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Functional role of *oppA* encoding an oligopeptide-binding protein from *Lactobacillus salivarius* Ren in bile tolerance

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Abstract *Lactobacillus salivarius* is a member of the indigenous microbiota of the human gastrointestinal tract (GIT), and some *L. salivarius* strains are considered as probiotics. Bile tolerance is a crucial property for probiotic bacteria to survive the transit through the GIT and exert their beneficial effects. In this work, the functional role of *oppA* encoding an oligopeptide transporter substrate-binding protein from *L. salivarius* Ren in bile salt tolerance was investigated. In silico analysis revealed that the *oppA* gene encodes a 61.7-kDa cell surface-anchored hydrophilic protein with a canonical lipoprotein signal peptide. Homologous overexpression of OppA was shown to confer 20-fold higher tolerance to 0.5 % oxgall in *L. salivarius* Ren. Furthermore, the recombinant strain exhibited 1.8-fold and 3.6-fold higher survival when exposed to the sublethal concentration of sodium taurocholate and sodium taurodeoxycholate, respectively, while no significant change was observed when exposed to sodium glycocholate and sodium glycodeoxycholate (GDCA). Our results indicate that OppA confers specific resistance to taurine-conjugated bile salts in *L. salivarius* Ren. In addition, the OppA overexpression strain also showed significant increased resistance to heat and salt stresses, suggesting the protective role of OppA against multiple stresses in *L. salivarius* Ren.

Keywords *Lactobacillus salivarius* · OppA · Function analysis · Overexpression · Bile salt tolerance

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Introduction

Lactobacillus salivarius is commonly isolated from the gastrointestinal tract (GIT), oral cavity and breast milk of humans [21, 31]. In the recent years, a variety of probiotic properties have been proposed for some strains, such as the ability to modulate gut microbiota, stimulate protective immune response and attenuate gastrointestinal inflammation [23, 26, 28]. Moreover, some *L. salivarius* strains could maintain high viability during cheese manufacturing, suggesting their potential use for the development of probiotic food [7]. To exert their beneficial effects, it is essential for *L. salivarius* strains to maintain a high survival and stable colonization in the GIT. However, after digestion, *L. salivarius* are exposed to various physico-chemical stresses, such as low pH in the stomach and bile salts in the intestine. Bile salts are known as biological detergents that exert strong antimicrobial activity by disrupting the lipid bilayer structure of cellular membranes, inducing protein misfolding and causing DNA oxidation [4]. Therefore, tolerance to bile stress is critical for *L. salivarius* to survive in the GIT.

Indigenous lactobacilli in the GIT have evolved numerous mechanisms to cope with the toxic effects of bile, such as the production of bile salt hydrolase (BSH), an enzyme which is responsible for deconjugation of glycine- or taurine-conjugated bile salts [5]. Another important

mechanism contributing to bile resistance is the active extrusion of bile acids through efflux pumps, such as lr1584 in *Lactobacillus reuteri* ATCC 55730 and four multidrug transporters in *Lactobacillus acidophilus* NCFM [29, 36]. In addition, changes in the architecture or composition of cell envelope also play an important role in bile resistance in lactobacilli [35]. A transcriptome study on *L. salivarius* UCC118 revealed that genes involved in carbohydrate transport and metabolism, energy production and conversion, and exopolysaccharide synthesis were differentially expressed after exposure to bile [9]. Notably, a gene cluster (LSL_1695–LSL_1699), encoding a putative oligopeptide (Opp) transporter, was approximately 20-fold up-regulated following exposure to cholate. Interestingly, a cell envelope proteome analysis of *Bifidobacterium longum* NCIMB 8809 also revealed that OppA, the extracellular substrate-binding protein of Opp transporter, was accumulated on the cell surface in the presence of bile [33]. Recently, an RNA-Seq transcriptomic analysis performed by our team showed that the gene *oppA* (LSR_0477) was threefold up-regulated upon oxgall exposure in *L. salivarius* Ren, a potential probiotic strain isolated from healthy centenarians living in Bama (China) [38]. Taken together, these findings suggest that the overproduction of OppA may represent a novel bile resistance strategy in intestinal bacteria.

