A synthetic promoter library for constitutive gene expression in *Lactobacillus plantarum*

Ida Rud,^{1,2} Peter Ruhdal Jensen,³ Kristine Naterstad¹ and Lars Axelsson¹

Correspondence

Lars Axelsson

lars.axelsson@matforsk.no

¹MATFORSK, Norwegian Food Research Institute, Osloveien 1, N-1430 Ås, Norway

²Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, PO Box 5003, N-1432 Ås, Norway

³Microbial Physiology and Genetics, BioCentrum, Technical University of Denmark, Kgs Lyngby, Denmark

A synthetic promoter library (SPL) for *Lactobacillus plantarum* has been developed, which generalizes the approach for obtaining synthetic promoters. The consensus sequence, derived from rRNA promoters extracted from the *L. plantarum* WCFS1 genome, was kept constant, and the non-consensus sequences were randomized. Construction of the SPL was performed in a vector (pSIP409) previously developed for high-level, inducible gene expression in *L. plantarum* and *Lactobacillus sakei*. A wide range of promoter strengths was obtained with the approach, covering 3–4 logs of expression levels in small increments of activity. The SPL was evaluated for the ability to drive β -glucuronidase (GusA) and aminopeptidase N (PepN) expression. Protein production from the synthetic promoters was constitutive, and the most potent promoters gave high protein production with levels comparable to those of native rRNA promoters, and production of PepN protein corresponding to approximately 10–15 % of the total cellular protein. High correlation was obtained between the activities of promoters when tested in *L. sakei* and *L. plantarum*, which indicates the potential of the SPL for other *Lactobacillus* species. The SPL enables fine-tuning of stable gene expression for various applications in *L. plantarum*.

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INTRODUCTION

Lactobacillus is a large genus of lactic acid bacteria (LAB) known for its heterogeneity and for its wide application in the food industry. Many lactobacilli are used as starter cultures in the production of a variety of fermented products (Axelsson, 2004). In addition, a variety of *Lactobacillus* species are members of the normal microbiota of the human gastrointestinal tract and oral cavity, and some also have probiotic effects in humans and animals. *Lactobacillus plantarum* is among the *Lactobacillus* species frequently encountered in such niches (Ahrné *et al.*, 1998), and strains with probiotic effects have been reported (Alander *et al.*, 1999; Schultz *et al.*, 2002; Mercenier *et al.*, 2003). *L. plantarum* is also an important industrial species, due to versatile properties and high flexibility. It is capable of degrading a wide range of carbohydrates, and tolerates relatively low pH. Another species of *Lactobacillus* important in industrial fermentation is *Lactobacillus sakei*, which survives a variety of adverse conditions, such as low temperature and pH, high salt concentration, smoke, ethanol, low water activity and radiation (Axelsson & Ahrné, 2000).

The importance of LAB in industrial fermentation and the GRAS (generally regarded as safe) status of most LAB have resulted in an increased interest in their genetics, and a variety of genetic tools have been developed over the last few decades in order to analyse and modify the metabolism of these bacteria. Metabolic engineering of the production of diacetyl and folate by *Lactococcus lactis* (Hugenholtz *et al.*, 2000; Sybesma *et al.*, 2003), and the production of acetaldehyde by *Streptococcus thermophilus* (Chaves *et al.*, 2002), are examples. Research involving LAB as cell factories, both for bulk production of chemicals and enzymes, and for *in situ* production in food, is another upcoming field. The potential of using certain species of LAB, such as *L. plantarum*, as an *in situ* producer and deliverer of therapeutic compounds in the human body, has also been investigated (Krüger *et al.*, 2002).

Most advances in the development of gene expression systems for LAB have been for *Lc. lactis*, and to a lesser extent for lactobacilli, although in recent years several systems have been published that relate to the latter. In particular, gene expression systems based on inducible promoters have been constructed (Pavan *et al.*, 2000; Axelsson *et al.*, 2003; Neu & Henrich, 2003; Sørvig *et al.*, 2003, 2005; Mathiesen *et al.*, 2004). Inducible expression can be preferable in cases where the aim is to overproduce a desired protein at high levels,

Abbreviations: LAB, lactic acid bacteria; SPL, synthetic promoter library.

e.g. at a specific moment during fermentation. However, in other applications, inducible systems are less suitable, i.e. during *in situ* production in the human body or when steady-state gene expression is required (Jensen *et al.*, 1993), and constitutive promoters could be an alternative for such applications.

Research has been done to identify and isolate constitutive promoters. The most common strategies have been to screen random chromosomal DNA fragments by cloning them in vectors that carry promoterless reporter genes, or to identify strong promoters from housekeeping genes (Pouwels & Leer, 1993; de Vos & Simons, 1994; Mercenier *et al.*, 1994). Jensen & Hammer (1998b) have developed a more efficient approach, in which a library of synthetic promoters for *L. lactis* is obtained by randomization of the spacer sequence that separates the consensus sequences of the promoter. In this library, a wide range of promoter activities is covered in small steps. The approach has been further refined (Solem & Jensen, 2002) and successfully employed for metabolic engineering and metabolic control analysis of *L. lactis*, where fine-tuned expression levels of the studied gene are needed (Koebmann *et al.*, 2002a, b; Solem *et al.*, 2003).

