Inducible Promoter-Repressor System from the *Lactobacillus casei* Phage ϕ FSW

Bernhard Binishofer,¹ Isabella Moll,¹ Bernhard Henrich,² and Udo Bläsi^{1*}

*Institute of Microbiology and Genetics, Vienna Biocenter, University of Vienna, 1030 Vienna, Austria,¹ and
Department of Biology, University of Kaiserslautern, 67653 Kaiserslautern, Germany²*

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With the aim to extend the presently available inducible gene expression systems for lactobacilli, we have isolated a thermoinducible promoter-repressor cassette from the temperate *Lactobacillus casei* phage ϕ FSW-TI in *Escherichia coli*. The ϕ FSW-TI promoter fragment was abutted to the plasmid-borne promoterless β -glucuronidase (*gusA*) reporter gene and shown to direct its transcription in *L. casei*. In addition, the functionality of the promoter-repressor system was verified in the *L. casei* ϕ FSW-TI lysogen by showing that the *gusA* reporter gene, controlled by the isolated ϕ FSW-TI promoter, was repressed at 28°C and expressed at 42°C. Moreover, a homology search revealed that the C terminus of the isolated ϕ FSW repressor shows a high similarity to the small *mutS*-related domain of the MutS2 protein family that is unprecedented for phage-encoded repressor proteins.

Given the economic importance of lactic acid bacteria, and more recently their prospective use as safe vaccines, a number of physiological traits as well as food-grade expression tools of lactobacilli and lactococci have been studied (9). Various inducible gene expression systems have been developed for *Lactococcus lactis* (reviewed in reference 3). In general, these transcriptional induction systems require the exogenous supply of inducers (18, 19, 21). In addition, a heat-inducible homologous expression system has been developed for *L. lactis* based on a genetically engineered thermolabile repressor of the lactococcal phage ϕ r1t (17). In lactobacilli, studies on the regulation of gene expression have focused mainly on carbon catabolism pathways, and an inducible-repressible expression system derived from the lactose operon of *Lactobacillus casei* has been described (5 and references therein).

The temperate phage ϕ FSW-TI was isolated from *L. casei* strain Shirota (S1) after chemical mutagenesis of a lysogen followed by heat induction (22, 24). Since the mutation responsible for the thermoinducibility was shown to reside in the phage DNA, it seemed likely that it could affect the stability of the phage repressor at the elevated temperature. In this study, we have characterized a promoter and a cognate repressor of ϕ FSW-TI with the potential to be exploited in a thermoinducible food-grade expression system for *L. casei*.

Isolation of a promoter and a cognate heat-labile repressor of ϕ FSW-TI in *Escherichia coli*. The following strategy was used to isolate the thermoinducible promoter-repressor cassette of phage ϕ FSW-TI. The ϕ FSW-TI DNA was first shotgun cloned into the *E. coli* promoter test vector pKK232-8 (Amersham Pharmacia) carrying a promoterless *cat* gene. Chloramphenicol (Cm)-resistant clones were cotransformed with a ϕ FSW-TI library generated in the compatible plasmid pK194 (7). The DNA sequence encoding the protein with

apparent repressor activity and its cognate promoter sequence were then obtained from clones that were sensitive and resistant to the antibiotic at 28 and 42°C, respectively.

Phage ϕ FSW-TI DNA was prepared as described earlier (23). The DNA was cleaved with either *Hae*III, *Rsa*I, *Hinc*II, or *Eco*RV, and the corresponding fragments were cloned into the *Sma*I site of the promoter test vector pKK232-8. The *E. coli* strain MC4100F' (25) was transformed with the ligation mix, and 51 candidate clones were selected on Luria-Bertani agar plates (14) containing 50 μ g of Cm/ml.

To isolate the cognate repressor gene, ϕ FSW-TI DNA was partially digested with the same enzymes used for the procedure described above. The restriction fragments were cloned into the *Sma*I site of plasmid pK194, and the ligation mix was transformed into *E. coli* strain MC4100F'. From the pool of the transformants obtained on Luria-Bertani agar supplemented with 50 μ g of kanamycin (Km)/ml, a batch plasmid isolation was performed. Then each of the 51 candidate promoter clones was transformed with the batch plasmid preparation. Cotransformants were selected on Cm/Km plates at 42°C, a temperature at which the putative repressor was predicted to be inactive, and thus, transcription of the *cat* gene in plasmid pKK232-8 was not supposed to be repressed. One hundred and fifty cotransformants were replica plated on Cm/Km at 28°C. The recombinant plasmids of five cotransformants unable to grow at 28°C were isolated from the corresponding colonies on the master plate and retransformed into MC4100F' in order to obtain colonies containing the individual plasmids. The inserts of two corresponding plasmid pairs were sequenced using an Applied Biosystems DNA sequencer. Both pairs contained the same core sequences. The plasmids with the shortest inserts were designated pKK232-8P (524-bp insert containing the putative promoter) and pK194R-1 (1,557-bp insert containing the putative repressor gene) and were used in further experiments.

