

High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors

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Vectors have been developed for inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* in which expression of the gene of interest is driven by strong, regulated promoters from bacteriocin operons found in *L. sakei* strains. The activity of these promoters is controlled via a two-component signal transduction system, which responds to an externally added peptide pheromone. The vectors have a modular design, permitting easy exchange of all essential elements: the inducible promoter, the cognate regulatory system, the gene of interest, the antibiotic resistance marker and the replicon. Various variants of these so-called 'pSIP' vectors were constructed and tested, differing in terms of the bacteriocin regulon from which the regulatory elements were derived (sakacin A or sakacin P), the regulated promoter selected from these regulons, and the replicon (derived from p256 or pSH71). Using β -glucuronidase (GusA) and aminopeptidase N (PepN) as reporters, it was shown that the best vectors permitted inducible, pheromone-dose-dependent gene expression at very high levels, while displaying moderate basal activities when not induced. The most effective set-up was obtained using a vector containing the pSH71 replicon, the *orfX* promoter from the sakacin P regulon, and the cognate regulatory genes, in a *L. sakei* host. GusA levels obtained with this set-up were approximately ten times higher than the levels obtained with prototype pSIP versions, whereas PepN levels amounted to almost 50% of total cellular protein.

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INTRODUCTION

Many *Lactobacillus* species are important in the food industry, where they are used in a variety of products. *Lactobacillus sakei* is used in meat and vegetable fermentations and has a broad range of possible habitats. The versatility of *L. sakei* can partly be explained by its ability to survive and grow under adverse conditions, such as low temperature and pH, high salt concentration, smoke, ethanol, low water activity and radiation. *Lactobacillus plantarum* is also found in many habitats, including dairy and meat products, plant and vegetable fermentations, and in the gastrointestinal tract and oral cavity of humans (Axelsson & Ahrné, 2000). Some *L. plantarum* strains have probiotic effects on human health (Alander *et al.*, 1999; Schultz *et al.*, 2002; Mercenier *et al.*, 2003). Lactobacilli have great potential as food-grade cell factories and as delivery vehicles for interesting proteins, such as antigens, antibodies and growth factors (Pouwels *et al.*, 1996, 2001; Pavan *et al.*, 2000; Krüger *et al.*, 2002; Scheppler *et al.*, 2002). Thus, there is considerable interest

in the development of genetic tools for efficient and controllable gene expression in lactobacilli (de Vos, 1999; Mercenier *et al.*, 2000).

Many lactic acid bacteria (LAB) produce antimicrobial peptides called bacteriocins, and their production is often regulated via quorum-sensing mechanisms based on a secreted peptide pheromone (Eijsink *et al.*, 2002; Quadri, 2002). The pheromone activates a two-component regulatory system consisting of a histidine kinase receptor and a cognate response regulator. In strains producing class I bacteriocins (lantibiotics such as nisin), the bacteriocin itself acts as pheromone (Kuipers *et al.*, 1995). Strains producing class II bacteriocins, such as sakacin A and sakacin P, produce and secrete a separate, non-modified peptide pheromone whose gene is usually co-transcribed with the genes encoding the histidine kinase and the response regulator (Nes & Eijsink, 1999). In both cases, the activated response regulator enhances transcription of all operons involved in bacteriocin production.

The promoters and regulatory genes from the *Lactococcus lactis* nisin gene cluster have been used to develop regulated

Abbreviations: LAB, lactic acid bacteria; MU, Miller Unit equivalents; NICE, nisin-controlled expression.

gene expression systems for lactococci (de Ruyter *et al.*, 1996), lactobacilli (Pavan *et al.*, 2000; Sørvig *et al.*, 2003) and other Gram-positive bacteria (Eichenbaum *et al.*, 1998; Bryan *et al.*, 2000). While being efficient and well regulated in lactococci, the plasmid-based nisin-controlled expression (NICE) systems often exhibit significant basal activity (i.e. activity without induction) in lactobacilli (Pavan *et al.*, 2000; Sørvig *et al.*, 2003). This problem can be circumvented by integrating the histidine kinase and response regulator genes in the chromosome (Pavan *et al.*, 2000), thus limiting the expression systems to specially designed host strains.

Recently, we have developed one-plasmid inducible expression systems based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin A (*sap* gene cluster) and sakacin P (*spp* gene cluster) (Axelsson *et al.*, 2003; Sørvig *et al.*, 2003). Sørvig *et al.* (2003) constructed prototypes of the modular so-called pSIP expression vectors (Fig. 1). These vectors contain a promoter element derived either from the sakacin A or from the sakacin P structural gene with an engineered *Nco*I site for translational fusion cloning, as well as the cognate two-component regulatory system. Using β -glucuronidase from

