JOURNAL OF BACTERIOLOGY, Dec. 1999, p. 7430–7438 Vol. 181, No. 24
0021-9193/99/$04.00+0

The Genetic Switch Regulating Activity of Early Promoters of the Temperate Lactococcal Bacteriophage TP901-1

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Received 7 June 1999/Accepted 17 September 1999

A functional analysis of open reading frame 4 (ORF4) and ORF5 from the temperate lactococcal phage TP901-1 was performed by mutant and deletion analysis combined with transcriptional studies of the early phage promoters p_R and p_L. ORF4 (180 amino acids) was identified as a phage repressor necessary for repression of both promoters. Furthermore, the presence of ORF4 confers immunity of the host strain to TP901-1. ORF5 (72 amino acids) was found to be able to inhibit repression of the lytic promoter p_L by ORF4. Upon transformation with a plasmid containing both ORF4 and ORF5 and their cognate promoters, clonal variation is observed: in each transformant, either p_L is open and p_R is closed or vice versa. The repression is still dependent on ORF4, and the presence of ORF5 is needed for the clonal variation. Induction of a repressed p_L fusion containing *orf4* and *orf5* was obtained by addition of mitomycin C, and the induction was also shown to be dependent on the presence of the RecA protein, even though ORF4 does not contain a recognizable autocleavage site. Our results suggest that the relative amounts of the two proteins ORF4 and ORF5 determine the decision between lytic or lysogenic life cycle after phage infection and that a protein complex consisting of ORF4 and ORF5 may constitute a new type of genetic switch in bacteriophages.

A temperate bacteriophage has a choice between two very different life cycles after infection of a sensitive host bacterium: it can enter either a lytic cycle or a silent state, transforming the host into a lysogenic bacterium. During the lytic cycle, phage genes are expressed in a temporal manner leading to production of new phages and cell lysis. In a lysogenic bacterium, the genome of the phage is present but only very few genes are expressed. The almost silent phage DNA is transferred to new daughter cells whether it is integrated in the genome, which seems to be the prevailing mechanism, or is present in the form of a plasmid, thus maintaining the lysogenic state of the host. The existence of these two life cycles argues for the presence of tightly regulated promoters and their cognate regulatory proteins in the phage genome. The paradigm for the early expressed promoters is represented by the p_L and the p_R promoters of bacteriophage λ from *Escherichia coli*. They are recognized by the host polymerase, and repression is established through the cI repressor protein, while the Cro protein, by competing for binding to the same operator sites as cI, first represses synthesis of cI from the p_{RM} promoter and later in infection also represses transcription from the p_R and the p_L promoters. The *cro* gene is the first gene transcribed from the λ p_R promoter, while the adjacent *c*I gene is divergently transcribed from the p_{RM} promoter (for a review, see reference 11).

TP901-1 is a temperate lactococcal phage belonging to the P335 group of phages infecting lactic acid bacteria, including lytic phage species (1). Analysis of the temporal transcriptional pattern during the lytic cycle resulted in localization of consecutive regions of early, middle, and late expressed genes on the phage genome (19). The early region was found to cover 13 kb of the 38.4-kb phage genome, and 6.4 kb of this region was sequenced and found to encode 11 ORFs as well as the phage attachment site *attP* (5, 19). The region was found to be divergently transcribed in accordance with the orientation of the ORFs, and two consensus promoters (p_L and p_R) were proposed from the sequence data. The start site for initiation of transcription for both promoters was identified by primer extension (19). The p_R promoter is oriented facing *attP*, and four ORFs were identified as being transcribed from p_R, the last of which (*orf1*) encodes the phage integrase (5). The lytic promoter p_L is located upstream of p_R, directing transcription in the opposite direction; the longest mRNA produced from this promoter reaches a size of 10 kb. The sequenced region covers ORF5 to ORF11 and part of ORF12 of the promoter-distal part of this mRNA. In this report, we present experimental evidence for the presence and regulation of these promoters obtained from promoter fusions and primer extension studies. The reported data identify ORF4 as a repressor protein for both promoters and furthermore show that ORF4 is sufficient for achievement of phage immunity. Finally, the data demonstrate that both ORF4 and ORF5 together with the divergent promoter region can function as a genetic switch, resulting in either repression or derepression of the lytic p_L promoter.

MATERIALS AND METHODS

DNA technology. Recombinant plasmid DNA from *E. coli* was isolated by the alkaline lysis technique. Plasmids were isolated from *Lactococcus lactis* subsp. *cremoris* by the alkaline lysis technique after incubation with lysozyme (20 mg/ml) in 20 min at 37°C. Preparative portions were further purified by passage through Qiagen columns as recommended by the supplier (Qiagen, Hilden, Germany). Pharmacia Biotech supplied restriction endonuclease enzymes, DNA polymerase Klenow fragment, T4 DNA ligase, and buffer systems. All enzymes were used as recommended by the supplier. PCRs were performed in a DNA thermal cycler (Perkin-Elmer Cetus) using Amplitaq polymerase and buffer supplied by Perkin-Elmer. DNA sequencing was performed by the method of Sanger and coworkers (24) as instructed by the protocol for the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio).

