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Effect of superoxide dismutase and manganese on superoxide tolerance in *Lactobacillus casei* strain Shirota and analysis of multiple manganese transporters

Masaki SERATA[1*], Emi YASUDA[1] and Tomoyuki SAKO[1]

[1]*Yakult Central Institute, 5-11 Izumi, Kunitachi-shi, Tokyo 186-8650, Japan*

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The *Lactobacillus casei*/*paracasei* group accumulates a high level of manganese, which works to scavenge superoxide anions produced during aerobic growth. The genome of *L. casei* strain Shirota, however, also codes the gene for superoxide dismutase (SOD), *sodA*, which catalyzes the dismutation of superoxide anion into hydrogen peroxide and oxygen. We anticipated that the SOD and/or manganese may contribute to the aerobic growth of *L. casei* Shirota and tried to clarify how *L. casei* Shirota can eliminate the toxicity of superoxide anion. When the *sodA* of *L. casei* Shirota was cloned and expressed in *Escherichia coli* as well as in *L. casei* Shirota, there was no increase in SOD activity detected, meaning that the protein is in an inactive form, even if it is produced in *L. casei* Shirota. We next focused on the role of the manganese transport system of *L. casei* Shirota. One ABC-type manganese transporter (*mtsCBA* cluster) and three NRAMP-type manganese transporters (*mntH1*, *mntH2*, and *mntH3*) are coded in the genome. To clarify the role of these genes, we disrupted one or more of these manganese transporter genes in different combinations and analyzed the intracellular manganese levels. As a result, we found that NRAMP-type manganese transporters coded by *mntH1* and *mntH2* and ABC-type manganese transporter coded by *mtsCBA* cluster are complementarily involved in the accumulation of intracellular manganese and are necessary for aerobic growth of *L. casei* Shirota. These results indicate that intracellular manganese accumulated by multiple complementary manganese transporters, but not SOD, plays a pivotal role in tolerance to superoxide in *L. casei* Shirota.

Key words: superoxide, superoxide dismutase, manganese transporter, oxidative stress, *Lactobacillus casei*

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyl radicals are produced when microorganisms grow under aerobic conditions. ROS are the main causes of so-called "oxidative stress" and have various adverse effects on the growth and metabolism of the organisms, and therefore they should be eliminated with an appropriate methodology when grown under aerobic growth conditions. Lactic acid bacteria are "aerotolerant anaerobes" that are able to grow in either the presence or absence of oxygen. It is known that individual species and strains of lactic acid bacteria harbor different mechanisms to tolerate oxidative stress under aerobic growth conditions [1–8].

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide into hydrogen peroxide and oxygen [9]. In lactic acid bacteria, lactococci and streptococci are reported to have manganese superoxide dismutase (Mn-SOD) activity [1, 4, 10], while it has been suggested that *Lactobacillus casei* and *L. plantarum* do not have SOD activity but instead accumulate millimolar levels of manganese in their cells that play the role of scavenging superoxide [11, 12]. In recent reports, however, there are contradictory results suggesting that *L. casei* has a *sodA* gene and SOD activity [13, 14]. When we looked at the genome sequence of *Lactobacillus casei* strain Shirota, it was clear that it possesses a superoxide dismutase gene, *sodA*, in its genome (private data).

There have been some studies in *L. plantarum* strains on the relationship between oxygen tolerance and manganese transporters. *L. plantarum* has three systems to uptake manganese in the cell [15]. The first is Mn^{2+}- and Cd^{2+}-specific P-type ATPase (MntA), which is a high-affinity uptake system for Mn^{2+} in Mn^{2+}-starved cells [16]. The second is the ATP-binding cassette-type (ABC-type) transporter, which is composed of solute-binding protein, ATP-binding protein, and integral membrane protein and is widely distributed in bacteria. The growth of solute-binding protein mutants in other bacteria is impaired in Mn-limited medium in the presence of oxygen [17–20]. The third is the natural resistance-associated macrophage protein (NRAMP) transporter [21]. Homologues of this protein are widely distributed in bacteria and have been characterized [22, 23]. A study using *L. plantarum* strains where one or two of these uptake genes were disrupted

*Corresponding author. Masaki Serata (E-mail: masaki-serata@yakult.co.jp)

revealed that there was no significant change in growth or manganese uptake, suggesting that Mn^{2+} homeostasis is regulated by multiple genes [15]. With regard to *L. casei*, on the other hand, there has been no report of the functions of these manganese transporters.

We previously reported that the thioredoxin–thioredoxin reductase system is essential for aerobic growth of *L. casei* strain Shirota [24] and discovered a novel hydrogen peroxide resistance gene [25]. In the current study, we further analyzed the contributions of *sodA* and manganese to superoxide tolerance in *L. casei* strain Shirota and the uptake of manganese using manganese transporter disruption mutants.

