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ORIGINAL ARTICLE

Cloning and heterologous expression of the manganese superoxide dismutase gene from *Lactobacillus casei* Lc18

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Abstract Many of the species of *Lactobacillus* can be considered to be "probiotics" with a variety of benefits, including imparting antioxidative effects to the host. *Lactobacillus* species evolve different mechanisms to defend themselves against oxygen toxicity, such as superoxide dismutases (SODs), hydroperoxidases and high intracellular levels of metal ions. The SODs provide a cellular defense mechanism against oxidative stress by scavenging O_2^-. Most *Lactobacillus* species appear to lack a *sod* gene. To date, only two species of *Lactobacillus* (*Lactobacillus casei* and *L. sakei*) may have *sod* genes, as evidenced by sequence analysis of the genome, but no experimental verification, including cloning and heterologous expression of the *Lactobacillus sod* gene, has been reported. It is therefore unknown whether these *sod* genes can express SOD or are functional. We have PCR-amplified the gene from *L. casei* Lc18 that encodes the SOD using primers designed according to the genome of *L. casei* ATCC 334 and ligated it into the vector pET-28a(+) for heterologous expression in *Escherichia coli* BL21(DE3). After being induced with IPTG, the fusion protein was efficiently expressed in a soluble form. The superoxide radical scavenging activity of the recombinant strain was found to be increased relative to that of the control strain. The SOD was also purified by nickel ion affinity chromatography and found to consist of a single band, as determined by SDS-PAGE analysis, with an activity of 39.97 U/mg. N-terminal amino acid sequence analysis indicated that it may be a manganese-containing SOD. This is the first report of a *sod* gene from *Lactobacillus* spp. being expressed in other prokaryotes.

Keywords *Lactobacillus casei* Lc18 · Antioxidative activities · Manganese superoxide dismutase · Heterologous expression

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Introduction

Probiotics are living microorganisms that impart health benefits to the host beyond inherent basic nutrition upon ingestion in certain numbers (Guarner and Schaafsma 1998). Lactobacilli are the most widely used probiotics and are considered to be key commensals involved in the promotion of the host's health. Various studies have indicated that lactobacilli may alleviate lactose intolerance, have a positive influence on the intestinal flora of the host, stimulate or modulate mucosal immunity, reduce blood cholesterol and impart antioxidative effects to the host (Vizoso Pinto et al. 2006; Wang et al. 2009). Among these potential health-promoting benefits, the antioxidative effect is one of the most interesting properties.

Oxidative stress is the adverse effect of oxidants on physiological function. Oxidative stress, which has been implicated in the progression of aging and disease, occurs when abnormally high levels of reactive oxygen species (ROS) are generated, resulting in DNA, protein and lipid damage (Kullisaar et al. 2002). During the past decade, oxidative stress and antioxidative potency have been identified as key points in the molecular regulation of

cellular stress responses. Free radicals, such as the superoxide radical anion (O_2^-) and hydroxyl radical ($OH^·$), and other ROS are considered to be highly potent oxidants that can react with all biomacromolecules in living cells and are associated with carcinogenesis and mutagenesis (Kodali and Sen 2008). The radicals may cause oxidative damage by oxidizing biomolecules, thereby resulting in cell death and tissue injury.

Various synthetic and natural antioxidants have been reported (Brioukhanov and Netrusov 2004; Sánchez-Venegas et al. 2009); however, the safety and long-term effects of synthetic antioxidants on health are uncertain. Accordingly, antioxidants from natural sources are both more desirable (Lin and Yen 1999) and more valuable. Among natural antioxidants, live microorganisms have the highest applicability. *Lactobacillus* spp., which are generally recognized as safe microorganisms with respect to human health (Adams and Marteau 1995), are commonly used in the fermentation of foods, including dairy, meat, vegetable and bakery products. Some of them are reported to have excellent antioxidative activities in the host, where they are able to decrease the risk of an accumulation of ROS during the ingestion of food, while others apparently lack these activities (Korpela et al. 1997).

