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\(_{zna} zitR\) 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...
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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence$^a$</th>
<th>Restriction site(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>czcD-f</td>
<td>CGGGGTACCGGATCCTGACGGGAGATAGTTGTTATACAGG</td>
<td>KpnI, SbfI</td>
</tr>
<tr>
<td>czcD-r</td>
<td>CAGCTCTTCTCTTTTTCCATGTTTCTTATCCTTGTATATAG</td>
<td>NcoI</td>
</tr>
<tr>
<td>gfp-f</td>
<td>CTATTAAAACAAAGTGAGAACATGGGAAAAGGAGAAGAGCTG</td>
<td>NcoI</td>
</tr>
<tr>
<td>gfp-r</td>
<td>GGAAGATCTATTAATCGAAATAGTTGTTACAGG</td>
<td>BglII</td>
</tr>
<tr>
<td>gfp-r2</td>
<td>CCCAGCTTCAAAATACGGGCAAGC</td>
<td>HindIII</td>
</tr>
<tr>
<td>mCherry-f</td>
<td>CGGGGTACCTCCGGTTGCAAATTTTG</td>
<td>KpnI</td>
</tr>
<tr>
<td>mCherry-r</td>
<td>GTACTCAGTGTCCTGCAAGCCATTAATAGG</td>
<td>PmlI</td>
</tr>
<tr>
<td>sczA-czcD-f</td>
<td>ATCAAGATCTAGAATAAGACAACTGAAGCTTTAC</td>
<td>BglII</td>
</tr>
<tr>
<td>sczA-czcD-r</td>
<td>AGATCCATGTTTCTATCCTTGTATATAG</td>
<td>NcoI</td>
</tr>
<tr>
<td>pIL-f</td>
<td>ATCAAGATCTACAGCAAAAGATGCGGAAACG</td>
<td>BglII</td>
</tr>
<tr>
<td>pIL-r</td>
<td>AATCGAATAGCTTGGCTGCAAGGTC</td>
<td>BglII</td>
</tr>
</tbody>
</table>

$^a$ Restriction sites engineered in the primers are underlined.
addition of zinc, SczA will move to motif 1 unblocking the transcrip-
tion (10).

Primers czcD-f and czcD-r (Table 1) were designed to amplify the regulator protein SczA and the P\textsubscript{czcD} region from the \textit{S. pneu-
moniae} 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\textsubscript{czcD}-GFP was generated by

FIG 3 (A) Growth (dotted lines) and GFP intensity (solid lines) after induction with different zinc concentrations. \textit{L. lactis} NZ9000(pCZG) was induced at an OD\textsubscript{600} of 0.06 with different concentrations of ZnSO\textsubscript{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\textsubscript{czcD} and P\textsubscript{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 \textit{L. lactis} NZ9000(pCZG). Fluorescent signal is shown as specific units per OD\textsubscript{600}. The fluorescent signal changed less sensitively at the concentration of ZnSO\textsubscript{4} above 0.3 mM. The standard errors are less than 15% for each value.
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). pNZ8048G was constructed based on pCZG, in which the gfp gene was controlled by P\_nics (Fig. 1B). Unfortunately, the low production level obtained after induction with zinc and the leakage in the noninduced state made P\_nics 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 location of pNZ8048. In pNZ8048G, GFP expression is under the control of P\_nics (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 ZnSO4 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, ZnSO4 was added after 0 h, 2 h, or 4 h of growth at a final concentration of 0.5 mM. The cell growth was measured monitoring the optical density at 600 nm (OD600), and the signal of GFP was measured as mentioned above. Cultures were induced with 0.7 mM ZnSO4 at an OD600 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 ZnSO4 or nisin. These data show that simultaneous overexpression 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 pHLZ-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 P\_L-f and P\_L-r (Table 1) in order to insert the BglII-Sacl 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 ZnSO4 was performed (Fig. 4). We measured the production of nisin using an activity test against L. lactis NZ9800 (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-

<table>
<thead>
<tr>
<th>ZnSO4 as inducer (0.7 mM)</th>
<th>Nisin as inducer (5 ng/ml 1 h later)</th>
<th>Fluorescent intensity (AU)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>181.00 ± 8.01, 0.67 ± 4.19</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>138.00 ± 2.49, 544.33 ± 17.68</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>5.33 ± 3.00, 612.00 ± 39.04</td>
</tr>
</tbody>
</table>

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\_nics controls the expression of mCherry, whereas P\_czcD controls the expression of GFP. To construct this vector, a fragment encompassing from P\_nics 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 ZnSO4 at an OD600 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 ZnSO4 or nisin. These data show that simultaneous overexpression of mCherry and GFP in this system cause around 23% and 11% reduction of the two fluorescent signals, respectively.
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 (PZn-zitR promoter developed for L. lactis) 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 PZn-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 developer 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) presented in this paper a suitable candidate for the production of alloenzymes. This advantage makes the expression system presented in this paper a suitable candidate for the production of alloenzymes.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
NZ9800/pNZ-nisA (5ng/ml Nisin) & NZ9800/pCZ-nisA (5ng/ml Nisin) & 0 mM Zn\(^{2+}\) & 0.1 mM Zn\(^{2+}\) & 0.3 mM Zn\(^{2+}\) & 0.5 mM Zn\(^{2+}\) \\
\hline
\end{tabular}
\caption{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\(_{cza1}\). 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.}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{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\(_{cza1}\). 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.}
\end{figure}

\section*{Acknowledgments}
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\section*{References}


