recent studies, some lactic acid bacteria in which the electron transfer system is activated by the addition of heme alone or together with menaquinone (vitamin K_2) and oxygen is consumed as the final electron acceptor have been reported [18–20]. Compared with anaerobic fermentation, in which ATP is obtained only from glycolysis, use of the electron transfer system increases the amount of ATP and improves growth rates. Additionally, organic hydroperoxide resistance protein transcriptional regulator (OhrR), which was initially found in the gram-negative bacterium *Xanthomonas campestris,* is involved in resistance to organic peroxide and H_2O_2 [21]. There are similar reports in the gram-positive bacterium *Bacillus subtilis* [22]. However, OhrR has not been reported in lactic acid bacteria. According to the information on lactobacilli published in KEGG, *L. casei* and *L. plantarum*, which are generally considered oxygen-resistant, carry *ohrR*, but oxygen-sensitive species such as *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* do not possess the gene. Thus, the antioxidant factors possessed by lactic acid bacteria vary depending on the genus and species, and the response to oxygen stress differs accordingly.

Comparative genomic analysis of *L. casei* and *L. paracasei* revealed that several genes involved in oxidative stress tolerance are shared between the species [23–31]. However, the functions of these genes remain to be clarified.

L. casei IGM394 has high immunostimulatory capacity, and it is used as a host for drug delivery systems [32, 33]. The bacterium also exhibits an extremely good growth rate under aerobic conditions. Similar to other *L. casei* group bacteria, this strain has multiple oxidative stress tolerance genes, and thus, it is predicted that it has complex mechanisms of oxygen stress tolerance. However, the details of these mechanisms are unclear. It is important to clarify the functions of genes involved in tolerance to oxidative stress in conducting applied research with this strain as the host.

In this study, we constructed 23 deficient mutants (deficient in a single gene, 14 strains; deficient in multiple genes, 9 strains) targeting antioxidant genes reported in other bacteria via a double-crossover method. The oxidative stress tolerance mechanisms of these strains were evaluated by examining oxygen resistance in shaking culture as well as based on the consumption and resistance to H_2O_2 generated in metabolic processes. As a result, although no differences were observed in the growth of most of the deficient mutants, the Δ*npr* strain had a decreased growth rate. We found that NADH peroxidase is an essential enzyme for H_2O_2 degradation in *L. casei* IGM394.

METHODS

Strains, plasmids, media, and growth conditions

The strains and plasmids used in this study are listed in Table 1. *L. casei* IGM394 was used as the wild type. The *L. casei* IGM394 was a derivative of *L. casei* ATCC 393, and the *L. casei* ATCC 393 was distributed by a European collaborator. The *L. casei* IGM394 exhibits high transformation efficiency. *Escherichia coli* DH5α (Toyobo, Osaka, Japan) was used as the competent cells for DNA transformation. The plasmid pBTE was used as a cloning vector for deficient mutants. Lactic acid bacteria were grown at 37°C in MRS medium (Becton, Dickinson and Company, Sparks, MD, USA) and LAPTg medium (2% glucose, 1% yeast extract, 1% Bacto Proteose Peptone No. 3, 0.1% Bacto Tryptone, 0.1% Tween 80, and 0.01% $MgSO_4 \cdot 7H_2O$). *E. coli* was grown at 37°C in LB Miller medium (Becton, Dickinson and Company). Erythromycin was added at a final concentration of 5 µg/mL for lactic acid bacteria. Ampicillin was added at a final concentration of 100 µg/mL for *E. coli*. The optical density of the culture was measured at 600 nm (OD_{600}) using a UV-1200 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The medium was dispensed into test tubes with loose aluminum caps (static condition) or silicon caps that allowed free air exchange (shaking condition). The cells were cultured with shaking at 180 rpm for the shaking condition. Growth analysis of wild-type and mutant strains was performed in three independent experiments under the static or shaking condition. The data are shown as the mean ± SE of three independent experiments.

Construction of deficient mutants

pBTE is a derivative of the shuttle and thermosensitive plasmid vector pBT2. The origin of replication for lactic acid bacteria cannot function at 42°C. Recombinant plasmids for deficient mutants were constructed as follows. The upstream and downstream fragments of the target gene were amplified by PCR using *L. casei* IGM394 genomic DNA as a template, PrimeSTAR Max DNA polymerase (Takara, Shiga, Japan), and the primer pairs listed in Table 2. The fragments were digested at both ends using appropriate restriction enzymes (Table 2). The fragments were cloned into pBTE, which had previously been digested using the same restriction enzymes. Recombinant plasmids were purified using NucleoSpin® Plasmid (Macherey-Nagel, Bethlehem, PA, USA) and transferred into *L. casei* IGM394 via electroporation. Cells were grown in 10 mL of MRS broth to the stationary phase and harvested via centrifugation, and they were then suspended in 10 mL of MRS broth containing 8% (w/v) glycine and incubated at 37°C for 90 min. The cells were subsequently washed twice with an equal volume of sterile water, followed by washing with an equal volume of 50 mM EDTA solution and washing twice with an equal volume of 0.3 M sucrose solution. They were then suspended in 1 mL of 0.3 M sucrose solution. Electroporation was done with a Gene Pulser (BTX, San Diego, CA, USA) using 100 µL of competent cells and 10 µL of plasmid DNA solution in a 2-mm electroporation cuvette at a capacitance, resistance, and voltage of 25 µF, 48 Ω, and 1.5 kV, respectively. Cells were transferred to 1 mL of MRS broth and then incubated at 37°C for 2 hr. After incubation, cells were plated onto MRS agar containing 5 µg/mL erythromycin and incubated at 37°C for 3 or 4 days under anaerobic conditions using AnaeroPouch®-Anaero (MGC, Tokyo, Japan). Erythromycin-resistant colonies were selected, and plasmid introduction was confirmed by PCR with appropriate primers (Table 2). To induce plasmid integration, transformants were incubated at 42°C in MRS broth containing 5 µg/mL erythromycin. After several cycles of subculture, cells were plated onto MRS agar containing 5 µg/mL erythromycin and incubated at 37°C for 3 or 4 days under anaerobic conditions using Anaero Pouch®-Anaero. A colony was selected at random, and plasmid integration was confirmed by PCR with appropriate primers (Table 2). The integrants were incubated at 37°C in MRS broth. After several cycles of subculture, cells were plated onto MRS agar and incubated at 37°C for 3 or 4 days under anaerobic conditions using AnaeroPouch®-Anaero. Colonies were selected at random, and gene disruption was confirmed by PCR with