Some protection against ROS in aerobes and facultative anaerobes (such as *Lactobacillus*) is provided by antioxidative defense enzymes, one of the most important of which is superoxide dismutase (SOD) (Brioukhanov and Netrusov 2004). The SODs (EC 1.15.1.1), which provide a cellular defense mechanism against the toxicity of oxygen by catalytically scavenging O_2^- (Archibald and Fridovich 1981), are metalloenzymes that catalyze the conversion of the superoxide anion into hydrogen peroxide and dioxygen. This significant antioxidative activity is the basis for the increased resistance of some *Lactobacillus* strains to toxic oxidative compounds, allowing them to survive in the host, and to serve as defensive components in the intestinal microbial ecosystem (Kullisaar et al. 2002).

The SOD enzymes can be distinguished into three types based on the composition of their metal center: manganese, copper–zinc or iron. SODs are found across a broad range of organisms that utilize one, two, or all three types to meet their antioxidant needs (Hassan 1997). The majority of SODs comprise two identical subunits, each containing a metal ion, a disulfide bridge, a sulfhydryl group and an acetylated terminal amino group. The molecular weights of Cu,ZnSODs are approximately 32 kDa, and they are homodimers. Prokaryotes were initially believed to possess FeSODs and/or MnSODs which, unlike the Cu,ZnSODs of eukaryotes, are insensitive to the cyanide radical (CN^-). However, Cu,ZnSODs have since also been identified in prokaryotes, and these are also inhibited by CN^-. FeSODs and MnSODs are highly homologous and have similar three-dimensional structures, molecular weights of approximately 40 kDa (2×20 kDa) and similar metal-binding ligands in the active site of the enzyme (Meile et al. 1995). MnSODs are cambialistic (functionally active with either metal) SODs of anaerobic microorganisms (Brioukhanov and Netrusov 2004); they were first isolated from *Escherichia coli* and found to be unlike the corresponding enzymes of eukaryotes with respect to all parameters except their catalytic activity (Keele et al. 1970).

Until recently, most lactobacilli were believed to lack SODs (Bruno-Bárcena et al. 2004); however, *Lactobacillus plantarum* and several other aerotolerant lactobacilli have been identified as possessing a nonenzymatic dismutation system based on the accumulation of high intracellular levels of Mn^{2+}, which can stoichiometrically remove O_2^- (Archibald and Fridovich 1981). Analyses of genome sequences indicate that *L. casei* and *L. sakei* likely contain the *sod* gene, but the presence of these genes and the expression and activity of SOD have not yet been demonstrated by experimental methods, such as cloning or heterologous expression.

In this study, the *sod* gene from *Lactobacillus casei* Lc18 was heterologously expressed in *E. coli* BL21(DE3). The fusion protein (SOD enzyme) was then purified and its antioxidative activity detected.

Materials and methods

Bacterial strains and plasmids

The bacterial strains, plasmids and primers used in this study are listed in Table 1. *Escherichia coli* DH5α was used for gene cloning and *E. coli* BL21(DE3) was used as a host for the expression of the recombinant proteins. *L casei* Lc18 and Lc2 and *L. acidophilus* LA11 and LA12 were provided by Shanghai Jiao Da Onlly Co. (Shanghai, PR China. The *L. casei* and *L. acidophilus* strains were cultivated in De Man, Rogosa and Sharpe (MRS) medium without shaking at 37°C for 18 h to reach the stationary phase. The *E. coli* strains were cultivated in Luria–Bertani (LB) broth with shaking (200 rpm) at 37°C for 12 h to reach the stationary phase. *E. coli* transformants were selected with 100 μg/ml of ampicillin or 50 μg/ml of kanamycin. As soon as the OD_{600} of recombinant *E. coli* BLSod reached 0.6, isopropyl β-*D*-1-thiogalactopyranoside (IPTG) was added to 1 mM to induce protein expression.

Chemicals and enzymes

Restriction enzymes (*Nde*I and *Bam*HI), Thermus aquaticus DNA Polymerase, LA Taq polymerase and T4 DNA ligase were purchased from TaKaRa (Shiga, Japan). All chemicals