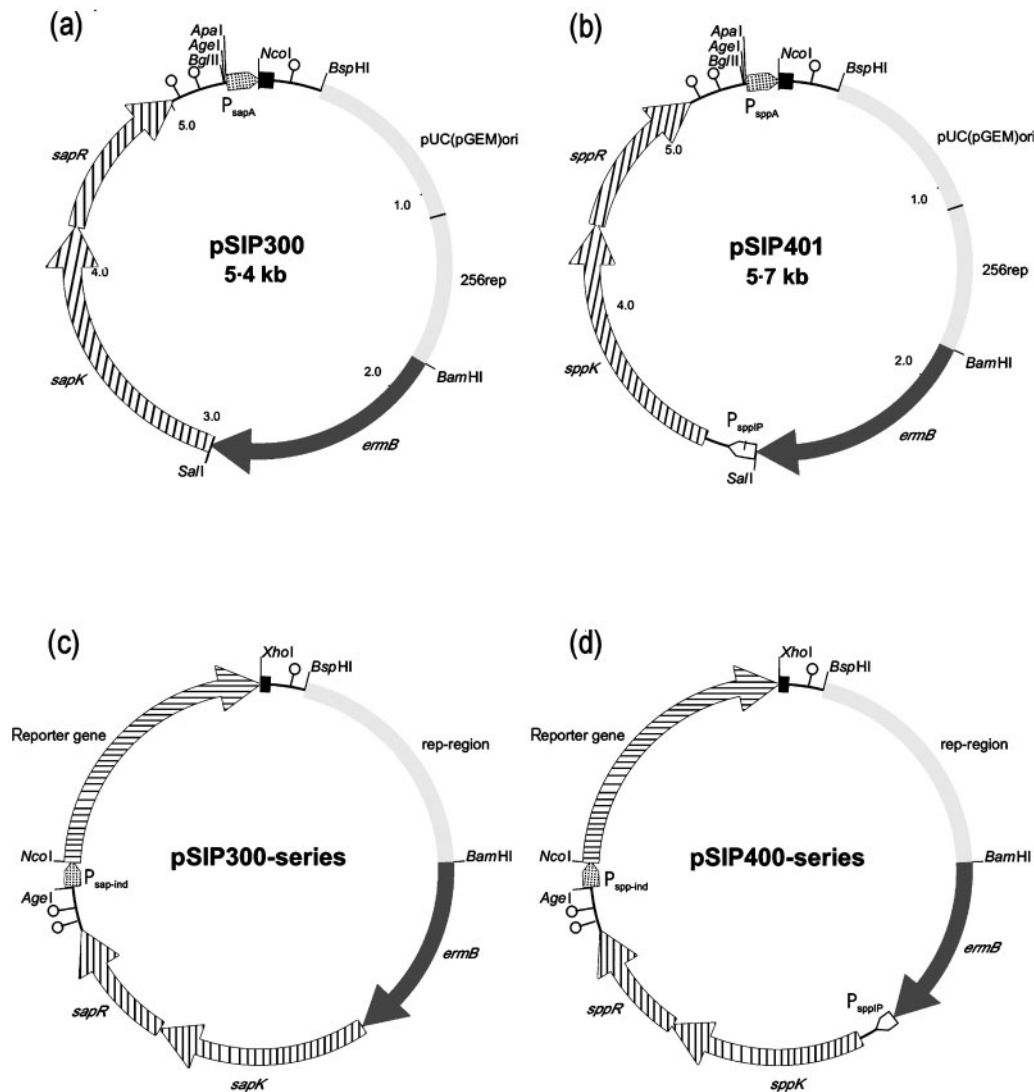


Fig. 1. Construction of plasmids. (a) and (b), Prototype expression vectors. Light-grey regions, replication determinants; dark-grey regions, erythromycin resistance marker; vertically hatched regions, histidine protein kinase and response regulator genes; dotted regions, inducible bacteriocin promoters; white region, inducible *sppIP* promoter; lollypop structures, transcriptional terminator; black boxes, multicloning site (*Sph*I, *Xba*I, *Xho*I, *Eco*RI, *Kpn*I, *Sma*I, *Nar*I, *Hind*III). All restriction sites shown are unique. (c) and (d), Schematic overview of the different vectors constructed in this study. Horizontally hatched regions, reporter gene (*gusA* or *pepN*); dotted regions, inducible promoters (P_{sapA} , P_{sapIP} and P_{orf1} in the pSIP300-series; P_{sppA} , P_{orf330} and P_{orfX} in the pSIP400-series); light-grey regions, replication determinants (256_{rep} or SH71 $_{rep}$). All other regions are identical to the prototype vectors. Relevant restriction sites are shown. See Table 1 and text for further description.

Escherichia coli (*gusA*) as a reporter gene, *sap*-derived (pSIP300 series) and *spp*-derived (pSIP400 series) vectors were compared, and in addition the effect of the promoter driving expression of the two-component regulatory system was studied (Sørvig *et al.*, 2003), since this had earlier been shown to be important in the development of vectors in the NICE system (Kleerebezem *et al.*, 1997). The prototype pSIP vectors showed promising results in terms of expression levels and the ability to control these levels, but the maximum expression of the β -glucuronidase reporter protein was relatively low compared with results obtained with the NICE system, for example (Sørvig *et al.*, 2003). The potential for the use of regulatory elements from the bacteriocin regulons in protein production was further illustrated by Mathiesen *et al.* (2004b), who constructed a plasmid containing the complete sakacin P regulon, in which the sakacin P structural gene and its cognate immunity gene had been replaced by the aminopeptidase N gene (*pepN*) from *Lactococcus lactis*. *L. plantarum* cells harbouring this construct produced PepN at levels amounting to 40% of the total cellular protein. Unfortunately, the type of construct used by Mathiesen *et al.* (2004b) was cumbersome to make, large (13.7 kb), difficult to change (e.g. reporter gene, replicon, marker) and unstable in one of the two *Lactobacillus* host strains used.

The goal of the present study was to further develop the pSIP vectors so that they would allow gene expression at levels as high as the highest levels attainable with existing systems, while still being easy to use and keeping background expression at a minimum. To achieve these goals, we have tested the effect of exchanging the inducible promoters present in the prototype pSIP vectors with other regulated promoters present in the *spp* and *sap* regulons. In addition, we have studied the effects of varying the reporter gene (*pepN* and *gusA*), the host strain (*L. sakei* and *L. plantarum*), and the replicon [the narrow-host-range *Lactobacillus* replicon from plasmid p256 (Sørvig *et al.*, 2005) and the broad-host-range, high-copy-number pSH71 replicon (de Vos, 1987)]. The resulting optimized pSIP vectors permitted controlled, high-level expression of β -glucuronidase and aminopeptidase N from *Lc. lactis* (*pepN*), yielding protein levels that were among the highest ever obtained in LAB.

METHODS

Bacterial strains and media. The bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown in BHI media (Oxoid) at 37 °C with shaking, *Lactococcus* strains were grown at 30 °C in M17 medium (Oxoid) supplemented with 0.5% glucose, and *Lactobacillus* strains were grown in MRS media (Oxoid) at 30 °C. Lactococci and lactobacilli were grown in unshaken cultures. Agar plates were made by using BHI agar (Oxoid) for *E. coli*, BSR (Holo & Nes, 1995) for *Lactococcus* and MRS agar (Oxoid) for *Lactobacillus* strains. When appropriate, erythromycin was added as follows: 200 $\mu\text{g ml}^{-1}$ for *E. coli* and 10 $\mu\text{g ml}^{-1}$ for lactococci and lactobacilli.

