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Original Research Article

Development of Zn^{2+}-controlled expression system for lactic acid bacteria and its application in engineered probiotics

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ABSTRACT

Lactococcus lactis and *Streptococcus thermophilus* are considered as ideal chassis of engineered probiotics, while food-grade genetic tools are limited in those strains. Here, a Zn^{2+}-controlled gene expression (ZICE) system was identified in the genome of *S. thermophilus* CGMCC7.179, including a transcriptional regulator *sczA*$_{st}$ and a promoter region of cation transporter *czcD* (P_{czcDst}). Specific binding of the $SczA_{st}$ to the palindromic sequences in P_{czcDst} was demonstrated by EMSA analysis, suggesting the regulation role of $SczA_{st}$ on P_{czcDst}. To evaluate their possibility to control gene expression *in vivo*, the *sczA*$_{st}$-P_{czcDst} was employed to drive the expression of green fluorescence protein (GFP) gene in *L. lactis* NZ9000 and *S. thermophilus* CGMCC7.179, respectively. Both of the transformants could express GFP under Zn^{2+} induction, while no fluorescence without Zn^{2+} addition. For optimal conditions, Zn^{2+} was used at a final concentration of 0.8 mM in *L. lactis* and 0.16 mM in *S. thermophilus* at OD_{600} close to 0.4, and omitting yeast extract powder in the medium unexpectedly improved GFP expression level by 2.2-fold. With the help of the ZICE system, engineered *L. lactis* and *S. thermophilus* strains were constructed to secret cytokine interleukin-10 (IL-10) with immunogenicity, and the IL-10 content in the supernatant of the engineered *L. lactis* was 59.37 % of that under the nisin controlled expression system. This study provided a tightly controlled expression system by the food-grade inducer Zn^{2+}, having potential in development of engineered probiotics.

1. Introduction

Lactococcus lactis and *Streptococcus thermophilus* are worldwide industrial workhorses used for dairy fermentation [1]. They have relatively simple carbon metabolism and high glycolytic flux to produce enough lactic acid for rapid acidification of raw milk, and also acetoin, diacetyl, acetaldehyde as aromatic end-products [2,3]. Due to the long history of safe use in food production, these two strains have "Generally Recognized as Safe'' (GRAS) status in USA and a Qualified Presumption of Safety (QPS) status in the European Union [4,5]. In addition, *L. lactis* and *S. thermophilus* have potential as probiotics, as demonstrated by various health effects, including transient survival, moderate adherence, anti-inflammatory and immunoregulation in the gastrointestinal tract [6,7]. Thanks to the GRAS status and the known probiotic effects, *L. lactis* and *S. thermophilus* are considered as ideally biotechnological chassis to deliver therapeutic proteins in the gastrointestinal tract [2,5,8]. Compared with oral administration or intramuscular injection, delivery of therapeutic proteins into intestinal tract by engineered probiotics are thought to preserve activity to a greater extent and reduce dose-dependent side effects [9,10]. In this context, food grade gene expression systems with tight-control properties are critical tools when achieving this biotechnological application.

Nowadays, the nisin controlled expression (NICE) system is the only commercial food grade expression system for lactic acid bacteria (LAB). The NICE system consists of the inducible promoter P_{nisA} and the two-component signal transduction components *nisR/K* belonging to the bacteriocin nisin biosynthesis gene cluster [11]. When a targeted gene is placed behind the P_{nisA}, efficient gene expression can be induced by the addition of sub-inhibitory amounts of nisin to the culture medium [12]. A lot of merits have been proposed for the NICE system, including the food-grade inducer, high level gene expression as well as wide range hosts (*L. lactis*, *S. thermophilus* and *Lactobacillus*), making this system the most widely used controllable expression system in LAB. However, there remains limitations, such as background expression, the sensitivity of

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nisin to proteinase and also its cost. With the explaining of molecular mechanism of sugar utilization, material transport and stress response, the relevant genetic elements were adopted to construct expression systems controlled by lactose, xylose, metal ions, temperature or acid in *L. lactis* [13–16] as well as *S. thermophilus* [17–21]. Thereinto, the genetic components of metal ions-controlled expression system are relatively simple, and metal ions as inducer are easy to prepare and manageable conditions. What's more, it is possible to avoid transcriptional leakage by using metal ions-controlled expression systems. However, the food grade expression systems under metal ions control are still limited for LAB.