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Table 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Phenotype of genotype	Source or reference
Strains		
L. casei		
IGM394	Wild-type	our collection
Δ*nox*	deficient of *nox* gene	This study
Δ*nox5*	deficient of *nox5* gene	This study
Δ*poxF*	deficient of *poxF* gene	This study
Δ*cidC*	deficient of *cidC* gene	This study
Δ*ahpC*	deficient of *ahpC* gene	This study
Δ*ohrR*	deficient of *ohrR* gene	This study
Δ*sod*	deficient of *sod* gene	This study
Δ*suf*	deficient of *suf* gene	This study
Δ*flp*	deficient of *flp* gene	This study
Δ*dpsB*	deficient of *dpsB* gene	This study
Δ*cydAB*	deficient of *cydAB* gene	This study
Δ*gshR1*	deficient of *gshR1* gene	This study
Δ*ipr*	deficient of *ipr* gene	This study
Δ*npr*	deficient of *npr* gene	This study
Δ*nox*::Δ*npr*	deficient of *nox* and *npr* gene	This study
Δ*nox5*::Δ*npr*	deficient of *nox5* and *npr* gene	This study
Δ*sod*::Δ*npr*	deficient of *sod* and *npr* gene	This study
Δ*gshR1*::Δ*npr*	deficient of *gshR1* and *npr* gene	This study
Δ*gshR2*::Δ*npr*	deficient of *gshR2* and *npr* gene	This study
Δ*ipr*::Δ*npr*	deficient of *ipr* and *npr* gene	This study
Δ*gshR1*::Δ*gshR2*::Δ*npr*	deficient of *gshR1*, *gshR2* and *npr* gene	This study
Δ*sod*::Δ*gshR1*::Δ*gshR2::Δnpr*	deficient of *sod*, *gshR1*, *gshR2* and *npr* gene	This study
Δ*ipr*::Δ*gshR1*::Δ*gshR2::Δnpr*	deficient of *ipr*, *gshR1*, *gshR2* and *npr* gene	This study
E. coli		
DH5a	Commercial strain purchased from Toyobo	
Plasmids		
pBTE	*E. coli*-gram positive bacteria shuttle vector carrying pBT2 ori region, pAMb1 erythromycin resistance gene, multi cloning sites and temperature sensitivity	our collection
pBTE::Δ*nox*	pBTE carrying deficient fragment of *nox*	This study
pBTE::Δ*nox5*	pBTE carrying deficient fragment of *nox5*	This study
pBTE::Δ*poxF*	pBTE carrying deficient fragment of *poxF*	This study
pBTE::Δ*cidC*	pBTE carrying deficient fragment of *cidC*	This study
pBTE::Δ*ahpC*	pBTE carrying deficient fragment of *ahpC*	This study
pBTE::Δ*ohrR*	pBTE carrying deficient fragment of *ohrR*	This study
pBTE::Δ*sod*	pBTE carrying deficient fragment of *sod*	This study
pBTE::Δ*suf*	pBTE carrying deficient fragment of *suf*	This study
pBTE::Δ*flp*	pBTE carrying deficient fragment of *flp*	This study
pBTE::Δ*dpsB*	pBTE carrying deficient fragment of *dpsB*	This study
pBTE::Δ*cydAB*	pBTE carrying deficient fragment of *cydAB*	This study
pBTE::Δ*gshR1*	pBTE carrying deficient fragment of *gshR1*	This study
pBTE::Δ*gshR2*	pBTE carrying deficient fragment of *gshR2*	This study
pBTE::Δ*ipr*	pBTE carrying deficient fragment of *ipr*	This study
pBTE::Δ*npr*	pBTE carrying deficient fragment of *npr*	This study

appropriate primers (Table 2).

Quantification of H_2O_2

A mixture of the chromogenic reagent DA64 (100 µM in PIPES buffer [0.1 M, pH 6.8, 0.5% Triton-X 100]) and horseradish peroxidase (100 units/mL) was used to measure H_2O_2 concentrations. Cultures of each strain were harvested via centrifugation (10,000 × *g*, 3 min). Each supernatant (20 µL) were added to the mixture, which was incubated at 37°C for 5 min. After incubation, OD_{727} was measured, and H_2O_2 content was quantified using the standard curve.

H_2O_2 consumption

Cells precultured at 37°C were inoculated into 10 mL of MRS medium at OD_{600} = 0.05. The cells were used after static culture at 37°C for 5 hr. They were then washed twice with PIPES buffer (pH 6.8) and resuspended in 10 mL of H_2O_2 adjusted to 50 µM, 100 µM, 300 µM with PIPES buffer. After incubation at 37°C for 1 hr under a static condition, the cells were harvested via centrifugation (10,000 × *g*, 3 min). Supernatants were used to

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Table 2. Primers sequence used in this study