DNA preparation and transformation. Plasmid DNA from *E. coli* was isolated using commercial kits (Qiagen). Plasmid DNA from *Lactobacillus* and *Lactococcus* strains was isolated by a modified

alkaline lysis method, as described previously (Axelsson *et al.*, 1993). Total DNA from *Lactobacillus* strains was isolated by a lysis procedure in combination with a phenol/chloroform extraction: cells were resuspended in 0.2 \times culture volume of 0.1 M Tris/HCl, pH 8.0, containing lysozyme (final concentration 20 mg ml⁻¹) and mutanolysin (final concentration 40 U ml⁻¹). After a 45 min incubation at 37 °C, SDS was added to a final concentration of 1%, the suspension was incubated at 65 °C for 15 min (for inactivation of DNases), and finally 3 M NaCl was added until precipitation occurred. After centrifugation, the lysate was extracted with phenol/chloroform and the DNA precipitated with 2-propanol.

E. coli XL10 Gold (Stratagene) and *Lc. lactis* MG1363 were used as host strains for the construction of plasmids. *E. coli* XL10 Gold cells (Stratagene) were transformed according to the manufacturer's procedure; *Lc. lactis* MG1363 and *Lactobacillus* cells were electrotransformed according to Holo & Nes (1989) and Aukrust & Blom (1992), respectively.

Construction of plasmids. Plasmids used and constructed in this study are listed in Table 1. Plasmids were constructed using standard molecular cloning and PCR fusion (overlap extension) techniques (Sambrook *et al.*, 1989; Horton & Pease, 1991). Primers used in PCR reactions are listed in Table 2. Restriction enzymes were purchased from Promega or New England Biolabs. Primers were purchased from MWG AG Biotech. PCR was performed with the Gene Amp PCR System 9700 (PE Biosystems) and Expand High Fidelity PCR System Polymerase (Roche Diagnostics) using standard procedures.

The prototype vectors used as a starting point for this study contained the minimal replicon from the *L. plantarum* plasmid p256. As described by Sørvig *et al.* (2005), this 688 bp fragment contains features which indicate that p256 replicates via a theta mechanism. As an alternative in this study, we used the pSH71 replicon (de Vos, 1987). This is a high-copy-number lactococcal replicon with a rolling-circle type replication mechanism, and has been widely used for the development of vectors, such as the well-known pNZ series (Kok, 1991), in lactococci and other LAB.

For exchange of the 256_{rep} replicon of pSIP300 and pSIP401 (Sørvig *et al.*, 2003), SH71_{rep} was PCR-amplified using primers Sip52c and Sip53 (Table 2), with pVS2 (Von Wright *et al.*, 1987) as template. The PCR product was digested with *NcoI* (a site for which was inserted at the 5' end by the Sip52c primer) and *Sau3AI* (a site occurring naturally at the 3' end of SH71_{rep}), and pSIP300 and pSIP401 were digested with *BspHI* and *BamHI* (restriction sites flanking the 256_{rep} fragment), thus generating fragments with compatible ends.

The *E. coli gusA* gene fragment (Schlaman *et al.*, 1994) was excised from pSIP302 (Sørvig *et al.*, 2003) using the 5' *NcoI* site that includes the start codon and the *XhoI* restriction site at the 3' end. The *pepN* gene was amplified with primers Sip32 and Sip41, which introduce *NcoI* and *XhoI* restriction sites at the 5' and 3' ends, respectively (Table 2), using pSTO10 (Stroman, 1992) as template. Thus, the *gusA* and *pepN* gene fragments generated could easily be introduced/exchanged using the *NcoI* and *XhoI* restriction sites present in the pSIP vectors (Fig. 1).

The P_{orfX} promoter fragment was obtained by PCR, using primers Sip56 and Sip57 (Table 2), and pMLS114 (Hühne *et al.*, 1996), as template. The primers introduce *AgeI* and *NcoI* restriction sites at the 5' and 3' ends of the promoter fragment, respectively. Since these two restriction enzymes also flank the P_{sapA} and P_{sppA} promoter fragments in other pSIP vectors (Fig. 1), exchange of promoter fragments is straightforward.

New pSIP vectors were generated by ligation of the appropriate fragments and subsequent transformation of *E. coli* XL10 Gold or *Lc.*

Table 1. Bacterial strains and plasmids

See Fig. 1 for details of the vectors constructed in this work. The constructs may be viewed as variants of pSIP300 or pSIP401, containing different replicons and/or promoters and/or reporter genes.

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
<i>Escherichia coli</i> XL10 Gold	Host strain	Stratagene
<i>Lactobacillus sakei</i> Lb790	Host strain, meat isolate; two indigenous plasmids of approximately 19 and 60 kb	Schillinger & Lücke (1989)
<i>Lactobacillus plantarum</i> NC8	Host strain, silage isolate; plasmid-free	Aukrust & Blom (1992)
<i>Lactococcus lactis</i> MG1363	Host strain, plasmid-free and prophage-cured derivative of <i>L. lactis</i> NCDO 712	Gasson (1983)
Plasmids		
pSIP300	<i>sap</i> -based expression vector; <i>sapKR</i> expression driven by <i>ermB</i> read-through; 256 _{rep} ; Em ^R	Sørvig <i>et al.</i> (2003)
pSIP401	<i>spp</i> -based expression vector; <i>sppKR</i> expression driven by <i>ermB</i> read-through and cognate promoter; 256 _{rep} ; Em ^R	Sørvig <i>et al.</i> (2003)
pVS2	Source of SH71 _{rep}	Von Wright <i>et al.</i> (1987)
pSTO10	Source of <i>pepN</i>	Chr. Hansen A/S, Denmark; Strøman (1992)
pMLS114	Source of P _{orfX}	Hühne <i>et al.</i> (1996)
pSIP302	pSIP300 with P _{sapA} :: <i>gusA</i>	Sørvig <i>et al.</i> (2003)
pSIP304	pSIP300 with SH71 _{rep} and P _{sapA} :: <i>gusA</i>	This work
pSIP305	pSIP300 with P _{sapA} :: <i>pepN</i>	This work
pSIP308	pSIP300 with SH71 _{rep} and P _{sapA} :: <i>pepN</i>	This work
pSIP403	pSIP401 with P _{sppA} :: <i>gusA</i>	Sørvig <i>et al.</i> (2003)
pSIP404	pSIP401 with SH71 _{rep} and P _{sppA} :: <i>gusA</i>	This work
pSIP407	pSIP401 with P _{sppA} :: <i>pepN</i>	This work
pSIP409	pSIP401 with P _{orfX} :: <i>gusA</i>	This work
pSIP410	pSIP401 with P _{orfX} :: <i>pepN</i>	This work
pSIP411	pSIP401 with SH71 _{rep} and P _{orfX} :: <i>gusA</i>	This work
pSIP412	pSIP401 with SH71 _{rep} and P _{orfX} :: <i>pepN</i>	This work

