Construction of vectors for inducible gene expression in 
*Lactobacillus sakei* and *L. plantarum*

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Abstract

We have constructed vectors for inducible expression of genes in *Lactobacillus sakei* and *Lactobacillus plantarum*. The key elements of these vectors are a regulatable promoter involved in the production of the bacteriocins sakacin A and sakacin P and the genes encoding the cognate histidine protein kinase and response regulator that are necessary to activate this promoter upon induction by a peptide pheromone. The vectors are built up of cassettes that permit easy exchange of all parts through restriction enzyme digestion and ligation. Using β-glucuronidase as a reporter enzyme, variants of these vectors were compared with each other, and with a corresponding system based on genes involved in the production of nisin. Several of the new vectors permitted tightly controlled and efficient expression of β-glucuronidase in both *L. sakei* and *L. plantarum*.

Keywords: *Lactobacillus*; Inducible expression; Vector

1. Introduction

Lactic acid bacteria (LAB) are used in a large variety of industrial food fermentations and are known for the beneficial effects they exert on the health of humans and animals. Lactobacilli are used as starter cultures in the production of fermented meats, cheeses, fermented plant foods, wine, beer, sourdough bread and silage. Lactobacilli are generally the most acid-tolerant of the LAB and will therefore terminate many spontaneous lactic fermentations such as silage and vegetable fermentations [1]. *Lactobacillus sakei* is used in meat and vegetable fermentations and has the ability to grow at low temperatures. *Lactobacillus plantarum* can be found in many habitats such as the gastrointestinal tract and the oral cavity of humans, and in a variety of fermented food products [2]. Some *L. plantarum* strains have also been reported to have probiotic effects on human health [3,4]. Considerable interest exists in the development of genetic tools that allow production of desired proteins from these microorganisms in order to create strains with improved protective and fermentative properties or in order to create ‘food-grade’ cell factories. Such expression systems should preferably permit inducible overproduction of a desired protein at any moment during fermentation.

The expression system based on the autoregulatory (quorum-sensing) properties of the *Lactococcus lactis* nisin gene cluster [5,6], is one of the most widely used [7,8]. Nisin induces the transcription of the genes under control of the *nisA* and *nisF* promoters via a two-component regulatory system, consisting of a histidine kinase (HK) and a response regulator (RR) encoded by the *nisK* and *nisR* genes [9–11]. A transferable nisin-controlled expression (NICE) system based on the combination of the *nisA* promoter and the *nisRK* regulatory genes has been developed for *L. lactis*. In this system the level of expression can be controlled by the amount of nisin added for induction [5]. For general use in other LAB than *Lc. lactis*, a system consisting of two compatible plasmids has been constructed: one plasmid carries the *nisRK* regulatory genes and a second plasmid carries the gene of interest under the control of the *nisA* promoter [12].
The production of some class II bacteriocins in lactobacilli is regulated by quorum-sensing mechanisms which differ from the mechanism employed for nisin regulation in *L. lactis*. These lactobacilli secrete a non-modified peptide whose primary function is to act as a pheromone and not as a bacteriocin. The regulatory operons in these systems consist of three genes, encoding the peptide pheromone precursor, a HK protein that senses the pheromone, and a cognate RR protein. Activation of the RR enhances transcription from regulated promoters that precede all operons involved in bacteriocin production, processing, secretion and immunity, as well as the regulatory operon itself [13,14]. A recent study describes the construction of a prototype inducible expression vector based on regulatory genes and promoters involved in the production of sakacin A by *L. sakei* Lb706 [15]. The present study is an expansion of this work and describes the systematic construction of a set of versatile expression vectors where the operational parts consist of an HK gene, an RR gene and a cognate bacteriocin promoter. The genes and the promoter are naturally involved in the production of sakacin A ([16], *sap* genes) or sakacin P ([17,18], *spp* genes). The vectors are built up of cassettes that permit easy exchange of all parts through restriction enzyme digestion and ligation, and they can be used as models for food-grade and ‘self-cloning’ systems. The functionality of these vectors was analyzed using β-glucuronidase as a reporter enzyme in *L. sakei* and *L. plantarum*. For comparative purposes, an analogous nisin-based vector system was developed.