MATERIALS AND METHODS

Strains, plasmids, media, and growth conditions

The strains and plasmids used are shown in Table 1. *L. casei* strain Shirota (YIT 9029) was used as the wild-type strain. *Escherichia coli* JM109 was purchased from Toyobo (Osaka, Japan) as competent cells for DNA transformation. *E. coli* BLR(DE3) was purchased from Merck (Tokyo, Japan). *L. casei* ATCC334, *L. lactis* ATCC19435, and *E. coli* K-12 HfrH were used for cloning the *sodA* genes. Plasmid pYSSE3 [26] was used as a cloning vector for deletion mutagenesis. Plasmid pYAP300 [26], which enables gene integration into the *L. casei* chromosome at the *attB* site for phage phiFSW and has a constitutive expression promoter, was used for expression of various *sodA* genes. Plasmid pET-21(+) (Merck) was used as an expression vector for recombinant proteins in *E. coli*. Lactic acid bacteria were grown at 37°C in MRS medium or Mn-free MRS medium supplemented with various concentrations of manganese. *E. coli* strains were grown at 37°C in LB medium [27]. Erythromycin (Abbott Japan, Tokyo, Japan) was added to a final concentration of 20 μg/ml for lactic acid bacteria and 500 μg/ml for *E. coli* if needed. The turbidity of the cultures was measured with a Klett-Summerson photoelectric colorimeter (Klett MFG, New York, USA) or a DU 800 spectrophotometer (Beckman Coulter, Tokyo, Japan).

Construction of gene disruption mutants

Gene disruption mutants were constructed by deletion of targeted genes as reported previously [24]. The primer pairs used to amplify fragments containing the 5′- and 3′- terminal ends of target genes are listed in Table 2. Recombinant plasmids were constructed with these two fragments and pYSSE3. These plasmids were introduced into *L. casei* strain Shirota, and gene deletion mutants were constructed using a stepwise double-crossover method.

Preparation of cell-free extracts

Cells from 2 ml of overnight cultures of lactic acid bacteria and *E. coli* were harvested by centrifugation. They were washed twice with 0.1 M phosphate buffer (pH 7.0). They were then suspended in 500 μl of the same buffer, to which had been added 0.3 g of glass beads (0.1 mm in diameter), and were disrupted by a FastPrep FP120 cell disrupter (Thermo, Tokyo, Japan) at speed of 6.5 m/sec for 30 sec. The solution was centrifuged at 18,000 *g* for 10 min, and the supernatant was used as a cell-free extract. The protein concentration was determined with a Bio-Rad Protein assay (Bio-Rad Laboratories, Tokyo, Japan).

Measurement of SOD activity

SOD activity was measured by the method of McCord and Fridovich [9] with some modifications. A mixture of 1.1 ml of 50 mM phosphate buffer (pH 7.8), 0.6 ml of 0.5 mM EDTA in phosphate buffer, 0.5 ml of 0.06 mM cytochrome *c* in phosphate buffer, 0.5 ml of 0.3 mM xanthine in phosphate buffer, and 0.1 ml of sample solution with or without 0.1 mM EDTA was prepared. The reaction was initiated by adding to the mixture 0.2 ml of xanthine oxidase solution at a concentration to produce reduction of cytochrome *c* at a rate of 0.025 absorbance units per minute at 550 nm. The mixture was then incubated at 25°C, and the increase in absorbance at 550 nm during 1 min incubation was measured. The amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome *c* by 50% was defined as 1 U of activity. The activity was measured without EDTA when we evaluated the effect of manganese.

Cloning and expression of sodA genes from different bacteria

Expression in L. casei

The *sodA* gene from *L. lactis* ATCC19435 or *L. casei* strain Shirota was amplified from the respective genomic DNA by PCR using KOD Plus DNA polymerase (Toyobo) with the primers indicated in Table 2 and was cloned into pYAP300 containing a constitutive expression promoter. pYAP300–*LlsodA* or pYAP300–*sodA* were introduced into *L. casei* strain Shirota in a manner similar to that for gene disruption.

Expression in E. coli

The *sodA* gene from *L. casei* strain Shirota or *E. coli* K-12 HfrH was amplified from the respective genomic DNA by PCR using KOD Plus DNA polymerase (Toyobo). Forward primers were designed containing the pET-21a(+) Shine-Dalgarno sequence (Table 2). The fragments of PCR products were digested at *Bam*H I/*Xho* I and cloned into pET-21(+), which had previously been digested with the same restriction enzymes. These plasmids (pET-21(+)–*sodA*M and pET-21(+)–*EcsodA*) were amplified in *E. coli* JM109 and transferred into *E. coli* BLR(DE3).