lactis MG1363. Vectors containing pUC(pGEM)ori/256_{rep} were transformed into *E. coli* and vectors containing SH71_{rep} were transformed into *L. lactis*. After plating on the appropriate agar containing erythromycin, recombinant plasmids were selected. The sequences of all constructs were verified using an ABI Prism 3100 Genetic Analyser, with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems), following the manufacturer's

recommendations. To prevent changes in codon usage, silent mutations were not accepted; thus, the DNA sequences of the reporter genes (*gusA* or *pepN*) were identical in all constructs. The final plasmids were electrotransformed into *L. sakei* Lb790 and *L. plantarum* NC8. The integrity of the vectors in *L. sakei* Lb790 and *L. plantarum* NC8 was confirmed by plasmid isolation as described above. DNA rearrangements involving the vectors were not detected in any instance.

Table 2. Primers used for PCR amplification

Restriction sites are underlined.

Primer	Restriction enzymes	Sequence (5'→3')	Target	Source and reference
Sip52c	<i>NcoI</i>	CTA <u>CCA TGG</u> ATC GAT TTT TTA TTA AAA CGT CTC AAA ATC G	sh71rep	pVS2; Von Wright <i>et al.</i> (1987)
Sip53	<i>Sau3AI</i>	CAG <u>GGA TCA</u> TTT TGT TTA TTG CAA TCG TAT TGC	sh71rep	pVS2; Von Wright <i>et al.</i> (1987)
Sip32	<i>NcoI</i>	GAT <u>CCC ATG GCT</u> GTA AAA CGT TTA ATT G	<i>pepN</i>	pSTO10; Chr Hansen A/S, Denmark; Strøman (1992)
Sip41	<i>XhoI</i> , <i>XbaI</i>	GTA <u>CCT CGA GTC TAG ACT</u> ACA ATT TTT CAG CAA TAT C	<i>pepN</i>	pSTO10; Chr Hansen A/S, Denmark; Strøman (1992)
Sip56	<i>AgeI</i>	CAG <u>ACC GGT</u> TTA ATT TGA AAA TTG ATA TTA GCG	P _{orfX}	pMLS114; Hühne <i>et al.</i> (1996)
Sip57	<i>NcoI</i>	CAG <u>CCA TGG</u> CTA AAA TCT CCT TGT AAT AG	P _{orfX}	pMLS114; Hühne <i>et al.</i> (1996)

Assay for GUS activity. A modified β -galactosidase assay (Miller, 1972) was used to determine β -glucuronidase activities in *Lactobacillus* strains harbouring vectors with *gusA*. Cultures were induced with 50 ng ml⁻¹ of the SapIP or SppIP inducing peptides (Molecular Biology Unit, University of Newcastle, UK) at OD₆₀₀ ~0.3, and grown to OD₆₀₀ ~1.8. Assays were performed as described by Axelsson *et al.* (2003). Activity was calculated as described by Miller (1972) and expressed as Miller Unit equivalents (MU). Dose-response studies were performed as described by Sørvig *et al.* (2003).

Assay for PepN activity. *Lactobacillus* strains harbouring vectors with *pepN* were induced with 50 ng ml⁻¹ of SapIP or SppIP at OD₆₀₀ ~0.3, and grown to OD₆₀₀ ~1.8. Cells were harvested, resuspended in a buffer consisting of 0.01 M KCl, 0.05 M NaHPO₄ and 0.001 M MgSO₄, and disrupted by glass beads (106 μ m and finer, Sigma), essentially as described by van de Guchte *et al.* (1991). The resulting cell-free extracts were used to assay aminopeptidase activity using L-lysine *p*-nitroanilide (Sigma) as substrate. PepN activity was determined according to the protocol described by Exterkate (1984), with the following modifications: reactions were conducted at 30 °C (instead of 37 °C), and in 0.1 M Tris/HCl, pH 8.5 (instead of a 0.1 M sodium phosphate buffer). Protein concentrations were determined using the Bio-Rad Protein Assay with BSA as standard.

Protein analysis. For protein analysis of GusA, strains were induced and cultured as described above. Cells were harvested, resuspended in 0.05 M NaHPO₄ and disrupted by glass beads (106 μ m and finer, Sigma). The cell-free extracts were analysed on 12% SDS-polyacrylamide gels. PepN-containing extracts prepared as described above were also analysed on 12% SDS-polyacrylamide gels, and the amounts of PepN were quantified as a percentage of the total intracellular protein content by scanning the gels with a densitometer (Gel Doc 1000, Bio-Rad).

Determination of plasmid copy-number. Plasmid copy-number was estimated in slot-blot experiments by hybridizing a plasmid-specific probe encompassing the *ermB* gene of the vectors to known amounts of total DNA isolated from *L. sakei* Lb790 or *L. plantarum* NC8, and to known amounts of purified plasmid DNA. All pSIP plasmids contain the *ermB* gene, allowing the use of the same probe in all hybridization reactions. The amount of plasmid DNA in a sample of total DNA was estimated by comparing the hybridization signal obtained with total DNA to the hybridization signal obtained

with plasmid DNA. By taking into account the respective sizes of chromosomal and plasmid DNA, the number of plasmids per chromosome was calculated (Gardner *et al.*, 2001).

RESULTS

Plasmid construction

Previous work (Sørvig *et al.*, 2003) had shown that the sakacin A-based vector pSIP300 and the sakacin P-based vector pSIP401 were the most promising for production of GusA in lactobacilli (Fig. 1). Thus, these vectors, and their *gusA*-containing derivatives pSIP302 and pSIP403, were used as a starting point for studying the effects of exchanging the replicon or the promoter, as well as for studying the expression of another reporter gene, the *Lc. lactis pepN* gene. Details of the plasmids constructed and tested in this study are provided in Table 1 and Fig. 1.