### 2. Materials and methods

#### 2.1. Bacterial strains and media

The bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in BHI medium (Oxoid, Hampshire, UK) at 37°C with shaking, while *Lactobacillus* strains were grown in MRS medium (Oxoid) at 30°C without shaking. Agar plates were made using BHI agar medium (Oxoid) for *E. coli* and MRS agar medium (Oxoid) for *Lactobacillus* strains. When appropriate, erythromycin was added as follows: 200 µg ml⁻¹ for *E. coli* and 10 µg ml⁻¹ for lactobacilli. 

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*Promoters are indicated by P, directly followed by the gene name; terminators are indicated by T, directly followed by the gene name; Em, erythromycin.*
2.2. Plasmid DNA preparation and transformation

Plasmid DNA from *E. coli* was isolated using commercial kits (Qiagen, Venlo, The Netherlands). Plasmid DNA from *L. sakei* and *L. plantarum* was isolated by a modified alkaline lysis method as described previously [19]. *E. coli* XL10 Gold (Stratagene, La Jolla, CA, USA) was used as a host strain for the construction of the plasmids, and cells were transformed according to the manufacturer’s procedures. *Lactobacillus* strains were electrotransformed according to Aukrust and Blom [20].

2.3. Construction of plasmids

Plasmids used directly or as template for polymerase chain reaction (PCR) reactions and plasmids constructed in this study are listed in Table 1. Plasmids were constructed using standard molecular cloning and PCR fusion (overlap extension) techniques [21,22]. All enzymes were purchased from Promega (Madison, WI, USA). Primers were purchased from MWG Biotech (Ebersberg, Germany) (see Appendix 1, http://www.matforsk.no/LAB/ESO/). PCR was performed with the Gene Amp PCR System 9700 (PE Biosystems, Foster City, CA, USA), with Expand High Fidelity PCR System polymerase (Roche Diagnostics, Mannheim, Germany) using standard procedures.

Plasmid pSIP200 (Fig. 1) was designed to contain the ‘backbone’ elements of the expression vectors: the replication determinants for *E. coli* (pUC(pGEM)ori), *L. sakei* and *L. plantarum* (256rep), an erythromycin resistance marker (*ermL*) and the pepN terminator (GenBank accession number M87840, nt 2781–3004) from pNZ8048 to prevent read-through from the reporter gene (see Table 1 for sources and Appendix 1 for primers). It also contains the *sapA* promoter followed by a *Nco*I site (allowing translational fusions) and a multicloning site (MCS). The *P*<sub>sapA</sub>*-NcoI-MCS fragment was constructed as a PCR fusion between *P*<sub>sapA</sub> from pSAK17B and parts of the MCS from pGEM<sup>®</sup>-7Zf<sup>+</sup> (Promega).

Plasmid pSIP300 contains *sapKR* directly 3’ of the erythromycin resistance gene, while pSIP301 contains *sapKR* preceded by a mutated, non-functional version of the native pheromone gene (*sapIP*) with its intact cognate promoter (Fig. 1). In addition the vectors contain a double terminator region directly 3’ of *sapKR* consisting of the terminator region found 3’ of *sapAsaA* in the native system and the catT94 terminator (GenBank accession number SAC194, nt 901–1036) from pNZ8048. These elements were obtained by PCR amplification (see Table 1 for sources and Appendix 1 for primers) and subsequently inserted into pSIP200 using restriction sites (Fig. 1). In this process a *Nco*I site in the beginning of *sapR* was mutated by a single nucleotide substitution using gene SOEing [22]. The change did not affect the amino acid sequence of *sapR*.