Opp transporters are membrane-associated five-protein complexes (oppABCDF) of the ATP-binding cassette (ABC) transporters superfamily and are found in both Gram-positive and -negative species [11]. Besides nutrient uptake, Opp systems are also involved in the initiation of competence in *Bacillus subtilis* [34], induction of conjugation in *Enterococcus faecalis* [20] and production of biofilm in *Vibrio fluvialis* [18]. Furthermore, analysis of an *oppA* mutant strain of *Listeria monocytogenes* suggests that OppA was involved in low temperature growth and intracellular survival in macrophages [6]. Moreover, OppA was also found up-regulated by acid stress in a proteomics study on *L. reuteri* ATCC 23272 [19]. In addition, it has been shown previously that Opp systems are implicated in the uptake of proline-containing compounds and confer osmoprotection on *B. subtilis* and *Sinorhizobium meliloti* [1, 37]. However, little information is available about the role of OppA in lactobacilli under stressed condition.

In this study, homologous overexpression of the *oppA* gene in *L. salivarius* Ren was applied to investigate its functional role in bile tolerance. Then, different kinds of bile salts were employed to evaluate the effect of OppA on substrate specificity in recombinant strains. Meanwhile, the resistance to acid, salt, heat and low temperature of recombinant strains was also investigated in this study. To the best of our knowledge, this work represents the first study on the pleiotropic effects of OppA in the genus *Lactobacillus*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *L. salivarius* Ren was grown anaerobically in man–rogosa–sharpe (MRS) medium at 37 °C. *Lactococcus lactis* NZ9000, used as a host in cloning experiments, was grown in M17 broth (Oxoid, Basingstoke, UK) supplemented with 0.5 % (w/v) D-glucose (GM17) at 30 °C without agitation. When appropriate, chloramphenicol was added to the media at a concentration of 5 μg/mL for both *L. salivarius* Ren and *L. lactis* NZ9000. Oxgall, sodium glycocholate (GCA), sodium glycodeoxycholate (GDCA), sodium taurocholate (TCA) and sodium taurodeoxycholate (TDCA) were obtained from Sigma (St Louis, MO, USA).

DNA manipulations and transformation

Isolation of plasmid DNA from *L. lactis* NZ9000 and *L. salivarius* Ren was performed using QIAGEN Miniprep Spin Kit (Qiagen Inc, Valencia, CA). Chromosomal DNA

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
L. salivarius Ren	Host strain, healthy centenarians isolate	CGMCC[a] 3606
L. lactis NZ9000	Plasmid-free derivative of *L. lactis* MG1363 *pepN::nisRK*	[15]
LSRCK	*L. salivarius* Ren containing p11-8148 plasmid	This work
LSRoppA	*L. salivarius* Ren containing p11-oppA plasmid	This work
Plasmids		
pNZ8148	Gene expression vector P_{nisA}, Cm^r	[24]
p11-8148	pNZ8148 derivative carrying constitutive promoter P11 instead of P_{nisA}	This work
p11-oppA	p11-8148 carrying the *oppA* gene under the control of P11 promoter	This work

[a] China General Microbiological Culture Collection Center (WDCM550)

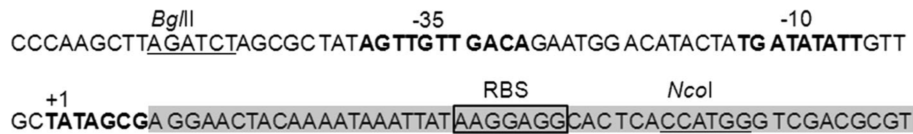

Fig. 1 Schematic representation of the promoter sequence used for plasmid construction. The transcriptional start site (+1), −35 and −10 regions are indicated in *bold type*. The Ribosomal-binding site (RBS) is indicated in *box*. Restriction sites are *underlined*. The sequence derived from pNZ8148 is indicated in *gray*