Construction of plasmids. The plasmids used in this study are listed in Table 1. Plasmid pPM92 was constructed by cloning a 2,787-bp *Xho*I fragment of pG5f4 (4) into the *Xho*I site of the *E. coli* vector pIC19R (20). This plasmid contains *orf4* to *orf9* of TP901-1. The following plasmids were constructed by cloning PCR products obtained by using pPM92 as a template, a vector primer (1211; see below), and primers located either in the region between *orf4* and *orf5* (24BamHI, P5A, and erm2ABamHI), within *orf5* (pextPstI), or downstream of *orf5* (orf4orf5PstI and porf7PstI). All clonings were performed in *L. lactis* subsp.

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TABLE 1. Plasmids used in this work

Plasmid	Description[a]	Promoter fusion	Relevant genotype	Reference
pIC19R				20
pCI372				10
pCI3340				10
pG5f4	pGEM5::*Eco*RV4 of TP901-1		*orf2–orf10*[b]	4
pAK80			*lacLM*	13
pNZ8010		p_{nisA}-*gusA*		7
pPM92	pIC19R::2787 bp *Xho*I pG5f4		*orf4–orf9*[b]	This work
pPM126	pAK80::*Pst*I PCR (2622–3458)	p_L-*lacLM*	*orf4*[b]	This work
pPM127	pAK80::*Hin*dIII PCR (2622–3900)	p_L-*lacLM*	*orf4 orf5*[b]	This work
pPM131	pAK80::*Hin*dIII PCR (2622–3900)	p_L-*lacLM*	*orf5*[b]	This work
pPM132	pAK80::*Hin*dIII PCR (2622–3900)	p_R-*lacLM*	*orf4 orf5*[b]	This work
pPM136	pAK80::*Pst*I PCR (2622–3458)	p_R-*lacLM*		This work
pPM137	pAK80::*Pst*I PCR (2622–3458)	p_R-*lacLM*	*orf4*[b]	This work
pPM138	pAK80::*Pst*I PCR (2622–3458)	p_L-*lacLM*		This work
pPM139	pAK80::*Hin*dIII PCR (2622–3900)	p_R-*lacLM*	*orf5*[b]	This work
pAB223	pCI3340::*Xho*I-*Hin*dIII (2621–3900)		*orf4 orf5*[b]	2
pAJ80	pCI372::*Bam*HI PCR (2622–3332)		*orf4*[b]	This work
pAJ93	pAK80::*Bam*HI PCR (3221–3332)	p_L-*lacLM*[c]		This work
pAJ94	pAK80::*Bam*HI PCR (3221–3332)	p_R-*lacLM*[c]		This work
pAJ98	pNZ8010::*Pst*I PCR (2622–3900)		*orf4 orf5*[b]	This work
pAJ115	pNZ8010::*Bam*HI *Pst*I PCR (2622–3370)	p_{nisA}-p_R-*orf4 gusA*	*orf4*[b]	This work
pAJ142	pNZ8010::*Bam*HI *Pst*I PCR (3338–3574)	p_{nisA}-*orf5 gusA*	*orf5*[b]	This work

[a] Coordinates in parentheses refer to the sequence Y14232.
[b] Open reading frame(s) of TP901-1 present.
[c] The −10 region of the p_R promoter is disrupted.

cremoris MG1363. Plasmids pAJ93 and pAJ94 were constructed by cloning a *Bam*HI-digested PCR product (primers 24BamHI and erm2ABamHI) into pAK80 (13) also digested with *Bam*HI. The resulting plasmids contain p_L (pAJ93) and p_R (pAJ94) fused to the *lacLM* genes. PCR products obtained by using primer 1211 and pextPstI were cloned into pAK80 after digestion with *Pst*I, giving rise to plasmids pPM126 and pPM137 carrying *orf4* as well as p_L and p_R fused to the *lacLM* genes, respectively. Plasmids pPM127 and pPM132 were constructed by cloning a *Hin*dIII-digested PCR product (primers 1211 and porf7PstI) into pAK80. The plasmids thus contain *orf4* and *orf5* as well as p_L (pPM127) or p_R (pPM132) fused to the *lacLM* genes. A frameshift mutation was introduced in the beginning of the *orf4* gene by filling in the *Spe*I site of pPM92 with Klenow polymerase. After ligation and redigestion with *Spe*I, two PCRs were performed on the ligation mixture. In the first PCR, primers 1211 and pextPstI were used, and a PCR product containing the mutated *orf4* gene was cloned into pAK80 after digestion with *Pst*I, giving rise to plasmids pPM138 and pPM136, carrying only the mutated *orf4* gene as well as p_L and p_R fused to the *lacLM* genes, respectively. In the second PCR, primers 1211 and orf7PstI were used. After digestion with *Hin*dIII, a PCR product containing *orf5* in addition to the mutated *orf4* was cloned in pAK80, and plasmids pPM131 and pPM139 were constructed. Plasmids pPM131 and pPM139 thus contain p_L and p_R, respectively, fused to the *lacLM* genes. In all cases, several independent clones giving rise to the same β-galactosidase specific activity were isolated. Furthermore, the inserts of plasmid pAJ93, pAJ94, pPM127, pPM126, and pPM132 were sequenced, as well as the mutation within the *orf4* gene in plasmids pPM131, pPM136, pPM138, and pPM139. The PCR product generated by use of primer 1211 and erm2ABamHI was digested with *Bam*HI and cloned into pCI372 (10) also digested with *Bam*HI, resulting in plasmid pAJ80, carrying p_L, p_R, and *orf4*. The PCR product produced by using primer 1211 and P5A was cloned into pNZ8010 (7) after digestion with *Bam*HI, giving rise to pAJ115 containing *orf4* and p_L fused to the *gusA* gene. PCR products produced by using primer 1211 and orf4orf5PstI or Orf5A and Orf5B were cloned into pNZ8010 (7) after digestion with *Pst*I and *Bam*HI-*Pst*I, respectively. Thereby plasmids pAJ98 and pAJ142 were constructed so as to contain *orf4* and *orf5* transcribed from p_R and p_L on a high-copy-number plasmid and *orf5* under the control of the nisin promoter, respectively. The inserts of pAJ80, pAJ98, pAJ115, and pAJ142 were sequenced.