Characterization of the promoter region. The DNA sequence of the 524-bp insert (National Center for Biotechnology Information [NCBI] accession no. AF458097) present in

* Corresponding author. Mailing address: Institute of Microbiology and Genetics, Vienna Biocenter, Dr. Bohrgasse 9, 1030 Vienna, Austria. Phone: 43-1-4277-54609. Fax: 43-1-4277-9546. E-mail: Udo.Blaesi@univie.ac.at.

FIG. 1. (A) Determination of the transcriptional start site of the isolated φFSW promoter (φFSW-P). Lane 1, the oligonucleotide V10 complementary to the sequence downstream of the predicted start point of φFSW-P was used to prime cDNA synthesis of RNA isolated from strain MC4100F'(pKK232-8P). The 3'-terminal nucleotide of the cDNA complementary to the 5' end of the transcript assigned to a G residue is marked by an arrow. The -10 hexamer is shaded. Reference sequencing reactions were performed with the same primer on double-stranded plasmid pKK232-8P as template. (B) Comparison of φFSW-P with the promoter consensus sequence for lactobacilli. Boldface type indicates identity with consensus (20). The transcriptional start site (TS) is underlined. Dots are used for the alignment of the -35 and -10 hexamer sequences.

plasmid pKK232-8P was analyzed for putative promoter sequences using the promoter search program available on the website <http://www.dot.imgen.bcm.tmc.edu:9331/cgi-b>. The highest score was obtained for a putative promoter spanning bp 275 (-35 hexamer) to bp 309 (predicted transcriptional start). To determine the transcriptional start site, primer extension was performed as described earlier (6) with total RNA prepared from *E. coli* MC4100F' harboring plasmid pKK232-8P containing the sequenced 524-bp insert with the putative promoter. The oligonucleotide V10, 5'-GACATGGGAGGCAC CAGC-3', which binds 58 bp downstream of the putative -10 region, was used as a primer for the reverse transcriptase reaction. As shown in Fig. 1A, the primer extension analysis revealed that the transcript starts with the G located 6 bp downstream of the -10 hexamer. The alignment with the previously defined *Lactobacillus* consensus sequence (20) showed five matches for both the -35 and -10 hexamers, which are separated by 18 bp (Fig. 1B). The distance between the -10 hexamer and the transcriptional start site is 5 bp (Fig. 1B), which is within the range found for other *Lactobacillus* promoters (13).

The functionality of the isolated φFSW promoter was verified in *L. casei* S1 (23) by transforming this strain with a plasmid in which the promoter (including upstream and downstream sequences: bp -99 to +35 with regard to the transcrip-

tional start [+1]) was abutted to the *gusA* reporter gene. The selected φFSW promoter was cloned 5' of the *gus* gene in plasmid pNZ8008 (18) as follows: the promoter fragment was obtained by PCR amplification using plasmid pKK232-8P and the forward primer 5'-TATCTGCAGTG-AATCAAAGCCG TGGCGTTA-3' together with the reverse primer 5'-CCGAA TTCGCGGCCAGCGCAATGAGCTT-3' containing a *Pst*I site and an *Eco*RI site, respectively. The purified PCR fragment was cleaved with *Pst*I and *Eco*RI and cloned into the corresponding sites of plasmid pNZ8008, resulting in plasmid pNZFSWP. The plasmid was transformed in *L. casei* S1 as described earlier (2). Colonies of *L. casei* S1 transformed with pNZFSWP showed β-glucuronidase activity, whereas colonies of *L. casei* S1 transformed with the parental vector pNZ8008 did not, demonstrating that the φFSW promoter was directing transcription of the *gus* reporter gene in the *L. casei* strain (not shown).

Characterization of the cognate repressor. As mentioned above, plasmid pK194R-1 contained a 1,557-bp insert of φFSW-TI DNA comprising five open reading frames (ORFs) (Fig. 2). To identify the repressor gene, four different restriction fragments (Fig. 2) were isolated from plasmid pK194R-1 and recloned into plasmid pK194. Plasmid pK194R-2 contained the 3' end of the 1,557-bp fragment covering ORF4 and ORF5, whereas plasmid pK194R-3 contained ORF3 in addi-

FIG. 2. Identification of the ORF encoding the repressor by deletion analysis. As described in the text, plasmid pK194R-1 bears a 1,557-bp DNA insert of ϕ FSW containing five putative ORFs. The restriction sites used for isolation of the different subfragments and for reinsertion into plasmid pK194 are indicated on top. The restriction sites are indicated by capital letters: H, *Hind*III; B, *Bam*HI; X, *Xma*I; E, *Eco*RI; and S, *Sma*I. The lengths of the DNA fragments present in plasmids pK194R-2 to -5 are schematically shown. + and - signs signify repressor activity (i.e., Cm sensitivity at 28°C) in strain MC4100F'(pKK232-8P) cotransformed with either of the depicted plasmids. ORF1 (indicated by a black bar) encodes the Smr-homologous ϕ FSW repressor.

tion to ORF4 and ORF5. Plasmid pK194R-4 harbored the 5'-proximal reading frames ORF1 to ORF3, while plasmid pK194R-5 contained only ORF1. These four plasmids were individually transformed into *E. coli* strain MC4100F' (pKK232-8P). The presence of either plasmid pK194R-4 or pK194R-5 in strain MC4100F'(pKK232-8P) conferred the same levels of Cm resistance at 42°C (expression of the *cat* gene due to thermal inactivation of the repressor) and resulted in Cm sensitivity at 28°C (repression of the *cat* gene due to the presence of active repressor protein). The repressor gene was therefore assigned to ORF1.