of *L. salivarius* Ren was extracted using TIANamp Bacteria DNA Kit according to the manufacturer's instructions (TianGen, Beijing, CN). Lysis of *L. salivarius* and *L. lactis* was performed by adding lysozyme to TES buffer (50 mM Tris–Cl, 1 mM EDTA, 25 % sucrose; pH 8.0) to a final concentration of 30 mg/mL, and the suspension was incubated at 37 °C for 1 h. PCR was carried out using KOD-Plus-Neo *Taq* polymerase according to the manufacturer's instructions (Toyobo, Osaka, Japan). Restriction endonuclease digestions and DNA ligation were conducted according to the supplier's instructions (Takara, Dalian, CN). Plasmids were introduced into *L. lactis* NZ9000 and *L. salivarius* Ren by electroporation method as described previously [2, 12]. DNA sequencing was performed with the Bigdye Terminator cycle sequencing kit (Sangon, Beijing, CN).

Plasmid construction

A 119-bp oligonucleotide sequence, which consists of the constitutive promoter P11 [32] and the ribosomal-binding site (RBS) sequence from pNZ8148 [24], was designed as Fig. 1. Restriction sites *Bgl*II and *Nco*I were introduced to the 5′- and 3′- ends, respectively. The sequence was synthesized and purified by Sangon (Beijing, CN) and then inserted into the vector pNZ8148 between *Bgl*II and *Nco*I. Subsequently, the ligation mixture was transformed into *L. lactis* NZ9000 by electroporation using Bio-Rad Gene Pulser Xcell™ (Bio-Rad, Richmond, USA) in a 0.2 cm cuvette at 2.0 kV, 25 µF and 400 Ω. The resulting plasmid with the promoter P_{NisA} replaced by the constitutive promoter P11 was designated p11-8148. The gene *oppA* was amplified by PCR from the chromosomal DNA of *L. salivarius* Ren using the primers LSR_0477F (5′-CATGCCATGGGAAAATTCAAGAAAG-3′) and LSR_0477R (5′-GCTCTAGATTACTTACCGAAATATGCTC-3′). Restriction sites used for subsequent cloning are underlined: *Nco*I and *Xba*I for LSR_0477F and LSR_0477R, respectively. The amplicon obtained was digested by *Nco*I and *Xba*I, and then inserted into the expression vector p11-8148. The resulting recombinant plasmid p11-oppA was sequenced and further analyzed with the DNAMAN software package. Subsequently, p11-oppA was introduced into *L. salivarius* Ren by electroporation in a 0.2 cm cuvette at 1.5 kV, 25 µF and 200 Ω. The recombinant strain with p11-oppA was designated LSRoppA. The plasmid p11-8148 was also introduced into *L. salivarius* Ren, resulting in recombinant strain LSRCK as a control.

Sequence analysis

DNA sequence analysis was performed from the genome of *L. salivarius* Ren (GenBank accession no. JNVE00000000). Putative promoter and terminator sequences were identified using the BPROM and Find Term prediction programs, respectively (http://www.softberry.com). The deduced protein sequence of OppA was searched against the UniProt database using BLAST algorithms. Theoretical, physical and chemical properties of OppA were assessed using the ProtParam tool (http://web.expasy.org/protparam/). Grand average of hydropathy (GRAVY) value was used to evaluate the hydrophilicity of OppA (http://gcat.davidson.edu/DGPB/kd/kyte-doolittle.htm) [16]. Pfam (http://www.sanger.ac.uk/Software/Pfam/) was used to determine conserved protein domains [3], and LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/) was used to predict lipoprotein signal peptides [14]. Sequences were manually searched for the presence of lipobox motif [L-(A/S)-(A/G)-C] [13].