Primers used in this study. For construction of plasmids, the following primers were used: 1211 (5′-GTAAAACGACGGCCAGT-3′), 24BamHI (5′-GCGCGGATCCTATAGCGCATCTTGAAC-3′), P5A (5′-GCGCGGATCCTAATCATAACTCATTTATGT-3′), erm2ABamHI (5′-GCGCGGATCCCACGTTTCATGAACTTT-3′), orf4orf5PstI (5′-GCGCCTGCAGCCCAAGCTTCATCAGTTC-3′), Orf5A (5′-GCGCGGATCCAAGAAAGGAGAAACATAAAT-3′), Orf5B (5′-GCGCCTGCAGTTAATGAACTTTTGCATTAA-3′), pextPstI (5′-AAAACTGCAGAGACACAGTTCTCTCTG-3′), and porf7PstI (5′-AAAAAACTGCAGATCCCCCATGTGCTTTCC-3′). For primer extensions, primer PE (5′-AGACACAGTTCTCTCTGAAAGCCCC-3′) was used for mapping of the p_L promoter, while primer 23 (5′-GGTCAGGAGATTGAACG-3′) was used for p_R.

Transformation and selection in *E. coli* and *L. lactis* subsp. *cremoris*. *E. coli* XL1-Blue was made competent with $CaCl_2$ and was transformed as described by Sambrook et al. (23). Selection was performed with 100 μg of ampicillin per ml. *L. lactis* subsp. *cremoris* MG1363 was transformed by electroporation according to the method described by Holo and Nes (12), using 0.03 to 0.5 μg of DNA per electroporation. When *L. lactis* subsp. *cremoris* 3107 was made competent, the growth medium (12) contained only 0.2 M sucrose, and 25 μg DNA was used per transformation. Transformants were selected on plates containing 5 μg of erythromycin per ml. Screening on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates was performed at a concentration of 90 μg/ml.

Bacteria and phages. Bacterial strains used in this study are listed in Table 2. The temperate bacteriophage TP901-1 was induced from *L. lactis* subsp. *cremoris* 901-1 (1) by the use of UV light as previously described (4).

Cell growth and enzyme assay. *E. coli* strains were grown with agitation at 37°C in Luria-Bertani broth (23) (Difco Laboratories, Detroit, Michigan). Bacto Agar (Difco Laboratories) was used at 1.5% (wt/vol) in solid media. *L. lactis* strains were propagated at 30°C without shaking in GM17 (M17 broth [Oxoid Limited, Basingstoke, Hampshire, United Kingdom] containing 0.5% [wt/vol] glucose) (26). For determination of β-galactosidase activity, exponential growing cells were diluted in fresh medium to an optical density at 600 nm (OD_{600}) of 0.05. At an OD_{600} of 0.8, 70 ml of culture was harvested and resuspended in 7 ml of Z-buffer, and cell extracts were obtained with a French press. The activity of β-galactosidase was determined according to Miller (21), and specific activity was calculated in Miller units.

Induction of transcription from the nisin promoter. Exponentially growing cells of strains LKH169 and LKH206 were diluted in fresh medium (GM17 containing 5 μg of erythromycin per ml and 5 μg of chloroamphenicol per ml) to an OD_{600} of 0.05. At an OD_{600} of 0.2, nisin was added to the culture to a final concentration of 1 ng/ml. Samples were withdrawn before and after addition of nisin, diluted, and plated on plates containing X-Gal but no nisin. After overnight incubation at 30°C, blue and white colonies were counted.

