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Zirex: a Novel Zinc-Regulated Expression System for *Lactococcus lactis*

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Here, we report a new zinc-inducible expression system for *Lactococcus lactis*, called Zirex, consisting of the pneumococcal repressor SczA and P_{czcD}. P_{czcD} tightly regulates the expression of green fluorescent protein in *L. lactis*. We show the applicability of Zirex together with the nisin-controlled expression system, enabling simultaneous but independent regulation of different genes.

Lactococcus lactis is a Gram-positive bacterium that has been intensively engineered for the production of heterologous proteins (1, 2). In addition, it is an organism generally recognized as safe (GRAS). To date, several promoters originally from *Lactococcus*, regulated by inducers or environmental factors, have been documented, including the *dnaJ* promoter, induced by heat shock (3); the PA170 promoter, which can be upregulated at a low pH during the transition to stationary phase (4); the *prtP* promoter, which is regulated by the peptide concentration in the medium (5); and the P_{Zn} *zitR* promoter, which responds to divalent cation starvation (6). The promoter of nisin, P_{nisA}, is the most widely used promoter for inducible protein expression in *L. lactis* (1, 7) and other Gram-positive bacteria (8). The expression from the P_{nisA} promoter is regulated by the two-component regulatory system NisRK, which is triggered by nisin. For the other promoters mentioned above, there are still some drawbacks, such as relatively low induction levels or high background level at the uninduced stage, which may complicate efforts to tightly control the expression or coexpression of one or two different proteins in the same cell (9). The aims of the present work were to develop a novel tightly controlled promoter for *L. lactis* and to investigate if such

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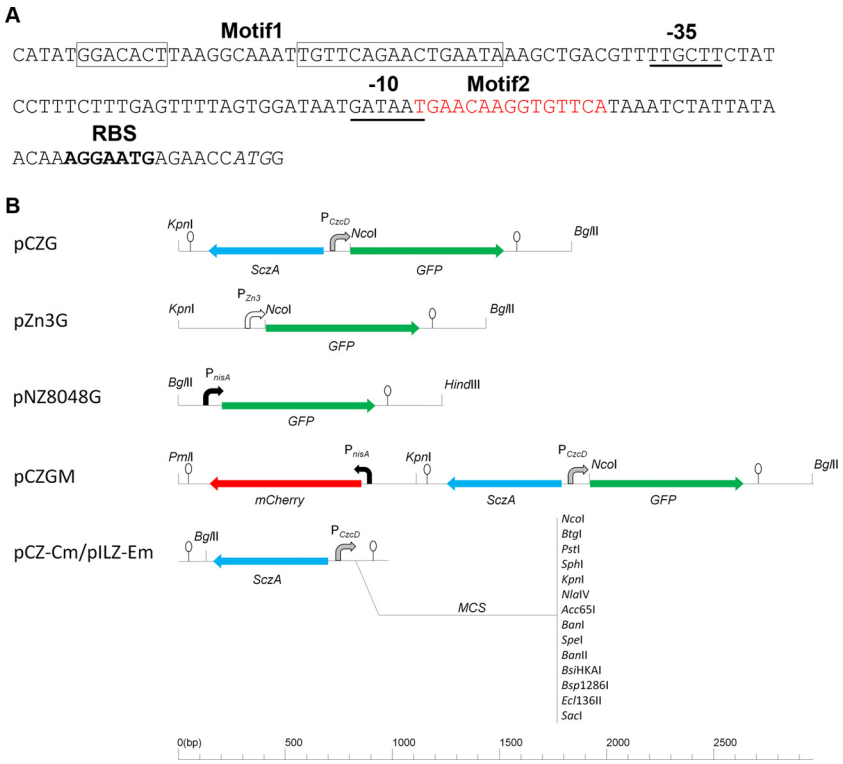

FIG 1 (A) Nucleic acid sequence of P_{czcD}. Motif1 (in boxes) and Motif2 (red letters) are the binding sites for the SczA regulator. The start codon of *czcD* is indicated in italics. The ribosome binding site (RBS) of P_{czcD} is shown in boldface. −35 and −10 sequences are underlined (10). (B) Expression systems used in this study. Hairpins represent terminators.