SDS-PAGE analysis

Overnight cultures of LSRCK and LSRoppA were inoculated into 20 mL of fresh MSR medium containing 5 µg/mL of chloramphenicol. Cells were harvested at OD_{600} of 1.0 (early stationary phase) by centrifugation at 8000*g* for 10 min at 4 °C, washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4) and resuspended in the same buffer. The cells were disrupted with an ultrasonic processor sonifier (Automatic Ultrasonic Processor UH-500A, Automatic Science Instrument Co., Ltd., Tianjin, China) and cell debris was removed by centrifugation at 13000*g* for 10 min at 4 °C. The protein concentration in each sample was determined using the Qubit Protein Assay (Invitrogen, Oregon, USA). Aliquots of 10 µg proteins from the control and the OppA overexpression strain were analyzed by SDS-PAGE using a 12 % polyacrylamide gel. PageRuler Prestained Protein Ladder (Thermo Scientific, Rockford, USA) was used as molecular mass standard. Protein bands were stained using Coomassie blue R-250.

Survival experiments of *L. salivarius* exposed to different kinds of bile salts

To determine the bile resistance of the *L. salivarius* recombinant strains, overnight cultures of LSRCK and LSRoppA were inoculated into 10 mL of fresh MSR medium containing 5 μg/mL of chloramphenicol. When cells were grown to an OD_{600} of 0.5, aliquots of 1 mL of each culture were collected and centrifuged at 6000*g* for 2 min, then resuspended in the same volume of fresh medium supplemented with different concentrations of oxgall (0.2, 0.3 and 0.5 %), GCA (0.5, 1, 2 %), GDCA (0.05, 0.1, 0.2 %), TCA (0.5, 1, 3 %) or TDCA (0.5, 1, 2 %). After incubation for 1 h at 37 °C, the numbers of colony-forming units per milliliter (CFU/mL) of each sample were determined by plating serial 10-fold dilutions on MRS agar containing chloramphenicol and then incubated at 37 °C for 48 h. Survival rates were calculated by dividing the CFU/mL after bile incubation by the value obtained immediately after resuspension. The bile tolerance of the recombinant strain conferred by *oppA* was evaluated by comparing the survival rates of LSRCK and LSRoppA incubated at the same bile salt concentration. All results were obtained by at least three independent experiments and each biological replicate was performed in triplicate.

Resistance assays of *L. salivarius* to heat, cold, acid and salt stresses

Overnight cultures of LSRoppA and LSRCK were, respectively, inoculated into 10 mL of MRS supplemented with 5 μg/mL chloramphenicol. When cell density reached an OD_{600} of 0.5, cultures were centrifuged at 6000*g* for 2 min and resuspended in fresh MRS with 5 μg/mL chloramphenicol. Heat challenge was carried out at 55 °C for 1 h and low temperature treatment was performed at 4 °C for 1 day. For acid stress assay, MRS was adjusted to pH 3.8 with lactic acid and the cells were incubated at 37 °C for 1 h. To estimate the level of salt resistance, cultures were resuspended in MRS containing 7.5 % (w/v) NaCl and 5 μg/mL chloramphenicol for 1 h at 37 °C. The numbers of viable cell counts after each treatment were determined by 10-fold serial dilutions and enumeration on MRS plates with chloramphenicol. Survival rates were calculated as described above. All results were obtained by at least three independent experiments and each biological replicate was performed in triplicate.

Statistical analysis

Student's *t* test was performed to investigate statistical differences. Differences between samples with p values <0.05 were considered to be statistically significant.

Results

In silico analysis of the *oppA* gene and its flanking DNA sequences

DNA sequencing result verified that the DNA length of the amplified gene *oppA* was 1617 bp, which predicted an open reading frame encoding 538 amino acids and a TAA stop codon. The nucleotide sequence of the amplified PCR product was 100 % homology with the *oppA* gene from *L. salivarius* Ren. Sequence analysis of the flanking regions of *oppA* revealed two divergent genes: *pepX* and *cysK*, encoding proline iminopeptidase and cysteine synthase, respectively (Supplemental Fig. 1). The *pepX–oppA* gene order is conserved in several lactobacilli strains, such as *L. acidophilus* NCFM, *Lactobacillus plantarum* ST-III and *Lactobacillus helveticus* IF03809. In addition, it is worthwhile noting that no obvious terminator was detected between *oppA* and *pepX*, suggesting that the gene products may also be physiologically linked, i.e., the peptides that imported by OppA may subsequently be hydrolyzed by PepX.