Induction by mitomycin C. Strains were grown in GM17 containing 5 μg of erythromycin per ml at 28°C, due to the temperature sensitivity of the *recA* strain. Exponentially growing overnight cultures were diluted to an OD_{600} of 0.05 in fresh medium, and growth was monitored. At an OD_{600} of 0.4, each culture was diluted into two separate cultures to an OD_{600} of 0.1. Subsequently, mitomycin C was added to a final concentration of 2.5 μg/ml to one of the cultures, while the other served as a control. Samples were removed at different time points, harvested, extracted, and assayed as described above for β-galactosidase activity.

Plaque assay. TP901-1 phages were diluted in A buffer (0.8% [wt/vol] NaCl, 10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 1 mM $CaCl_2$) and plated on lawns of *L. lactis* subsp. *cremoris* 3107 transformed with either pPM126, pPM138, pPM127, or pPM131. As a control, *L. lactis* subsp. *cremoris* 3107 was used as an indicator

TABLE 2. Bacterial strains used in this study

Strains	Relevant description or genotype	Source or reference
E. coli XL1-Blue		Stratagene
L. lactis subsp. *cremoris*		
3107	Indicator strain for TP901-1	1
901-1	Lysogenic for TP901-1	1
MG1363		9
NZ3900	MG1363 *pepN*::*nisRK*	6
VEL1122	MG1363 Δ*recA*::tet	8
PM75	MG1363/pPM126	This work
PM80	MG1363/pPM131	This work
PM81	MG1363/pPM132[a]	This work
PM85	MG1363/pPM136	This work
PM86	MG1363/pPM137	This work
PM87	MG1363/pPM138	This work
PM88	MG1363/pAK80	This work
PM92	MG1363/pPM139	This work
PM93	MG1363/pPM127[b]	This work
PM94	MG1363/pPM127[c]	This work
PM95	MG1363/pPM132[d]	This work
PM97	3107/pPM138	This work
PM99	3107/pPM131	This work
LKH48	3107/pPM127[c]	This work
LKH49	3107/pPM127[d]	This work
LKH51	3107/pPM126	This work
LKH68	VEL1122/pPM127[c]	This work
LKH148	NZ3900/pPM127[b]	This work
LKH149	NZ3900/pPM127[c]	This work
LKH169	NZ3900/pNZ8010 pPM127[c]	This work
LKH206	NZ3900/pAJ142 pPM127[c]	This work

[a] White strain on plates containing X-Gal (90 μg/ml) after transformation with pPM132.
[b] Very blue strain on plates containing X-Gal (90 μg/ml) after transformation with pPM127.
[c] White strain on plates containing X-Gal (90 μg/ml) after transformation with pPM127.
[d] Light blue strain on plates containing X-Gal (90 μg/ml) after transformation with pPM132.

strain. After incubation at 30°C overnight, plaques were counted. For each strain, the efficiency of plaquing (EOP) was calculated as the number of plaques formed on a lawn of the strain of interest divided by the number of plaques formed on *L. lactis* subsp. *cremoris* 3107.

Extraction of RNA. RNA was isolated from strains PM93, PM94, and PM80 growing exponentially in defined medium SA (14) (1.0% glucose [wt/vol], 5 μg of erythromycin per ml), using the method described by Johnsen et al. (15) as modified by Madsen and Hammer (19).

Primer extensions. Primers PE and 23 were phosphorylated by using T4 polynucleotide kinase (Gibco BRL) and [^{32}P]ATP (Amersham) as described by Sambrook et al. (23); 25 μg of total RNA isolated from strain PM93, PM94, or PM80 was mixed with 1 pmol of phosphorylated primer in a volume of 10 μl and incubated at 70°C for 10 min. The mixture was allowed to cool to 37°C over a 1-h period. Then 1 μl of Superscript reverse transcriptase (Gibco), 4 μl of 5× first-strand buffer (Gibco), 2 μl of 0.1 M dithiothreitol (Gibco), and dATP, dGTP, dCTP, and dTTP to a final concentration of 500 μM were added, and the mixture was incubated at 37°C for 1 h.

RESULTS

ORF4 is a negative regulator of p_L and p_R. Previously, we identified two divergently orientated promoters (p_L and p_R) located between *orf4* and *orf5* of the temperate bacteriophage TP901-1 (19). To investigate the expression and regulation of the promoters, we cloned a phage DNA fragment of 837 bp, containing both promoters and *orf4*, in both orientations in the promoter probe vector pAK80. The *lacLM* genes contained in this plasmid give rise to β-galactosidase activity when a functional promoter is inserted in the cloning region, and stop codons in all three reading frames ensure that a transcriptional fusion is constructed. When p_L (pPM126) and p_R (pPM137) are located upstream of the *lacLM* genes, low β-galactosidase activities (1.0 and 0.5 Miller units, respectively) can be measured when the plasmids are present in *L. lactic* subsp. *cremoris* MG1363 (Fig. 1). These enzyme levels are, however, significantly different from the basal level of 0.02 Miller units measured in MG1363 containing the pAK80 vector, which is in agreement with the existence of the divergently orientated promoters p_L and p_R on this phage DNA fragment. The effect of the presence of *orf4* on the activity of the promoters was investigated by introduction of an early frameshift mutation at codon position 6. The β-galactosidase activities of the resulting plasmids, carrying the p_L fusion (pPM138) and the p_R fusion (pPM136) were measured as 62 and 15 Miller units, respectively, in MG1363. These results demonstrate the presence of divergently orientated promoter activities on the cloned phage DNA fragment and clearly show that the presence of a functional ORF4 protein represses the expression of both phage promoters. The levels of repression are 62- and 31-fold for p_L and p_R, respectively (Fig. 1).