GusA expression

Previous work in our laboratories with similar expression systems has shown that maximum levels of GusA expression are obtained when the lactobacilli are harvested at OD₆₀₀ 1.5–2.0 (Axelsson *et al.*, 2003; Sørvig *et al.*, 2003). Thus, in the present work, cells were harvested at OD₆₀₀ ~1.8 to ensure maximum or near-maximum expression levels. Fig. 2 shows the results with the *gusA*-containing expression vectors in the host strains *L. sakei* Lb790 and *L. plantarum* NC8. The exchange of the 256_{rep} replicon with the SH71_{rep} replicon led to an approximately twofold increase in the levels of GUS activity for the sakacin P-based vector in both host strains (pSIP404 versus pSIP403). For the sakacin A-based vector the increase was approximately threefold in *L. sakei* Lb790, whereas no significant effect was observed in *L. plantarum* NC8 (pSIP304 versus pSIP302). Exchange of the P_{sppA} promoter with the P_{orfX} promoter from the same *spp* regulon (Brurberg *et al.*, 1997; Risøen *et al.*, 2000) clearly

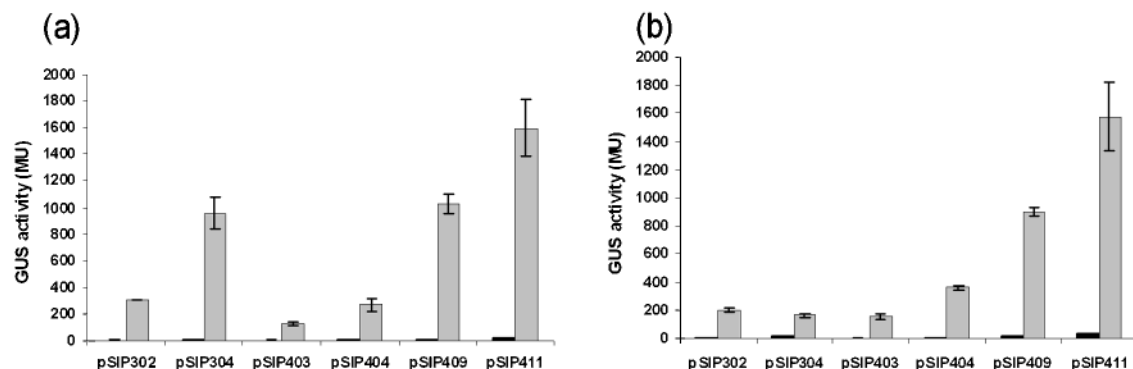


Fig. 2. GusA activity in *L. sakei* Lb790 (a) and *L. plantarum* NC8 (b) strains harbouring various plasmids, measured at OD₆₀₀ ~1.8. Grey bars, cultures induced with 50 ng inducing peptide ml⁻¹; black bars, non-induced cultures. GusA activity is expressed as Miller Unit equivalents (MU), which equals the ΔA_{405} value (sample value minus culture medium value) divided by the OD₆₀₀ value of the culture sample. All data are the mean of three independent experiments; the error bars indicate the standard deviation.

enhanced GusA production in both host strains (pSIP409 versus pSIP403). The highest production levels were obtained with pSIP411, which is based on the P_{orfX} promoter and the SH71_{rep} replicon. By changing from pSIP403 to pSIP411, GusA production in *L. sakei* Lb790 and *L. plantarum* NC8 was increased 12- and 10-fold, respectively. SDS-PAGE of cell-free extracts of GusA-producing strains showed a band representing the GusA protein, and confirmed that the highest levels of GusA were obtained in strains harbouring pSIP411 (Fig. 3a, b).

Several other promoters were tested in an analogous fashion: P_{sapIP} (previously known as P_{orf4}) and P_{orfI} from the *sap* regulon (Axelsson & Holck, 1995), and P_{orf330} , a sakacin P-type promoter sequence obtained from the *L. sakei* Lb790 chromosome (GenBank accession no. AJ626710; Møretro *et al.*, 2005). All constructs containing these promoters yielded clearly lower GusA levels than those obtained with pSIP302 and pSIP403 (results not shown).

As observed previously (Sørvig *et al.*, 2003), the levels of basal activity and, linked to them, the levels of induction factors, depend on both the vector and the host strain. Table 3 shows the basal activity and the induction factors for the different constructs, based on the expression of GusA activity, as depicted in Fig. 2. For comparison, results from experiments with the two-plasmid NICE system were included (Kleerebezem *et al.*, 1997; Sørvig *et al.*, 2003). It can be seen that (i) in *L. sakei*, only pSIP411 of the pSIP vectors has a basal activity significantly higher than the background level for strains without the *gusA* gene, which is about 6 MU (Sørvig *et al.*, 2003); (ii) the *L. plantarum* variants generally show higher basal activity with all

constructs and therefore generally lower induction factors than *L. sakei*; and (iii) the NICE system shows high levels of expression, but has higher basal activity.

Dose–response studies showed that the amount of GusA produced was dependent on the inducing peptide concentration in the 0.1–5 ng ml⁻¹ range for both host strains, as observed previously for pSIP302 and pSIP403 (Sørvig *et al.*, 2003) (results not shown).

PepN expression

The experimental set-up assumed to yield near-maximum GusA expression was also applied to the expression of PepN. Fig. 4 shows the results obtained with constructs containing the *pepN* reporter gene. The lowest PepN levels were obtained with the *pepN* analogue of pSIP302, pSIP305, in both *L. sakei* Lb790 and *L. plantarum* NC8. Similarly to that which was observed with the *gusA* reporter gene, the exchange of 256_{rep} with SH71_{rep} (creating vector pSIP308) increased PepN production in *L. sakei* Lb790 (threefold), while PepN levels in *L. plantarum* NC8 were not affected (pSIP308 versus pSIP305, Fig. 4a, b).