The recombinant SOD proteins were overproduced in *E. coli* in accordance with the pET system manual (Merck), with some modifications. *E. coli* BSM4 (harboring pET-21(+)–*sodA*M) and BES3 (harboring pET-21(+)–*EcsodA*) were grown at 30°C in LB medium containing 100 μg/ml ampicillin until OD_{600} = 0.6. Then, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for SOD induction,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
Strains		
Lactobacillus casei		
Shirota (YIT 9029)	Wild-type	Our collection
TM1002	Deletion in *mntH1* gene	This study
TM1003	Deletion in *mntH2* gene	This study
TM1004	Deletion in *mtsA* gene	This study
TM1005	Deletion in *mntH1* and *mntH2* gene	This study
TM1006	Deletion in *mntH1* and *mtsA* gene	This study
TM1010	Deletion in *mntH2* and *mtsA* gene	This study
TM1011	Deletion in *mntH1, mntH2,* and *mtsA* gene	This study
TM1012	Deletion in *mntH1, mntH2,* and *mntH3* gene	This study
MS1001	*attB*::pYAP300–*LlsodA,* Em^r	This study
MS1002	*attB*::pYAP300–*sodA,* Em^r	This study
ATCC334	Strain isolated from cheese	ATCC
Lactococcus lactis		
ATCC19435	Type strain	ATCC
Escherichia coli		
JM109	Commercial strain purchased from Toyobo Co., Ltd.	
K-12 HfrH	High-frequency recombination strain of K-12	Our collection
BLR(DE3)	Commercial strain purchased from Merck Ltd.	
BSM4	BLR(DE3) harboring pET-21(+)–*sodA*M	This study
BES3	BLR(DE3) harboring pET-21(+)–*EcsodA*	This study
Plasmids		
pET-21(+)	Commercial plasmid purchased from Merck Ltd.	
pET-21(+)–*sodA*M	pET-21(+) carrying wild-type *sodA* with the *E. coli* Shine-Dalgarno sequence	This study
pET-21(+)–*EcsodA*	pET-21(+) carrying *E. coli sodA* with the *E. coli* Shine-Dalgarno sequence	This study
pYSSE3	*E. coli* cloning vector carrying the pUC19 *ori* region, pAMβ1 erythromycin resistance gene, and multicloning sites	[26]
pYSSE3–Δ*mntH1*-del	pYSSE3 carrying an upstream region with the N terminus of *mntH1*and downstream region with the C terminus of *mntH1*	This study
pYSSE3–Δ*mntH2*-del	pYSSE3 carrying an upstream region with the N terminus of *mntH2*and downstream region with the C terminus of *mntH2*	This study
pYSSE3–Δ*mntH3*-del	pYSSE3 carrying an upstream region with the N terminus of *mntH3*and downstream region with the C terminus of *mntH3*	This study
pYSSE3–Δ*mtsA*-del	pYSSE3 carrying an upstream region with the N terminus of *mtsA* and downstream region with the C terminus of *mtsA*	This study
pYAP300	*E. coli* cloning vector carrying the p15A *ori* region, pAMβ1 erythromycin resistance gene, phiFSW *attP* site and *int,* multicloning site, and synthetic promoter sequence active in lactobacilli upstream of the multicloning site	[26]
pYAP300–*LlsodA*	pYAP300 carrying *L. lactis* ATCC19435 *sodA*	This study
pYAP300–*sodA*	pYAP300 carrying wild-type *sodA*	This study

and the culture was incubated overnight at 20°C or 30°C. Cell-free extracts and whole cells were analyzed by SDS-PAGE with Perfect NT Gels (5–20%) (DRC, Tokyo, Japan). Concentrated protein precipitated from 50 µl of cell-free extracts by trichloroacetic acid or whole cells from 100 µl of culture were loaded onto the gels.

Treatment with paraquat

Overnight cultures grown in Mn-free MRS medium were inoculated at 1% (v/v) into Mn-free MRS medium with or without 1 µg/ml manganese. Paraquat at 0.5, 1, or 2 mM was added to the respective cultures. The cultures were grown at 37°C and the turbidity of the cultures was measured by using a Klett-Summerson photoelectric colorimeter (Klett MFG).