Since the spacer between the initiation sites of the p_L and p_R transcripts is only 101 bp, it is possible that binding of the RNA polymerase to one of them affects transcription of the other. To exclude this possibility, a 111-bp PCR fragment carrying the p_L promoter but only the −35 region of the p_R promoter was cloned in both orientations upstream of the *lacLM* genes in the promoter probe vector pAK80. The specific activity of β-galactosidase of *L. lactis* subsp. *cremoris* MG1363 containing either pAJ94 (p_R fusion) or pAJ93 (p_L fusion) was measured. The presence of pAJ94 (p_R fusion) gave rise to only a background level of β-galactosidase activity (0.14 Miller units), as expected due to the absence of the −10 region of the p_R promoter. MG1363 containing pAJ93 (p_L fusion), in which the p_R promoter is not active, showed the same specific β-galactosidase activity (66.5 Miller units) as when the larger p_L fusion (pPM138) carrying an active p_R promoter was present. Thus, the activity of the p_L promoter is the same regardless of whether the p_R promoter is present, showing that the activity of the p_R promoter does not affect the efficiency of the p_L promoter.

Antirepressing activity of ORF5. In bacteriophage λ, the Cro and the cI repressors compete for the λ operator sites and thereby regulate expression of the p_{RM}, p_R, and p_L promoters. To investigate the influence of both ORF4 and ORF5 on the p_L and p_R promoters of TP901-1, we cloned a 1,279-bp phage DNA fragment carrying both *orf4* and *orf5* in both orientations in pAK80, resulting in plasmids pPM127 (p_L fusion) and pPM132 (p_R fusion). The transformants arising from both plasmids exhibited clonal variation when screened on GM17 plates containing X-Gal. Of 200 transformants arising from pPM127 (p_L fusion), strong blue colonies were observed in 191 cases and the remaining 9 colonies were white, giving a frequency of 4.5% of white colonies. This frequency is comparable to the frequency of lysogenization of approximately 1 to 3% by TP901-1 (3). When plasmid pPM132 (p_R fusion) was used, the same type of clonal variation was observed but with opposite frequencies: 95% white colonies and only 5% light blue transformants were observed. The phenotype was stable for both plasmids when restreaked to single colonies more than three consecutive times and also after freezing and restreaking. When plasmid DNA was isolated from either blue or white transformants and used for retransformation of MG1363, segregation into white and blue transformants was observed again with the same frequencies as seen before. The phenotype of a transformant was thus independent of the phenotype of the strain from which the plasmid was prepared.