Target gene	Primer name	Primer sequence (5’ to 3’)	Restriction enzyme site
Construction of deficient mutants			
nox	nox_A-forward	CAACCTGCAGTTTTTGCTGTTGATTAATATGTTTGAAAAT	*Pst* I
	nox_A-reverse	TGTGAAGGAGTGTTTAACTATCCATTCGAATTGCAAACAA	
	nox_B-forward	TTGTTTGCAATTCGAATGGATAGTTAAACACTCCTTCACA	
	nox_B-reverse	TTC GAAGCTTGTTATCCGCAACGTGCCGT	*Hind* III
*nox*5	nox5_A-forward	CCATGGATCCCCCGTGAAGCGTAGTTGTTG	*BamH* I
	nox5_A-reverse	ACGAATTCATAATTTTCCCCCAGCATCTGCCTTCCTTTCA	
	nox5_B-forward	TGAAAGGAAGGCAGATGCTGGGGGAAAATTATGAATTCGT	
	nox5_B-reverse	GCC AAAGCTTCTTGATCGGCTCGTCTGATC	*Hind* III
*pox*F	poxF_A-forward	GTTGGGATCCAGCCAATGGCGACTTCTGGA	*BamH* I
	poxF_A-reverse	ACTTTTTGGGAGGGATTCTTCTAGTGATTAAAAAAGAGAT	
	poxF_B-forward	ATCTCTTTTTTAATCACTAGAAGAATCCCTCCCAAAAAGT	
	poxF_B-reverse	TGCACTGCAGGGCTTGGCAGTGCCGAA	*Pst* I
*cid*C	cidC_A-forward	GCTAGGATCCCAGCGTGACGGCTTTTTATA	*BamH* I
	cidC_A-reverse	TGATACAAGCTAATCGAAAAATCAAAATCTCCTTTATCGC	
	cidC_B-forward	GCGATAAAGGAGATTTTGATTTTTCGATTAGCTTGTATCA	
	cidC_B-reverse	GACAAAGCTTGGGACACAATATGCTGAGGC	*Hind* III
*ahp*C	ahpC_A-forward	GCTAGGATCCGCTACATTCTCGATATCGGT	*BamH* I
	ahpC_A-reverse	CTAAAAATGGAGGTAATATCAACGGTGCTTTGATCAGCTA	
	ahpC_B-forward	TAGCTGATCAAAGCACCGTTGATATTACCTCCATTTTTAG	
	ahpC_B-reverse	GATTAAGCTTATGTCCGTGATCGTCCGTTTATTAAGAATC	*Hind* III
ohrR	ohrR_A-forward	CAAGCTGCAGGAGCGATGATGCCTAG	*Pst* I
	ohrR_A-Reverse	CCTTATTTTTGGGCGGCTGCTTCCTCCTAA	
	ohrR_B-forward	TTAGGAGGAAGCAGCCGCCCAAAAATAAGG	
	ohrR_B-Reverse	GCACAAGCTTAAGGTTGATGGATCAGGATG	*Hind* III
sod	sod_A-forward	CTGACTGCAGCGCTGAAATTGCGCAAAATC	*Pst* I
	sod_A-reverse	AGTGACCAAACATCGAGGAATCAACCTTTC	
	sod_B-forward	GAAAGGTTGATTCCTCGATGTTTGGTCACT	
	sod_B-reverse	GGTTAAGCTTGGTAAATTACCATCCCCAAAAGGTG	*Hind* III
suf	suf_A-forward	AATAGGATCCTTGTGGTCCAAGCATCAGGAC	*BamH* I
	suf_A-reverse	TTGGAGGCAAGTGTCCTGGTTGACTTAAAA	
	suf_B-forward	TTTTAAGTCAACCAGGACACTTGCCTCCAA	
	suf_B-reverse	GCATCTGCAGAATCATGCTAAAATGTTGGC	*Pst* I
flp	flp_A-forward	GCTAGGATCCCAAGCACAGACCCATTTG	*BamH I*
	flp_A-reverse	CATCACTCGGCAACCGTTGCCACCTCCTAA	
	flp_B-forward	TTAGGAGGTGGCAACGGTTGCCGAGTGATG	
	flp_B-reverse	TCGGCTGCAGGCCCGATGACCTCAAC	*Pst I*
*dps*B	dpsB_A-forward	GGTCGGATCCAATGCCTTACGGTTACGGCA	*BamH* I
	dpsB_A-reverse	CGCTCTGTCAACAAGCTTGAGTTCTTCTCCTCTTAACCGC	
	dpsB_B-forward	GCGGTTAAGAGGAGAAGAACTCAAGCTTGTTGACAGAGCG	
	dpsB_B-reverse	GCGGCTGCAGGAGAAGCAATTGTTGAAAGTCAGT	*Pst* I
cydAB	cydAB_A-forward	CCTCGGATCCCCTACTCACTATCATTC	*BamH I*
	cydAB_A-reverse	TCTTTTCGCCGCCGCTTTGATCACCTCTAT	
	cydAB_B-forward	ATAGAGGTGATCAAAGCGGCGGCGAAAAGA	
	cydAB_B-reverse	TAGACTGCAGCCTGAACACGTGCATACTG	*Pst I*
gshR1	gshR-1_A-forward	CAATGTCGACGCTTCGGTATCCCGG	*Sal I*
	gshR-1_A-reverse	ACAAGGAGGATCAATGGTTGTAGCGGCAAT	
	gshR-1_B-forward	ATTGCCGCTACAACCATTGATCCTCCTTGT	
	gshR-1_B-reverse	GTTGAAGCTTTGATCGGCGCGCCG	*Hind III*
gshR2	gshR-2_A-forward	AACAGGATCCCAATTAAGGTCATATCATCCC	*BamH I*
	gshR-2_A-reverse	CACAGAGACGAAGGAAGGCGTAATTGATAC	
	gshR-2_B-forward	GTATCAATTACGCCTTCCTTCGTCTCTGTG	
	gshR-2_B-reverse	GCGGGTCGACGCATCACGGCCTAAG	*Sal I*
ipr	ipr_A-forward	CCGACTGCAGCGTATTAATCACGTTGC	*Pst I*
	ipr_A-reverse	TTTGGAGGCGTAACGATAAAGTTGCTTGAT	
	ipr_B-forward	ATCAAGCAACTTTATCGTTACGCCTCCAAA	
	ipr_B-reverse	GGCCAAGCTTATGAGGTACAGCTTCGC	*Hind III*
npr	npr_A-forward	CAATGGATCCATCGGAGCATATCCCTTCAG	*BamH I*
	npr_A-reverse	AATTAGGAGGAATTTACTTTTAAAAAGACA	
	npr_B-forward	TGTCTTTTTAAAAGTAAATTCCTCCTAATT	
	npr_B-reverse	GATACTGCAGATTTGGCCGGGACAAGTG	*Pst* I

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Table 2. continued.

Target gene	Primer name	Primer sequence (5' to 3')	Restriction enzyme site
Confirmation of deficient mutants			
nox	279962_F	GTAGCATCGGCAATTGTCATGTAGTGTCAC	
	282934_R	CTGTTTTGAGTCATACCGTGCAACCCG	
nox5	177892_F	CTGCGGTTCGATGGTGCTAAGGTCACCTTC	
	180877_R	GTTTTGACGCATTCATCGAATCGAGTCGCG	
poxF	2268013_F	GTCTGACTAATATGCAGTGGCGCAAAGTGAG	
	2270930_R	CGAGGCAGCCAAAGCTTTCGTTAAGAAGCAC	
cidC	498129_F	CGTTGCTTCGATCATGGTCTGGCAGAATTC	
	501457_R	GGCCAGTGGCATTCCTGATTACACCGAG	
ahpC	2611951_F	GAATAACCATAGAAAGAAGGGAGGCAGTTG	
	2614120_R	AATTATTACCAGCCGGACCCGAGCACAAAG	
ohrR	1040892_F	CAATTTTAGATCCGGATACCATGGCGATTTC	
	1043371_R	CTCCATTGCACACAAATTGCACACAAATTC	
sod	2004800_F	CAATCGCATGCTCGGAAATGAGTTTCAAAC	
	2007409_R	GGAAATAGGTATGCGATATTCATTTACGAC	
flp	69484_F	CTTATGGAGGAGGTTTCGATCCTATAGAAC	
	71774_R	GCAGTATACCAACGTTCCAACCGCTATC	
dpsB	68755_F	GAAAAGGTGATGTTTGTCGGTGACGGGATC	
	70973_R	GTATTTAAAAAACATCACTCGGCAACCTCACCAAG	
cydAB	14573_F	GAAGCTTAGAGTGACGGCTAATGAAC	
	18661_R	CCGCAAAATGGACGGGTATTATCCATC	
gshR1	2311599_F	CAATGGGTTGCGGTTCGCATTCCTGAC	
	2314729_R	CTGTCGGAACGTTACTCGTCATGCTTG	
gshR2	2748254_F	CAGTGACCAAAGATTTTGACCATCATAAAC	
	2751189_R	GTTGATCCAACGAGCGGCGTCATC	
ipr	706255_F	GGGTAATAAACCAGCAATGACCACAAGACG	
	708795_R	CTAGAATTCAATCGAAATAATATTCGGATTGTCGG	
npr	464266_F	CCAATTTTTTCTGCAAAGTCCTTTTGAGAG	
	467233_R	CGTTTTACAAGCATGGGAAAATACGGC	
qRT-PCR			
NADH peroxidase		ACGGCAATCCACAAGTTTGC	
		TTGTTGTTGAACGGCGAGTG	
Elongation factor Tu		AACCGCGAACAAGTTGAACG	
		ACGGCCACCTTCTTCTTTTG	
Glyceraldehyde 3-phosphate dehydrogenase		AACACGATTCCTCACAGCAC	
		ACAACAGAAACACGCTGTGC	

measure H_2O_2 concentrations.