Maintenance of bacterial intracellular homeostasis of metal ions is dependent on metal ion uptake and efflux systems. Researches in pathogenic *Streptococcus pneumoniae* established a Zn^{2+} efflux system, which consisted of a cation diffusion facilitator CzcD and the adjacent TetR family regulator SczA that activates expression of the *czcD* gene in the presence of Zn^{2+} [22]. Food grade *S. thermophilus* has a close phylogenetic relationship with *S. pneumoniae* [23,24]. The possibility that the similar Zn^{2+} efflux system existing in *S. thermophilus* is yet to be verified. In this work, putative genetic elements for Zn^{2+} efflux system were found in the genome of *S. thermophilus* CGMCC7.179, and cloned to develop a new rigorous gene expression system controlled by Zn^{2+} in *L. lactis* and *S. thermophilus*. The influences of various conditions on the system were evaluated to obtain the optimal gene expression level. Finally, the system was applied in the expression of IL-10 with immunogenicity by the host cells *L. lactis* NZ9000 and *S. thermophilus* CGMCC7.179.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains and plasmids used in this work are summarized in Table S1. *Escherichia coli* was cultured aerobically in Luria Bertani broth at 37 °C. *L. lactis* NZ9000 [25] and its derivatives were cultivated in M17 broth (Oxoid, Basingstoke, United Kingdom) containing 0.5 % glucose (GM17) at 30 °C under static conditions. *S. thermophilus* CGMCC7.179 and its derivatives were cultivated in M17 broth containing 1.0 % lactose (LM17) at 42 °C under static conditions. If necessary, chloramphenicol (Sangon, China) was used at 5 μg/mL for *L. lactis*, 5 μg/mL for *S. thermophilus* and 10 μg/mL for *E. coli*, ampicillin (Sangon, China) 100 μg/mL for *E. coli*. Nisin (Sigma, USA) was added to a final concentration of 10 ng/mL.

2.2. Sequence analysis

The SczA and P_{czcD} from *S. thermophilus* CGMCC7.179 were named as $SczA_{st}$ and P_{czcDst}. Amino acid sequence of the $SczA_{st}$ as well as DNA sequence of the P_{czcDst} were aligned with those from *S. pyogenes* MGAS2221, *S. agalactiae* A9 and *S. pneumoniae* R6, respectively. Multiple-sequence alignments were performed using Clustal W and ESPript 3.0. Protein structure of the $SczA_{st}$ was modeled by Alphafold2. The promoter prediction was conducted in the website of Softberry (http://www.softberry.com/).

2.3. Plasmid and recombinant strain construction

The primers used in this work are listed in Table S2. The coding sequence of IL-10 fused with the signal peptide Usp45 was synthesized as stated in Table S3. DNA synthesis and sequencing were performed by RuiBotech Biological Biotechnology., Ltd (China). All molecular manipulations were carried out as standard techniques [26]. Plasmid DNA and genomic DNA were extracted according to the instructions of the Plasmid Mini Kit (Omega) and TIANamp Bacteria DNA kit (TIANGEN).

DNA fragment of the $sczA_{st}$ was PCR amplified from the genome of *S. thermophilus* CGMCC7.179 with primers SczAF/SczAR, and the product was subcloned into the corresponding sites of the pET-15b. The resultant plasmid pETSczA was transformed into chemically competent *E. coli* BL21 cells, generating the recombinant strain *E. coli*/pETSczA.

The P_{czcDst}-$SczA_{st}$ fragment was PCR amplified from the genome of CGMCC7.179 with primers SF/PR, and inserted into the compatible sites of the plasmid pNZ8148 [27], generating the basic ZICE vector pNZST. To express *gfp* gene, the DNA fragment of *gfp* was PCR amplified from pGFP [28] using primers GF/GR, and inserted into the *Nco*I/*Kpn*I sites of pNZST and pNZ8148, generating the recombinant plasmids pNZST-gfp and pNZ-gfp, respectively. To deliver human IL-10, DNA fragment of the usp45-IL10 was PCR amplified from the synthetic template with primers IF/IR, and inserted into pNZST and pNZ8148, generating the recombinant plasmid pNZST-IL and pNZ-IL, respectively. The recombinant plasmids were electroporated into the host *L. lactis* NZ9000 or *S. thermophilus* CGMCC7.179 to obtain the corresponding recombinant strains according to the previous methods [29,30].

2.4. Purification of the $SczA_{st}$ protein and gel mobility shift assay (EMSA)

The *E. coli*/pETSczA was grown in LB broth until OD_{600} reached 0.4. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and the temperature was lowered to 16 °C for the induction of $SczA_{st}$ overexpression. After 16 h of induction, the cells were harvested and the $SczA_{st}$ was purified by the HisTrap FF column (GE Healthcare) according to the manufacturer's instructions.

The gel mobility shift assay was performed as previous description with some modification [31]. 133-bp DNA fragment of the promoter P_{czcDst} was PCR amplified from the genome of CGMCC7.179 with primers PF/PR2. DNA fragments containing 1 - 62 bp (F1) and 63 - 133 bp (F2) of the P_{czcDst} were PCR amplified from the plasmid pNZST-gfp using primers M1F/M1R and M2F/M2R, respectively. DNA fragments of F3 and F4 with site mutations in F1 and F2 were PCR amplified from the plasmid pNZST-gfp using primers M3F/M1R and M2F/M3R. The lactate dehydrogenase promoter, used as control, was PCR amplified from the genome of CGMCC7.179 using primers L1F/L1R. 100 ng DNA probes were added to 20 μL EMSA reaction mixtures containing the purified $SczA_{st}$ protein and binding buffer at a concentration of 0, 25, 50, 75 or 100 ng per reaction. Immediately after incubation for 30 min at room temperature, samples were loaded onto a 6 % nondenaturing polyacrylamide gel. Gel electrophoresis was performed at 100 V for 60 min in 0.5 × Tris-acetate-EDTA (TAE) buffer. The binding complex was visualized by ethidium bromide staining.