The highest levels of PepN were obtained with pSIP401 derivatives. In *L. sakei* Lb790, the 256_{rep}-containing constructs pSIP407 and pSIP410 yielded approximately equal amounts of PepN, indicating that the P_{sppA} and P_{orfX} promoters function equally well. PepN production was increased twofold by exchanging the 256_{rep} replicon in pSIP410 with the SH71_{rep} replicon (yielding pSIP412). The *L. sakei* strain containing pSIP412 produced massive amounts of PepN when harvested at OD₆₀₀ ~ 1.8, and it

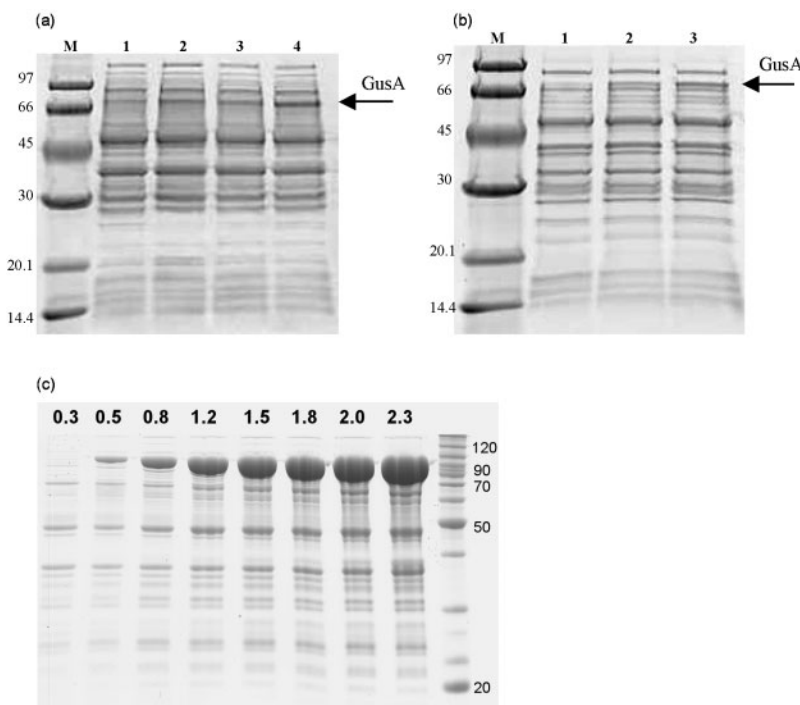


Fig. 3. Coomassie blue-stained gels after SDS-PAGE of cell-free extracts of *Lactobacillus* strains. All cell cultures were induced with 50 ng inducing peptide ml⁻¹. (a) GusA production in *L. sakei* Lb790 harbouring various plasmids and harvested at OD₆₀₀ ~ 1.8; lane M, molecular mass marker (kDa); lane 1, pSIP300 negative control; lane 2, pSIP304; lane 3, pSIP409; lane 4, pSIP411. (b) GusA production in *L. plantarum* NC8 harbouring various plasmids and harvested at OD₆₀₀ ~ 1.8; lane M, molecular mass marker (kDa); lane 1, pSIP300 negative control; lane 2, pSIP409; lane 3, pSIP411. (c) PepN production in *L. sakei* Lb790/pSIP412 harvested at the indicated OD₆₀₀ values; the sample to the far right is the molecular mass marker (kDa). Arrows indicate the location of the GusA protein (calculated molecular mass 68 kDa). The calculated molecular mass of PepN is 95 kDa.

Table 3. GusA activity in Miller Unit equivalents (MU) in induced (50 ng inducing peptide ml⁻¹) or non-induced *L. sakei* Lb790 and *L. plantarum* NC8 cultures containing various plasmids

Mean values and standard deviations were based on measurements of at least three independent cultures. The induction factor was calculated as the maximum mean GusA activity in the induced state divided by the maximum mean GusA activity in the non-induced state.

Strain	Maximum GusA activity without induction (MU)	Maximum GusA activity with induction (MU × 10 ²)	Induction factor
Lb790 (pSIP302)	6*	3.0 ± 0.01	50
Lb790 (pSIP304)	9.6 ± 5.0	9.6 ± 1.2	100
Lb790 (pSIP403)	6*	1.3 ± 0.1	22
Lb790 (pSIP404)	6*	2.7 ± 0.5	45
Lb790 (pSIP409)	8.0 ± 4.7	10.2 ± 0.7	127
Lb790 (pSIP411)	18.3 ± 6.2	15.9 ± 2.1	87
Lb790 (pNZ8048gus + pNZ9530)†	32.9 ± 17	15.0 ± 1.1	46
NC8 (pSIP302)	10.3 ± 4.4	2.0 ± 0.1	20
NC8 (pSIP304)	15.2 ± 8.1	1.6 ± 0.1	11
NC8 (pSIP403)	6*	1.6 ± 0.2	27
NC8 (pSIP404)	7.8 ± 3.8	3.7 ± 0.2	47
NC8 (pSIP409)	15.0 ± 5.4	9.0 ± 0.3	60
NC8 (pSIP411)	35.7 ± 8.2	15.8 ± 2.5	44
NC8 (pNZ8048gus + pNZ9530)†	71.2 ± 7.6	16.8 ± 1.2	24

*Background values for control strains containing vectors without the *gusA* gene in some cases amounted to up to 6 MU, which is near the detection limit of the assay. The measured values marked with a * were between 4.0 and 5.3, with high standard deviations. These values were all set to 6 MU.

†The original two-plasmid NICE-system as described by Kleerebezem *et al.* (1997) expressing GusA (Sørvig *et al.*, 2003).