Repressive effect of H_2O_2 on bacterial growth

Cells precultured overnight at 37°C were inoculated into fresh MRS medium at OD_{660} = 0.05. These bacterial cultures were aliquoted (180 µL/well) into a 96-well plate, and 20 µL of H_2O_2 solution was added to a final concentration of 0.5, 1.0, or 2.0 mM; the plate was then incubated at 37°C for 24 hr. The OD_{600} was measured using a multiplate reader, and the value at each concentration was calculated on the basis of the value at 0 mM for each strain. Significance was indicated by $p<0.05$ (for each wild-type concentration).

RNA isolation

H_2O_2 treatment was performed as follows. Samples precultured at 37°C were inoculated into 20 mL of MRS medium at OD_{600} = 0.05. The cultures were grown for 5 hr at 37°C under static conditions and divided into two 10 mL aliquots. The cells were harvested via centrifugation (10,000 × *g*, 3 min) and washed twice with PIPES buffer (pH 6.8). One aliquot was resuspended in 10 mL of PIPES buffer and incubated at 37°C for 1 hr. The other aliquot was resuspended in H_2O_2 adjusted to 0.5 mM with PIPES buffer and incubated at 37°C for 1 hr. The 10 mL cultures were added to 20 mL of RNAprotect Bacteria Reagent (Qiagen). The mixtures were kept at room temperature for 5 min. The cells were then harvested via centrifugation for 10 min at 5000 × *g*, suspended in 500 µL of TE buffer (50 mM Tris–HCl, pH 8.0) containing 5 mg/mL lysozyme and 20 µL/mL mutanolysin, and incubated at 37°C for 30 min. Total RNA was purified using a Direct-zol™ RNA MiniPrep kit (Zymo Research) according to the manufacturer's protocol. DNA was digested using DNase I in the purification step. RNA was isolated from three independent cultures.

Quantitative real-time PCR assays

cDNA was synthesized using a PrimeScript RT reagent kit (Takara) according to the manufacturer's protocol. In total, 0.1 mg of total RNA was used as a template. Quantitative real-time PCR assays were performed using a CFX96 Real-Time PCR Detection System (Bio-Rad) with THUNDERBIRD SYBR

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qPCR Mix (Toyobo). The primers were designed to amplify products of approximately 80 bp in length (Table 2). The reaction mixture contained 25 µL of THUNDERBIRD SYBR qPCR Mix, 1 µL of 15 µM forward primer, 1 µL of 15 µM reverse primer, 1 µL of 50× ROX reference dye, 20 µL of dH_2O, and 2.5 µL of diluted cDNA templates. All reactions were run in duplicate for each of the three independent RNA samples. The gene expression values were normalized using the elongation factor Tu and glyceraldehyde 3-phosphate dehydrogenase as an internal standard. Standard curves for both the internal standard and target genes were generated by amplifying 10-fold serial dilutions of cDNA. The gene expression data from quantitative real-time PCR were analyzed using Student's t-test.

RESULTS

Construction of mutants deficient in genes involved in oxygen and ROS tolerance in L. casei *IGM394*

To elucidate the mechanisms of oxygen and ROS tolerance in *L. casei* IGM394, we constructed gene-deficient mutants targeting enzymes or factors that are expected to be involved in oxygen tolerance (Table 3). The target gene was completely deleted via the double-crossover method using a thermosensitive suicide vector. Fourteen out of 16 targeted genes were successfully disrupted in mutants. However, disruptants were not obtained for *trxB2* and the chaperone protein gene *groEL*.

Table 3. Targeting enzymes or factors that are expected to be involved in oxidative stress tolerance

	Gene name
NADH peroxidase	*npr*
Organic hydroperoxide resistance protein transcriptional regulator	*ohrR*
NADH oxidase (H_2O - forming)	*nox*
NADH oxidase (H_2O_2 - forming)	*nox5*
Pyruvate oxidase	*poxF*
Pyruvate oxidase	*cidC*
Alkyl hydroperoxide reductase subunit C	*ahpC*
Superoxide dismutase	*sod*
Fe-S cluster assembly protein	*suf*
Probable transcriptional regulator	*flp*
DNA binding protein	*dpsB*
Cytochrome bd ubiquinol subunit I, II	*cydAB*
Glutathione reductase	*gshR1*
Iron-dependent peroxidase	*ipr*
Thioredoxin reductase	*trxB2*
Chaperon protein	*groEL*

Growth of deficient mutants under static and shaking conditions

We evaluated the growth rates of 23 deficient mutants under static and shaking conditions (Fig. 1A, 1B). The growth rate of Δ*nox*, which is an NADH oxidase (*nox*, H_2O-forming) gene-deficient mutant, was decreased under both culture conditions. Compared with the findings for the wild type, the OD decreased from 2.2 to 1.5 under the static condition and from 2.2 to 1.6 under the shaking condition after 24 hr of culture. In the Δ*npr* mutant, the growth rate was decreased only under shaking culture. Under the static condition, the OD of Δ*npr* was 2.0, which was similar to that of the wild type (2.2). However, under the shaking condition, the OD of Δ*npr* was 1.5, whereas that for the wild type was 2.2. Conversely, the ODs of four strains, namely the NADH oxidase (*nox5*, H_2O_2-forming) gene-deficient mutant Δ*nox5*, pyruvate oxidase gene-deficient mutants Δ*poxF* and Δ*cidC*, and DNA-binding protein gene-deficient mutant Δ*dpsB*, were slightly increased (approximately 0.1–0.2) under the shaking condition. The other eight deficient mutants did not exhibit different growth rates versus the wild type under either of the culture conditions. Decreased growth under shaking was observed only for Δ*npr*. Therefore, we constructed mutants deficient in multiple genes using Δ*npr* as a host and evaluated the effect on viability. We constructed six double-deficient mutants, one triple-deficient mutant, and two quadruple-deficient mutants. The target genes of the six double-deficient mutants were *nox*, *nox5*, *sod*, glutathione reductase (*gshR1* or *gshR2*), and iron-dependent peroxidase (*ipr*). The triple-deficient mutant featured mutations of *gshR1* and *gshR2*. Finally, the quadruple-deficient mutants featured mutations of *sod* or *ipr* using the triple-deficient mutant Δ*gshR1*::Δ*gshR2*::Δ*npr* as a host. However, these deficient mutants exhibited the same growth rate as Δ*npr* (Fig. 1B). These results indicated that the Npr gene is important for the growth of *L. casei* IGM394 under the shaking condition. Measuring the amount of H_2O_2 accumulated in LAPTg medium after 24 hr, H_2O_2 was detected only in Δ*npr* and nine multiple-deficient mutants (data not shown). In addition, the evaluation by bacterial turbidity included dead cells, and there was a possibility that the results might be inaccurate. Therefore, we measured colony-forming units, and the results were in line with the measured OD values. Thus, only the results for turbidity are presented.