OppA is a putative cell surface-anchored hydrophilic protein

The *oppA* gene encodes a protein of 538 amino acids with a calculated molecular weight of 61.7 kDa and a theoretical isoelectric point (pI) of 9.44. The GRAVY value of OppA is negative (−0.62), together with the Kyte–Doolittle plot, indicating that OppA may be a hydrophilic protein. The N-terminal domain of OppA contains a canonical lipoprotein signal peptide, which may be involved in the type-II signal peptidase recognition (Supplemental Fig. 1). A lipoprotein attachment site was found at position 23 (Cys residue), indicating that OppA could be a lipoprotein. Meanwhile, a type-I signal peptidase cleavage site between amino acid number 30 and 31 was also predicted, suggesting that the OppA protein could also be secreted into the medium, which was reported in *B. subtilis* previously [27]. The deduced protein sequence of OppA was subjected to UniProt database for homology search. Protein Blast results demonstrated that OppA exhibits 99, 65 and 52 % identity to oligopeptide ABC transporter substrate-binding proteins from *L. salivarius* ATCC 11741 (HMPREF0545_0764, GenBank accession no. EEJ74264.1), *L. acidophilus* NCFM (LBA1958, GenBank accession no. AAV43752.1) and *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (Ldb1060, GenBank accession no. CAI97862.1), respectively. All these results suggest that OppA is a putative cell surface-anchored lipoprotein, which may be involved in the uptake of oligopeptides in *L. salivarius* Ren.

Overexpression of OppA improves oxgall resistance of *L. salivarius* Ren

SDS-PAGE analysis revealed the overproduction of a 58-kDa protein in LSRoppA (Fig. 2a, lane 2), which is the expected size of the OppA mature protein, indicating the successful overexpression of OppA in *L. salivarius* Ren. The appearance of the overproduced OppA in cell-free extract further confirmed our prediction that OppA is a hydrophilic protein. To investigate the functional role of OppA in bile salt tolerance, a survival experiment of LSRCK and LSRoppA exposed to different concentrations of oxgall was employed. When the recombinant strains were incubated with 0.3 % oxgall for 1 h, the viable cell counts of LSRCK and LSRoppA were 5.5 and 6.2 log CFU/mL, respectively. When incubated with 0.5 % oxgall, LSRCK viable counts decreased to 4.6 log CFU/mL, whereas LSRoppA still remained 6.0 log CFU/mL (Fig. 2b). After treated with 0.3 or 0.5 % oxgall, the survival rate of LSRoppA was 6.7-fold or 20-fold higher than that of the control, respectively. These results indicate that the overexpression of OppA improved the survival of LSRoppA in oxgall stress conditions and conferred increased resistance to oxgall.