Here, we generalize the Jensen–Hammer approach for obtaining synthetic promoters, and exemplify the method for *L. plantarum*. A consensus promoter sequence for *L. plantarum* was derived by aligning its rRNA promoters, and this sequence used as the basis for constructing a synthetic promoter library for *L. plantarum*. The promoter library obtained provided a wide range of promoter activities with stable protein production, and the most potent promoters yielded protein production at levels comparable to those of native rRNA promoters.

METHODS

Bacterial strains and plasmids. *Escherichia coli* XL10 Gold (Stratagene) was used as host for the cloning strategies, whereas the plasmid-free strain *L. plantarum* NC8 (Aukrust & Blom, 1992) was the main host for the synthetic promoter library. The library was also investigated in a plasmid-free variant of *L. sakei* Lb790, Lb790-X (Schillinger & Lücke, 1989; Møretrø *et al.*, 2005). The high-level, inducible vector pSIP409 (Sørvig *et al.*, 2005), developed for lactobacilli, was used as reference vector and as template for the construction of the library. A derivative of pSIP409, pSIP410, harbouring *pepN* as reporter gene (Sørvig *et al.*, 2005), was used for constructions of plasmids with selected promoters fused to *pepN*.

Growth conditions. *E. coli* strains were grown at 37 °C in BHI medium (Oxoid) with vigorous shaking. The lactobacilli were grown at 30 °C in MRS medium (Oxoid) without shaking, or in a defined medium for lactobacilli (DML) (Møretrø *et al.*, 1998), with the following modifications: the medium was supplemented with 20 mM glucose and the succinate buffer was replaced with 0.1 M MES buffer. Stability experiments were performed with batch cultures (flasks) in 100 ml DML. Slow stirring with magnets was used to keep the cultures homogeneous. BHI agar and MRS agar (both Oxoid) were used as solid media for *E. coli* and lactobacilli, respectively. Erythromycin was added to a concentration of 200 µg ml⁻¹ for *E. coli* and 10 µg ml⁻¹ for lactobacilli.

Plasmid DNA preparation and transformation. Plasmid DNA from *E. coli* and lactobacilli was isolated using the QIAprep Mini-prep kit (Qiagen). An additional lysis step was introduced for *L. plantarum* and *L. sakei* cells, before adding the lysis buffer from the QIAprep kit: *L. plantarum* and *L. sakei* cells were incubated at 37 °C for 10 and 25 min, respectively, in buffer (50 mM glucose, 25 mM Tris/HCl, pH 8.0, 10 mM EDTA) with lysozyme (20 mg ml⁻¹), mutanolysin (40 U ml⁻¹) and RNase (100 µg ml⁻¹). *E. coli* XL10 Gold cells were transformed according to the manufacturer's procedure, and *Lactobacillus* strains were electrotransformed according to Aukrust & Blom (1992).

Cloning of synthetic promoter library (SPL) fragments. The SPL was constructed using standard molecular cloning techniques (Sambrook *et al.*, 1989). Restriction enzymes and T4 DNA ligase were purchased from Promega. PCR was performed with the Expand High Fidelity PCR System Polymerase (Roche Diagnostics) using the standard procedure. The inducible P_{orfX} promoter fragment was excised from pSIP409 using the *Bgl*II site in the 5' end of the promoter and the *Nco*I site included in the *gusA* start codon. The synthetic promoter fragments were obtained by PCR with pSIP409 plasmid DNA as template. The forward primer SPL1 (DNA Technology A/S, Denmark) (Fig. 2A) carries the *Bgl*II site and has homology to the 5' end of the *gusA* mRNA. The reverse primer *gusRT1* (5'-GACTTCGCGCTGATACCAG-3') (MWG AG Biotech) has homology to the interior region of the *gusA* gene. The resulting PCR product, containing synthetic promoters followed by part of the *gusA* gene, was digested with *Bgl*II and *Nco*I, and ligated to compatible ends of the digested pSIP409. After transformation into *E. coli*, all the transformant colonies (approx. 300–400) were pooled and inoculated into 30 ml of medium. After overnight growth, plasmid DNA was isolated from 3 ml of culture and subsequently transformed into *L. plantarum*. Screening for different promoter activities was achieved by plating the cells onto agar that contained 200 µg ml⁻¹ X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; Biosynth AG), on which the developing colonies ranged in colour from white to dark blue.

Cloning of rRNA promoters. Putative rRNA promoters from the genome of the sequenced strain *L. plantarum* WCFS1 were identified (Fig. 1). Cloning of selected rRNA promoters, rRNA1-a, rRNA3-a, rRNA4-a, rRNA2-b and rRNA4-b, was performed with the same molecular cloning strategy as that of the SPL, but with specific forward primers for each promoter. The forward primers (MWG) were identical to the SPL1 primer (Fig. 2) in length, and at the 5'-end restriction site and the 3' end, but contained the interior region sequences from positions -2 to -48 (Fig. 1).

Promoter sequence analysis. The promoter sequences of the selected colonies were determined based on standard PCR products generated from isolated plasmid DNAs. The PCR products were generated with the primers sip3 (5'-CCCAGAAATACCCGTCTAAG-GAATTGTCAGATAGGC-3') and *gusRT1*, the first primer upstream of the *Bgl*II restriction site. Both primers were supplied by MWG. Sequencing was performed using an ABI Prism 3100 Genetic Analyser with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, following the manufacturer's recommendations (Applied Biosystems).