Table 1 Bacterial strains, plasmids and primers used in this study

Strain, plasmid or primer	Characteristic(s)	Reference or source
Strains		
Escherichia coli DH5α	*rec* cloning strain	TaKaRa (Shiga, Japan)
E. coli BL21(DE3)	Protein expression strain	Novagen (Madison, WI)
E. coli BLSod	Recombinant BL21(DE3) harboring pETsod	This study
Lactobacillus casei Lc18		This study
L. casei Lc2		This study
Plasmids		
pMD19-T	TA-cloning	TaKaRa
pET-28a(+)	Expression vector, T7 promoter, His-tag	Novagen
pETsod	0.69-kb sod PCR amplicon from L. casei Lc18 cloned into pET-28a(+)	This study
Primers		
SS (*Nde*I restriction site underlined)	5'-CAAACAAGAAAGGTTGATTCATATG-3'	This study
SR (*Bam*HI restriction site underlined)	5'-AAATTTACGGATCCTTTTTCGG-3'	This study
M13-47	5'-CGCCAGGGTTTTCCCAGTCACGAC-3'	TaKaRa
RV-M	5'-GAGCGGATAACAATTTCACACAGG-3'	TaKaRa

and antibiotics used were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Construction of the expression vector

Lactobacillus genomic DNA was isolated according to Sambrook et al. (2001) with some modifications. Briefly, 50 μl of proteinase K was added during the lysing step, after which the mixture was incubated for 30 min at 37°C, followed by the addition of 600 μl of phenol/chloroform/isoamyl alcohol (25:24:1). The extracted DNA was electrophoresed on a 1% agarose gel, quantified spectrophotometrically at 260 nm and diluted to 50 ng/μl for PCR.

The coding sequence of SOD was subjected to PCR amplification from the chromosomal DNA template of *L. casei* Lc18 using a forward primer containing a *Nde*I restriction site SS and a reverse primer containing a *Bam*HI restriction site SR. These two primers were derived from the whole-genome sequences of *L. casei* ATCC 334 using Primer Premier software, ver. 5.0 (PREMIER Biosoft Int, Palo Alto, CA).

The PCR reactions were carried out in a 50-μl volume containing 100 ng DNA template solution, 5 μl of 10× LA PCR Buffer II (Mg^{2+} plus), 2.5 U of LA Taq Polymerase, 10 nmol of each deoxyribonucleoside triphosphate and 10 pmol of each primer. The PCR reaction was performed under following conditions: an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 40 s, and extension at 72°C for 40 s, with a final elongation step at 72°C for 10 min. All PCRs were carried out with Eppendorf Mastercycler ep gradient S (Hamburg, Germany). The PCR product, which was 700 bp, as expected, was purified by using the Agarose Gel DNA Purification kit ver.2.0 (TaKaRa), ligated into the vector pMD19-T and used to transform competent cells of *E. coli* DH5α with heat shock according to the manufacturer's instructions. Transformants were identified using primers M13-47 and RV-M. The nucleotide sequences were determined by AmlipTaq FS DNA polymerase fluorescent dye terminator reactions by means of an Applied Biosystems 3730 automated sequencer (Foster City, CA).

The purified PCR products were digested with *Nde*I and *Bam*HI and ligated to the pET-28a(+) expression vector, yielding the pETsod plasmid (Fig. 1). Restriction enzymes, T4 ligase and other DNA-modifying enzymes were used according to the protocols of the manufacturers. The recombinant plasmid pETsod was introduced into competent *E. coli* BL21(DE3) according to Tu et al. (2005). Three positive transfomants were grown in liquid LB medium containing 50 μg/ml kanamycin and identified through PCR and restriction enzyme digestion.

Preparation of dialyzed cell-free extracts

Fresh single colony-forming units (CFUs) of each test strain were inoculated into 10 ml of proper culture medium. After incubation at 37°C, total cell numbers were adjusted to 10^{10} CFU/ml, then cells of each strain were harvested by centrifugation at 5000 *g* for 10 min. Cell pellets were then quickly washed twice and resuspended in deionized water followed by ultrasonic disruption (5 min, 200 W, 50% working time) on a Sonicator JY96-II (Scientz Biotechnology, Ningbo, China) with constant cooling on ice, The supernatant