2.5. Determination of relative fluorescence unit (RFU)

The recombinant *L. lactis* and *S. thermophilus* strains were cultivated overnight at the conditions as mentioned above. The overnight culture was diluted 100-fold in 5 mL of fresh medium. To find proper induction conditions for *L. lactis*, the concentration of inducer $ZnSO_4$ was added in 0.2 mM increment from 0 to 1.0 mM, and inductions were initiated at the time of inoculation or OD_{600} reaching 0.2, 0.4, 0.6, 0.8 or 1.0. For *S. thermophilus*, $ZnSO_4$ was added from 0 to 0.24 mM. After induction for 4 h or more at 18 °C, the cells were harvested, washed twice and resuspended in phosphate-buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Whole cell fluorescence intensity was measured on LS-50B spectrofluorometer (Perkin-Elmer, USA) by excitation at 488 nm and emission at 511 nm. The relative fluorescence unit (RFU) was calculated as fluorescence intensity/OD_{600}.

2.6. Western blotting

The recombinant *L. lactis* strains NZ-ZICEIL and NZ-NICEIL, and recombinant *S. thermophilus* strain ST-ZICEIL were cultivated and induced for protein expression at the proper Zn^{2+} conditions. Then, the extracellular proteins of each culture were collected as previous method [32]. Protein samples were subjected to SDS-PAGE, and the IL-10 protein was

detected by western blot using an anti-IL-10 monoclonal antibody (Proteintech).

2.7. Statistical analysis

Experimental data were reported as the mean ± standard deviation. Statistical significance between treatment and control conditions was assessed by unpaired 2-tailed Student's t-tests. $P < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Putative Zn^{2+} efflux system in S. thermophilus

Genome-wide search of *S. thermophilus* CGMCC7.179, focused on cation diffusion systems, led to the identification of a putative Zn^{2+} efflux system which was composed of two genes transcribed oppositely, a TetR family regulator $SczA_{st}$ and a cation diffusion facilitator $CzcD_{st}$ (Fig. 1A). Further search revealed that the Zn^{2+} efflux system was highly conserved and widely equipped in other *S. thermophilus* strains, such as CNRZ1066, LMG 18311, JIM8232, ND03, MN-ZLW-002, ASCC1275 and S9. Besides, the genetic organization of the putative Zn^{2+} efflux systems from *S. thermophilus* was similar to that of pathogenic *S. pneumoniae* R6 [22].

Sequence alignment showed that the amino acid sequence of the $SczA_{st}$ had 74 % identity with that from *S. pneumoniae* R6, and conserved amino acid residues R6 and K10 existed in the N-terminal region of $SczA_{st}$ (Fig. 1B). The dibasic residues could enhance DNA binding through contact with the phosphate backbone, which were thought to be vital to transcriptional regulator function [22]. Protein structure of the $SczA_{st}$ modeled by Alphafold2 predicted an N-terminal DNA-binding domain and a variable C-terminal regulatory domain (Figure S1), suggesting that the $SczA_{st}$ might act as a transcriptional regulator. The 133 bp sequence of intergenic region between the $sczA_{st}$ and $czcD_{st}$ was subjected to promoter prediction, and the promoter elements (-35 region, −10 region and ribosome binding site) were found (Fig. 1C). And two palindromic sequences (motif1 and motif2) were located in the intergenic region, which have been proved to act as the SczA binding sites in *S. pneumoniae* R6 [22,33]. Therefore, the 133 bp sequence (renamed as P_{czcDst}) might contain the operator sequence of the $czcD_{st}$, that was transcriptional regulated by the $SczA_{st}$.

3.2. In vitro binding of the $SczA_{st}$ to the P_{czcDst}

To test specific binding of the $SczA_{st}$ to the P_{czcDst}, the $SczA_{st}$ with N-terminal His_6-tag was purified after overexpressed in *E. coli*. As shown in Fig. 2A, the protein band about 25 kDa was observed, corresponding to the theoretical molecular weight (MW) of $SczA_{st}$ (179 aa, 21.5 kDa) plus the N-terminal His_6-tag. Using the purified $SczA_{st}$ protein, EMSA analysis exhibited that the shifts of P_{czcDst} were gradually retarded along with the increase of $SczA_{st}$ protein, so were the two truncations P_{czcDst}-1 lacking motif2 and P_{czcDst}-2 lacking motif1 (Fig. 2B). These results indicated the specific binding between the $SczA_{st}$ and the individual DNA fragment, and binding sites existing in both of the two truncations. Moreover, binding of the $SczA_{st}$ to P_{czcDst} or P_{czcDst}-1 resulted in complexes of higher MW, while to P_{czcDst}-2 yielded the complexes of low MW. This difference might result from the monomeric $SczA_{st}$ binding to the P_{czcDst}-2 (containing motif2) while the oligomeric $SczA_{st}$ binding to the P_{czcDst} or P_{czcDst}-1 (containing motif1), as results reported in *S. pneumoniae* [22]. When the base mutations were introduced into the palindromic sequences of motif1 and moitf2, the interaction of the $SczA_{st}$ with P_{czcDst}-1 were greatly weakened, with P_{czcDst}-2 were abolished, implying the palindromic sequences were crucial for the interaction (Fig. 2C). The shifts of P_{czcDst}-3 showed a bit of retardation with the increase of $SczA_{st}$ protein, as the seven bases at the 5' end of motif1 could act as another binding sites for $SczA_{st}$ [22]. Therefore, the *in vitro* EMSA analysis demonstrated that the $SczA_{st}$ specifically bond to the palindromic sequences in P_{czcDst}, suggesting a regulation role of $SczA_{st}$ on P_{czcDst}.