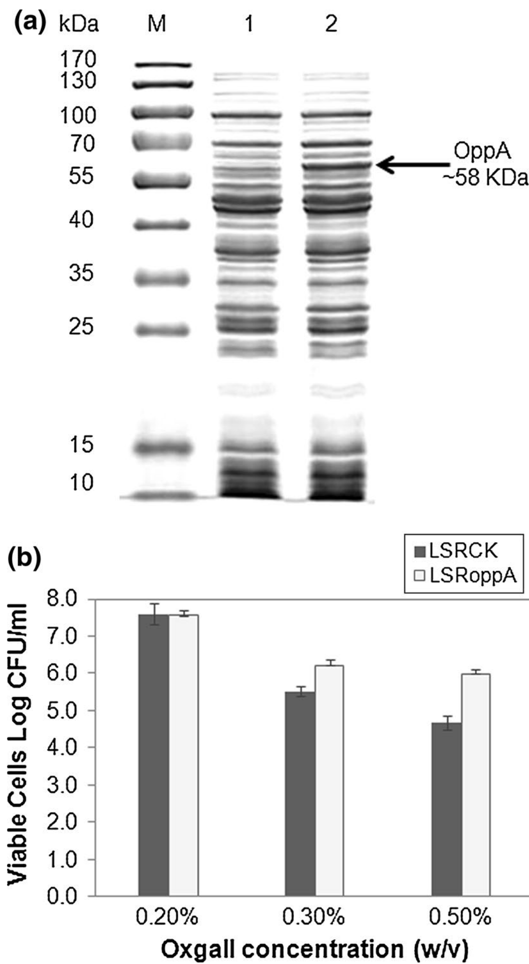

Fig. 2 The overexpression of OppA in *L. salivarius* Ren detected by Coomassie *blue-stained* SDS-PAGE and the survival of *L. salivarius* strains in the presence of different concentrations of oxgall. **a** SDS-PAGE of cell-free extracts of LSRCK and LSRoppA. Samples were loaded in aliquots of 10 μg per lane and separated by 12 % polyacrylamide gel. *Lane M*: PageRuler Prestained Protein Ladder (Thermo Scientific); *lane 1*: LSRCK; *lane 2*: LSRoppA. The *arrow* indicates the overproduced OppA protein. **b** Survival of *L. salivarius* strains in the presence of different concentrations of oxgall. LSRCK and LSRoppA were treated by 0.2, 0.3 or 0.5 % oxgall for 1 h. All results were obtained by at least three independent experiments and each biological replicate was performed in triplicate. *Error bars* correspond to the values of standard errors of the means (SEM)

OppA confers specific resistance to taurine-conjugated bile salts in *L. salivarius* Ren

To investigate the protective role of OppA towards different individual bile salts, GCA, GDCA, TCA and TDCA were selected to assess the bile tolerance specificity. The sublethal concentrations of individual bile salts for LSRCK were determined as 3 % TCA, 0.5 % TDCA, 1 % GCA and 0.1 % GDCA by viable cells counting. After incubated for 1 h in the presence of TCA, TDCA, GCA and GDCA with sublethal concentrations, the survival rate of LSRCK was 35.86, 12.67, 29.67 and 19.67 %, respectively. However, the corresponding survival rate of LSRoppA was 66.14, 46.00, 40.17 and 15.33 %, respectively. These results revealed that LSRoppA showed 1.8- and 3.6-fold higher survival than that of the control strain when exposed to TCA and TDCA, respectively, while no significant change was observed when exposed to GCA or GDCA (Fig. 3). These results indicate that the overexpression of gene *oppA*

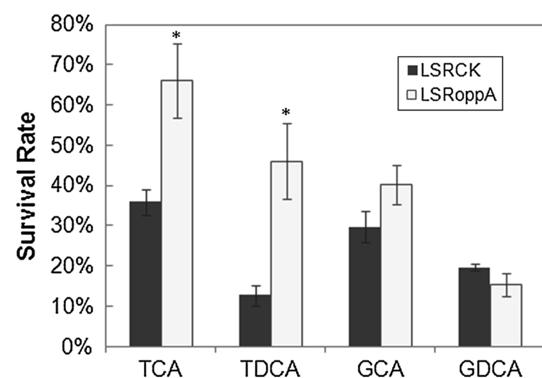

Fig. 3 Survival rate of LSRCK and LSRoppA treated by 3 % TCA, 0.5 % TDCA, 1 % GCA and 0.1 % GDCA for 1 h, respectively. *Asterisks* represent significant differences at $p < 0.05$ by the Student's *t* test. Survival rate was calculated by dividing the number of CFU/mL after bile incubation by the value obtained immediately after resuspension. All results were obtained by at least three independent experiments and each biological replicate was performed in triplicate. *Error bars* correspond to the SEM

mainly contributed to increase the resistance of *L. salivarius* Ren to taurine-conjugated bile salts.