GusA assay. The promoter activity was investigated with regard to β-glucuronidase (GusA) activity, which was expressed by the *gusA* reporter gene fused to the promoter. Cultures were grown in defined medium and samples were collected in the exponential phase. The GusA assay was done as described by Axelsson *et al.* (2003), except for the plasmids that harboured the weakest promoters, for which larger aliquots were concentrated and used in the assay. Activity was calculated as described for β-galactosidase by Miller (1972) and expressed as Miller Unit equivalents (MU). The detection limit of

	-48	-40	-35		-15	-10	+1
rRNA1-a	TTAAATAAA	AGTTGTT	GACAAGG	AATGATGAA	ATGATATTAT	GTAAT	AGTTGTTG
rRNA2-a	TTATAAAAA	AGTTGTT	GACAAGAT	GTTGATAAC	TTGATATGAT	GATTA	AGTTGCG
rRNA3-a	TTATAAAAA	AGATGTT	GACAGCT	TGTTCTGATG	ATGATAAACT	TTAAT	AGTTGCG
rRNA4-a	TTAAAAAAA	AGTTGTT	GACAAGAT	TATTGATTAAGAT	GTTATGAT	GATTA	AGTTGTTG
rRNA5-a	TAAAAAAA	AGTTGTT	GACAAGAT	TATTGATTAAGAT	GTTATGAT	GATTA	AGTTGTTG
rRNA1-b	AGCAAAAA	AGTTATT	GACA	TGAGTAAT	CGCTAATGATA	AAGATATTT	CTTGTCCG
rRNA2-b	TGCATTTT	AGGGCTT	GCAA	TTTATCAGG	ATAATTGGTAAAT	TAGTATAC	CGTTGTT
rRNA3-b	TTCATAAAA	AGGGTTG	ACC	CGGGTCGTT	TATTCTTGGTATT	TATTTCTT	GTCGTT
rRNA4-b	ATTGGATT	AATTGTT	GACA	GTTTCATA	ACTGGCTGGTAT	TATAGTAA	ACGTTGCT
rRNA5-b	TTCAGAAAA	AGTTGTT	GACA	CTTTTACTT	CAATTGGTAT	TATAGTAA	ACGTTGCT
Consensus	-----	AGTTGTTGACA	-----	-----	TGRTAWDNT	-----	-----

Fig. 1. Alignment of rRNA promoters and derivation of consensus sequences. The conserved bases are indicated in black boxes and the derived consensus sequences are in bold type. For details see text. Semi-conserved bases: R = A or G; W = A or T; D = A, G or T; N = A, G, T or C. Positions from the transcriptional start site (+1) are shown.

the GusA assay has previously been determined to be 6 MU (Sørvig *et al.*, 2003). Mean measurements of the activities from at least four individual cultures of each strain are presented. The stability experiment was performed with two individual cultures of each selected strain.

Construction of PepN strains with selected promoters. Three promoters from the promoter library (P9, P11 and P27) were excised from their respective pSIP409 vectors by digesting with *Bgl*II and *Nco*I, and ligated to compatible ends of digested pSIP410 harbouring the *pepN* reporter gene.

PepN assay. Strains harbouring pSIP410-P9, P11 and P27 were investigated with regard to aminopeptidase activity using L-lysine *p*-nitroanilide (Sigma) as substrate. The cells were harvested by centrifugation, resuspended in buffer (0.01 M KCl, 0.05 M NaHPO₄, pH 7.0, 0.001 M MgSO₄) and disrupted by glass beads (106 µm and finer, G-4649, Sigma), essentially as described by van de Guchte *et al.* (1991). The resulting cell-free extracts were used in the PepN assay.

PepN activity was determined according to the protocol described by Exterkate (1984), with the following modifications: reactions were conducted at 30 °C and in 0.1 M Tris/HCl, pH 8.5. Protein concentrations were determined using the RC DC Protein Assay (Bio-Rad) with BSA as standard. The stability experiment was performed with one culture of each selected strain. Each culture was divided in two before harvesting and the corresponding cell-free extracts were prepared and measured for PepN activity on two separate days.

SDS-PAGE analysis. For protein analysis of PepN, two control strains harbouring pSIP410 were cultivated, and one was induced with 50 ng ml⁻¹ of the SapIP induction peptide (Molecular Biology Unit, University of Newcastle, UK) at OD₆₀₀ ~0.3. All the cells were grown to OD₆₀₀ ~1.6 before harvesting. Cell-free protein extracts were obtained as above, and analysed on 12% SDS-polyacrylamide gels (Bio-Rad). PepN was quantified as a percentage of the total intracellular protein content by scanning the gel with a densitometer (Gel Doc 1000, Bio-Rad).

Fig. 2. Construction of the synthetic promoter library (SPL). (A) Oligonucleotide sequence (SPL1) with consensus sequence (underlined and in bold) and randomized spacer sequences (N = 25% of A, G, C or T). Semi-conserved bases: R = 50% of A or G; W = 50% of A or T; D = 33% of A, G or T. A restriction site is included in the 5' end and the part homologous to P_{orfX} is in the 3' end. (B) Primers (SPL1 and gusRT1) annealing to the pSIP409 vector containing P_{orfX} and parts of *gusA*. The position from the transcriptional start site (+1) and the ribosome-binding site (RBS) are indicated. The resulting PCR fragments, including restriction sites, were used to replace the P_{orfX} promoter.

RESULTS

In this work, an SPL for *L. plantarum* was made. The approach involved derivation of consensus sequences, design of a synthetic oligonucleotide and construction of the SPL in the pSIP expression system.