3.3. Controlled gfp expression at optimal Zn^{2+} conditions in L. lactis

To find out whether the $SczA_{st}$ could regulate the expression of gene downstream the P_{czcDst} *in vivo*, the $sczA_{st}$-P_{czcDst} was used to drive the *gfp* expression in *L. lactis*. As shown in Fig. 3A, the relative fluorescence unit (RFU) was almost same in the strain NZ-ZICEgfp as that in the control strain NZ-ZICE when they were cultured in GM17 broth during 0 h to 6 h, suggesting the *gfp* was not expressed. When 0.2 mM to 1.0 mM $ZnSO_4$ was added at the time of inoculation, the RFU in the NZ-ZICEgfp exhibited obvious increase, and the highest value was obtained by 1.0 mM $ZnSO_4$ for 6 h induction. These results indicated that Zn^{2+} addition could induce the expression of gene under the P_{czcDst} in *L. lactis*. The possible mechanism would be proposed that the $SczA_{st}$ bound to the motif1 but disaggregated from the motif 2 of the P_{czcDst} by Zn^{2+} addition to generate transcriptional activation of gene downstream P_{czcDst}, as suggest in *S. pneumoniae* [22,33].

When $ZnSO_4$ was added at the time of inoculation, growth of the NZ-ZICEgfp was clearly affected by 1.0 mM $ZnSO_4$, and a lesser extent by 0.6 mM and 0.8 mM (Fig. 3B), as excess of metal ions can be deleterious to the cell [34]. To relieve this adverse impact, $ZnSO_4$ at a final concentration of 0.8 mM was added to the culture when OD_{600} reached 0.2, 0.4, 0.6, 0.8 or 1.0. The results showed that the growth inhibition was gradually removed with the retardation of Zn^{2+} addition (Figure S2A), and GFP expression level increased by 42.8 % when Zn^{2+} was added at $OD_{600} = 0.4$ compared with that at the time of inoculation (Fig. 3C).

Using the transcriptional regulator $SczA_{st}$ and promoter P_{czcDst}, a Zn^{2+} controlled expression (ZICE) system was constructed in *L. lactis*. At the optimal Zn^{2+} condition, the expression strength of ZICE was about 45 % of that produced with the NICE system (Figure S2B). Although like this, zinc salt as an inducer could be much cheaper than nisin used for the NICE system [35]. Notably, the control by the ZICE system was rigorous, so it would be favorable to produce proteins toxic to the host without leakage problem.

3.4. Feasibility of the ZICE system in S. thermophilus

To test feasibility of the ZICE system in *S. thermophilus*, the plasmids pNZST and pNZSTgfp were introduced into the host strain CGMCC7.179, yielding the ST-ZICE (control) and ST-ZICEgfp. As shown in Fig. 4, the two recombinants had similar RFU under 0 mM Zn^{2+} conditions, indicating no leakage occurred in the ST-ZICEgfp. When Zn^{2+} from 0.08 to 0.2 mM was added to the culture of strain ST-ZICEgfp at $OD_{600} = 0.4$, the RFU increased with the increase of Zn^{2+} levels. Considering that 0.2 mM Zn^{2+} severely inhibited the growth of ST-ZICEgfp, 0.16 mM Zn^{2+} was used to induce gene expression in *S. thermophilus*. Compared with *L. lactis*, *S. thermophilus* showed better sensitivity to Zn^{2+}, as 0.16 mM Zn^{2+} induced ST-ZICEgfp and 0.8 mM Zn^{2+} induced NZ-ZICEgfp resulted in similar GFP expression levels, indicating that *S. thermophilus* showed better response to Zn^{2+}. We supposed that *S. thermophilus* with Zn^{2+} efflux system in the chromosome would enhance the cell sensitivity to Zn^{2+}.

The adaptability of the ZICE system was also tested in *Lactobacillus casei* BL23, but it did not work (data not shown). Comparison with the NICE system adapted in *L. lactis* and *Lb. casei*, the ZICE system could be applied in *L. lactis* and *S. thermophilus*.