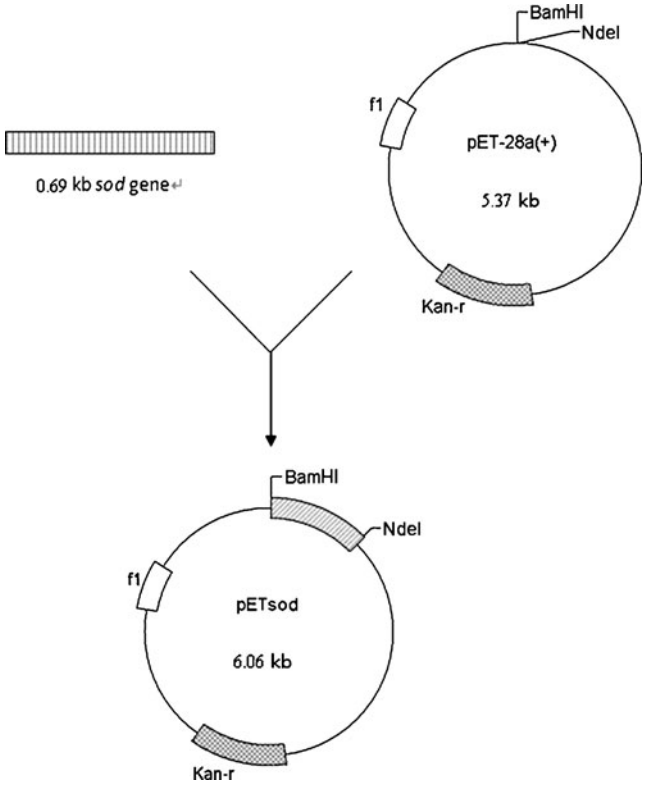

Fig. 1 Construction of the pETsod plasmid. *BamHI*, *NdeI* Restriction enzymes, *sod* superoxide dismutase, *kan* kanamycin, *r* resistance

(cell-free extract, CFE) was recovered and used immediately in subsequent experiments.

Antioxidant activity assays

Measurement of superoxide radical scavenging activity

Superoxide radical scavenging activity was measured by the method of Nishikimi et al. (1972). Briefly, 1 ml of nitroblue tetrazolium (NBT) solution (156 μM in 100 mM phosphate buffer, pH 7.4), 1 ml of coenzyme I reduced disodium salt (NADH) solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of fresh sample or distilled water (control) were mixed. The reaction was started by adding 100 μl of phenazine methyl sulfate (PMS) solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The mixture was then incubated at 25°C for 5 min and the absorbance measured at 560 nm. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The scavenging activity of superoxide radical was calculated as follows:

$$\text{Superoxide radical scavenging activity}\,(\%) = \left[1 - A_{\text{Sample}}/A_{\text{Control}}\right] \times 100.$$

Measurement of hydrogen peroxide scavenging activity

Hydrogen peroxide scavenging activity was measured according to Pick and Keisari (1980) with minor modification. Briefly, 50 μl of 5 mM H_2O_2 solution and 50 μl of fresh sample or distilled water (control) were mixed and incubated at room temperature for 20 min. Then, 100 μl of horseradish peroxidase (HRP)–phenol red solution (300 μg/ml HRP and 4.5 mM phenol red in 100 mM phosphate buffer) was added and after 10 min of incubation, the sample absorbance was monitored at 610 nm. The scavenging effect was calculated according to the following equation:

$$\text{Hydrogen peroxide scavenging activity}\,(\%) = \left[1 - A_{\text{Sample}} / A_{\text{Control}}\right] \times 100.$$

Expression, purification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the recombinant protein

A single colony of recombinant *E. coli* BLSod was inoculated in 2 ml LB medium containing 50 μg/ml kanamycin and cultivated at 37°C at 200 rpm. The overnight culture was diluted 100-fold into 50 ml of fresh LB medium containing 50 μg/ml kanamycin, grown to an OD_{600} of about 0.6 and then induced with 1 mM isopropylthiogalactoside (IPTG). The culture was subsequently incubated to express the fusion protein. After 9 h, wet cells were collected from 50 mL of bacteria culture by centrifugation and resuspended in 5 ml of Native Binding Buffer (50 mM NaH_2PO_4, 0.5 M NaCl, pH 8.0); 8 mg lysozyme was then added and the mixture incubated on ice for 30 min and subjected to ultrasonication. In order to determine whether the recombinant SOD protein was expressed, or not, and if expressed whether it is in the form of inclusion bodies, the bacteria cells were sonicated and separated into soluble and insoluble fractions by centrifugation at 10,000 *g* at 4°C for 10 min. Both the soluble and insoluble fractions were analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to the protocol of the manufacturer (Bio-Rad, Hercules, CA). The ultrasonicated supernatant (soluble fraction) was then subjected to His-tag purification and purified by the Ni-NTA Purification system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Both the crude and purified protein fractions were boiled for 5 min and analyzed by SDS–PAGE. Proteins were visualized by Coomassie brilliant blue staining.