TABLE 1 Primers used in this study

Primer	Sequence[a]	Restriction site(s)
czcD-f	CGGGGTACCGGATCCTGCAGGCAGATATAGTTGATAATCAAGG	KpnI, SbfI
czcD-r	CAGCTCTTCTCCTTTTCCCATGGTTCTCATTCCTTTGTTATAATAG	NcoI
gfp-f	CTATTATAACAAAGGAATGAGAACCATGGGAAAAGGAGAAGAGCTG	NcoI
gfp-r	GGAAGATCTATTAATCGAAATACGGGCAGAC	BglII
gfp-r2	CCCAAGCTTCGAAATACGGGCAGAC	HindIII
mCherry-f	CGGGGTACCTCCTGGTTGCAAATTTTG	KpnI
mCherry-r	CGTACTCACGTGCTGCAAGGCGATTAAGTTG	PmlI
sczA-czcD-f	ATCAAGATCTAGAAATAAGACAACTGAAGCTTTAC	BglII
sczA-czcD-r	AGATCCATGGTTCTCATTCCTTTGTTATAATAG	NcoI
pIL-f	ATCAAGATCTACAGCAAAGAATGGCGGAAACG	BglII
pIL-r	AATCGATAAGCTTGGCTGCAGGTC	

[a] Restriction sites engineered in the primers are underlined.

an inducible promoter system could be coupled to the P_{nisA} promoter to create a dual-promoter-regulated production system for different proteins. First, we searched in the genome of *L. lactis* MG1363 (NCBI reference sequence NC_009004.1) for proteins putatively involved in cation transport that may be regulated by the presence of cations. A putative promoter, namely, P_{Zn3}, preceding the translation of a cationic ion efflux protein (NCBI reference sequence YP_001032214.1) in *L. lactis* was further investigated (see below). Additionally, we explored the genome of other related Gram-positive bacteria for cation-regulated promoters. In the case of *Streptococcus pneumoniae*, a zinc-inducible promoter was previously described by Kloosterman et al. (10) and Eberhardt et al. (11). *sczA* and *czcD* are transcribed divergently (Fig. 1A). The promoter of *czcD* gene is regulated by SczA. SczA binding to the motif 2 sequence located downstream of the −10 sequence of P_{czcD} blocks transcription of *czcD* in the absence of zinc. After the