OppA confers tolerance to heat and salt stress in *L. salivarius* Ren

To further investigate the tolerance of LSRoppA to other stresses, we examined the response of this OppA-overproducing strain to different stress conditions, including heat, cold, acid and salt. As shown in Fig. 4, no significant differences in survival were observed for LSRoppA and LSRCK exposed to acid (pH 3.8) or low temperature (4 °C) stresses. However, the survival rate of LSRoppA was 3.8-fold higher than that of the control strain under 7.5 % (w/v) NaCl stress. In addition, the viability of both LSRCK and LSRoppA was affected dramatically following incubation at 55 °C for 1 h. Nevertheless, LSRoppA displayed significantly higher heat resistance than the control, i.e., a 4-log CFU/mL increase in viable cell counts was observed (Fig. 5). Based on these results, it can be concluded that the overexpression of OppA not only enhanced oxgall tolerance to *L. salivarius* Ren but also improved its resistance to heat and NaCl.

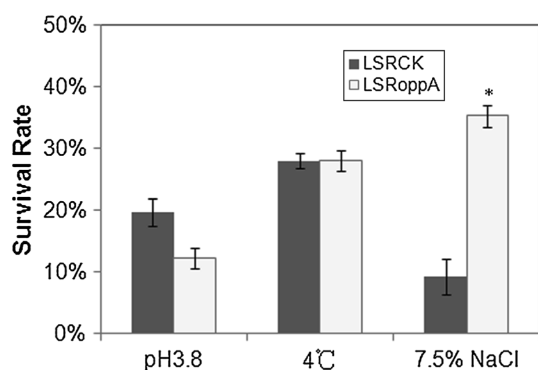

Fig. 4 Survival of LSRCK and LSRoppA exposed to acid (pH 3.8), low temperature (4 °C) and salt stress (7.5 % NaCl) conditions. *Asterisk* represents significant differences ($p < 0.05$) determined by the Student's *t* test. All results were obtained by at least three independent experiments and each biological replicate was performed in triplicate. *Error bars* correspond to the SEM

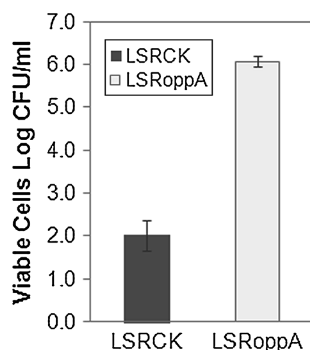

Fig. 5 Survival of LSRCK and LSRoppA exposed to heat stress (55 °C) for 1 h. The results were obtained by at least three independent experiments and each biological replicate was performed in triplicate. *Error bars* correspond to the SEM

Discussions

Bile tolerance is a crucial property for enteric bacteria, which allows them to inhabit the human GIT and determines their survival, colonization ability and activities. Recently, our team has obtained the transcription profile of *L. salivarius* Ren in response to bile salts using the next-generation sequencing platform Illumina HiSeq 2000. The transcription of nearly 200 genes was detected to be associated with bile stress, including genes involved in carbohydrate and amino acid metabolism, cell envelope and fatty acid biogenesis, transcription and translation. The gene LSR_0477 encoding OppA was threefold up-regulated upon oxgall exposure (data accessible at NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/), accession GSE68033). In this study, homologous overexpression of the LSR_0477 gene in *L. salivarius* Ren was employed to analyze its functional role in bile tolerance. The synthetic promoter P11, which has shown high activities in *Lactobacillus* sakei and *L. plantarum* [32], was used to drive the expression of *oppA* in *L. salivarius* Ren. Our results indicate that the overexpression of OppA significantly enhanced bile salt tolerance in *L. salivarius* Ren, and a substrate preference for taurine-conjugated bile salts was also observed in the recombinant strain. Conjugated bile salts are considered as detergent-like compounds because of their amphipathic structure [17]. Compared to glycine-conjugated bile salts, taurine-conjugated bile salts display improved surfactant properties, due to the low pKa of the sulfonic acid group of taurine [8]. The Opp component from *Escherichia coli* has been reported to be implicated in resistance to surfactant [25]. Therefore, we presume that the OppA protein of *L. salivarius* Ren may play a role in the recognition of the negatively charged sulfonic acid group of taurine-conjugated bile salts. In addition, a cell membrane hemolysin-like protein (TlyC1) from *B. longum* BBMN68 has also been shown to display a strong preference for taurine-conjugated bile salts [22]. It is speculated that cell envelope-located proteins, such as OppA and TlyC1, may mainly be responsible for binding taurine-conjugated bile salts and then reduce the internalization of these toxic compounds into the cells.