Measurement of purified SOD activity

Enzyme activity was measured as described above. One unit of SOD activity was defined as the amount of purified

enzyme that consumed 1 μmol of PMS from the substrate per minute. The specific activity was defined as units of activity per milligram of purified protein (purified SOD).

The protein concentrations were determined by the Lowry method (Liong and Shah 2005) using bovine serum as the standard.

Amino acids sequence analysis

The deduced amino acid sequence of the *sod* gene was analyzed using BioEdit software ver. 7.0.1.

Nucleotide sequence accession number

The *sod* gene nucleotide sequences and the 16S rDNA gene of *L. casei* Lc18 and Lc2 has been deposited in the GenBank database under accession numbers HM070825, HM070024, HM070025.

Results

Construction and Identification of recombinants

The 700-bp PCR-fragment cloned from *L. casei* Lc18 was identified as the *sod* gene by a BLAST search of the GenBank database. The cloned *sod* gene contained an 618-bp open reading frame (ORF) that encoded a protein of 205 amino acids with a predicted molecular weight of 23.3 kDa. The recombinant plasmid pETsod was demonstrated to be correct by restriction enzyme digestion (Fig. 2) and DNA sequencing (nucleotide sequence accession number HM070825). Figure 2 shows that the recombinant plasmid pETsod was successfully constructed and transformed into *E. coli* BL21(DE3) to form the recombinant strain *E. coli* BLSod.

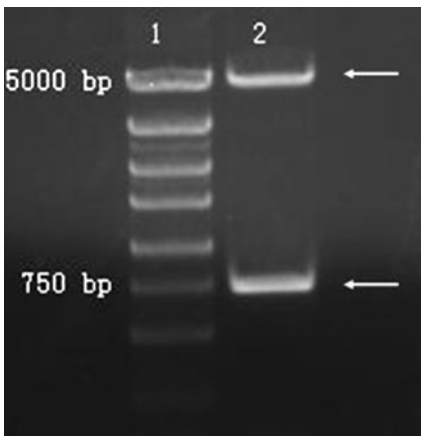

Fig. 2 Identification of recombinant plasmid pETsod by restriction enzyme digestion. *Lanes*: *1* 5-kb DNA ladder, *2* digestion of pETsod. *Arrows* Two digestion products; *upper band* digested plasmid vector, *lower band sod* gene

Expression and purification of the recombinant protein

Using the T7 promoter and the N-terminal His-tag of pETsod in recombinant *E. coli* BLSod, the expression of the fusion protein was induced by incubation in the presence of 1 mM IPTG at 37°C for 9 h when the OD_{600} reached 0.6. SDS–PAGE analysis of the soluble and insoluble fractions revealed that most of the induced protein was present in the soluble fraction. SDS–PAGE also revealed a distinct band of the expected size (about 24 kDa) in the recombinant *E. coli* BLSod lane following induction with 1 mM IPTG (Fig. 3, lane 4) and the absence of this band in the controls, namely, BL21(DE3) (Fig. 3, lane 1), BL21(DE3) harboring pET-28a(+) (Fig. 3, lane 2) and BLSod without induction (Fig. 3, lane 3). These findings indicate that the SOD protein may be expressed normally in *E. coli* BLSod with the induction of IPTG. We then used the Ni-NTA purification system to confirm these results. Following nickel ion metal affinity chromatography, the final eluate (the purified target protein) produced a single distinct band (Fig. 3, lane 7) that had the same size as the expected SOD (about 24 kDa) in lane 4 of Fig. 3; there was no similar band in the other two controls, BL21(DE3) (Fig. 3, lane 5) and BL21(DE3) harboring pET-28a(+) (Fig. 3, lane 6). An enzyme activity test of the purified SOD revealed that the enzyme activity of O_2^- scavenging was 39.97 U/mg. An additional band of about 27 kDa was visualized in both lanes 4 and 7, which was interpreted as an endogenous protein of BL21(DE3) containing several seriate histidines that was induced by IPTG.