3.5. Effects of nitrogen and carbon sources on gene expression level under the ZICE system

To detect effects of nitrogen source on gene expression level under the ZICE system, soya peptone, tryptone, beef extract and yeast extract powder were omitted from the GM17 broth respectively. After the strain

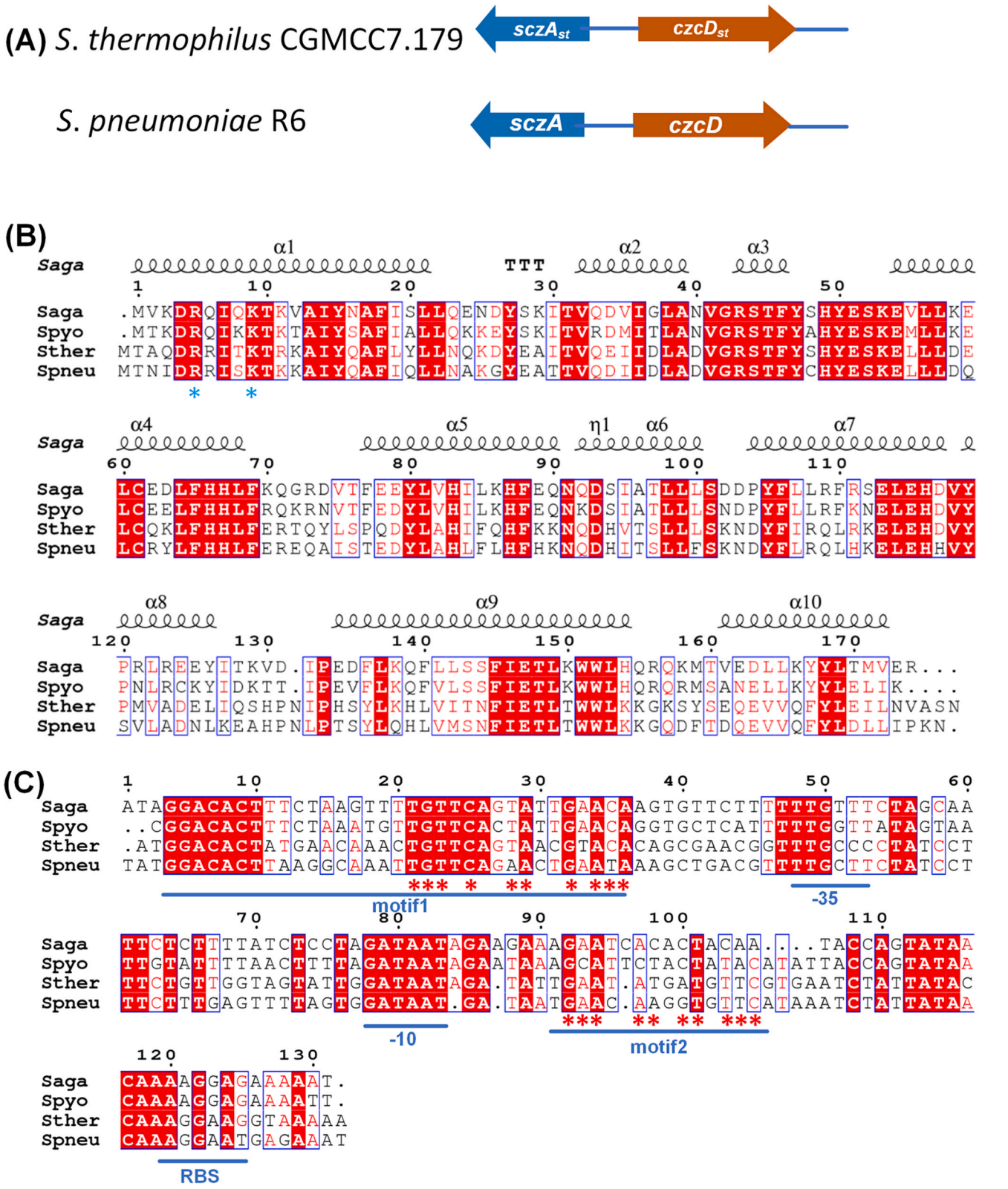

Fig. 1. Prediction of putative Zn^{2+} efflux system from *S. thermophilus* CGMCC7.179. (A) Genetic organization of the putative Zn^{2+} efflux system from *S. thermophilus* CGMCC7.179 and that from *S. pneumoniae* R6. (B) Multiple sequence alignment of the SczA from *S. pyogenes* MGAS2221 (Spyo), *S. agalactiae* A9 (Saga), *S. thermophilus* CGMCC7.179 (Sther), *S. pneumoniae* R6 (Spneu). The alignment was generated using ClustalW, and the figure was prepared using ESPript. Secondary structural elements of *S. agalactiae* SczA are shown as α-helices (coils; α1-α10) and residue numbering across the top refers to the *S. agalactiae* SczA sequence. The blue stars indicate the conserved basic residues R6 and K10. (C) Multiple sequence alignment of the 133 bp sequence of intergenic region between the $sczA_{st}$ and $czcD_{st}$ (Sther) with those from *S. pyogenes* MGAS2221 (Spyo), *S. agalactiae* A9 (Saga) and *S. pneumoniae* R6 (Spneu). Motif1 and motif2 are the predicted binding sites for the SczA regulator in *Streptococcus*. The red stars indicate the palindromic sequences in motif1 and motif2. RBS, ribosome binding site.