Alignment of rRNA promoters from *L. plantarum* and derivation of consensus sequences

The consensus sequences of the synthetic oligonucleotide were based on the alignment of 16S rRNA promoters from the genome-sequenced strain *L. plantarum* WCFS1 (Fig. 1). Five rRNA genes have been identified in the genome, and we identified two putative promoters (rRNA-a and rRNA-b) upstream of each gene. The rRNA-a promoters were identified nearest to the rRNA genes. TTGACA was the most conserved -35 consensus sequence, and was found in all of the rRNA-a promoters. The -10 sequence was less well conserved in the rRNA promoters, and only three bases (TAXXXT) were conserved. The semi-conserved bases were derived from the rRNA promoters and resulted in a TAWDNT sequence (W=A or T, D=A, T or G, N=A, T, G or C), where N was included to allow for a slightly higher diversity in the -10 sequence. Upstream of the -10 sequence, a conserved TG motif was present at the -15 position of all the rRNA promoters, and the semi-conserved R base (A or G) was included based on the rRNA promoters with a 17 bp spacer sequence. A conserved A base was also identified in the -40 position of the rRNA promoters, and the AGTTG motif was found in almost all of the rRNA-a promoters.

Construction of the SPL

Inducible expression vectors, designated pSIP (Sørvig *et al.*, 2003, 2005) have previously been developed in our laboratory, and show high expression of GusA and PepN in lactobacilli. The pSIP409 vector was selected for the construction of the SPL, because of its low basal activity and high dynamic range of GusA expression. The construction of the SPL involved PCR with the synthetic oligonucleotide SPL1 (Fig. 2A) as the forward primer. A restriction site was included in the 5' end of the oligonucleotide, followed by the derived consensus sequences encompassing the 14 bases of randomized spacer sequence. The 3' end was designed to hybridize to the part of the inducible P_{orfx} promoter that has homology to the 5' end of the *gusA* mRNA (Fig. 2B). This assured the presence of the same *gusA* mRNA in both expression systems, and made them directly comparable. The derived PCR fragments, containing a mixture of potential promoter structures, were used to replace the P_{orfx} promoter through cloning into the two restriction sites.

GusA activity of the SPL and comparison with rRNA promoters

Different promoter activities were obtained by screening colonies of *L. plantarum* NC8 exhibiting different intensities of blue and white when growing on agar plates containing X-Gluc. The colonies were investigated for GusA activity (Fig. 3). A wide range of promoter activities was covered, from below 10 MU to more than 1600 MU, corresponding to a span of 3–4 logs of promoter activity. Comparison with five native rRNA promoters showed that the SPL contained

Fig. 3. Synthetic promoter library (SPL) compared to rRNA promoters in *L. plantarum* NC8. Activities of the SPL (grey bars) and rRNA promoters (dark-grey bars, numbered with asterisk) are measured as GusA activity. All data are mean values of at least four independent experiments; the error bars indicate the standard deviation.

promoters with activities that were in the same range as those of the native rRNA promoters. Interestingly, the activities of the native rRNA promoters were not equally high, but varied from approximately 750 MU to more than 1600 MU. By comparing the most potent promoters from the SPL with maximum induction of P_{orfX} in pSIP409 (Sørvig *et al.*, 2005), it was shown that some of the constitutive promoters had even higher activities than the inducible system.

Promoter sequences

Selected colonies from agar plates were analysed for promoter sequence (Fig. 4). Comparing their DNA sequences with the synthetic oligonucleotide resulted in a number of observations. Twenty-four of the 35 promoters had DNA sequences identical to that of the synthetic oligonucleotide. These included promoters with activities as low as 50 MU and as high as 1600 MU. The remaining promoters had errors in their consensus sequence or a different spacer length than that of the synthetic oligonucleotide, as observed previously (Jensen & Hammer, 1998b). As expected, these errors were most frequent in promoters with the lowest

activities. For instance, the entire spacer region and the -35 region were missing in the weakest promoter. Other promoters had deletions or base pair changes in the consensus sequences (-35 and -40) or deletions in the spacers (outside -40 , between -35 and -10 , or outside -10). Errors in the -15 region were not identified, and only one promoter had an error in the -10 region. However, this was the second weakest of the promoters, and had a base pair change in the -10 sequence, from the conserved A to a T. Two of the promoters (P14 and P45=P46) were not included in Fig. 4, since the inserts were not derived from the oligonucleotide sequence. However, putative -10 sequences were identified, and might explain promoter activities.

Stability of expression

The SPL was designed to generate expression vectors with constitutive promoters. The stability of GusA and PepN expression was tested with three selected promoters during the exponential phase (Fig. 5). Stable GusA and PepN expression was measured from all three promoters, perhaps