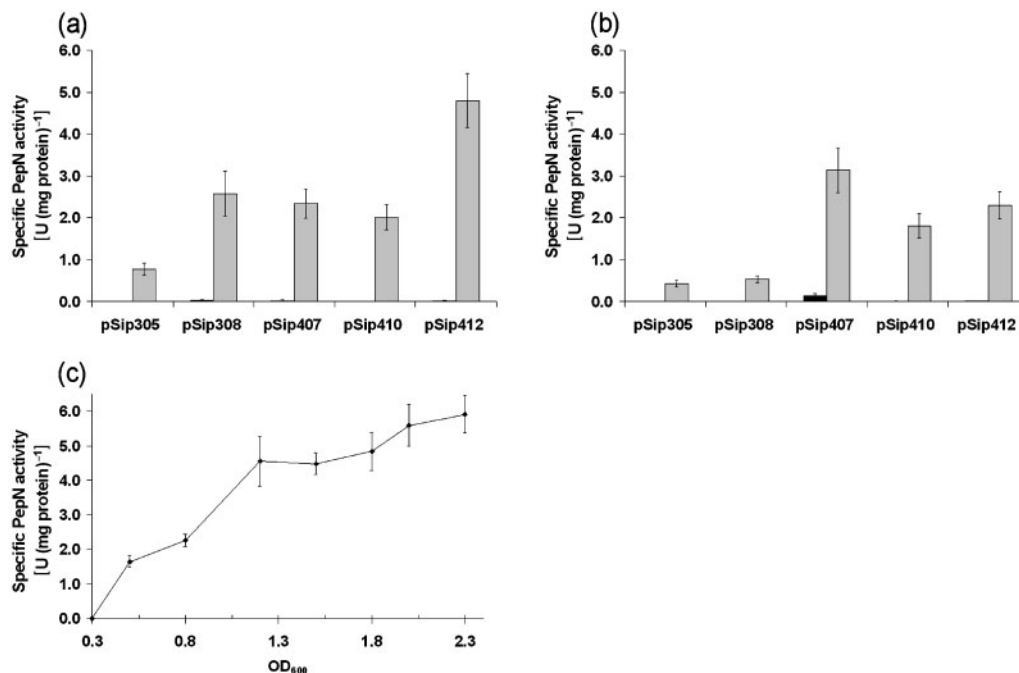


Fig. 4. PepN activity in *L. sakei* Lb790 (a) and *L. plantarum* NC8 (b) strains harbouring various plasmids, measured at OD₆₀₀ ~ 1.8. Grey bars, cultures induced with 50 ng inducing peptide ml⁻¹; black bars, non-induced cultures. (c) PepN activity measured during growth of *L. sakei* Lb790/pSIP412. One unit (U) of PepN activity represents production of one micromole *p*-nitroanilide per minute. All data are the mean of three independent experiments; the error bars indicate the standard deviation.

was therefore selected for a more-detailed analysis of the production pattern. Cells were grown up to $OD_{600} \sim 2.3$ and harvested at selected intervals, and as seen in Fig. 4c, the PepN activity increased steeply up to $OD_{600} 1.2$ and then levelled off. The amounts of the PepN protein appeared to increase up to $OD_{600} \sim 2.3$, as observed after SDS-PAGE of cell-free extracts of induced *L. sakei* Lb790/pSIP412 (Fig. 3c). Densitometric analyses indicated that at $OD_{600} \sim 2.3$, PepN constitutes approximately 46% of the total amount of intracellular proteins.

In *L. plantarum* NC8, pSIP407 (P_{spPA} promoter, 256_{rep}) gave the highest level of PepN activity, while lower activities were observed with plasmids pSIP410 (P_{orfX} promoter, 256_{rep}) and pSIP412 (P_{orfX} promoter, $SH71_{rep}$).

In contrast to the GusA constructs, the increase in maximum PepN activity going from pSIP305 (corresponding to GusA construct pSIP302) to the pSIP412 vector (corresponding to pSIP411) in *L. sakei* was not accompanied by an increase in basal activity in non-induced strains. The induction factors with the *pepN* constructs generally followed the same trend as for GusA (Table 3), that is, the *L. sakei* variants had higher induction factors ranging from 60 to 300, compared to the *L. plantarum* variants with induction factors from 40 to 120 (results not shown). However, for the PepN variants, the basal activities were essentially the same in the two species, and the lower induction factors in *L. plantarum* were therefore due to generally lower maximum expression levels. Worthy of note is that for pSIP407, for which the highest PepN activity for *L. plantarum* was obtained, the basal activity was five- to tenfold higher than for the other constructs.

Copy number analysis

Copy number analysis was performed according to Gardner *et al.* (2001), as described in Methods. The chromosome sizes of *L. sakei* Lb790 and *L. plantarum* NC8 were taken as 1.9 and 3.3 Mb, respectively (Dudez *et al.*, 2002; Kleerebezem *et al.*, 2003). The copy numbers of pSIP302 and pSIP403 (plasmids containing 256_{rep}) were estimated to be approximately three in *L. sakei* Lb790 and approximately six in *L. plantarum* NC8. This corresponds well with the estimated copy number of the p256 plasmid from *L. plantarum* NC7 (Sørvig *et al.*, 2005), from which 256_{rep} originates. After exchanging the 256_{rep} replicon with the $SH71_{rep}$ replicon (yielding pSIP304 and pSIP404), copy numbers in *L. sakei* Lb790 increased threefold, whereas the copy number in *L. plantarum* NC8 increased eightfold for pSIP304 and fourfold for pSIP404. Thus, the general picture is that vectors based on the $SH71_{rep}$ replicon have higher copy numbers than vectors based on the 256_{rep} replicon.

DISCUSSION

In this paper, we describe the development of a set of versatile, inducible expression vectors and their use for controlled high-level expression of GusA and PepN in *L.*

sakei and *L. plantarum*. The expression systems are based on recently developed prototype vectors (Sørvig *et al.*, 2003), which have a 'cassette'-like structure, permitting easy exchange of all parts through restriction-enzyme digestion and ligation.

Mathiesen *et al.* (2004b) have recently described a sakacin P-based expression plasmid yielding PepN levels amounting to approximately 40% of total intracellular protein in *L. plantarum* NC8. Mathiesen and co-workers kept the natural organization of the *spp* gene cluster more or less intact in the vector they created, because previous studies had shown that this could be important to obtain maximum expression levels. The best pSIP vectors developed in the present study show that equally high, or even slightly higher, expression levels may be obtained with a much more simple, smaller and versatile vector, in which all 'non-essential' DNA has been removed, and in which the natural organization of the regulatory gene cluster has been rearranged. One example is the vector pSIP412, which gave PepN levels amounting to close to half of the total intracellular protein content in *L. sakei* Lb790. These PepN levels are similar to the amounts reported for overexpression of PepN in its natural host, *L. lactis*, using the NICE system (de Ruyter *et al.*, 1996). Another example is pSIP411, which yielded GUS levels that were five to ten times higher than the levels obtained with the best prototype vectors (Sørvig *et al.*, 2003) and that were comparable to the levels obtained with the NICE system (Table 3). Previous studies have shown that nisin-based plasmid expression systems used in lactobacilli are poorly regulated (Pavan *et al.*, 2000; Sørvig *et al.*, 2003), and that integration of the regulatory genes into the chromosome may be necessary to sustain the inducibility of the vectors and to avoid basal activity (Pavan *et al.*, 2000). The work presented in the present study demonstrates that it is possible to obtain controlled, high-level expression with minor basal activities with a one-plasmid system and to sustain inducibility without integrating genes into the chromosome of the host.