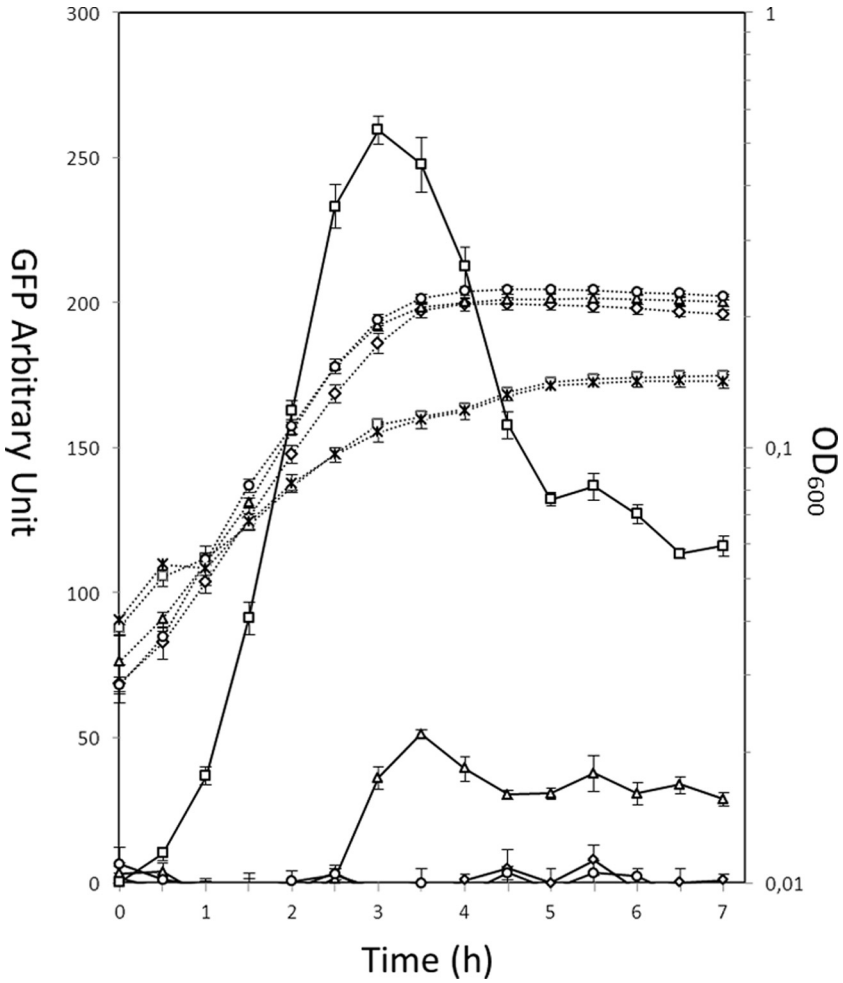

FIG 2 Growth (dotted lines) and GFP expression level (solid lines) after induction with 0.5 mM zinc at different time points. NZ9000(pCZG) induced with $ZnSO_4$ at a final concentration of 0.5 mM at 0 h (□), 2 h (△), and 4 h (○). Two control strains were run in parallel: NZ9000(pNZ8048) uninduced (◇) and induced with 0.5 mM $ZnSO_4$ at 0 h (×). The values represent the means from three independent measurements.