Growth of the wild-type and Δnpr strains under the shaking condition and H_2O_2 concentrations in the culture

H_2O_2 concentrations in the MRS culture medium of wild-type and Δ*npr* cultures were measured over time under the shaking condition (Fig. 2). Although H_2O_2 was not detected in the wild-type culture, following overnight culture of Δ*npr*, approximately 1,000 µM H_2O_2 had accumulated. In addition, no accumulation of H_2O_2 was observed in either strain under the static condition (data not shown). These results suggest that *L. casei* IGM394 converts oxygen in its growth process under shaking and that the generated H_2O_2 is degraded by NADH peroxidase.

Growth suppression by H_2O_2

The effect of H_2O_2 on the growth of each mutant was evaluated under various H_2O_2 concentrations (0–2.0 mM) in MRS medium (Fig. 3). The suppressive effect of H_2O_2 on growth was concentration dependent. In particular, 0.5 mM H_2O_2 had little effect on growth, whereas 1.0 mM H_2O_2 reduced proliferation. Bacterial growth was completely inhibited by 2.0 mM H_2O_2. The influence of 0.5 and 1.0 mM H_2O_2 on the growth rates of nine mutants was similar to that observed in the wild-type strain. However, the growth rate of Δ*npr* was reduced by 0.5 mM H_2O_2, and the rate was significantly lower than that of the wild type in the presence of 1.0 mM H_2O_2. The growth of Δ*ohrR* was not suppressed by 1.0 mM H_2O_2. In addition, Δ*ohrR* also proliferated in the presence of 2.0 mM H_2O_2, and H_2O_2 resistance was improved by the deletion of *ohrR*. From these findings, it was presumed that *ohrR* is one of the tolerance mechanisms in *L. casei* IGM394.

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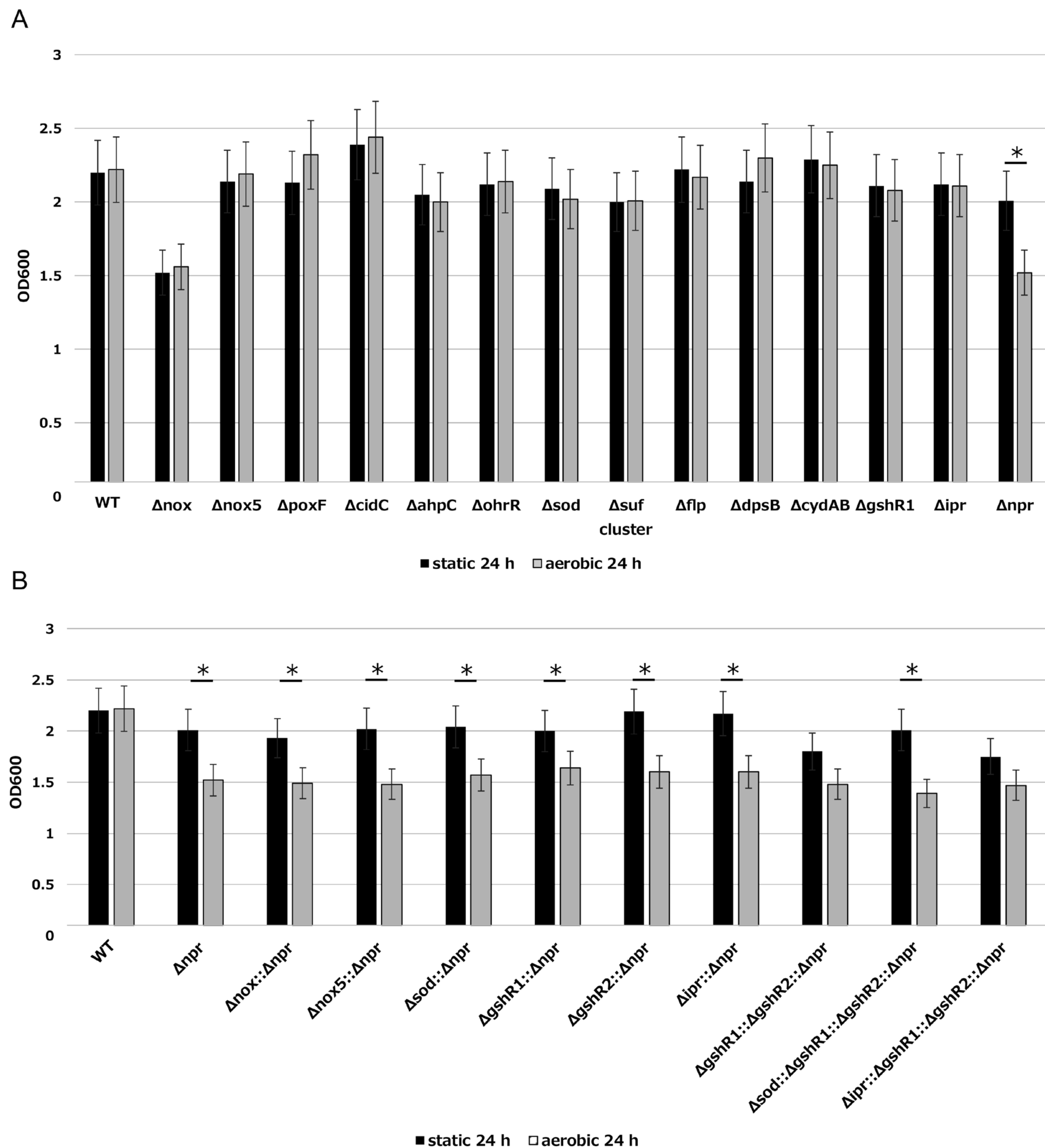

Fig. 1. (A) Growth rates of wild type and deficient mutants under static and shaking conditions. Strains precultured overnight at 37°C were inoculated into fresh LAPTg medium at a final OD_{600} of 0.05. After 24 hr, we measured the OD_{600} using a spectrophotometer. The black bar shows the static condition, and the gray bar shows the shaking condition. (LAPTg medium has no ability to consume H_2O_2.) The data are shown as the mean ± SE of three independent experiments. asterisk (*) Student's t-test; p<0.05. (B) Growth rates of Δnpr and multiple deficient mutants under static and shaking conditions. Strains precultured overnight at 37°C were inoculated into fresh LAPTg medium at a final OD_{600} of 0.05. After 24 hours, we measured the OD_{600} using a spectrophotometer. The black bar shows the static condition, and the gray bar shows the shaking condition. (LAPTg medium has no ability to consume H_2O_2.) The data are shown as the mean ± SE of three independent experiments. asterisk (*) Student's t-test; p<0.05.

