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Short communication

A new salt inducible expression system for *Lactococcus lactis*

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ABSTRACT

A new expression system for *Lactococcus lactis* based on the salt inducible *BusA* promoter and the *BusR* repressor gene of *L. lactis* MG1363 was developed. To achieve salt induction, the expression of *BusR* was modulated by introducing mutations to its promoter sequence. An activity of 6.0 μkat l^{-1} of the model enzyme *Lactobacillus amylovorus* α-amylase was achieved in the bioreactor cultivation. The major advantage of the current expression system is that no additions of inducing agents are needed into bioreactor cultivations.

1. Introduction

Lactococcus lactis is a homofermentative lactic acid bacterium that is widely used in the food industry for the production of fermented milk products. The "generally regarded as safe" (GRAS) status of *L. lactis* makes it an attractive host for the production of proteins for the food industry or for therapeutic purposes. In addition, *L. lactis* strains producing heterologous proteins as antigens have been studied for use as live mucosal vaccines [1–3].

Most of the promoters reported for the production of recombinant proteins in *L. lactis* are constitutive [4–6]. The few inducible expression systems described include a phosphate starvation inducible expression system [7], a system based on the bacteriophage T7 promoter combined with the T7 polymerase gene fused to the *L. lactis lac* operon promoter [8] and an expression system controlled by zinc availability [9]. The most widely used inducible expression system for lactic acid bacteria is the nisin controlled expression system (NICE) [10].

High salt concentrations cause osmotic stress to microbes and expose cells to dehydration. A common strategy for microbial cells is to accumulate high intracellular concentrations of organic osmolytes, such as glycine betaine, in response to osmotic stress [11]. Many *L. lactis* strains possess an ABC transporter system for glycine betaine uptake named BusA (or OpuA). The activity of the BusA transporter is enhanced by high osmolarity of the culture medium. In addition, the *BusA* gene is inducible by salt. At elevated salt concentrations the BusR repressor protein is released from the *BusA* promoter area, which results in the expression of the *BusA* gene. Binding of BusR to *BusA* promoter depends on the ionic strength of the cytoplasm and not on the presence of specific osmolytes [12,13].

In this work we have isolated the *BusA* promoter and its regulatory gene *BusR* from *L. lactis*, modulated the expression level of *BusR*, and used the system for heterologous production of a secreted α-amylase. We show that the new expression system is salt inducible and that in bioreactor cultivations addition of inducible agents is not required for triggering the recombinant enzyme production.

2. Materials and methods

2.1. Bacterial strains and plasmids

The plasmid carrying the phosphate starvation inducible expression system and *Lactobacillus amylovorus* α-amylase gene [7] derived from pNZ8032 (NIZO food research, Netherlands [14]) was used in cloning the expression system. *L. lactis* MG1363 (Institute of Food Research, UK, [15]) was the expression host.

2.2. Plasmid construction

The primers used in this work are shown in Table 1 and the organization of the genetic constructs is presented schematically in Fig. 1. The promoter of *L. lactis BusA* gene and the *BusR* gene were amplified using the genomic DNA of the MG1363 strain as the tem-

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Table 1
Primers used in this work. The restriction sites are shown in italics.

Primer	Sequence	Vector construct
BusAp_BglII_FW	5′-ATATAT*AGATCT*AGACATCGTTTACAAAACGATGCTTT-3′	pBA
BusAp_NcoI_REV	5′-TTATAT*CCATGG*AATAACTCTCCTTCATTCTATTACTCATGAGC-3′	pBA, pBRA, pBMRA1–pBMRA87
BusRp_BglII_FW	5′-TATCATATAGATCTGCTTCTCATATTTAGAAGCTA TTTTTAGTCCAGC-3′	pBRA
Degen_BglII_BusRp_FW	5′-TAGTACTAT*AGATCT*TGGGGNNNNNTGGTATAG ATTGAAANNNNNNNNNNNNNNNCGRTATAATNNNNNGGAATTTTGTTTTGCTTAGGTAAAG-3′ N = 25% A, 25% T, 25% G, 25% C R = 50% A, 50% G	pBMRA1–pBMRA87

plate. Two forward primers (BusAp_BglII_FW and BusRp_BglII_FW) and one reverse primer (BusAp_NcoI_REV) were used in the reactions. A PCR fragment containing the putative *BusA* promoter and another PCR fragment containing the *BusR* promoter, *BusR* and the *BusA* promoter were generated. *Bgl*II and *Nco*I restriction sites were introduced into the 5′- and 3′-ends of both PCR fragments, respectively.

To modulate the expression of *BusR* using the strategy described previously [16], a set of PCR fragments containing the *BusA* promoter and the *BusR* gene with mutations in its promoter area were amplified. The mutations were created using a mixture of forward primers shown in Table 1 (Degen_BglII_BusRp_FW) and the same reverse primer as with the first two constructs.