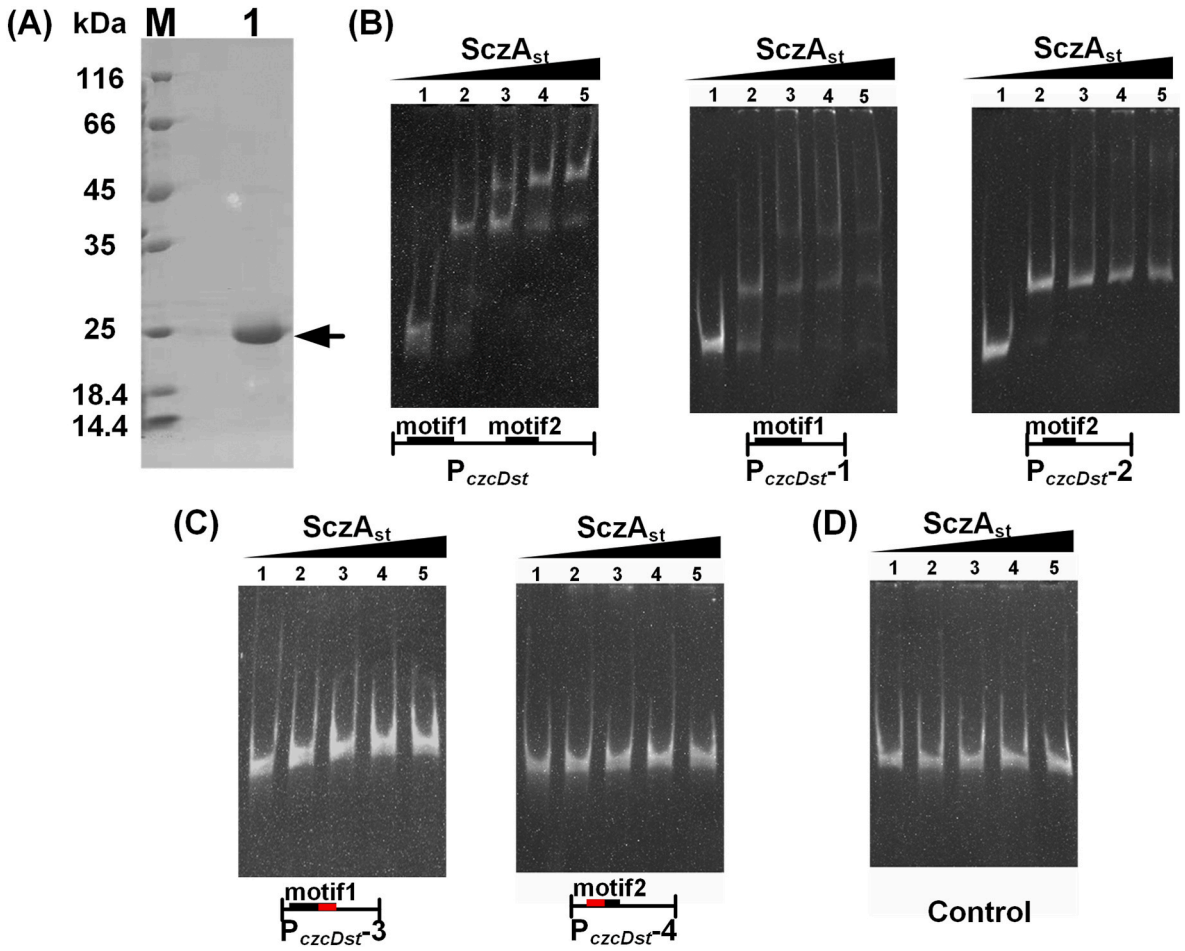

Fig. 2. ***In vitro*** **interaction of the SczA$_{st}$ with the P$_{czcDst}$.** (A) SDS-PAGE of the SczA$_{st}$ protein (indicated by arrow) purified by HisTrap FF column. (B) EMSA analysis of the binding between the SczA$_{st}$ and the P$_{czcDst}$ or its truncations P$_{czcDst}$-1, P$_{czcDst}$-2. (C) EMSA analysis the SczA$_{st}$ binding to the P$_{czcDst}$-3 and P$_{czcDst}$-4 which contained site mutations in the palindromic sequences of motif1 and motif2. The red bars indicate mutant bases. (D) The lactate dehydrogenase promoter was used as a negative control.