H_2O_2 consumption in PIPES buffer

H_2O_2 accumulation in the medium was estimated for 14 deficient mutants under the shaking condition, and H_2O_2 was only detected in the Δ*npr* culture. This illustrated that only Δ*npr* could not consume H_2O_2. H_2O_2 accumulation in the medium under the shaking condition was confirmed for Δ*npr*, and Δ*npr* could not consume H_2O_2 generated during the growth process (Fig. 4). Therefore, the ability to consume added H_2O_2 in PIPES buffer was measured. In this experiment, the wild-type, Δ*npr*, and Δ*ohrR* strains were used after 5 hr of logarithmic growth. After the cells were exposed to 0, 50, 100, or 300 µM H_2O_2 for 1 hr under the static condition, H_2O_2 concentrations in PIPES buffer were measured. On average, the H_2O_2 concentration was decreased by 84% compared with the initial concentration in the

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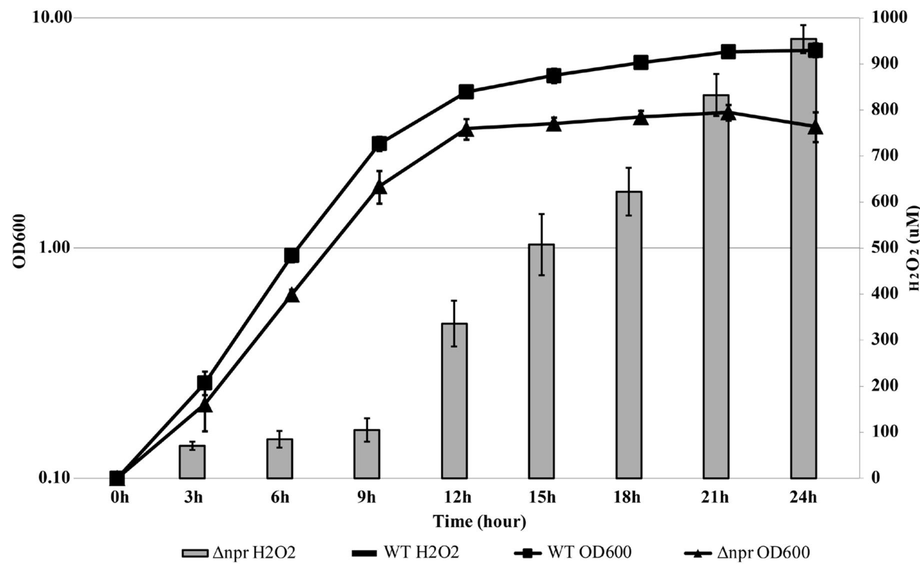

Fig. 2. Growth and accumulated H_2O_2 concentration of wild type and Δ*npr* under the shaking condition.

Strains precultured overnight at 37°C were inoculated into fresh MRS medium at a final OD_{600} of 0.05. We measured the OD_{600} using a spectrophotometer every 3 hr. Subsequently, 1 mL of the culture was collected and centrifuged (10,000 g, 3 min), and 20 μL of the supernatant was used for measuring the H_2O_2 concentration. After measuring the wavelength at 727 nm, chromogenic reagent DA64 was used to quantify H_2O_2 based on the standard curve. The black square represents the OD value of wild type, and the black triangle represents that of Δ*npr*. The gray bar shows the concentration of H_2O_2 in the Δ*npr* culture. The data are shown as the mean ± SE of three independent experiments.

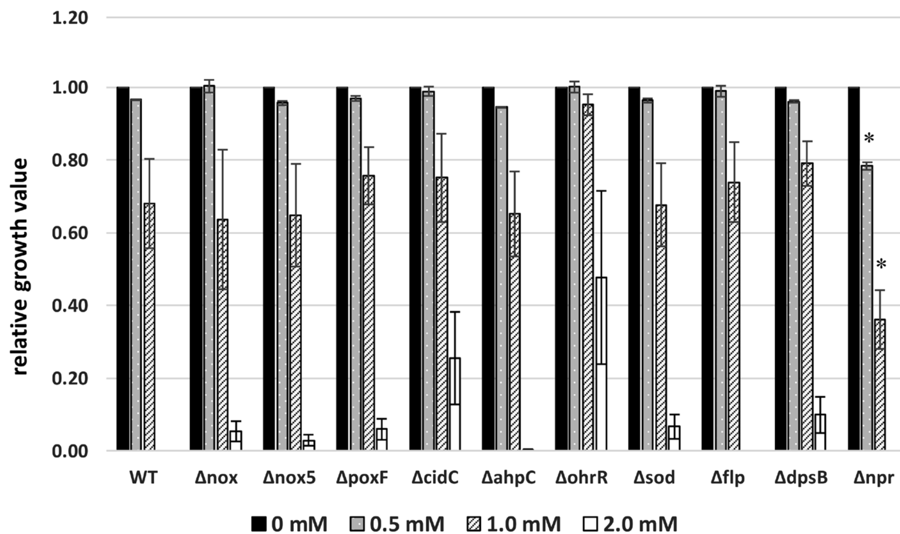

Fig. 3. Effects of adding H_2O_2 on the growth of the wild type and ten mutants.

Strains precultured at 37°C were inoculated into MRS medium at a final OD_{600} of 0.05, and these culture media were aliquoted (180 μL/well) to a 96-well plate. Twenty microliters of H_2O_2 solution was added to a final concentration of 0.5, 1.0, or 2.0 mM, and the plate was incubated at 37°C. After 24 hr, the OD_{600} was measured using a multiplate reader. Relative growth values were calculated using the following equation. The data are shown as the mean ± SE of three independent experiments.
Relative growth value = OD_{600} value at each H_2O_2 concentration/OD_{600} value at 0 mM H_2O_2.
*Student's t-test; $p<0.05$ (for each wild type concentration).

presence of wild-type cells. The wild-type strain decreased the supplemented H_2O_2 concentration from 50 to 0 μM, from 100 to 25 μM, and from 300 to 204 μM. The Δ*npr* strain could not consume H_2O_2 efficiently. The H_2O_2 concentration for the Δ*npr* strain after 1 hr of incubation was similar to or slightly higher than the control level. In Δ*ohrR* culture buffer, H_2O_2 could not be detected after adding 50 or 100 μM H_2O_2. The Δ*ohrR* strain completely consumed 100 μM H_2O_2 and decreased the H_2O_2 concentration from 300 to 174 μM. This indicated that the H_2O_2 consumption ability of the Δ*ohrR* strain was greater than that of

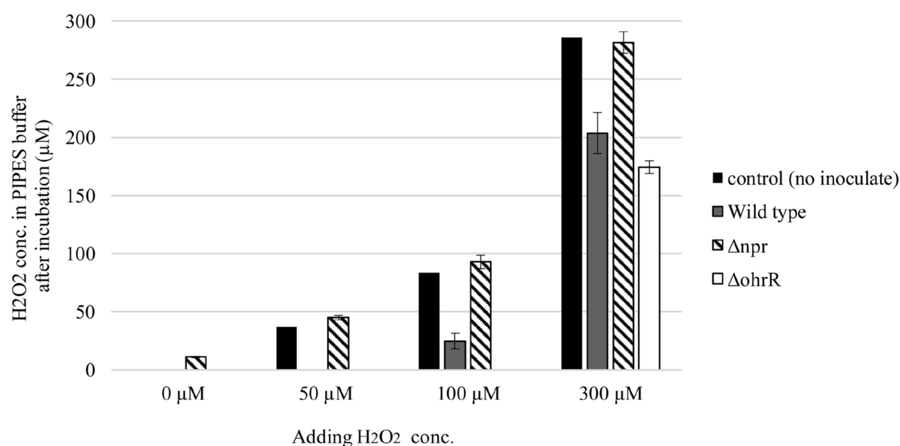

Fig. 4. H_2O_2 concentration of wild type and Δ*npr* in PIPES buffer.