The *pstF* promoter flanking the *L. amylovorus amyA* gene was removed from the plasmid ([7], construct *e*) by digestion with *Bgl*II and *Nco*I (New England Biolabs, USA). The vectors were named as follows: the vector carrying only the *BusA* promoter in front of the *amyA*: pBA; the vector carrying the original *BusR* promoter, *BusR* and the *BusA* promoter in front of the *amyA*: pBRA; and the vectors carrying the mutated *BusR* promoters, *BusR* and the *BusA* promoter in front of the *amyA*: pBMRA1-pBMRA87 (Fig. 1.).

L. lactis MG1363 was transformed with the plasmids by electroporation [17].

2.3. Media and cultivation conditions

Chloramphenicol was used at a concentration of 9 μg ml^{-1} for the selection of the vectors.

In small scale and cloning experiments *L. lactis* MG1363 (and its derivatives) were cultivated at 30 °C in GM17 medium (Difco, USA) supplemented with 2% (w/v) glucose. The different constructs were tested for salt induction of the α-amylase production in small scale experiments. Overnight cultures (240 μl) of the *L. lactis* MG1363 strains harboring the new constructs were used to inoculate 12 ml of GM17 medium. After 2.5 h of cultivation, the cultures were divided into two portions of which the other was supplemented with 3.7 M KCl to reach the final concentration of 0.5 M. The cultures were first incubated for 4 h at 30 °C and then overnight at room temperature.

Further experiments in bioreactor scale were conducted for the selected mutant construct (pBMRA20) and the control strains (pBA and pBRA). The medium used in the bioreactor cultivations comprised 80 g l^{-1} glucose, 19.75 g l^{-1} casitone (BD, France), 11.25 g l^{-1} yeast extract (Lab M, UK), 1 g^{-1} K_2HPO_4, 1 g l^{-1} KH_2PO_4 and 0.2 g l^{-1} $MgSO_4$. The bioreactor cultivations were conducted in a Biostat MD (B. Braun Biotech International, Germany) reactor with a working volume of 1 l. The bioreactor was inoculated with 50 ml of overnight culture. The pH was controlled at 7.0 by the addition of 3 M NaOH. The temperature was set at 32 °C and the stirring rate at 250 rpm. The cells were cultivated for 9.5 h after inoculation.

2.4. Analytical methods

Extracellular α-amylase activity was determined from the culture supernatants at 37 °C using the Phadebas® Amylase Test (Magle Life Sciences, Sweden) according to the instructions by the manufacturer.

Online, *in situ* optical cell density probe (TruCell, Finesse Instruments, USA) was used for monitoring the bioreactor cultivations. Cell dry weight (CDW) was determined at four time points (two parallel samples) of one bioreactor cultivation to obtain a correlation between the online measurement of optical density and CDW. An eight-point linear calibration curve of CDW versus online optical density (600 nm) with an R^2 of 0.986 was obtained.

3. Results and discussion

3.1. Performance of different constructs in small scale experiments

The expression constructs preliminarily tested for salt induction are presented in Fig. 1 and the α-amylase activities achieved are shown in Table 2. The concentration of the inducing agent KCl was chosen as 0.5 M on the basis of previous studies on the *BusR-BusA* system [13]. The addition of KCl to the culture medium of the transformant harboring the vector pBA (with only the *BusA* promoter in front of the *AmyA* gene) did not increase but decreased the α-amylase yield, because of growth inhibition by salt (data not

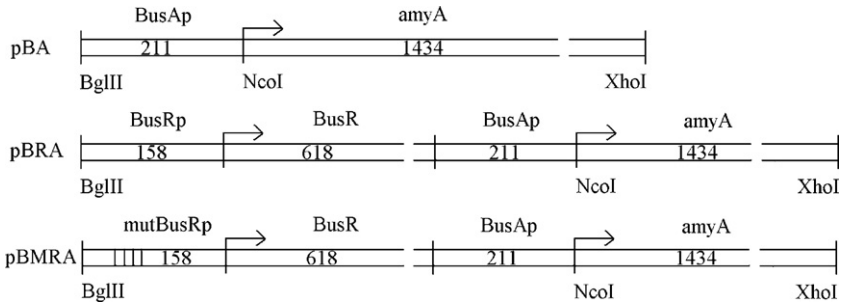

Fig. 1. The expression constructs used in this study. pBA: the BusA promoter and the *amyA* coding sequence. pBRA: the *BusR* promoter, *BusR* coding sequence, *BusA* promoter and the *amyA* coding sequence. pBMRA: the mutated *BusR* promoter, *BusR* coding sequence, *BusA* promoter and the *amyA* coding sequence. Translation start sites are shown with arrows.