		-40	-35		-15	-10	+1
Oligo seq	AGATCTNNNNNNNN	AGTTGTTGAC	NNNNNNNNNNNNNNNNNN	TGRTAWDNT	NNNNNN	TATAGCG	
P15	AGATCTACG----	AGTTGT	-----	TGGTATTGTGGCGG	TATAGCG		
P20	AGATCTGTCGTGGCAGTTGTTGACA	ACCTGTGGGCGGTT	TGATTGTTCTTGGT	TATAGCG			
P17	AGATCTAGGGGTTGAGT--	TTGACACTGATCCCGGCTGGT	TGGTAAATTTTCGTT	TATAGCG			
P13	AGATCTGGGGT---	AGTTGTTGACAGCGTGGGTGGTGC	TGGTAAATTTTCGCGT	TATAGCG			
P41	AGATCTAGGGAGTCAGTTG--	TGACAGGGCTGTGATGGTCTGGTATTGTTT	TGCTATAGCG				
P43	AGATCTGTCGGTCGAGTTGTTGACA	GGATAAAGGTCGCTGGTATGGTCTCAAT	TATAGCG				
P40	AGATCTTACCGGACA--TTGTT	CAACATACATGGATCG--	TGGTATGTTTCCAAT	TATAGCG			
P44	AGATCTGGGGTG--AGTTGTTGACA	CTGGAGGGTTTGATGGTAATCTCGCGT	TATAGCG				
P10	AGATCTGAGCTGCCAGTTGTTGACA	TGTTAATGGCCCCGATATATTTGGCC	TATAGCG				
P34	AGATCTCGCGATTAGTTGTTGACA	GACAAACATAAGGATGATATGCTCCAGT	TATAGCG				
P9=12	AGATCTACCCAGCCAGTTGTTGACA	GGGCTGAGCTGACCTGGTATGGT--	ATATATAGCG				
P42	AGATCTGGTCCGCCAGTTGTTGACA	GAGTTGTCTGGTATGATATTGCTCAAT	TATAGCG				
P5	AGATCTCGGCCGGGAGTTGTTGACA	CACAAAACCAGACATGGTATTAT--	AATCTATAGCG				
P33	AGATCTCCGGTTACAGTTGTTGACA	TGTGACCCGTTTTTATGGTATTATGGGGC	TATAGCG				
P21	AGATCTGTCCAGGAGTTGTTGACA	ATTCGGGGCATCTCGTGGTATAATTGTGCT	TATAGCG				
P23=26	AGATCTGTTAACGA----	TTGGACAAGTGATAAAACGGG	TGATATGATAGATATAGCG				
P22	AGATCTGGCCAGGCAGTTGTTGACA	GATTAGGGCGGTCATGGTAAATGGGAC	TATAGCG				
P18=27	AGATCTCGGGGTTAGTTGTTGACA	GGGAGGCTTCGTTGTGATAAGATGGTAC	TATAGCG				
P47	AGATCTGGGGGCCAAGTTGTTGACA	GACGCGGAGAAATGGTAAAGTTTACG	TATAGCG				
P16	AGATCTGGAAGGGG--GTTGTTGACA	TCCCCCTCTCTTTCTGGTATAATTGGGT	TATAGCG				
P3=28	AGATCTGTTGTGACAGTTGTTGACA	AAAGAGCCGGGTTTGGTATAATGGGCG	TATAGCG				
P29	AGATCTTGGGTGTTAGTTGTTGACA	GTTCTGGCTGGATATGGTAAACTTGTGCT	TATAGCG				
P6=7	AGATCTACCAGACAAGTTGTTGACA	GGCAGCCATCTCTATGGTAAATGTTGCT	TATAGCG				
P38	AGATCTGTGTCAGTAGTTGTTGACA	GAAATGTTTTTGTAGTGGTATAATGTATT	TATAGCG				
P30	AGATCTGAGGGCGGAGTTGTTGACA	GCCGAGGTACCATCTGGTATAATCCCGA	TATAGCG				
P31	AGATCTGGAGAACCAGTTGTTGACA	AAAGTCCAGGGTATGATATACTCTGAC	TATAGCG				
P8	AGATCTGGCGTTGTAGTTGTTGACA	AAATCCGGTGTCTATGGTATTCTCGGT	TATAGCG				
P25	AGATCTGTGGGGTTAGTTGTTGACA	TAAAGGCACATCATGATATGCTTAAAT	TATAGCG				
P35	AGATCTGGGACGTAAGTTGTTGACA	TGACGGGAGTTTGTGGTATAATGGGCC	TATAGCG				
P1=2	AGATCTGGACGAGGAGTTGTTGACA	GGGATATTAGAGTATGGTATGCTTGCCG	TATAGCG				
P4	AGATCTGCTCGGGGAGTTGTTGACA	CGCGCAGCAAGGCATGATATAATCTCGG	TATAGCG				
P11	AGATCTAGCGCTATAGTTGTTGACA	GAAATGGACATACTATGATATAATGTTGCT	TATAGCG				
P48	AGATCTGCATCGTAAGTTGTTGACA	TGGAACGAGGAATCTGATAATCTGTGAC	TATAGCG				

Fig. 4. Promoter sequences of the synthetic promoters. The promoters are ordered according to strength. Sequence matches to the oligonucleotide are indicated with black boxes. Deletions are indicated with hyphens and base-pair changes in the consensus sequences are shown with white boxes.

Fig. 5. Stability of expression during growth measured as (A) GusA activity and (B) PepN activity from P9 (●), P11 (■) and P27 (▲). Standard deviations are included.

with a slightly lower expression from the potent P11 promoter at high cell densities.

PepN production from the potent P11 promoter

The PepN activity expressed from the potent P11 promoter in pSIP410 was approximately 1 U ml⁻¹ (Fig. 5B). This is slightly lower (by approx. 0.2 U ml⁻¹) than the activity we achieved with maximum induction of P_{orfX} in pSIP410. However, SDS-PAGE of cell-free extracts of pSIP410-P11 and maximally induced pSIP410 shows a high amount of PepN protein (Fig. 6). Densitometric analysis indicated that PepN constitutes approximately 10–15% of the total amount of intracellular proteins of *L. plantarum* NC8 (pSIP410-P11) and of induced *L. plantarum* NC8 (pSIP410).