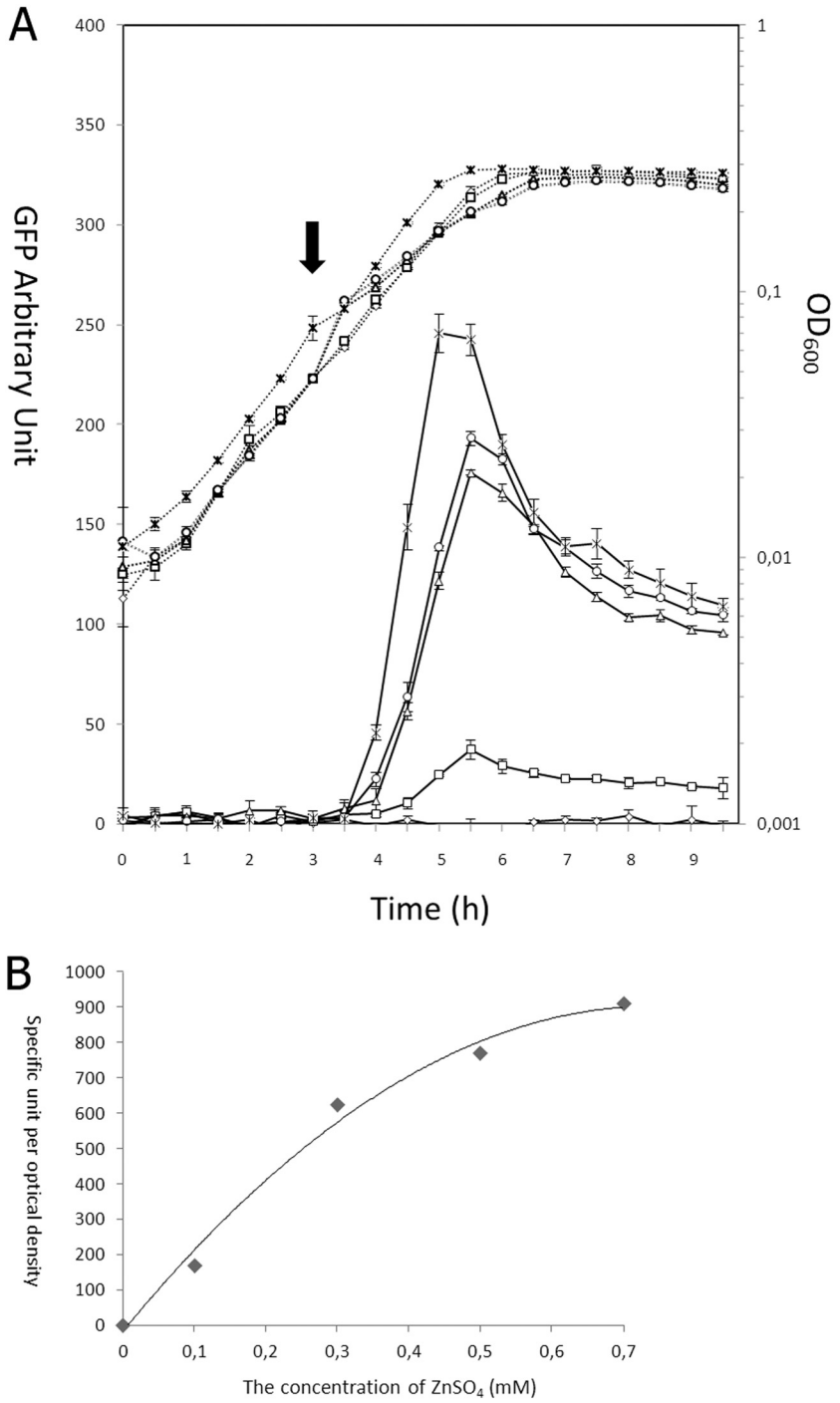

FIG 3 (A) Growth (dotted lines) and GFP intensity (solid lines) after induction with different zinc concentrations. *L. lactis* NZ9000(pCZG) was induced at an OD_{600} of 0.06 with different concentrations of $ZnSO_4$: 0 (◇), 0.1 mM (□), 0.7 mM (△), or 1.0 mM (○). A control, NZ9000(pNZ8048G) (×), induced with 5 ng/ml of nisin was also used to compare the production levels of P_{czcD} and P_{nisA}. The arrow indicates the time point for induction. These values represent the means from three independent measurements. (B) Dose-response curve of GFP expression of *L. lactis* NZ9000(pCZG). Fluorescent signal is shown as specific units per OD_{600}. The fluorescent signal changed less sensitively at the concentration of $ZnSO_4$ above 0.3 mM. The standard errors are less than 15% for each value.

addition of zinc, SczA will move to motif 1 unblocking the transcription (10).

Primers czcD-f and czcD-r (Table 1) were designed to amplify the regulator protein SczA and the P_{czcD} region from the *S. pneumoniae* D39 (12) genome, including the restriction sites KpnI and NcoI, respectively. The gene coding for green fluorescent protein (GFP) with its own terminator was amplified from pJWV102_gfp (a kind gift from J. W. Veening) by PCR with primers gfp-f and gfp-r. A BglII site was added on the 5′ end of primer gfp-r. czcD-r and gfp-f were designed to be reverse complementary by overlapping the 5′ ends of each other, and an NcoI site was inserted in both primers. The fragment SczA-P_{czcD}-GFP was generated by

spliced overlap extension PCR with primers czcD-f and gfp-r using the mixture of SczA-P_{czcD} and GFP-specifying amplicons as the templates (13). After digestion with KpnI and BglII, SczA-P_{czcD}-GFP was cloned into pNZ8048 (7), digested with the same enzymes to create the plasmid pCZG (Fig. 1B). pZn3G was constructed based on pCZG, in which the *gfp* gene was controlled by P_{Zn3} (Fig. 1B). Unfortunately, the low production level obtained after induction with zinc and the leakage in the noninduced state made P_{Zn3} an unsuitable candidate for further characterization (data not shown). pNZ8048G was created by cloning *gfp* amplified with the primers gfp-f and gfp-r2 (Table 1) in the NcoI-HindIII sites of pNZ8048. In pNZ8048G, GFP expression is under the control of P_{nisA} (Fig. 1B).

The expression assays were carried out in *L. lactis* NZ9000 (9), which was transformed with pCZG (containing SczA-P_{czcD}-GFP) according to Holo and Nes (14). All of the expression assays were conducted at 30°C in a chemically defined medium for prolonged cultivation (CDMPC) without $ZnSO_4$ supplemented with 10 μg/ml chloramphenicol (B. Teusink, F. Santos, O. P. Kuipers, C. E. Price, J. Kok, and D. Molenaar, unpublished data). Each assay was repeated in triplicate in a 96-well microtiter plate and monitored with an Infinite 200 Pro microplate spectrophotometer (Tecan Group, Ltd., Mannedorf, Switzerland).

First, we investigated the optimal induction moment. For this purpose, $ZnSO_4$ was added after 0 h, 2 h, or 4 h of growth at a final concentration of 0.5 mM. The cell growth was monitored measuring the optical density at 600 nm (OD_{600}), and the signal of GFP was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm (15). NZ9000(pNZ8048) was used as a negative control. We observed that the earlier we induced, the stronger the displayed signal was, with no induction observed in the stationary phase (Fig. 2). It should be noted that 0.5 mM $ZnSO_4$ showed comparatively low toxicity when it was introduced before inoculation (0 h) of the strains with (pCZG) or without (pNZ8048) GFP. The addition after 2 h of growth does not cause a visible reduction in growth. To assess if the slower growth when the cells were induced at 0 h was caused by the Zn salt used in the study, we also studied the growth tendencies and fluorescent signals of cells induced with the same amount of Zn^{2+} using $ZnCl_2$. The growth curves and the expression profiles were almost the same as those after induction with $ZnSO_4$ (data not shown). These results indicate that different Zn^{2+} sources do not affect the toxicity or potency of the induction.