Measurement of manganese concentration

Overnight cultures grown in Mn-free MRS medium supplemented with 1 µg/ml manganese were inoculated at 1% (v/v) into 30 ml of Mn-free MRS medium supplemented with various concentrations of manganese. Cells were grown at 37°C for 24 hr and harvested by centrifugation at 5,000 *g* for 10 min. Cells were washed twice with H_2O and suspended in the same volume of H_2O. The amount of intracellular manganese was measured by inductively coupled plasma

Table 2. Primers used in this study

Target gene	Primer sequence (5′→3′)		Restriction enzyme site
	Forward	Reverse	
Construction of deletion mutants			
mntH1-5′	TATTCTAGAAGTGAGCGGTAAGCAG	TATCTGCAGGCGTATCTTCGAAGTGAAT	*Xba* I/*Pst* I
mntH1-3′	AATCTGCAGTGCTCATCTTCGCGAT	AATAAGCTTTTGATGCATGGACA	*Pst* I/*Hin*d III
mntH2-5′	AACAAGCTTCTCCAGTGAGGACAG	TAAGAGCTCACAATAACTCTCCCCT	*Hin*d III/*Sac*I
mntH2-3′	ATAGAGCTCACATCTTGCAGCTGTAGC	TAATCTAGAACGGTGCCAGCCGCC	*Sac*I/*Xba* I
mntH3-5′	CGCGGATCCGTCATCACAATGCCTAAAG	CCGGAATTCATAACCAACAGCAACCAAGG	*Bam*H I/*Eco*R I
mntH3-3′	CCGGAATTCATGTGACGGCTTTTCAAGG	GTTGCTGCAGCATTGGCAGG	*Eco*R I/*Pst* I
mtsA-5′	AATCTGCAGGCTTGATCGGAAGCT	TATGTCGACCCCTGCAACCATGGTT	*Pst* I/*Sal* I
mtsA-3′	ATAGTCGACGGTTTGGCGGAATA	TAATCTAGAGCAACGTTCCAGTCTC	*Sal* I/*Xba* I
Expression in *L. casei*			
sodA	TCCCCCGGGGAAAGGTTGATTCCTATGAC	GCTCTAGATCAGGCGTTTGTATCGGG	*Sma* I/*Xba* I
LlsodA	TCCCCCGGGTAAAAGGAGAAATTACTATGG	GCTCTAGATTATTTTGCTTTAGCATAAAG	*Sma* I/*Xba* I
Expression in *E. coli*			
sodA	CGGGATCCaagaaggagatatacatATGACATTTGTTTTGCCAGAT	CCGCTCGAGGGCGTTTGTATCGGGATGC	*Bam*H I/*Xho* I
EcsodA	CGGGATCCaagaaggagatatacatATACATATGAGCTATACCCTGCCAT	CCGCTCGAGTTTTTTCGCCGCAAAACGTG	*Bam*H I/*Xho* I

The restriction enzyme sites are indicated (underlined).
The sequences derived from pET21a(+) containing the Shine-Dalgarno sequence are indicated by lowercase letters.

optical emission spectrometry (ICP-OES) (Vista-PRO, Agilent Technologies, Tokyo, Japan).

Multiple alignment

Multiple alignment was performed by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

RESULTS

SOD of L. casei *strain Shirota*

L. casei strain Shirota has a *sodA* gene in its genome. The predicted amino acid sequence of SOD of *L. casei* strain Shirota was of high similarity to Mn-, Fe-, and Fe/Mn-SOD of other organisms (Fig. 1). The amino acid residues at the active sites and metal ligands [4, 28] were fully conserved (Fig. 1). The identity with Mn-SOD was slightly higher than that with Fe-SOD (e.g., *Bacillus subtilis* Mn-SOD, 56%; *L. lactis* Mn-SOD, 53%; *E. coli* Mn-SOD, 50%; *E. coli* Fe-SOD, 41%; and *P. aeruginosa* Fe-SOD, 41%). In addition, since the SOD of *L. casei* strain Shirota has the typical amino acid residues of Mn-SOD [28], *L. casei* SOD was predicted to be Mn-SOD. Further, the amino acid sequence of *L. casei* SOD also has high similarity with *B. anthracis* Fe/Mn-SOD (55% identity), which was recently reported as cambialistic SOD [29].

SOD activity of wild-type strain and recombinants

Table 3 shows the SOD activity of wild-type strain and recombinants. While the SOD activity of *L. lactis* was 45.1 U/mg protein, *L. casei* wild-type strains did not show any detectable activity. When we expressed *L. lactis sodA* and *L. casei sodA* in *L. casei* strain Shirota, *L. lactis sodA* showed SOD activity in *L. casei* strain Shirota (MS1001), while *L. casei sodA* did not show any activity even under the constitutively active promoter (MS1002).

The production of *sodA* protein was further confirmed in *E. coli* using the pET system. The SOD protein was not found in cell-free extract when the recombinant cells were cultured at or higher than at 30°C, probably due to the formation of inclusion bodies. The SOD of *L. casei* strain Shirota remained in soluble form when expression of recombinant protein was induced overnight in the presence of 1 mM IPTG at 20°C (Fig. 2). However, the SOD activity in the cell-free extract of *E. coli* BSM4 (*L. casei* SOD-expressing recombinant) did not vary regardless of induction by IPTG (Table 3). In contrast, the SOD activity in the cell-free extract of *E. coli* BES3 (*E. coli* SOD-overexpressing recombinant) was increased by IPTG induction (Table 3). From these results, it was suggested that the SOD protein of *L. casei* strain Shirota, even if it is produced, is in an inactive form.