GusA activity in *L. plantarum* versus *L. sakei*

The inducible pSIP409 expression system is actively used in *L. plantarum* and *L. sakei* in our laboratory, and we wanted to investigate whether the strength of the synthetic promoters in *L. plantarum* is species-dependent or if their activities have comparable levels in *L. sakei*. Six promoters, covering a wide range of promoter activities in *L. plantarum* NC8, were also measured in *L. sakei* Lb790-X (Fig. 7).

Fig. 6. SDS-PAGE of cell-free extracts of *L. plantarum* NC8 strains harbouring pSIP410 and pSIP410-P11. The location of the PepN protein is indicated with an arrow. Lane M, molecular mass marker (kDa); lane 1, uninduced pSIP410; lane 2, pSIP410-P11; lane 3, induced pSIP410 with 50 ng inducing peptide ml⁻¹.

Fig. 7. Promoter activity in *L. plantarum* versus *L. sakei*. GusA activities from P1, P5, P11, P27, P31 and P44 promoters were measured in *L. plantarum* NC8 (grey bars) and *L. sakei* Lb790-X (white bars). Standard deviations from two independent cultures are shown.

A good correlation in the promoter activities of all the promoters was seen between the two species.

DISCUSSION

In certain research fields, such as metabolic control analysis, a steady-state gene expression is required. We have previously performed stability experiments with the inducible pSIP409 system, and the most stable GusA production is obtained when a culture is induced and recultured in induced media before the cells leave the exponential phase (data not shown). Synthesis of the SPL was chosen to circumvent the problems of stability of expression that are encountered with the inducible system, by obtaining constitutive promoters.

The Jensen–Hammer approach, which has successfully been used in *Lc. lactis* (Jensen & Hammer, 1998b), was used as the basis for generating the SPL. Jensen & Hammer (1998b) extracted extended consensus sequences from a range of sequenced *Lc. lactis* promoters. In our approach, the consensus sequences were derived by aligning rRNA promoters extracted from the sequenced *L. plantarum* genome. A comparison of the derived consensus sequences with sequences from the *L. plantarum* genome resulted in matches with a high number of putative promoters of known genes (data not shown). This indicates that the derived consensus sequence is likely to be active in *L. plantarum*. The reason for choosing rRNA promoters in finding a consensus sequence is that these are often available or can easily be obtained, even for organisms for which the genomes have not yet been sequenced. In addition, rRNA promoters are known to be among the strongest promoters in most bacteria.

The Jensen–Hammer approach is based on the fact that the spacer sequence between the –35 and the –10 consensus sequences plays an important role in promoter strength. The spacer sequence affects the three-dimensional structure of the promoter, which then affects the efficiency of interactions between the RNA polymerase holoenzyme and the promoter sequence. The spacer sequence was also shown to be important when generating the SPL in *L. plantarum*. A wide range of promoter activities was obtained, harbouring consensus sequences identical to those of the synthetic

oligonucleotide, but with completely different spacer sequences. However, most of the weak promoters had mutations in the consensus sequences or deletions of base pairs in the spacer sequences.

The comparison of the SPL with native rRNA promoters showed that the SPL contains some promoters with activities that are equally high as those of rRNA promoters and others with higher activities. This demonstrates that the range of the SPL also covers promoter strengths that probably are high in nature, since rRNA promoters are known to be among the strongest promoters in bacteria. However, it should be mentioned that the context of the rRNA promoters has been changed compared to their native context on the chromosome, which might affect their strength. The most potent promoters of the SPL also gave higher GusA activities than that measured with maximum induction of pSIP409 (Sørvig *et al.*, 2005). The GusA activities measured are comparable, since both expression systems generate an identical sequence of the *gusA* mRNA. The amount of PepN protein from the potent P11 promoter was also high (approx. 10–15 % of the total intracellular protein in *L. plantarum*), but slightly below the amount we found with maximum induction of pSIP410. Higher production of PepN has been obtained previously, reaching 28 and 40 % in *L. plantarum* (Kahala & Palva, 1999; Mathiesen *et al.*, 2004); however, stable production of PepN has not been reported in these examples.

The stable production of GusA and PepN from selected promoters during exponential growth (Fig. 5) indicates the constitutive potential of the SPL. A weak tendency of decreased GusA and PepN production can be seen from the potent P11 promoter at high cell densities. This has not been investigated, but might perhaps involve changes in plasmid copy number or stability of the reporter gene. However, we have never experimentally observed differences in plasmid copy number. The importance of the reporter gene, e.g. *gusA* and *pepN*, has been discussed previously (Sørvig *et al.*, 2005).

A promoter does not necessarily have the same activity in different organisms. The SPL developed for *Lc. lactis* has been shown to exert a different activity in *E. coli* (Jensen & Hammer, 1998b). Also, among species of *Lactobacillus*, promoters have been reported to be species dependent, i.e.

Lactobacillus rhamnosus versus *Lactobacillus fermentum* and *L. plantarum* (McCracken *et al.*, 2000) and *Lactobacillus helveticus* versus *Lactobacillus casei* (Chen & Steele, 2005). Interestingly, when comparing the activities of synthetic promoters in *L. plantarum* versus *L. sakei* (Fig. 7), a high correlation in promoter activities was shown. This corresponds to previous experiments with versions of the pSIP expression vector that harbour different inducible promoters, in which there is a correlation between *L. plantarum* and *L. sakei* in the production of GusA (Sørvig *et al.*, 2005). Three of the promoters of the SPL were also successfully introduced into another strain of *L. plantarum*, but fused to another reporter gene, yielding approximately the same ratios of protein expression between the synthetic promoters (data not shown).