Strains precultured overnight at 37°C were inoculated into fresh MRS medium at a final OD_{600} of 0.05. The cells were used after static culture at 37°C for 5 hr. They were washed twice with PIPES buffer (pH 6.8) and resuspended in 10 mL H_2O_2 adjusted to 0 to 300 µM with PIPES buffer. After incubation at 37°C for 2 hr, the cells were harvested by centrifugation (10,000 g, 3 min). Twenty microliters of the supernatant was used for measuring the H_2O_2 concentration. After measuring the wavelength at 727 nm, the chromogenic reagent DA64 was used to quantify H_2O_2 based on the standard curve. The data are shown as the mean ± SE of three independent experiments.

the wild-type strain.

Gene expression analysis of NADH peroxidase via quantitative real-time PCR

The aforementioned results revealed that H_2O_2 consumption was mainly performed by Npr and that deletion of *ohrR* eliminated the growth-suppressing effects of H_2O_2 in *L. casei* IGM394. It was presumed that OhrR was involved in H_2O_2 consumption; therefore, we examined the expression level of *npr* in the wild-type and Δ*ohrR* strains via quantitative real-time PCR. In addition, we observed that the expression level changed depending on the presence or absence of H_2O_2. In this experiment, the treatment conditions were exposure to H_2O_2 adjusted to 0.5 mM with PIPES buffer for 1 hr at 37°C. In the wild-type strain, the expression level of *npr* was constant regardless of the presence of H_2O_2. However, the expression level of *npr* in the Δ*ohrR* strain was 2.5-fold higher in the absence of H_2O_2 and 3-fold higher than that of the wild-type strain in the presence of H_2O_2.

DISCUSSION

Aerobic organisms have various tolerance mechanisms against oxygen and ROS. Lactic acid bacteria, which are facultative anaerobes, do not require oxygen to grow, but they can grow in the presence of oxygen. Several factors have been reported to be involved in oxidative stress tolerance, but the mechanisms differ by species and strain. The similar growth of *L. casei* IGM394 (wild type) under static and shaking conditions observed in this study indicated that this strain has multiple mechanisms to respond to oxidative stress.

SOD, which converts highly toxic superoxide substrates into H_2O_2, is important in the mechanism of oxidative tolerance. Serata *et al.* reported that *sod* of *L. casei* Shirota was transcribed but that its protein was inactive, and they reported that superoxide was eliminated via the intracellular accumulation of Mn^{2+} [34]. The possibility that *L. casei* IGM394 has the same Mn^{2+} accumulation mechanism as *L. casei* Shirota could explain why *sod* disruption did not affect the growth rate of the former bacterium (Fig. 1A).

Higuchi *et al.* reported that AhpC degrades H_2O_2 into water and that H_2O_2 is produced by Nox as a byproduct of oxygen consumption in *S. mutans* [3]. *L. casei* IGM394 expresses Nox5, which produces H_2O_2 in a manner similar to that observed in S. mutans. However, H_2O_2 was not detected in the culture medium of Δ*ahpC* under shaking. It is predicted that *L. casei* IGM394 carries a number of enzymes for degrading H_2O_2, such as NADH peroxidase, and that these enzymes complement the function of AhpC to degrade H_2O_2 under shaking.

There are reports that Fnr-like protein (Flp) is a potential sensor protein and regulator, although the genes it regulates in *Lc. lactis* and *L. casei* remain to be clarified [35, 36]. When Flp is oxidized, an intramolecular disulfide bond is formed, thereby conferring the ability to bind to the promoter region. Although double deletion of *flpA* and *flpB* leads to hypersensitivity to H_2O_2 in *Lc. lactis* ssp. *cremoris* MG1363 [37], Δ*flp* of *L. casei* IGM394 exhibited the same growth rate as the wild type under static and shaking conditions (Fig. 1A) or in the presence of 2 mM H_2O_2. It is unclear whether the different responses of the two strains are due to different functions of Flp. The DNA-binding protein Dps is an H_2O_2 resistance factor in *E. coli* that has been identified as a nonspecific DNA-binding protein accumulated in stationary cells [38]. It has been reported that Dps forms a ferritin-like complex, binds to DNA to form an extremely stable complex, and protects DNA against H_2O_2 [39, 40]. The *suf* cluster may participate in Fe-S cluster assembly or repair. Under oxidative stress, OxyR (regulator protein) activates the expression of the *suf* cluster in *E. coli* [41]. Cytochrome bd oxidase (CydAB) is the terminal electron acceptor that finally reduces oxygen to water. In all lactic acid bacteria, *cydAB* is clustered [42, 43]. GshR is one of the enzymes constituting the glutathione-ascorbic acid cycle, a metabolic pathway that detoxifies H_2O_2 generated in the process of metabolism. Yamamoto *et al.* reported that GshR may be important in protecting *S. mutans* against oxidative stress

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[44, 45]. Despite reports of their involvement in oxidative stress resistance, deletion mutants of these genes (Δ*dpsB*, Δ*suf*, Δ*cydAB*, Δ*gshR*) had similar growth rates as the wild-type strain under static and shaking conditions. It is presumed that the mechanisms of oxygen consumption or tolerance were complemented by other mechanisms in *L. casei* IGM394.It should be noted, however, that we predicted the presence of genes that are essential for growth under oxidative stress conditions but are not complemented by other mechanisms. In addition, we tried to disrupt *trxB2*, which plays a significant role in cellular redox processes, including protein repair and defense against oxidative stress, but these efforts were unsuccessful. Serata *et al.* succeeded in constructing a *trxB2*-deficient mutant in *L. casei* Shirota, and the ability of this strain to grow under aerobic conditions was significantly reduced. This suggested that TrxB2 is an important enzyme for oxygen tolerance in *L. casei* Shirota [13]. Our different findings may be due to the fact that we did not perform experimentation under an anaerobic condition.