Effect of SOD and manganese on SOD activity and oxidative stress resistance

We evaluated the effect of SOD and manganese with respect to oxidative stress. We examined the effect of paraquat, which is a superoxide generator, on growth by using the wild-type *L. casei* strain Shirota and the recombinant strain MS1001 (*L. casei* expressing *L. lactis sodA*), with or without 1 μg/ml manganese (Fig. 3). Growth of both strains was partly inhibited in Mn-free medium. Further, the growth of the wild-type strain in the absence of manganese was inhibited by the addition of paraquat, whereas the growth inhibition caused by paraquat was partially recovered by expression of the *L. lactis sodA* gene (Fig. 3c, 3d). However, in the presence of 1 μg/ml manganese, the addition of paraquat did not affect growth in either strain (Fig. 3a, 3b).

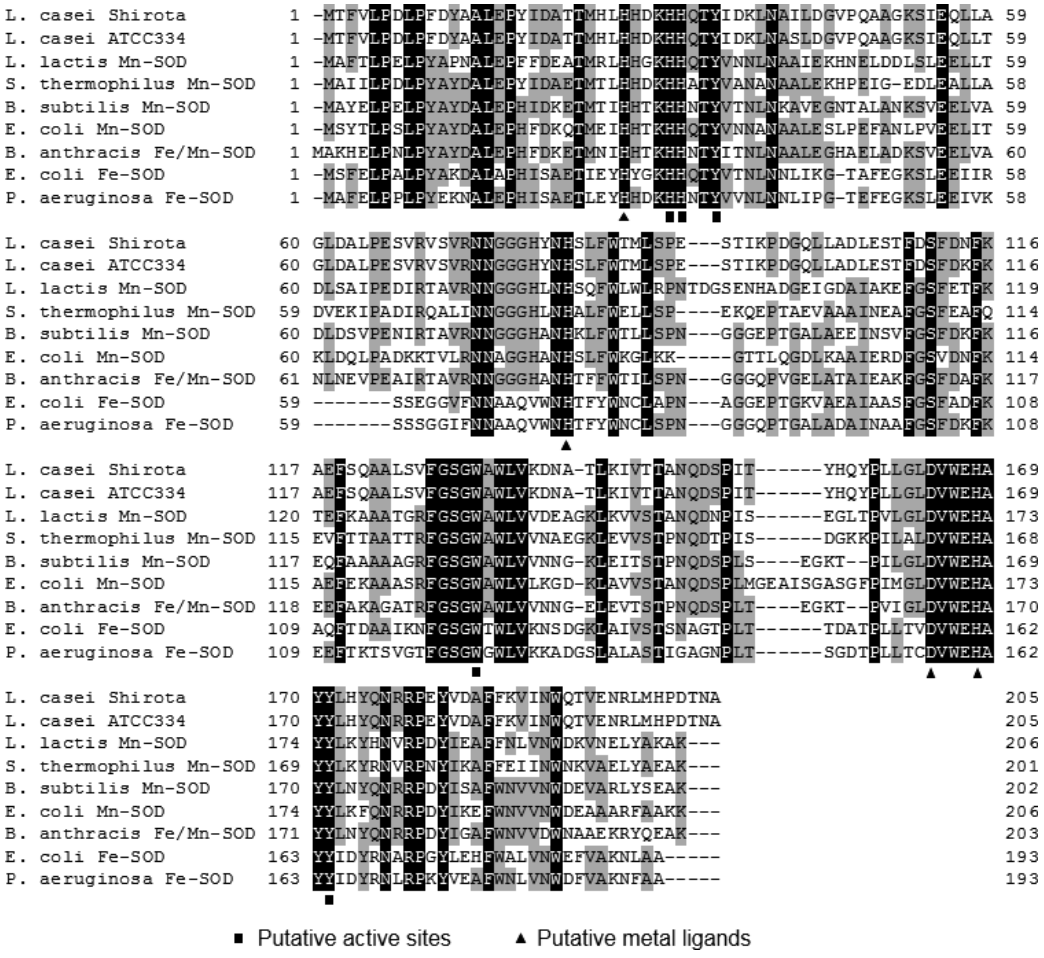

Fig. 1. Multiple alignment of the amino acid sequences of superoxide dismutase in various species. The residues with a black background represent residues conserved in all sequences; those with a grey background represent residues conserved in at least 50% of all sequences. Putative active sites and putative metal ligands are indicated by square and triangle symbols, respectively.