There are several advantages to the SPL developed here. The wide range of promoter activities in the SPL allows for the fine-tuning of gene expression, which is important for applications such as metabolic optimization and metabolic control analysis (Jensen & Hammer, 1998a). Another advantage is that the promoters are constitutive, making stability of production easier to achieve than with an inducible system. The stability is also important when performing metabolic control analysis. The fact that some of the synthetic promoters are very strong also makes them well suited for high-level production of proteins. In addition, with constitutive promoters, it is possible to avoid the addition of inducer compounds in large-scale fermentations, and the consequent high cost. The synthetic promoters also have great potential when using live bacteria as antigen delivery vehicles for oral vaccination purposes, especially since the use of *L. plantarum* and other *Lactobacillus* species for such purposes has received a lot of attention in recent years (Pouwels *et al.*, 1996, 1998; Havenith *et al.*, 2002). The expression vector pSIP409 was excellent as a basis for constructing the SPL; for example, it has a 'cassette-like' structure, which makes it easy to exchange all parts through digestion and ligation. By exchanging the antibiotic resistance marker with a food-grade marker, it can become food grade. It should be mentioned that the synthetic promoters are also regarded as food grade (Jensen & Hammer, 1998b).

In conclusion, we have generalized the Jensen–Hammer approach for obtaining synthetic promoters. The SPL developed in *L. plantarum* contains promoters with a wide range of promoter activities and with constitutive qualities, which allows for the fine-tuning of steady-state gene expression. The constitutive promoters are also useful for achieving stable protein production, especially when inducible systems are not applicable.

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REFERENCES

- Ahrné, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A. E. & Molin, G. (1998). The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. *J Appl Microbiol* **85**, 88–94.
- Alander, M., De Smet, I., Nollet, L., Verstraete, W., von Wright, A. & Mattila-Sandholm, T. (1999). The effect of probiotic strains on the microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). *Int J Food Microbiol* **46**, 71–79.
- Aukrust, T. & Blom, H. (1992). Transformation of *Lactobacillus* strains used in meat and vegetable fermentations. *Food Res Int* **25**, 253–261.
- Axelsson, L. (2004). Lactic acid bacteria: classification and physiology. In *Lactic Acid Bacteria: Microbiological and Functional Aspects*, 3rd edn, pp. 1–66. Edited by S. Salminen, A. von Wright & A. Ouwehand. New York: Marcel Dekker.
- Axelsson, L. & Ahrné, S. (2000). Lactic acid bacteria. In *Applied Microbial Systematics*, pp. 365–386. Edited by F. G. Priest & M. Goodfellow. The Netherlands: Kluwer Academic Publishers.
- Axelsson, L., Lindstad, G. & Naterstad, K. (2003). Development of an inducible gene expression system for *Lactobacillus sakei*. *Lett Appl Microbiol* **37**, 115–120.
- Chaves, A. C., Fernandez, M., Lerayer, A. L., Mierau, I., Kleerebezem, M. & Hugenholtz, J. (2002). Metabolic engineering of acetaldehyde production by *Streptococcus thermophilus*. *Appl Environ Microbiol* **68**, 5656–5662.
- Chen, Y. S. & Steele, J. L. (2005). Analysis of promoter sequences from *Lactobacillus helveticus* CNRZ32 and their activity in other lactic acid bacteria. *J Appl Microbiol* **98**, 64–72.
- de Vos, W. & Simons, G. (1994). Gene cloning and expression systems in lactococci. In *Genetics and Biotechnology of Lactic Acid Bacteria*, pp. 52–105. Edited by M. Gasson & W. de Vos. Oxford: Chapman and Hall.
- Exterkate, F. A. (1984). Location of peptidases outside and inside the membrane of *Streptococcus cremoris*. *Appl Environ Microbiol* **47**, 177–183.
- Havenith, C. E. G., Seegers, J. F. M. L. & Pouwels, P. H. (2002). Gut-associated lactobacilli for oral immunisation. *Food Res Int* **35**, 151–163.
- Hugenholtz, J., Kleerebezem, M., Starrenburg, M., Delcour, J., de Vos, W. & Hols, P. (2000). *Lactococcus lactis* as a cell factory for high-level diacetyl production. *Appl Environ Microbiol* **66**, 4112–4114.
- Jensen, P. R. & Hammer, K. (1998a). Artificial promoters for metabolic optimization. *Biotechnol Bioeng* **58**, 191–195.
- Jensen, P. R. & Hammer, K. (1998b). The sequence of spacers between the consensus sequences modulates the strength of prokaryotic promoters. *Appl Environ Microbiol* **64**, 82–87.
- Jensen, P. R., Westerhoff, H. V. & Michelsen, O. (1993). The use of *lac*-type promoters in control analysis. *Eur J Biochem* **211**, 181–191.
- Kahala, M. & Palva, A. (1999). The expression signals of the *Lactobacillus brevis* *slpA* gene direct efficient heterologous protein production in lactic acid bacteria. *Appl Microbiol Biotechnol* **51**, 71–78.
- Koebmann, B. J., Andersen, H. W., Solem, C. & Jensen, P. R. (2002a). Experimental determination of control of glycolysis in *Lactococcus lactis*. *Antonie van Leeuwenhoek* **82**, 237–248.
- Koebmann, B. J., Solem, C., Pedersen, M. B., Nilsson, D. & Jensen, P. R. (2002b). Expression of genes encoding F₁-ATPase results in uncoupling of glycolysis from biomass production in *Lactococcus lactis*. *Appl Environ Microbiol* **68**, 4274–4282.
- Krüger, C., Hu, Y., Pan, Q. & 10 other authors (2002). In situ delivery of passive immunity by lactobacilli producing single-chain antibodies. *Nat Biotechnol* **20**, 702–706.