In order to assess the optimal concentration of $ZnSO_4$ for the induction, NZ9000(pCZG) was induced at an OD_{600} of 0.06 (the middle of the exponential phase) with a final concentration of 0, 0.1, 0.3, 0.5, 0.7, or 1.0 mM $ZnSO_4$. NZ9000(pNZ8048G) grown in CDMPC was induced with 5 ng/ml of nisin at an OD_{600} of 0.06 as a reference. The growth rate was not affected by the addition of $ZnSO_4$, and the GFP signal produced by NZ9000(pNZ8048G) or NZ9000(pCZG) reached the highest level after 2 h or 2.5 h of induction, respectively (Fig. 3A). The highest intensity of GFP produced using the zinc-inducible system was almost 80% of that produced with the nisin-inducible system. Moreover, the GFP signal in the induced cells increased nearly proportionally with the $ZnSO_4$ concentration in the range between 0 and 0.3 mM (Fig. 3B). Furthermore, almost no fluorescent signal was detected under uninduced conditions, which demonstrated that this pneumococcal system is also effectively repressed in the absence of zinc in *L. lactis*.

TABLE 2 Simultaneous production of GFP and mCherry

$ZnSO_4$ as inducer (0.7 mM)[a]	Nisin as inducer (5 ng/ml 1 h later)[a]	Fluorescent intensity (AU)[b]	
		GFP (2.5 h)	mCherry (2.5 h)
+	−	181.00 ± 8.01	0.67 ± 4.19
+	+	138.00 ± 2.49	544.33 ± 17.68
−	+	5.33 ± 3.00	612.00 ± 39.04

[a] +, inducer present; −, inducer absent.
[b] AU, arbitrary units.

pCZGM was constructed to observe the effect of the induction with nisin and zinc at the same time. In pCZGM, P_{nisA} controls the expression of mCherry, whereas P_{czcD} controls the expression of GFP. To construct this vector, a fragment encompassing from P_{nisA} to the terminator of mCherry was amplified from pHK35C (a generous gift from H. Karsens) using the primers mCherry-f and mCherry-r, containing at their 5′ end a KpnI site and a PmlI site, respectively. After digestion with KpnI and PmlI, the fragment was inserted into pCZG cut with the same enzymes, resulting in pCZGM (Fig. 1B). The signal of mCherry was measured using an excitation wavelength of 590 nm and an emission wavelength of 620 nm, and GFP was measured as mentioned above. Cultures were induced with 0.7 mM $ZnSO_4$ at an OD_{600} of 0.06 and with 5 ng/ml nisin 1 h later. Uninduced controls lacking either nisin or zinc were run in parallel. In Table 2, we can observe the expression level of GFP or mCherry achieved after 2.5 h of the induction with $ZnSO_4$ or nisin. These data show that simultaneous overexpressions of mCherry and GFP in this system cause around 23% and 11% reduction of the two fluorescent signals, respectively.

Based on the results described above, we created pCZ-Cm for general use as a chloramphenicol-resistant expression vector for *L. lactis*. In this vector, the multiple-cloning site (MCS) of pNZ8048 was fused behind P_{czcD}. For this purpose, the region SczA-P_{czcD} was amplified from pCZG with the primers sczA-czcD-f and sczA-czcD-r (Table 1). After digestion with BglII and NcoI, the fragment SczA-P_{czcD} was inserted into pNZ8048 digested with the same restriction enzymes, rendering pCZ-Cm (Fig. 1B). An additional expression vector, termed pILZ-Em, containing this zinc-inducible expression system with the same MCS and erythromycin resistance was also constructed from the plasmid pIL253 (Fig. 1B) (16). A BglII site was inserted into pIL253 by round PCR with the primers pIL-f and pIL-r (Table 1) in order to insert the BglII-SacI region from pCZ-Cm.

In order to assess the usefulness of this double inducible system, the structural gene of nisin, *nisA*, was cloned into plasmid pCZ-Cm under the control of P_{czcD} and transformed into NZ9800 (17). In this strain, the enzymes responsible for the maturation and modification of nisin are controlled by P_{nisA}. Comparison of the production of nisin in CDM medium (18, 19) with a constant concentration of nisin and various amounts of $ZnSO_4$ was performed (Fig. 4). We measured the production of nisin using an activity test against *L. lactis* NZ9000 (20). The activity assay clearly shows that nisin can be successfully expressed in the system in a tightly regulated fashion when the gene *nisA* is controlled by P_{czcD} and the modification enzymes are regulated by nisin (Fig. 4).