Table 3. SOD activity of lactic acid bacteria and *E. coli.*

Strain	SOD activity (U/mg protein)		
	IPTG+	IPTG–	
L. casei Shirota	ND	-	-
L. casei ATCC334	ND	-	-
L. lactis ATCC19435	45.1	-	-
L. casei MS1001	11.0	-	-
L. casei MS1002	ND	-	-
E. coli BES3 (20°C)	-	100.4	55.6
E. coli BSM4 (20°C)	-	32.8	38.4

ND: not detected.
Temperatures in parentheses are those when the gene expression was induced.

The SOD activity of wild-type *L. casei* and MS1001 was measured with or without EDTA in the reaction mixture (Fig. 4). The SOD activity with EDTA indicates the activity caused by an enzymatic reaction, while that without EDTA indicates the activity caused by both enzymatic and nonenzymatic (manganese) reactions. We did not detect SOD activity in the wild-type *L. casei* in the presence of EDTA in the reaction mixture, while the SOD activity of MS1001 was detected after adding EDTA to the reaction mixture. This SOD activity remained the same regardless of the manganese concentration of the medium. However, when the reaction mixture contained no EDTA, the SOD activities of both strains increased in proportion to the concentration of manganese in the medium. In Mn-rich medium in particular, the SOD activity derived from manganese was markedly higher than the activity derived from the enzyme.

Effect of manganese transporters on manganese accumulation

We found one ABC-type manganese transporter (*mtsCBA*)

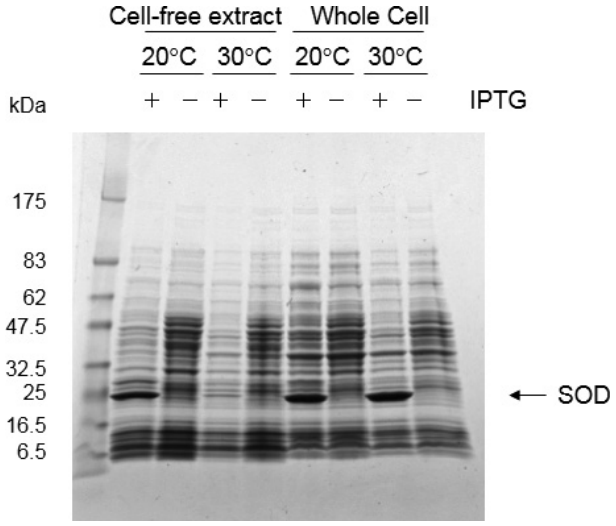

Fig. 2. SDS-PAGE analysis of *L. casei* strain Shirota SOD expression in *E. coli*. *E. coli* BSM4 (harboring pET-21(+)–*sodA*M) was grown at 30°C in LB medium containing 100 μg/ml ampicillin until OD_{600}=0.6. Then, IPTG (1 mM) was added for SOD induction, and the culture was grown overnight at 20°C or 30°C. Concentrated protein was precipitated from 50 μL of cell-free extracts by trichloroacetic acid, and whole cells from 100 μL cultures were loaded onto the gel. The arrow indicates SOD proteins.

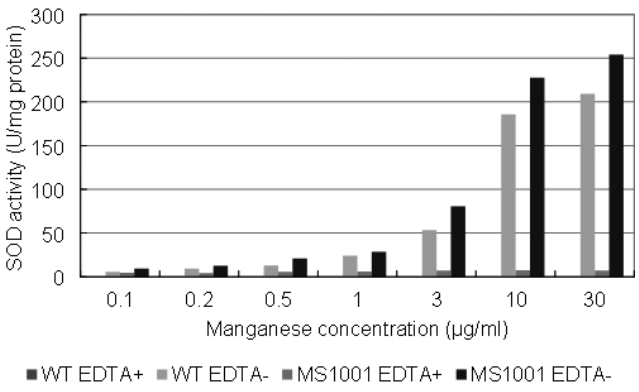

Fig. 4. SOD activity of *L. casei* strain Shirota (WT) and *L. casei* MS1001 (expressing *L. lactis sodA*). Cell-free extracts of cells grown overnight were measured with or without EDTA in the mixture.

and three NRAMP-type manganese transporters (*mntH1*, *mntH2*, and *mntH3*) in the genome of *L. casei* strain Shirota. However, we did not find the P-type ATPase *mntA*. In order to clarify the role of these transporters, we disrupted *mntH1*, *mntH2*, *mntH3*, and *mtsA*, which codes a solute-binding protein of the ABC-type transporter, in different combinations. Then, we measured the intracellular manganese concentrations in the wild-type strain and in manganese transporter–deficient