- Mathiesen, G., Sørvig, E., Blatny, J., Naterstad, K., Axelsson, L. & Eijsink, V. G. H. (2004). High-level gene expression in *Lactobacillus plantarum* using a pheromone-regulated bacteriocin promoter. *Lett Appl Microbiol* **39**, 137–143.
- McCracken, A., Turner, M. S., Giffard, P., Hafner, L. M. & Timms, P. (2000). Analysis of promoter sequences from *Lactobacillus* and *Lactococcus* and their activity in several *Lactobacillus* species. *Arch Microbiol* **173**, 383–389.
- Mercenier, A., Pouwels, P. & Chassy, B. (1994). Genetic engineering of lactobacilli, leuconostocs and *Streptococcus thermophilus*. In *Genetics and Biotechnology of Lactic Acid Bacteria*, pp. 252–293. Edited by M. Gasson & W. de Vos. Oxford: Chapman and Hall.
- Mercenier, A., Pavan, S. & Pot, B. (2003). Probiotics as bio-therapeutic agents: present knowledge and future prospects. *Curr Pharm Des* **9**, 175–191.
- Miller, J. M. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Møretrø, T., Hagen, B. F. & Axelsson, L. (1998). A new, completely defined medium for meat lactobacilli. *J Appl Microbiol* **85**, 715–722.
- Møretrø, T., Naterstad, K., Wang, E., Aasen, I. M., Chaillou, S., Zagorec, M. & Axelsson, L. (2005). Sakacin P non-producing *Lactobacillus sakei* strains contain homologues of the sakacin P gene cluster. *Res Microbiol* **156**, 949–960.
- Neu, T. & Henrich, B. (2003). New thermosensitive delivery vector and its use to enable nisin-controlled gene expression in *Lactobacillus gasseri*. *Appl Environ Microbiol* **69**, 1377–1382.
- Pavan, S., Hols, P., Delcour, J., Geoffroy, M. C., Grangette, C., Kleerebezem, M. & Mercenier, A. (2000). Adaptation of the nisin-controlled expression system in *Lactobacillus plantarum*: a tool to study in vivo biological effects. *Appl Environ Microbiol* **66**, 4427–4432.
- Pouwels, P. H. & Leer, R. J. (1993). Genetics of lactobacilli: plasmids and gene expression. *Antonie van Leeuwenhoek* **64**, 85–107.
- Pouwels, P. H., Leer, R. J. & Boersma, W. J. (1996). The potential of *Lactobacillus* as a carrier for oral immunization: development and preliminary characterization of vector systems for targeted delivery of antigens. *J Biotechnol* **44**, 183–192.
- Pouwels, P. H., Leer, R. J., Shaw, M., Heijne den Bak-Glashouwer, M. J., Tielen, F. D., Smit, E., Martinez, B., Jore, J. & Conway, P. L. (1998). Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. *Int J Food Microbiol* **41**, 155–167.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schillinger, U. & Lücke, F.-K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Appl Environ Microbiol* **55**, 1901–1906.
- Schultz, M., Veltkamp, C., Dieleman, L. A., Grenther, W. B., Wyrick, P. B., Tonkonogy, S. L. & Sartor, R. B. (2002). *Lactobacillus plantarum* 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm Bowel Dis* **8**, 71–80.
- Solem, C. & Jensen, P. R. (2002). Modulation of gene expression made easy. *Appl Environ Microbiol* **68**, 2397–2403.
- Solem, C., Koebmann, B. J. & Jensen, P. R. (2003). Glyceraldehyde-3-phosphate dehydrogenase has no control over glycolytic flux in *Lactococcus lactis* MG1363. *J Bacteriol* **185**, 1564–1571.
- Sybesma, W., Starrenburg, M., Kleerebezem, M., Mierau, I., de Vos, W. M. & Hugenholtz, J. (2003). Increased production of folate by metabolic engineering of *Lactococcus lactis*. *Appl Environ Microbiol* **69**, 3069–3076.
- Sørvig, E., Grönqvist, S., Naterstad, K., Mathiesen, G., Eijsink, V. G. & Axelsson, L. (2003). Construction of vectors for inducible gene expression in *Lactobacillus sakei* and *L. plantarum*. *FEMS Microbiol Lett* **229**, 119–126.
- Sørvig, E., Mathiesen, G., Naterstad, K., Eijsink, V. G. & Axelsson, L. (2005). High-level, inducible gene expression in *Lactobacillus sakei* and *Lactobacillus plantarum* using versatile expression vectors. *Microbiology* **151**, 2439–2449.
- van de Guchte, M., Kok, J. & Venema, G. (1991). Distance-dependent translational coupling and interference in *Lactococcus lactis*. *Mol Gen Genet* **227**, 65–71.