To test qualitatively whether the identified ϕ FSW-TI pro-

motor is controllable as well by the ϕ FSW-TI repressor in *L. casei*, the *L. casei* ϕ FSW-TI lysogen was transformed with plasmid pNZFSWP bearing the *gusA* reporter gene under transcriptional control of the isolated ϕ FSW-TI promoter. Since no β -glucuronidase activity was detected at 28°C, whereas thermal induction at 42°C resulted in expression of the reporter gene (not shown), we concluded that the thermoinducible promoter-repressor system is functional in *L. casei*.

As revealed by a PSI-Blast search available on the website http://www3.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-psi_blast, the isolated 113-amino-acid (aa) ϕ FSW-TI repressor protein (NCBI accession no. AF406970) shows a high similarity ($E =$

ϕ FSWR	1	---	MK	PTDEM	EKIKPS	QOSAAQRPV	KVSGGMSGP	STTEYL	LRGERY	DOAMA	LDQYID
<i>S. pyogenes</i>	666	MTLT	QDEFSLVR	QEEQ	VKNKQIN	VVKRADG	--SGP	EARL	DLRG	KRYEAM	QELDHID
<i>L. monocytogenes</i>	21	----	MKI	KTADLEYIQ	PEKPKQRI	ITSVHSSG	SPAKSBL	DLRG	RYEDAL	QKVDKYID	
<i>S. pneumoniae</i>	675	-----	QAQ	QEKPVKKQ	QVNVKRTSG	--RG	QARL	DLRG	KRYEAM	NELDTHID	
<i>L. lactis</i>	671	-----	DEF	EVIESPEQ	IAKTINVS	SKVT--	SKV	KAQL	DLRG	KRYEAM	LELDNYID
<i>B. halodurans</i>	675	-----	MKE	ASDIQLID	POPVET	PIATIR	SDHHV	KPEL	DLRG	KRYEAM	LVKVEYID
<i>S. aureus</i>	674	-----	MKL	PIEDLEKKQ	KBKVP	PTKMTV	EQ--NR	QTIK	TBLD	LRG	KRYEAM
Smr consensus											

FIG. 3. Primary sequence of the Smr-homologous repressor of phage ϕ FSW (ϕ FSWR) and multiple alignment of the sequence shared by the repressor and the carboxy-terminal domain (Smr domain) of the MutS-like or MutS2 proteins from *Streptococcus pyogenes* (AE006610; 776 aa), *Streptococcus pneumoniae* (AE007352; 778 aa), *L. lactis* (AE006395; 776 aa), *Bacillus halodurans* (AP001517; 785 aa), *Staphylococcus aureus* (AP003132; 782 aa), and the Smr-like protein of *Listeria monocytogenes* (AJ133006; 131 aa). Given in parentheses are the NCBI accession number and the length of the corresponding proteins in number of amino acids. The Smr consensus sequence is shown at the bottom. The homologous regions of the protein domains were identified by BlastP 2.2.1 (1). Multiple alignments were done with CLUSTALW (26) and created using BOXSHADE. Numbers at the left correspond to distances (in number of amino acids) from the N terminus of the respective proteins. Identical amino acids are highlighted in black, whereas amino acids in grey symbolize conservative exchanges.

2×10^{-18}) to the small *mutS*-related (Smr) consensus motif (15). The Smr protein in turn has significant homology to the C terminus of MutS2 proteins (Fig. 3), which are presumably involved in DNA mismatch repair (4, 15). Smr homologs have been found in a number of proteobacteria. The apparent absence of Smr proteins in all species that have *mutS2* genes and their presence in species that have *mutS1*-homologous genes have led to the speculation that the Smr protein may play a role in mismatch repair through an interaction with MutS1 (15). Malik and Henikoff (12) have suggested that the Smr domain in MutS2 proteins and Smr protein linked via MutL to MutS might act as a nicking endonuclease, thus replacing the *E. coli* MutH nuclease.

Several *E. coli* phage λ -homologous repressors have been identified from temperate phage of lactobacilli (8, 10) and *L. lactis* (16). In general, these repressor proteins undergo self-cleavage upon DNA damage via a RecA-mediated pathway, which results in prophage induction (11). In contrast, the ϕ FSW prophage was not inducible by either UV irradiation or mitomycin treatment (22), underlining that the prophage status of ϕ FSW-TI is not controlled by a canonical repressor. To our knowledge this is the first time that a repressor activity has been attributed to an Smr-homologous protein. Since the Smr protein and/or domain has apparent DNA binding activity, it is conceivable that the phage has acquired from its host the *smr* gene, which may then have gained repressor function. It remains to be seen whether the isolated ϕ FSW-TI repressor represents the founding member of a new family of repressors containing a region of homology to Smr proteins.

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