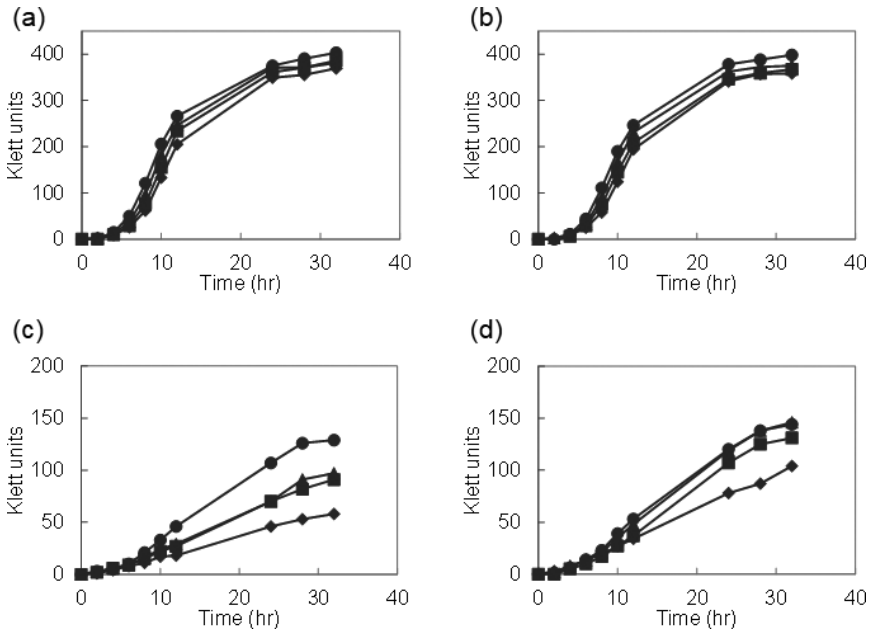

Fig. 3. Effect of paraquat on the growth of *L. casei* strain Shirota and *L. casei* MS1001 (expressing *L. lactis sodA*). Overnight cultures grown in Mn-free MRS medium were inoculated at 1% (v/v) into Mn-free MRS medium with or without 1 μg/ml manganese. The growth of *L. casei* strain Shirota with manganese (a) or without manganese (c). The growth of *L. casei* MS1001 with manganese (b) or without manganese (d). ●, no paraquat; ▲, 0.5 mM paraquat; ■, 1.0 mM paraquat; and ♦, 2 mM paraquat.

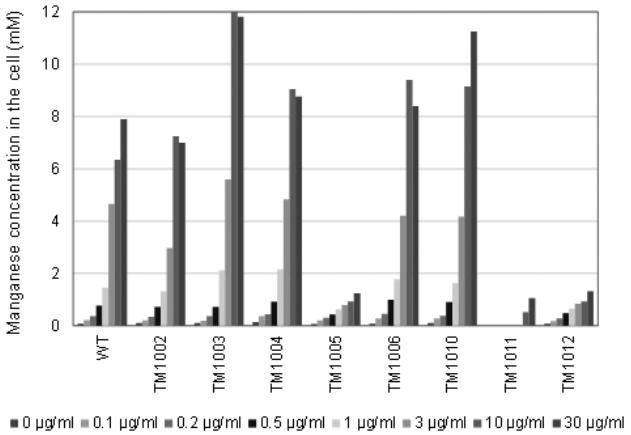

Fig. 5. Intracellular manganese accumulation by *L. casei* strain Shirota and manganese transporter–disrupted mutants. Cells were grown in Mn-free MRS medium supplemented with various concentrations of manganese. Manganese concentrations in the cells are represented as mmol per whole washed pellet volume.

mutants grown overnight in Mn-free MRS medium with various concentrations of manganese (Fig. 5). The wild-type cells incorporated manganese in a concentration-dependent manner, and almost all manganese in the medium up to 1 μg/ml was incorporated into the cells. However, at higher concentration of manganese, the uptake increased gently. The uptake of manganese was not affected in the mutant cells in which only one of the four predicted manganese transporter genes was disrupted (TM1002, TM1003, TM1004), even though a variation in uptake was seen at high concentrations of manganese. On the other hand, TM1005, which had deletion mutations in two genes (Δ*mntH1*/Δ*mntH2*), showed marked reduction of manganese uptake. Furthermore, TM1011, a triple mutant (Δ*mntH1*/Δ*mntH2*/Δ*mtsA*), could not grow and could not accumulate manganese in the medium supplemented with lower manganese concentrations (≤3 μg/ml). However, TM1011 grew marginally and showed residual manganese incorporation activity in the medium supplemented with higher manganese concentrations. On the other hand, the manganese uptake pattern of another triple mutant, TM1012 (Δ*mntH1*/Δ*mntH2*/Δ*mntH3*), was quite similar to that of TM1005 (Δ*mntH1*/Δ*mntH2*).