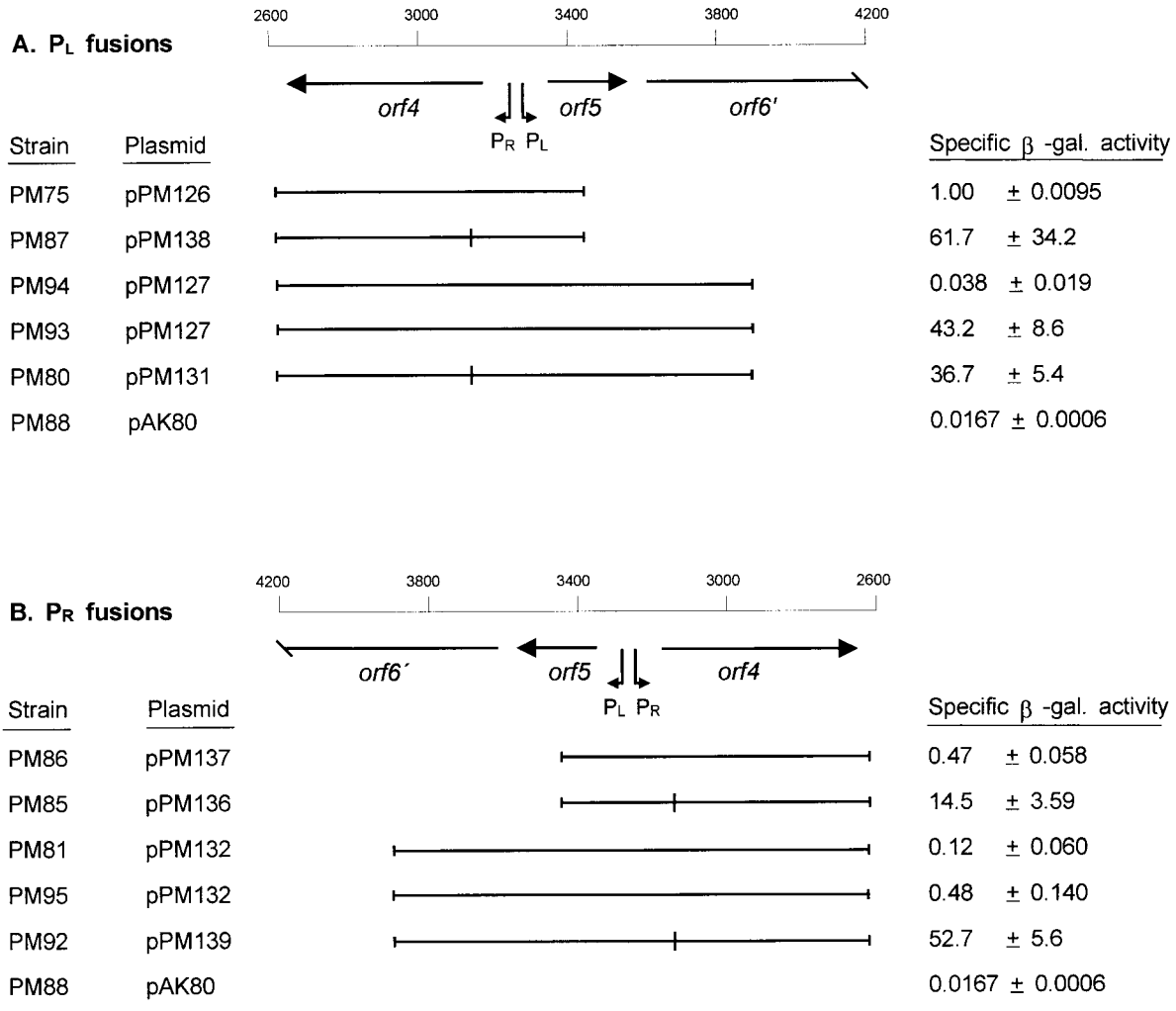

FIG. 1. Effects of *orf4* and *orf5* on expression from p_L (A) and p_R (B). Fragments cloned upstream of the *lacLM* genes of the pAK80 vector are indicated by lines. The *lacLM* genes are located downstream of p_L (A) or p_R (B). Small vertical lines indicate a frameshift mutation in *orf4*. Plasmid numbers and specific activities of β-galactosidase (β-gal) of the corresponding strains are indicated. For details of cell growth and the β-galactosidase assay, see Materials and Methods. For PM80 and PM95, only two independent experiments were performed; the remaining measurements are averages of three to five experiments.

In light of the observed variation of phenotype when both *orf4* and *orf5* were present, we repeated the transformation of MG1363 with plasmids pPM126 (p_L fusion) and pPM137 (p_R fusion), containing only *orf4*. Screening of the transformants on 90 μg of X-Gal per ml made it possible to visualize the difference in enzyme levels of 0.5, 1.0, and 62 Miller units as light blue, blue, and very blue colonies, respectively, all being distinctively different from the white colonies. Again 200 transformants containing either pPM126 (p_L fusion) or pPM137 (p_R fusion) were obtained and all were either light blue (pPM137) or blue (pPM126), corresponding to the measured enzyme levels of 0.5 and 1.0 Miller units (Fig. 1). Clonal variation was therefore not observed in the presence of only ORF4, suggesting that ORF5 is necessary for this phenomenon.

To assess the effect of the repression mediated by ORF4 in the presence of ORF5, *orf4* mutants were constructed in both plasmids by filling in the *Spe*I site in the N-terminal region of *orf4*. β-Galactosidase activities of the transformants containing the clonal variants of pPM127 (p_L fusion) and of the clonal variants of pPM132 (p_R fusion) as well as the plasmids carrying the *orf4* mutation were measured (Fig. 1). First, let us consider expression from the lytic promoter p_L. In the presence of *orf4* and *orf5*, the expression from p_L observed in PM94 is about 1,000-fold repressed compared to the activity found in the derepressed state (PM93) (Fig. 1). Furthermore, the p_L promoter activity in PM93 (containing *orf4* and *orf5*) is as high as in the *orf4* mutant (PM80); thus, PM93 is phenotypically an *orf4* mutant (Fig. 1). Expression from p_R is also subject to clonal variation. However, in the presence of both *orf4* and *orf5*, the difference between the repressed state of the p_R promoter in strain PM81 and the derepressed state in strain PM95 is only fourfold. When a mutation was introduced into *orf4*, the expression from p_R becomes even more derepressed, 439-fold compared to the expression found in strain PM81 (Fig. 1). Thus, also in the presence of ORF5, ORF4 is needed for repression of both p_R and p_L.