Antioxidative activity of the tested strains

Table 2 shows antioxidative activities, including superoxide radical scavenging activity and hydrogen peroxide scavenging activity, from both intact cells and CFEs of *E. coli* BL21 (DE3), *E. coli* BLSod, *L. casei* Lc18, *L. casei* Lc2, *L. acidophilus* LA11 and *L. acidophilus* LA12.

The results suggested that both the intact cells and CFEs of all six strains had some antioxidative ability and that the abilities of the CFEs were generally higher than those of intact cells. The antioxidative abilities of both *L. acidophilus* are quite low compared to the other four strains. The superoxide radical scavenging activity of *E. coli* increased from 37.17 to 43.24% (intact cells) and from 44.31 to 55.20% (CFEs) after recombination with the *sod* gene; the hydrogen peroxide scavenging activity also increased, from 52.08 to 55.37% (intact cells) and from 51.04 to 58.15% (CFEs) after recombination with *sod* gene.

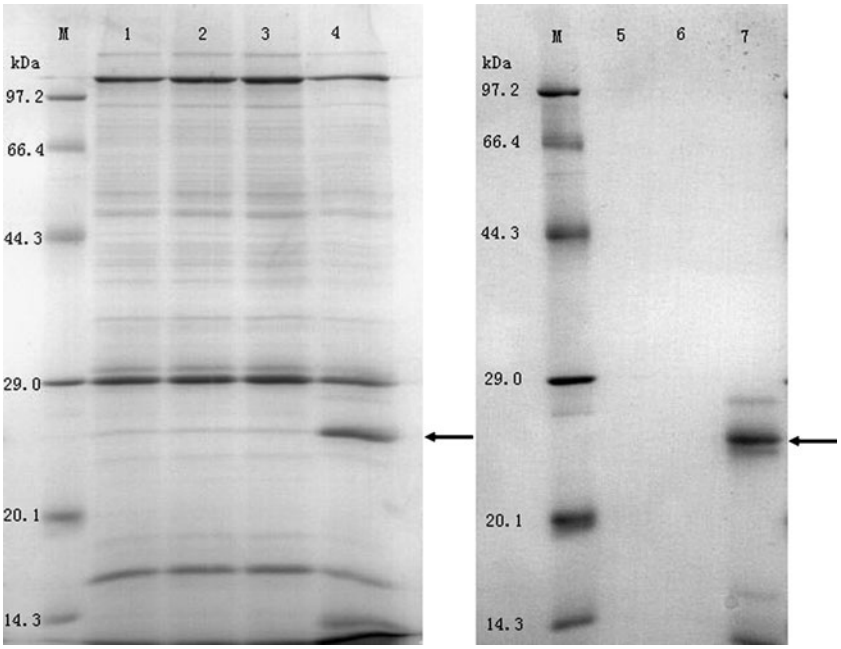

Fig. 3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of SOD heterologous expression in *Escherichia coli* BLSod. Isopropyl β-*D*-1-thiogalactopyranoside (IPTG) was added to the growth medium at a concentration of 1 mM for SOD induction, and the culture was then incubated at 37°C for 9 h prior to the analysis of cellular proteins. *Lanes*: *M* pre-stained protein molecular weight marker, *1* total protein of control strain [*E. coli* BL21(DE3)], *2* total protein of control strain [*E. coli* BL21(DE3) harboring pET-28a(+)], *3* total protein of recombinant strain BLSod [*E. coli* BL21(DE3) harboring pETsod] without induction of IPTG, *4* total protein of recombinant strain BLSod [*E. coli* BL21(DE3) harboring pETsod] with induction of IPTG, *5* purified protein of control strain [*E. coli* BL21(DE3)] by Ni-NTA purification system, *6* purified protein of control strain [*E. coli* BL21(DE3) harboring pET-28a(+)] by Ni-NTA purification system, *7* purified recombinant SOD of strain BLSod with IPTG induction by Ni-NTA purification system. *Arrows* Target SOD proteins