Plasmids pSIP400 and pSIP401 are the *spp* versions of pSIP300 and pSIP301, respectively, whereas pSIP500 and pSIP501 are the *nis* versions (Fig. 1). In analogy to pSIP301, pSIP401 contains a mutated, non-functional version of the native pheromone gene with its intact cognate promoter directly upstream of *sppKR*. In pSIP501, *nisR* is preceded by its cognate (constitutive) promoter. In the process of constructing pSIP500/pSIP501 a *Sal*I site in the beginning of *nisK* was mutated by a single nucleotide substitution using gene SOEing [22]. The change did not affect the amino acid sequence of *nisK*.

The *E. coli gusA* gene encoding β-glucuronidase (GUS [23]) was translationally fused to the regulated promoter in the vectors using the *Nco*I site. The *gusA* gene used in this work contained a 3-bp deletion in the 3’ end compared to the *gusA* with GenBank accession number S69414. This mutation leads to a deletion of a glutamate, but leaves GUS activity intact [15].

Appropriate recombinant plasmids were selected after transformation of *E. coli* XL10 Gold followed by plating on BHI agar containing 200 μg ml<sup>-1</sup> erythromycin. All constructs were verified by sequence analysis using an ABI Prism<sup>®</sup> 3100 Genetic Analyzer, with the ABI Prism<sup>®</sup> BigDye<sup>®</sup> Terminator Cycle Sequencing Ready Reaction kit, following the manufacturer’s recommendations (PE Biosystems). Verified constructs were electrotransformed into *L. sakei* Lb790 and *L. plantarum* NC8.

2.4. Assay for GUS activity

A modified β-galactosidase assay [24] was used to determine the β-glucuronidase activity of the *Lactobacillus* strains harboring the different vectors. The strains were induced at an OD<sub>600</sub> of approximately 0.3 and the assays were performed as described in Axelsson et al. [15]. Activity was calculated as described by Miller [24] and expressed as Miller Unit equivalents (MU). The sakacin A and sakacin P induction peptides, SapIP and SppIP, respectively [25,26], were purchased from the Molecular Biology Unit, University of Newcastle Upon Tyne (UK). Nisin was purchased from Sigma (St. Louis, MO, USA).

2.5. Dose response

 Cultures were induced with different concentrations of inducing peptide and the GUS activity was monitored over time. The maximum activity for each inducer concentration was used to create a dose–response curve.

3. Results

3.1. Plasmid construction

The vectors constructed in this work are shown in Fig. 1. The potential of the expression systems was assessed using the *E. coli* *gusA* gene as a reporter, creating plasmids
pSIP302, pSIP303, pSIP402, pSIP403, pSIP502 and pSIP503 (Table 1).

3.2. GUS activity

Repeated assays with *L. sakei* Lb790 and *L. plantarum* NC8 wild-type strains without the *gusA* gene showed background levels up to 6 MU, hence the detection limit of the GUS assay was set at 6 MU. To determine the maximum levels of expression, cell cultures were induced with relatively high amounts of inducing peptide (IP) (50 ng ml$^{-1}$ of SapIP and SppIP, and 5 ng ml$^{-1}$ of nisin) [12,15,26]. For all vectors, cells harvested at an OD$_{600}$ of 1.7–2.1 for *L. sakei* Lb790 and 1.2–1.8 for *L. plantarum* NC8 showed the highest GUS activities in these tests (data not shown). Fig. 2 shows that the sakacin A-based vector pSIP302 gave the highest levels of GUS activity in both *L. sakei* Lb790 and *L. plantarum* NC8 and that the maximum activity was higher in *L. sakei* Lb790 than in *L. plantarum* NC8. The nisin-based vector pSIP503 also gave high levels of activity in both strains. It did, however, exhibit a considerable basal activity (i.e. activity without induction), especially in *L. plantarum* NC8 (e.g. see $t=0$ points in Fig. 2). The induction factors were calculated by dividing maximum activity of an induced culture by the maximum activity of the parallel uninduced culture. If the
maximum activity of the uninduced culture was lower than the detection limit of 6 MU, the detection limit was used in the calculations. The induction factors for the sakacin A- and sakacin P-based vectors ranged from 7 for pSIP303 in L. plantarum NC8 and 9 for pSIP402 in L. sakei Lb790, to 32 for pSIP302 in L. sakei Lb790. The nisin-based vectors gave induction factors ranging from 1.3 in L. plantarum NC8 to 5 in L. sakei Lb790.