DISCUSSION

SOD is an enzyme that catalyzes dismutation of superoxide anion and is important for defense against oxygen toxicity. Many lactobacilli do not have SOD activity; however, oxygen-tolerant *L. casei* and *L. plantarum* are able to scavenge superoxide with high intracellular levels of accumulated manganese [11, 12]. Lin *et al.* [13] and Liu *et al.* [14] recently reported that some strains of *L. casei* encode a *sodA* gene in the genome and have SOD activity. As we describe in this report, it is true that *L. casei* strain Shirota possesses the *sodA* gene sequence in its genome. The predicted amino acid sequence of the *sodA* gene was highly similar to those of Mn-, Fe-, and Fe/Mn-SOD of other bacterial species, and the amino acid residues of putative metal ligands and putative active sites were conserved, although we could not identify its cofactor through homology analysis. When the *sodA* of *L. casei* strain Shirota was expressed in *L. casei* strain Shirota or in *E. coli*, we failed to detect its activity. Because the activity of SOD derived from *L. lactis* used as a positive control could be detected in *L. casei* strain Shirota and because the SOD protein of *L. casei* strain Shirota expressed in *E. coli* could be detected in soluble form, it is most probable that the product is not functional even though the protein is synthesized.

In *L. casei* and *L. plantarum*, the ability of manganese to scavenge superoxide was determined from the results of experiments in which the SOD activity was inhibited by dialysis and treatment with EDTA [11, 12]. In this study, although the SOD activity of *L. casei* strain Shirota was lost when EDTA was added, the activity was increased depending on the manganese concentration added to the medium in the absence of EDTA, and a correlation between the SOD activity and the concentration of manganese in the cells was found (Fig. 4). Furthermore, when we examined tolerance to superoxide of the wild-type strain and a recombinant strain that express *L. lactis sodA*, suppression of growth by paraquat was observed only when they were cultured in Mn-free medium. The presence of *L. lactis sodA* partially recovered their growth (Fig. 3), meaning that both manganese and SOD are involved in quenching superoxide toxicity. Because the SOD activity derived from *L. lactis sodA* was less than the superoxide elimination activity caused by manganese in the recombinant (Fig. 4), it is considered that the effect of *L. lactis* SOD was not detected when enough manganese was added to the medium. Although there are reports that heterologous SOD expression improves oxidative stress resistance in some lactic acid bacteria [30, 31], most of the species examined in those studies were sensitive to oxidative stress or were species that did not have SOD activity. Meanwhile, although *L. casei* strain Shirota has a *sodA* gene, its SOD activity is suspected to have been lost in the course of evolution because the lost SOD activity is sufficiently compensated for by the much stronger superoxide elimination activity of intracellular manganese.

Recently, some *L. casei* strains have been reported to have SOD activity [13, 14]. Our results supported the results of Archibald *et al.* indicating that *L. casei* had no SOD activity [11, 12]. On the other hand, the *sodA* sequences of *L. casei* strain Shirota and *L. casei* Lc18 are perfectly identical, and it is not clear what caused the difference in the results of Liu *et al.* [14]. One possibility is that the SOD assay methods used by the two groups were different from each other. In particular, since free manganese has superoxide elimination activity and has a strong influence on the apparent SOD activity, it is important to eliminate the possible influence of manganese by adding EDTA in the assay system. Therefore, we think that there is a necessity to reevaluate whether those

activities were derived from true SOD or from superoxide scavenging activity of intracellular manganese.

We further investigated the mechanism of manganese uptake of *L. casei* strain Shirota. Because single-gene disruption mutants did not affect the phenotype, multiple genes were suggested to have complementary functions (Fig. 5). The quantity of manganese uptake of the Δ*mntH1*/Δ*mntH2* double disruption mutant was markedly reduced, and the triple disruption mutant with additional *mtsA* disruption was not able to take up manganese at concentrations of up to 3 μg/ml in the medium (Fig. 5). Therefore, these three genes are suggested to have a pivotal role in enabling sufficient accumulation of intracellular manganese. However, the Δ*mntH1*/Δ*mntH2*/Δ*mtsA* triple disruption mutant can grow and take up manganese slightly under high manganese concentration conditions (Fig. 5). Thus, other relevant genes might still function. However, as the triple gene disruption mutant could hardly grow at all, we conclude that NRAMP-type manganese transporters coded by *mntH1*and *mntH2* and an ABC-type manganese transporter coded by the gene cluster of *mtsCBA* are the transporters that are responsible for accumulating all or majority of the intracellular manganese in *L casei* strain Shirota.

Taken together, SOD is defective in *L. casei* strain Shirota and intracellular manganese accumulated by multiple manganese transporters mainly functions to scavenge superoxide.

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