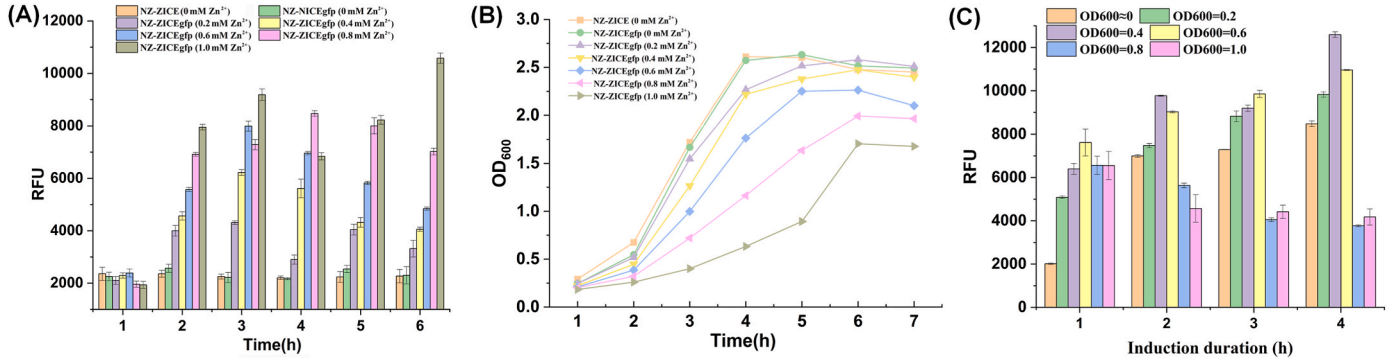

Fig. 3. Zn^{2+} controlled *gfp* expression in *L. lactis*. (A) GFP expression levels of *L. lactis* NZ-ZICE and *L. lactis* NZ-ZICEgfp when Zn^{2+} of different final concentrations were added at the time of inoculation. (B) Growth curves of *L. lactis* NZ-ZICE and *L. lactis* NZ-ZICEgfp when Zn^{2+} of different final concentrations were added at the time of inoculation. (C) GFP expression levels of *L. lactis* NZ-ZICEgfp induced by 0.8 mM Zn^{2+} added at the time of inoculation (OD600 ≈ 0) or OD$_{600}$ close to 0.2, 0.4, 0.6, 0.8 or 1.0.

NZ-ZICEgfp was cultivated in the broth omitting each of the nitrogen source to OD$_{600}$ close to 0.4, Zn^{2+} at a final concentration of 0.8 mM was added to the culture, and the cell densities as well as RFU were measured. As shown in Fig. 5, the absence of the nitrogen sources resulted in lower biomass after 4 h induction, ranging from 37.5 % (tryptone absent) to 71.8 % (beef extract absent) of the normal GM17 broth. The absence of yeast extract powder elevated the RFU by 2.2-fold compared with the normal GM17 broth. The yeast extract powder plays important roles in the bacterial growth and intracellular metabolism. Unexpectedly, omitting of yeast extract powder enhanced the GFP expression level of the NZ-ZICEgfp induced by Zn^{2+}, agreeing with the previous report that nitrogen sources impacted the promoter P$_{srfA}$ activity in LAB [36]. The reason for this effect needs further experiments.

The effects of carbon sources on gene expression level under ZICE

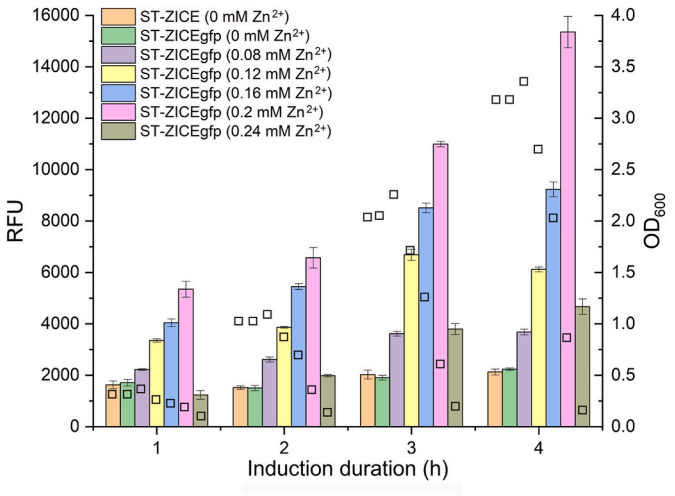

Fig. 4. Zn^{2+} controlled *gfp* expression in *S. thermophilus*. *S. thermophilus* ST-ZICEgfp was induced by Zn^{2+} at different final concentrations when OD_{600} close to 0.4. Squares indicate OD_{600}.

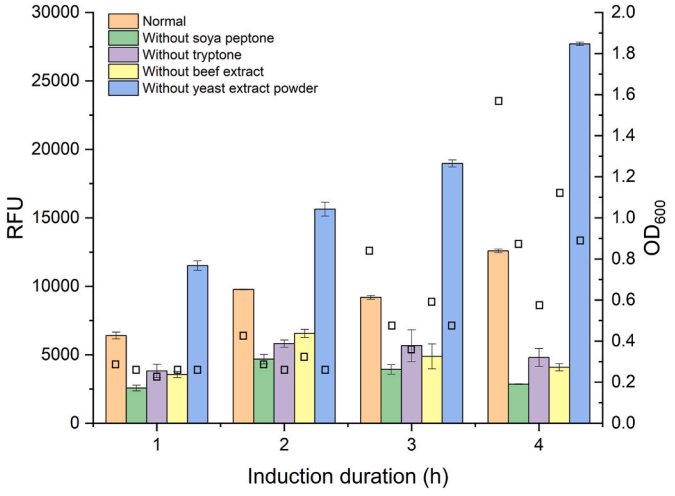

Fig. 5. The effects of nitrogen source on GFP expression level under the ZICE system. *L. lactis* NZ-ZICEgfp was induced by Zn^{2+} at a final concentration of 0.8 mM when OD_{600} close to 0.4. Squares indicate OD_{600}.

system were also tested by cultivating the NZ-ZICEgfp in the medium containing glucose, fructose, sucrose, lactose and maltose as the sole carbon source. However, the four carbon sources had no obvious effect on GPF expression levels (data not shown).