To compare the constructed vectors with the two-plasmid NICE system, the gusA gene was cloned in front of the nisA promoter in pNZ8048 (Table 1). The resulting plasmid (pNZ8048gus) and pNZ9530 (Table 1) were transformed into both L. sakei Lb790 and L. plantarum NC8 and GUS assays were performed. The NICE system gave GUS activities of approximately 1600 and 1800 MU in L. sakei Lb790 and L. plantarum NC8, respectively, and the vectors also showed a considerable basal activity (37 MU for L. sakei Lb790 and 78 MU for L. plantarum NC8) (data not shown). The induction factor for the NICE system was 23 in L. plantarum NC8 and 43 in L. sakei Lb790.

Studies with different concentrations of IP were performed on the sakacin A- and sakacin P-based vectors in L. sakei Lb790 and L. plantarum NC8. In both strains, the GUS activity was found to be IP dose-dependent, essentially in the range of 0.1–5 ng ml⁻¹ (Fig. 3). There was no or very low activity in uninduced cultures. The nisin-based vectors showed a considerable basal activity, and were not used in the dose-response assays.
4. Discussion

In this work, a set of inducible expression vectors, designed for use in *L. sakei* and *L. plantarum*, is described. Two sets of vectors were constructed, based on the genes whose natural functions are to regulate the production of the class II bacteriocins sakacin A and sakacin P (Fig. 1). For comparative purposes, a set of analogous nisin-based vectors was developed. The vectors are built up of cassettes that permit easy exchange of all parts through restriction enzyme digestion and ligation.

One of the most widely used expression systems for LAB is based on the autoregulatory properties of the *Lc. lactis* nisin gene cluster, where the combination of the *nisA* promoter, the *nisRK* regulatory genes and externally added nisin controls the expression. In *Lactobacillus* species, this inducible NICE system has been used either as a two-plasmid system [12], or as a one-plasmid system with a designed host strain containing the *nisRK* genes integrated in the chromosome [27,28]. A two-plasmid system is somewhat laborious to use and integration of genes into the chromosome limits the system to specially designed host strains. The expression systems described in this work are one-plasmid systems with replication determinants for *E. coli*, *L. sakei* and *L. plantarum*. This makes it possible to do all the construction work in *E. coli* and the systems should work in any strain of *L. sakei* and *L. plantarum*. The replication determinants of our vectors can easily be changed, meaning that the system can be made to function in other lactobacilli and potentially also in bacteria belonging to other genera. Although not completely tested in this study, the systems described can also be used (with some modifications) as ‘self-cloning’ expression systems. This is certainly true for *L. sakei*, for which the sakacin-based systems are native. Similar systems can be envisaged for *L. plantarum* using e.g. the plantaricin signal transducing system [29]. Since the vectors are built up of cassettes, one could, in theory, create ‘self-cloning’ systems for any bacteria using native regulatory elements and replication determinants. If the goal is a food-grade expression system the antibiotic resistance marker can easily be exchanged with a food-grade marker.