Sequence analysis and comparison

The results of the antioxidant activity assays and recombinant protein analysis demonstrated that the *sod* gene was amplified successfully from the genome DNA of *L. casei* Lc18. A BLAST search of the GenBank database revealed that the *sod* gene of *L. casei* Lc18 is quite different from that of many other species, including humans, animals, plants and other bacteria. However, a homology comparison of the sequence of the *L. casei* Lc18 *sod* gene with other SOD protein sequences revealed that the SOD protein sequence deduced from the *sod* gene of *L. casei* Lc18 is more similar to the three

Table 2 Antioxidative activities of the tested strains

Strain	O_2^- scavenging activity (%)	H_2O_2 scavenging activity (%)
Intact cells		
BL21(DE3)	37.17±0.47	52.08±1.73
BLSod	43.24±2.29	55.37±3.71
L. casei Lc18	49.37±2.58	17.40±1.37
L. casei Lc2	69.95±3.27	43.59±2.11
L. acidophilus LA11	10.42±2.86	5.48±1.50
L. acidophilus LA12	5.48±2.74	9.07±1.27
Cell-free extracts		
BL21(DE3)	44.31±1.09	51.04±2.94
BLSod	55.20±3.11	58.15±4.70
L. casei Lc18	58.91±2.63	21.74±3.37
L. casei Lc2	75.21±3.41	47.37±1.88
L. acidophilus LA11	7.14±1.24	3.44±0.29
L. acidophilus LA12	9.00±2.01	6.74±0.98

Data are given as the mean ± standard deviation of three independent experiments.

MnSOD protein sequences from *Streptococcus thermophilus* (Bruno-Bárcena et al. 2004), *L. lactis* (Sanders et al. 1995) and *E. coli* (Roy et al. 1993) (Fig. 4) than to the two FeSODs from *Pseudomonas ovalis* (Isobe et al. 1987) and *Photobacterium leiogathi* (Barra et al. 1987). The iron- and manganese-containing SODs can be distinguished by analysis of their primary structures (Parker and Blake 1988). The size of the *L. casei* Lc18 SOD (205 amino acid residues) is within the range of the size of SODs from the five aforementioned bacteria (194–206 residues) (Fig. 4). The amino acids involved in the binding of metal ligands are present in the *L. casei* Lc18 SOD (His-27, His-82, Asp-174 and His-178; Fig. 4), as are the postulated active-site residues (Parker et al. 1987; Parker and Blake 1988). The *L. casei* Lc18 SOD contains the typical residues (Gly-76, Gly-77, Phe-85, Gln-153, Asp-154; Fig. 4) of MnSODs and none of the typical residues (Gla-76, Gln-77, Thr-85, Gla-153, Gly-154; Fig. 4) of FeSODs (Parker and Blake 1988), which indicates that the *L. casei* Lc18 SOD belongs to the MnSODs.

Discussion

The SODs are very important because they provide a cellular defense mechanism against the toxicity of oxygen by catalytically scavenging O_2^-. During the past 20 years, many *sod* genes have been cloned from various organisms, including *E. coli* (Roy et al. 1993), *L. lactis* (Sanders et al. 1995), *P. ovalis* (Isobe et al. 1987), *S. thermophilus* (Bruno-Bárcena et al. 2004), *Trichoderma harzianum* (Yang et al. 2009), *Deschampsia antarctica* (Sánchez-Venegas et al. 2009), and humans (Tibell et al. 1987). For some widely used probiotics, such as *L. acidophilus*, that have no SOD and low antioxidative activities, heterologous expression of the *sod* gene from *L. casei* or *L. sakei* is of critical importance in the construction of genetic engineering of *Lactobacillus* with higher SOD activity. The functional gene from *L. casei* is more suitable for expression in other *Lactobacillus* because *L. casei* is a Gram-positive non-spore-forming organism belonging to the same group that has a low GC content; as such the *L. casei sod* gene is close to that of other strains in the *Lactobacillus* group. In this study, the *sod* gene from *Lactobacillus* was heterologously expressed in another prokaryote for the first time.