In our study, we introduced the streptococcal promoter P_{czcD} together with the gene coding for its regulatory protein, SczA, in *L. lactis*, yielding an effective zinc-regulated expression system, called Zirex. Our results clearly show that this system can effectively control the overexpression of proteins in response to mod-

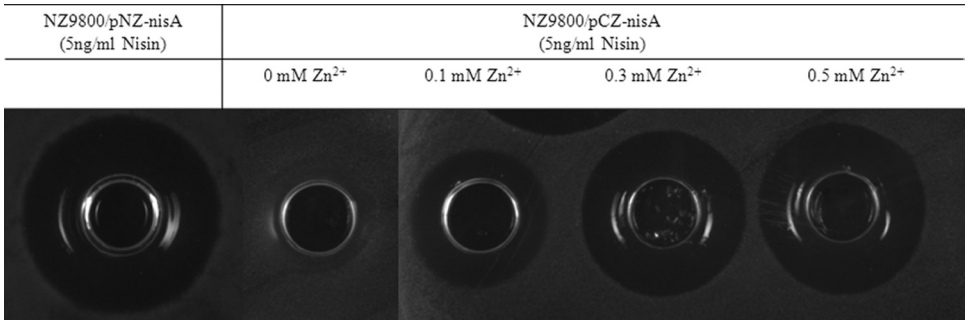

FIG 4 Expression of nisin measured in terms of activity. In the control strain, NZ9800(pNZ-nisA) (20), *nisA* and the modification enzymes that process nisin are controlled by P_{nisA}. In NZ9800(pCZ-nisA), *nisA* was controlled by P_{czcD}. A constant concentration of nisin (5 ng/ml) was used to induce both strains. In NZ9800(pCZ-nisA), no activity was detected in the absence of Zn^{2+}, whereas increasing amounts of Zn^{2+} led to the production of nisin proportionally to the concentration used.

est and nontoxic zinc additions to the medium in *L. lactis*. The very low basal expression without inducer suggests that SczA is also expressed in *L. lactis* and tightly represses the system in the absence of zinc. Notably, *L. lactis* showed high tolerance to zinc in the millimolar range when induced in the exponential phase (6). Previously, a zinc-repressed expression system (P_{Zn} *zitR* promoter) was reported. It was based on the *L. lactis zit* operon, which encodes an emergency Zn^{2+} uptake ABC transporter (6). The presence of Zn^{2+} can repress the expression of the emergency Zn^{2+} uptake ABC transporter, which partly explains the high tolerance of *L. lactis* to Zn^{2+}. The initiation of the P_{Zn} *zitR* promoter is caused by the addition of a chelating agent, which reduces the available zinc in the medium, therefore activating the transcription of the emergency uptake system (6). A drawback of this system is that the induction based on the depletion of Zn^{2+}, which is achieved with the addition of EDTA, can hamper the overexpression of proteins or enzymes that require cations. The zinc-inducible system presented here constitutes, to our knowledge, the first zinc-inducible promoter developed for *L. lactis*. It can be extremely useful for the overproduction of enzymes such as lanthipeptide cyclases, which require Zn^{2+} to be active, or other metalloenzymes. This advantage makes the expression system presented in this paper a suitable candidate for the production of lanthipeptides (21). So far the nisin-inducible expression (NICE) system is the most widely used and potent protein expression system in *L. lactis*. Compared to the nisin-inducible system, the common drawbacks of other regulated expression systems found in *L. lactis* are their low expression level and/or high leakage (9). The zinc-inducible system presented here achieves a high expression level comparable to that of nisin (ca. 80%), which is higher than the expression level obtained with the P_{Zn} *zitR* promoter (20% of that achieved with nisin) (6). Moreover, we demonstrate that it is possible to combine both inducible promoters for the expression of different proteins at different times during cell growth. This can be a useful tool for the overexpression of proteins or the creation of controlled gene regulatory circuits in *L. lactis* and expands the toolbox available for this bacterium. Moreover, the plasmid described here could be directly applicable for use in other Gram-positive hosts, as is the case for the NICE system, although this has to be further investigated to assess the specific characteristics.

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