Since the level of expression of the HK and RR genes has been shown to affect the performance of the NICE system [12] and the efficiency of the sakacin P production [17], two versions of each system were constructed in this study. In the first, the HK/RR genes are transcribed by direct read-through from the erythromycin resistance gene (Fig. 1; pSIP300, pSIP400 and pSIP500). In the second, the HK/RR genes are transcribed from the native promoter (Fig. 1; pSIP301, pSIP401 and pSIP501). Note that in the sakacin A and sakacin P systems, these native promoters are activated by the response regulator upon induction, in the same manner as *P_{sapA}* and *P_{sppA}*. To ensure that no IP was produced endogenously by the system, the cognate IP genes (*sapIP* and *sppIP*) were mutated in pSIP301 and pSIP401. In the native nisin system, the pheromone gene, *nisA*, is not co-transcribed with the R and K genes and the RK operon is preceded by a constitutive promoter [30]. Somewhat surprisingly, the results were not consistent with respect to which of the two types of vectors (with direct read-through or with cognate promoter) yielded the highest expression levels. Of the sakacin A-based vectors, the pSIP300-derived pSIP302 (direct read-through) gave the highest expression levels in both *L. sakei* Lb790 and *L. plantarum* NC8 (Fig. 2). For the other two systems, the pSIP401- and pSIP501-derived pSIP403 and pSIP503 (cognate promoter) showed the highest levels (Fig. 2). These results indicate that optimal expression levels are not related to HK/RR production in a straightforward manner but require a subtle balance between expression of the HK/RR genes and other factors.

The nisin-based vectors pSIP502 and pSIP503 showed high levels of GUS activity and a large basal activity,

![Fig. 3. Dose-response curves of gusA expression in L. sakei Lb790 (A) and L. plantarum NC8 (B) harboring pSIP302 (■), pSIP303 (▲), pSIP402 (●) and pSIP403 (▲). The cultures were induced with 0.1, 0.5, 1, 2.5, 5, 10 and 25 ng ml⁻¹ IP at OD₆₀₀ ≈ 0.3. Each data point shows maximum GUS activity at a given concentration of IP and is the average of three separate experiments.](https://academic.oup.com/femsle/article-abstract/229/1/119/473068)
especially in L. plantarum NC8 (Fig. 2). To compare our newly developed vectors with the two-plasmid NICE system as originally described by Kleerebezem et al. [12], the gusA gene was cloned in front of the nisA promoter in pNZ8048 (a derivative of the pNZ8030 series [9]). The resulting plasmid (pNZ8048-gus) and pNZ9530 (Table 1) were transformed into both L. sakei Lb790 and L. plantarum NC8 and GUS assays were performed. The NICE system gave up to six times higher GUS activity than the pSIP302 vector, but also exhibited a considerable basal activity in both strains. This correlates with previous findings where the two-plasmid NICE system exhibits basal activity in L. plantarum [27]. Bryan et al. [31] have constructed a one-plasmid, NICE-based expression vector for use in Gram-positive bacteria, which has thus far been tested only in enterococci. They found that the vector gave high expression levels, but as with our pSIP502/503 vectors and the two-plasmid NICE system, it exhibits some basal activity. By creating a designed host strain containing the nisRK genes integrated in the chromosome, however, the nisin-based expression system gives high expression levels in a tightly regulated manner in lactobacilli [27,28,32]. While giving lower maximum activity levels, all sakacin A- and sakacin P-based vectors (pSIP302/pSIP303 and pSIP402/pSIP403) gave no or very little basal activity (data not shown). Thus, in lactobacilli these vectors could be an alternative to the nisin-based systems when a simple, one-plasmid system for direct use is preferred and tight regulation is important.

Preliminary results obtained in our laboratories indicate that maximum expression levels obtained with the pSIP vectors may be increased by exchanging the 256rep replication determinant with a replication determinant that gives rise to a higher copy number (e.g. the pSH71-derived determinant used in nisin plasmids such as pNZ8048). To investigate the leakage of the promoters, a more sensitive reporter system should preferably be used.

Further studies of the sakacin A- and sakacin P-based systems showed that GUS activity was IP dose-dependent in the 0.1–5 ng ml⁻¹ range (Fig. 3). Thus, the present vectors enable both temporal and quantitative regulation of gene expression in Lactobacillus, indicating that they may be useful in a variety of applications. Work is in progress to further optimize these systems, e.g. by changing the promoters and/or the replication determinants, in order to create vectors that may be used to produce high amounts of a desired protein in a tightly controlled manner.

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