The vectors described in this paper are easy to transform into the host strains and should function in any strain of *L. sakei* and *L. plantarum*. The present study and previous studies (Kleerebezem *et al.*, 1997; Eichenbaum *et al.*, 1998; Kahala & Palva, 1999; Mathiesen *et al.*, 2004a) show, however, that expression levels in both the induced and non-induced situations depend to a considerable extent on the host and reporter genes used. These differences may be due to factors such as mRNA stability, codon usage in the reporter gene, the level of transcription of the two-component regulatory system (Kleerebezem *et al.*, 1997; Pavan *et al.*, 2000; Sørvig *et al.*, 2003), differences in protein folding efficiency and turnover, host cell physiology, plasmid copy-number, and potential negative effects of the reporter gene product on the host cell. For example, in this work, the expression of *gusA* yielded less protein than the expression of *pepN*, although otherwise identical vectors were used (Fig. 3). This could be due to the lactococcal *pepN*

gene being better adapted to the *Lactobacillus* expression machinery than the *gusA* gene from *E. coli*. Indeed, the codon adaptation index (CAI), calculated using a web tool developed at UMBC, Maryland (<http://www.evolvingcode.net/codon/CalculateCAIs.php>), was significantly higher for *pepN* than for *gusA* (~0.6 versus 0.4) using highly expressed genes from *L. plantarum* as the reference set (Kleerebezem *et al.*, 2003).

While effects of reporter genes on expression levels are well known and to some extent explainable, other variables are more difficult to rationalize. For example, in the present study, results obtained with the *gusA* reporter gene indicated in almost all instances that exchanging the P_{sppA} promoter with the P_{orfX} promoter as well as increasing the plasmid copy-number was beneficial for maximizing protein production. However, in the case of the *pepN* reporter gene, the results were inconsistent and more strain-dependent. Remarkably, exchange of the promoter had no significant effect on *PepN* production, whereas it clearly affected *GusA* production. Thus, the effect of the promoter exchange depended on the reporter gene in both host strains. Another remarkable finding concerns the fact that increasing the plasmid copy-number had clear positive effects in the *L. sakei* Lb790 host, but not in the *L. plantarum* NC8 host. Given the fact that the integrity of the vectors was maintained in the lactobacilli hosts, recombination events with indigenous plasmids (only relevant for *L. sakei* Lb790) or the chromosome seem unlikely, and do not appear to offer an explanation for these results.

Generally, the *L. plantarum* host showed less-consistent results than the *L. sakei* host. A complicating factor in the case of *L. plantarum* NC8 comes from the presence of bacteriocin regulatory genes on the chromosome, which was discovered very recently (Maldonado *et al.*, 2004). The induction factor and histidine protein kinase genes are novel, but the response regulator gene is identical to the response regulator involved in the production of plantaricins by *L. plantarum* C11 (Diep *et al.*, 1995). It has been shown previously that the products of the regulatory C11 genes can act on the regulated promoters in the sakacin P regulon (Brurberg *et al.*, 1997; Risøen *et al.*, 2000). Although the chromosomal system is not induced under the conditions used in this study (Maldonado *et al.*, 2003, 2004) (G. Mathiesen & V. G. H. Eijsink, unpublished observations), it is possible that low background expression of one or more of the regulatory genes affects the performance of the pSIP vectors in the *L. plantarum* NC8 host. Although *L. sakei* Lb790 performed somewhat more predictably than *L. plantarum* NC8 in this study, it should be noted also that several *L. sakei* strains, including Lb790, harbour genes related to bacteriocin gene clusters in their chromosomes, although some of these are mutated and non-functional (Møretrø *et al.*, 2005).

In several of the settings used in this study, the P_{orfX} promoter outperforms the P_{sppA} promoter in terms of protein production upon induction. In previous studies,

based on constructs in which the promoters were translationally coupled (but not fused) to a *cat* reporter gene, the P_{sppA} and P_{orfX} were found to be equally strong and strictly regulated (Risøen *et al.*, 2000). Like P_{sppA} , P_{orfX} drives expression of a bacteriocin structural gene (Mathiesen *et al.*, 2005). The present study based on translational fusions reveals differences that may be due to one or more subtle differences in the promoter sequences. These include a one-nucleotide exchange in the characteristic direct repeat sequences upstream of the -10 region (Brurberg *et al.*, 1997), a one-nucleotide difference in the spacing between the putative ribosome-binding site (AGGAG in both cases) and the start codon, and sequence differences in the spacer regions. It is well known that changes in the length and/or sequence of the window between the ribosome-binding site and the start codon may affect expression (van de Guchte *et al.*, 1992; Vellanoweth & Rabinowitz, 1992), and this has also been observed in an earlier variant of a P_{orfX} -based expression system (Mathiesen *et al.*, 2004a).

In conclusion, we present a set of easy-to-use vectors which permit dose-dependent high-level gene expression in lactobacilli and which can easily be adapted for a particular purpose by exchanging one or more 'cassettes'. The current set of pSIP vectors is based on two types of replicons and two types of pheromones, and similar vectors based on other replicons and other pheromones may be developed (Eijsink *et al.*, 1995). For the practical purpose of expressing a gene, it may sometimes be useful to try several of the developed vectors, because it seems that the performance of the expression system depends on the combination of the gene of interest, the promoter, the replicon, and the host strain. Work is now in progress in our laboratories to exploit the new expression systems for the expression of proteins of industrial and medical importance.

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