ORF4 and ORF5 are *trans* acting. The effect of ORF4 and ORF5 donated from a plasmid in *trans* to the promoter fusions contained on the compatible pAK80 plasmid was investigated. As observed in Table 3, the presence of *orf4* on the donor plasmid results in repression of both a p_L fusion (pPM138) and a p_R fusion (pPM136) in the recipient strain, whereas the

TABLE 3. Effect of donation of *orf4* and/or *orf5* in *trans* to promoter fusion plasmids measured as the color of transformants on X-Gal plates

Recipient strain			Color of transformants[a]			
Strain	Plasmid	Genotype	pCI372[b]	pAJ80 (low copy no.; *orf4*)	pAJ115 (high copy no.; *orf4*)	pAB223 (low copy no.; *orf4 orf5*)
PM85	pPM136	p_R-*lacLM*	B	W	ND	94% W, 6% B
PM87	pPM138	p_L-*lacLM*	B	W	ND	1.2% W, 98.8% B
LKH148	pPM127	*orf4 orf5* p_L-*lacLM*[c]	B	B	92% W, 8% B	ND
LKH149	pPM127	*orf4 orf5* p_L-*lacLM*[d]	W	ND	ND	W[e]

[a] B, blue colonies on plates containing X-Gal (90 μg/ml) after transformation with the indicated donor plasmid; W, white colonies on plates containing X-Gal (90 μg/ml) after transformation with the indicated donor plasmid; ND, not determined.
[b] Donor plasmid for transformation.
[c] Very blue strain on plates containing X-Gal (90 μg/ml) after transformation with pPM127.
[d] White strain on plates containing X-Gal (90 μg/ml) after transformation with pPM127.
[e] Transformants were also white when the high-copy-number plasmid pAJ98 (*orf4 orf5*) was used as the donor plasmid.

vector plasmid itself (pCI372) does not change the constitutive expression monitored as blue colonies. Thus, ORF4 is able to repress both p_L and p_R when given in *trans*. Subsequently, the effect of donation of a plasmid containing both *orf4* and *orf5* (pAB223) to either the p_R or the p_L fusion strain was investigated. The result clearly showed clonal variation, with the majority of the p_L fusions turning blue and the majority of the p_R fusions turning white. Thus, the same effect of ORF4 and ORF5 was observed whether *orf4* and *orf5* were donated in *trans* or were located on the same plasmid as the promoters (pPM127 and pPM132). Regulation of p_L and p_R thus seems to be dependent on ORF4 and ORF5, both being able to function in *trans*.

The state of p_L can be changed. The clonal variation mediated by the presence of both ORF4 and ORF5 was found to be stably inherited. Particularly, expression from p_L is affected by the choice of configuration, since an approximately 1,000-fold difference in the specific activities of β-galactosidase of strains PM93 (p_L open) and PM94 (p_L closed) was found (Fig. 1). The ratio between the amount of ORF4 and ORF5 could determine the choice of configuration of p_L, and we therefore examined whether the state (repressed or open) of the p_L promoter could be changed by introduction of additional amounts of either ORF4 or ORF5. For this purpose, strain LKH148 carrying pPM127 (*orf4 orf5*), in which p_L is open, was transformed with plasmids expressing *orf4* from the p_R promoter, and the color of the transformants was observed on plates containing X-Gal. In the presence of *orf4* on a low-copy-number plasmid (pAJ80), only blue transformants were seen, whereas 92% white and 8% blue colonies were found when *orf4* was present on a high-copy-number plasmid (pAJ115) (Table 3). It is thus possible to change an open state of the p_L promoter to a repressed state by introduction of a sufficient amount of additional ORF4.

In the situation where p_L is closed (strain LKH149 carrying pPM127 [*orf4 orf5*]) we investigated the effect of increasing the expression of *orf5* first by introducing a plasmid containing both *orf5* and *orf4* transcribed from p_L and p_R, respectively (pAB223). However, this did not change the repressed state of the p_L promoter located on pPM127 in LKH149, even when a high-copy-number plasmid was used (Table 3). This was expected since a surplus of ORF4 is able to shut down expression of the p_L promoter and *orf5* expression is dependent on p_L in pAB223. To be able to produce a surplus of ORF5, a plasmid containing *orf5* under the control of the inducible promoter p_{nisA} was therefore constructed (pAJ142). Addition of the inducer nisin will lead to a rapid increase in *orf5* expression. For this experiment we used strain LKH206, which contains pAJ142 in addition to the p_L fusion on pPM127 (*orf4 orf5*). The advantage of this experimental setup is that the influence of a short induction period with nisin in the liquid culture on the final choice of the phenotype of a colony originating from a cell diluted from this culture and plated without nisin can be tested. Thus, after dilution, the color of LKH206 was examined on plates containing X-Gal but no nisin before and after nisin was added to the culture (Fig. 2). Before addition of nisin, 4 to 6% blue colonies were seen, probably due to a low frequency of initiation of transcription of the nisin promoter also in the absence of nisin. After 5 min of growth in the presence of nisin, 51% of the colonies were blue if samples were removed and plated. This frequency of blue colonies was increased further to 84% after 10 min, and after 40 min all colonies were blue. The phenotype of the blue colonies obtained in this experiment was stable after restreaking to single colonies. This shows that a repressed state of p_L can be changed into an open state if the amount of ORF5 is increased only momentarily (10 min)