Our finding that only Δ*nox* exhibited decreased growth versus the wild type indicated that Nox may have different functions than other oxygen-consuming enzymes. One reason for this is that Nox converts oxygen to water without producing H_2O_2. Other oxygen-consuming enzymes, such as NADH oxidase (Nox5, H_2O_2-forming) and pyruvate oxidase (PoxF, CidC), convert oxygen to H_2O_2. It is considered that the oxygen consumption function depends on the H_2O_2-generating enzyme following the deletion of Nox, and that the influence of H_2O_2 or ROS produced by the Fenton reaction explains the decreased growth rate of Δ*nox.* One other reason is that Nox works to maintain the redox potential in cells. Futhermore, the increased growth rates observed for Δ*nox5*, Δ*poxF*, and Δ*cidC* suggested that the decreased production of H_2O_2 by these proteins eased the stress on cells.

Δ*npr*, in which the NADH peroxidase gene was disrupted, only displayed decreased growth under the shaking condition relative to the wild type. As NADH peroxidase is an H_2O_2-degrading enzyme, it was considered that Δ*npr* could not degrade the H_2O_2 generated as a byproduct of oxygen consumption under the shaking condition. A high concentration of H_2O_2 was detected in the Δ*npr* culture under the shaking condition. The H_2O_2 concentration increased with the incubation time and reached about 500 µM after 15 hr and about 950 µM after 24 hr. However, in cultures of the wild-type and all other deficient mutant strains, H_2O_2 was not detected under either condition (Fig. 2). This revealed that the loss of H_2O_2 degradation could not be compensated for by other genes in *L. casei* IGM394. Other mutants featuring deficiencies of multiple genes displayed no changes in phenotype under shaking. From this finding, it was suggested that the oxidative stress tolerance mechanisms of *L. casei* IGM394 are multiple and diverse, and thus, no effect on growth was observed because missing functions could be complemented by other genes. However, the data indicated that H_2O_2 consumption is critical for the oxidative stress tolerance mechanism in this strain because decreased growth under the shaking condition was only observed for Δ*npr*. As lactic acid bacteria cannot synthesize heme, there is no catalase-based H_2O_2 degradation system. Previous studies reported that *L. plantarum* carries manganese catalase, which uses manganese as a cofactor [46], and that *L. sakei* synthesizes heme catalase when heme is added to the medium [47]. Genomic data revealed that *L. casei* IGM394 possesses four peroxidases: NADH peroxidase, iron-dependent peroxidase, glutathione peroxidase, and thiol peroxidase; however, the bacterium does not carry manganese catalase. Our findings revealed that peroxidases other than NADH peroxidase cannot efficiently degrade H_2O_2.

Interestingly, Δ*ohrR*, which is a deficient mutant of the transcriptional regulator gene (*ohrR*), showed strong resistance to H_2O_2 (Fig. 3). The Δ*ohrR* could grow under 0.5 mM and 1.0 mM H_2O_2 supplemented conditions as well as 0 mM supplemented conditions. Furthermore, Δ*ohrR* could grow under even 2.0 mM supplemented conditions, that is, conditions in which wild type could not grow. OhrR is a transcriptional repressor of organic hydroperoxide resistance protein (OhrA). As the *ohrR* disruption resulted in the constitutive expression of the OhrA protein, it induced strong resistance to H_2O_2 in Δ*ohrR*. The OhrR gene was first identified in *Xanthomonas campestris* [20] and subsequently reported in many gram-negative bacteria. In gram-positive bacteria, the Ohr family was reported to be involved in resistance to organic peroxide and H_2O_2 in *Bacillus subtilis* [21], and OhrA overexpression induced H_2O_2 tolerance. In *B. subtilis*, OhrR repressed *ohrA* expression by binding to the inverted repeat (IR) sequence (TACAATT-N-AATTGTA) presented upstream of *ohrA*. However, there is no detailed report on the Ohr family in lactic acid bacteria, and a similar IR sequence upstream of *ohrA* was not detected in *L. casei* IGM394. Our results suggested that deletion of *ohrR* induced greater H_2O_2 resistance, and these effects appear to be related to the constitutive expression of *ohrA*. Meanwhile, deletion of *ohrR* induced higher expression of NADH peroxidase (Fig. 5). In *L. casei* IGM394, *ohrA* expression might be regulated by the recognition of different IR sequences or a different mechanism from that observed for *ohrR* in *Bacillus*. The association between the constitutive expression of *ohrA* and the higher expression of NADH peroxidase is unclear at present. However, it was suggested that the OhrR protein participates in the mechanisms combating oxygen and ROS in lactic acid bacteria. Analyses of *ohrA* expression control and function will be required in the future.

The findings of decreased growth under the shaking condition

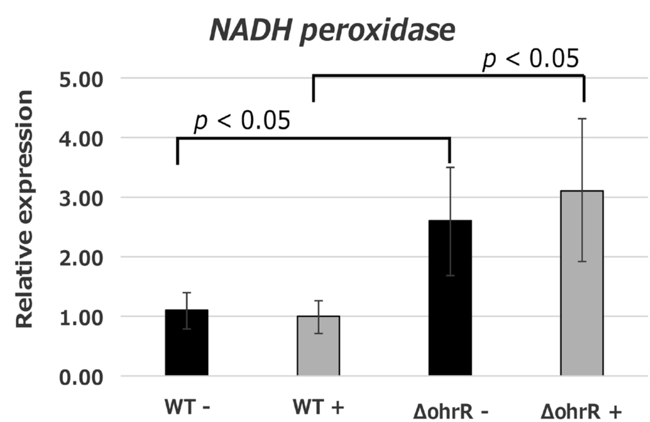

Fig. 5. NADH peroxidase expression level with and without H_2O_2 in the wild type and Δ*ohrR*.

Target gene: NADH peroxidase gene (*npr*).
Housekeeping gene: elongation factor Tu gene and glyceraldehyde 3-phosphate dehydrogenase gene.
The relative expression levels were calculated using 2 housekeeping genes. Black bars represent untreated; gray bars represent H_2O_2 treated. The data are shown as the mean ± SE of three independent experiments.

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and the loss of H_2O_2 consumption following disruption of *npr* were similar to those reported for *L. casei* Shirota [16]. Although *npx* expression was increased by approximately 10-fold in *L. casei* Shirota in response to H_2O_2 exposure according to quantitative real-time PCR, the expression level of *npr* in *L. casei* IGM394 was constant under the shaking condition. Serata *et al.* reported that *L. casei* Shirota exhibited the ability to consume H_2O_2 only after 1 hr of pretreatment with 0.5 mM H_2O_2 added to the culture medium. However, *L. casei* IGM394 could consume H_2O_2 without this pretreatment. This difference in the regulation of H_2O_2 consumption remains to be clarified.

According to our study, *L. casei* IGM394 has multiple oxygen consumption mechanisms, and disruption of a single gene is not sufficient to eliminate the ability to consume oxygen or alter growth. It is presumed that NADH oxidase efficiently converts oxygen to water in the wild-type strain, and thus, multiple H_2O_2 consumption mechanisms may not be necessary. NADH peroxidase plays a key role in H_2O_2 consumption, and other genes could not compensate for its function. Thus, it was concluded that the NADH peroxidase has a critical role in the oxidative stress response mechanisms in *L. casei* IGM394.

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