3.6. IL-10 delivery of engineered LAB under ZICE system

L. lactis is a well-known host for bioproduction of fine chemicals and food ingredients [2]. Besides, recent studies have also focused on the construction of *S. thermophilus* recombinant strains to improve healthy and functional characteristics of food [5]. Considering their abilities to survive the intestinal passage and to be metabolically active in gastrointestinal tract, *L. lactis* and *S. thermophilus* are promising chassis for delivering various biological molecules to the gastrointestinal tract [8]. Here, we applied the ZICE system to direct the delivery of cytokine interleukin-10 (IL-10) by *L. lactis* and *S. thermophilus* (Fig. 6A). Western blot using anti-IL-10 monoclonal antibody showed that the bands corresponding to IL-10 could be detected in the supernatant of *L. lactis* NZ-ZICEIL at 4 h postinduction by Zn^{2+}, as well as in the supernatants of *L. lactis* NZ-NICEIL at 4 h postinduction by nisin (Fig. 6B). Densitometry analysis of the bands indicated that IL-10 content induced by the ZICE system was 59.73 % of that induced by the NICE system. For the recombinant *S. thermophilus* ST-ZICEIL, IL-10 was detected in the culture supernatant, of which the IL-10 content was 57.47 % of that in the supernatant of *L. lactis* NZ-ZICEIL (Fig. 6C). These results confirmed that the ZICE system could drive the production of active proteins in LAB hosts. Compared with the widely used NICE system, the ZICE system developed here showed a lower expression level of IL-10. However, nisin, inducer of the NICE system, is sensitive to the enzymolysis of trypsin commonly distributed in the intestinal juice [37]. Zn^{2+}, a kind of inorganic ion, would be more stable and suitable for controlling gene expression in the intestinal environment, which might be provided by dietary supplement or rectal injection.

4. Conclusion

In this work, a new Zn^{2+} controlled expression (ZICE) system was developed for *L. lactis* and *S. thermophilus*. The ZICE system had a great deal to recommend it, since it avoided transcriptional leakage, originated from a food-grade strain and used a cheap and stable inducer. Therefore, the system facilitated to generate recombinant probiotic strains with IL-10 delivery which was thought to cope with intestinal inflammation. Further works are needed such as the improvement of gene expression level controlled by the ZICE system as well as

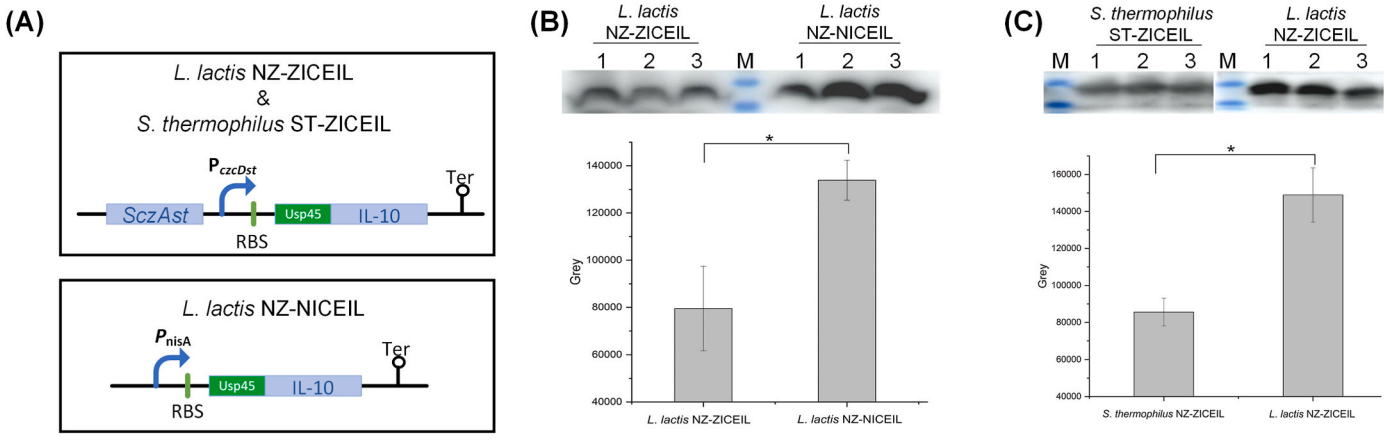

Fig. 6. IL-10 delivery by engineered LAB under the ZICE system. (A) Schematic representation of the expression cassette of IL-10 under the ZICE system as well as the NICE system. (B) Western blot and densitometric analysis of IL-10 in the 50-fold concentrated extracellular fractions of *L. lactis* NZ-ZICEIL and *L. lactis* NZ-NICEIL. (C) Western blot and densitometric analysis of IL-10 in the 100-fold concentrated extracellular fractions of *S. thermophilus* ST-ZICEIL and *L. lactis* NZ-ZICEIL. *$P <$ 0.05. In panels (B) and (C), 1, 2, and 3 represent three independent experiments.

application of the system to produce heterologous proteins in different environments such as intestinal environment.

CRediT authorship contribution statement

Xiaoning Xu: Investigation, Methodology, Visualization. **Lingwen Zhang:** Investigation, Methodology. **Yue Cui:** Investigation, Methodology. **Jian Kong:** Supervision, Resources, Writing – review & editing. **Tingting Guo:** Conceptualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.01.009.

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