An unexpected finding of this work is that OppA may play a role in the plasmid transformation of *L. salivarius* Ren, since obvious phenotypic difference between

LSRoppA and LSRCK was observed after electroporation. Generally, it will take 2–3 days for LSRCK to form detectable colonies on MRS plates containing chloramphenicol after electroporation. However, colonies of LSRoppA could be observed only after 1 day of electroporation, and the size of the colonies was larger than that of the control strain grown for 2 days. However, no difference in transformation efficiency between LSRoppA and LSRCK was observed. Previous studies have demonstrated that Opp systems are involved in the development of competence in *B. subtilis* and *Streptococcus thermophilus*, and it is proposed that OppA may participate in the import of specific substrates, such as competence-stimulating factors (CSF) or pheromones, which are required for activating of specific transduction signal pathway for the initiation of competence in these bacteria [10, 34]. Notably, the recombinant *L. lactis* NZ9000 strain harboring p11-oppA did not exhibit the same phenotype as LSRoppA after electroporation. The reason might be that signaling molecules involved in the initiation of competence are different between *L. lactis* NZ9000 and *L. salivarius* Ren, or the heterologous OppA protein from *L. salivarius* Ren could not recognize other Opp components in the host *L. lactis* NZ9000. However, the mechanism involved in the modulation of competence is complex and poorly documented in lactobacilli. Further studies are needed to investigate the underlying mechanisms by which OppA senses extracellular pheromones and triggers the competence state in lactobacilli.

Cell surface-associated proteins are the prime contact point of the bacteria with their environment, and are thought to be related to the ability of bacteria to survive in diverse environments. Hence, we further examined the response of the OppA-overproducing strain to different stress conditions. Our results show that LSRoppA exhibited a 4-log CFU/mL increased viable cell counts than the control strain under heat stress. A previous report suggests that OppA might have a chaperone-like function in protein folding and renaturation [30]. Therefore, we hypothesize that the overproduced OppA in *L. salivarius* Ren may play a role in protection proteins against thermal denaturation, and thus may confer heat resistance in the recombinant strain LSRoppA. Survival of *L. salivarius* Ren was also enhanced by the overexpression of OppA when exposed to 7.5 % NaCl. In silico analysis of the *L. salivarius* Ren genome sequence revealed that *oppA* is adjacent to *pepX*, a gene encoding proline iminopeptidase that specifically cleaves N-terminal X-Pro dipeptides from tri- and oligopeptides. The *pepX*–*oppA* gene order is conserved in several lactobacilli strains and no obvious terminator was detected between these two genes, suggesting that the gene products may also be physiologically linked. It has been demonstrated that Opp systems are implicated in the uptake of proline-containing peptides or proline betaine in *S. meliloti* and *B. subtilis* [1, 37]. L-proline liberated from these imported compounds may serve as water-attracting osmolyte and confers osmoprotection on bacteria cells. Taken together, we presume that under salt stress condition, the overexpression of OppA might improve the import of proline-containing peptides, which could be subsequently hydrolyzed by endogenous peptidases, such as PepX, to release osmoprotectant proline in *L. salivarius* Ren. In the present study, our results indicate that the overexpression of OppA increased resistance to heat and salt stress in *L. salivarius* Ren, suggesting that OppA plays important roles in protection against multiple stresses.

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