The antioxidative effects of every bacterial strain tested in this study were examined in intact cells and in intracellular extracts (CFEs). The results shown in Table 2 show that all of the tested bacterial strains had some antioxidative activity and that the antioxidative effects of disrupted cells were higher than those of intact cells. A

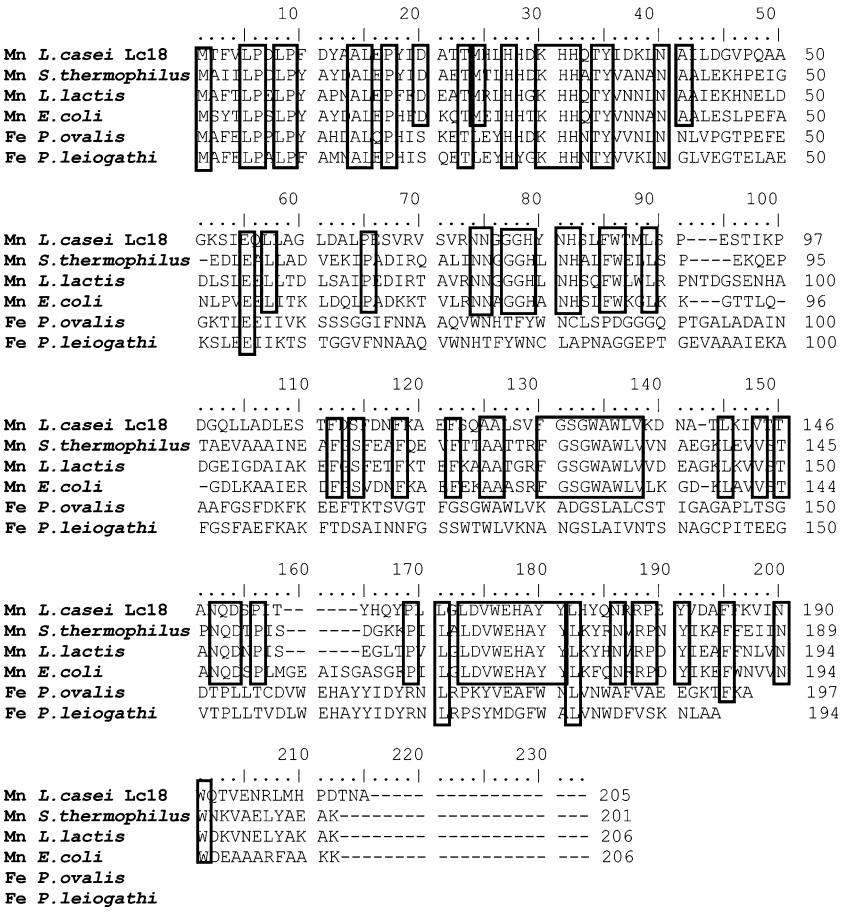

Fig. 4 Aligned amino acid sequences of manganese and iron SODs. Invariant residues are *boxed*

similar finding was previously reported by Saide and Gilliland (2005) who found that intracellular extracts of lactobacilli possessed higher antioxidative activity than intact cells. The higher antioxidative activity of the intracellular extracts could be due to the better accessibility of antioxidative enzymes or metal ions to the oxidant substrates.

Although the antioxidative activities of *L. casei* Lc2 were higher than those of *L. casei* Lc18, we failed to amplify the *sod* gene from *L. casei* Lc2 using the same PCR conditions employed for *L. casei* Lc18. The PCR primers were designed based on the genome of *L. casei* ATCC 334. Therefore, the inability to amplify the *sod* gene from *L. casei* Lc2 may indicate that this strain was genetically altered during its evolution or did not contain a *sod* gene at all. However, this strain still displayed high superoxide radical scavenging activity and hydrogen peroxide scavenging activity, which might have been due to the high concentration of Mn^{2+} in its cells or other unknown antioxidative mechanisms.

The O_2^- scavenging activity and H_2O_2 scavenging activity of the recombinant *E. coli* BLSod was slightly higher than that of *E. coli* BL21(DE3). The slight increase in hydrogen peroxide scavenging activity may be explained by the positive interaction between SOD and catalase. Superoxide radicals can inactivate catalase; therefore, SOD can protect catalase due to its O_2^- scavenging activity (Brioukhanov and Netrusov 2004).

The aim of this study was to clone the *sod* gene from *L. casei* into *E. coli* BL21(DE3) for ectopic expression in order to demonstrate that the SOD from *Lactobacillus* is able to function in a heterologous prokaryote with considerable enzyme activity. Further work is required to clone the *sod* of *L. casei* into other species of *Lactobacillus*, such as *L. acidophilus*, which is widely used as a probiotic but lacks the SOD protein and has low antioxidative activity.

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