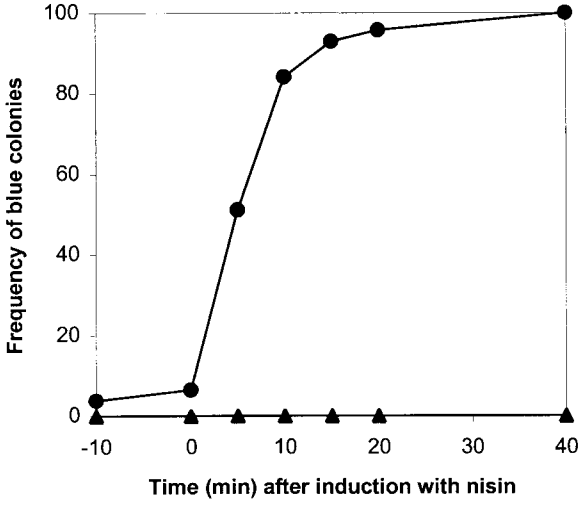

FIG. 2. Change of the p_L promoter from the closed to the open configuration by a burst of induction of synthesis of ORF5 from the nisin promoter. Strains LKH169 and LKH206 both contain pPM127 carrying the p_L promoter in the closed configuration. At time zero, nisin (1 ng/ml) was added to exponentially growing cultures. At the indicated times, samples were withdrawn, diluted, and plated on GM17 plates containing X-Gal. After incubation at 30°C, the number and color of the colonies were determined and the frequency of blue colonies was calculated. Circles, strain LKH206 containing pPM127 and pAJ142 (*orf5* under the control of the nisin promoter); triangles, strain LKH169 containing pPM127 and pNZ8010 (the vector of pAJ142).

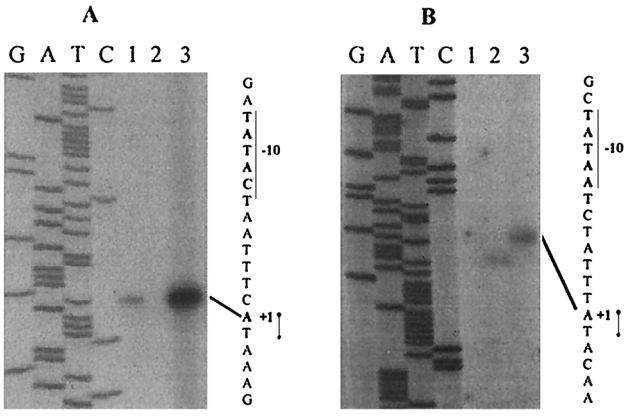

FIG. 3. Primer extension analysis of regulation of p_L and p_R. Total RNA used in the analysis was isolated from the following strains, all derivatives of MG1363: lanes 1, PM93 containing pPM127 (p_L open); lanes 2, PM94 containing pPM127 (p_L closed); lanes 3, PM80 containing pPM131 (p_L and p_R open). Lanes G, A, T, and C contain the sequence reactions performed with the primers used for the analysis. (A) Mapping of the p_L promoter; (B) mapping of the p_R promoter. To the right of each lane, the sense strand sequence is given; the −10 and +1 positions of the promoters are also indicated.

and that once established, this configuration can be maintained without further nisin-induced ORF5 production.

Next we wanted to show that overproduction of ORF5 is also able to inhibit the ORF4-mediated repression of the p_L promoter in the short fusion pPM126, which contains only 145 bp of the p_L transcript, in contrast to the 587 bp in pPM127. Strains containing pPM126 (*orf4* p_L fusion) and pAJ142 (carrying *orf5* under the control of the p_{nisA} promoter) or pNZ8010 (vector of pAJ142) were grown in the presence of nisin, and the specific activity of β-galactosidase was measured. An 11-fold increase in expression from p_L in the presence of expressed *orf5* was found. Thus, ORF5 is also able to inhibit the ORF4-mediated repression of the p_L promoter in the short fusion contained in pPM126.

Study of the regulation of promoters by primer extension. In a previous study, locations of the divergent promoters p_L and p_R in the region between the divergent reading frames in the early expressed region of TP901-1 were determined by primer extension (19). The results from the transcriptional fusions presented in the present report have also demonstrated the presence as well as the regulation of divergent promoter activity. By primer extension analysis, we were furthermore able to simultaneously monitor expression from the p_R and p_L promoters in the same strain. Therefore, we decided to map the transcriptional start sites from both promoters by primer extension using mRNA prepared from three strains: PM93 and PM94, both containing pPM127 but carrying the p_L promoter in the derepressed state and the repressed state, respectively, as well as PM80, containing the corresponding plasmid with a mutation in *orf4* (pPM131). In PM93 (*orf4* and *orf5* present), expression from the p_L promoter is high, as shown by the β-galactosidase measurements. The mRNA prepared from this strain shows an mRNA start site from the p_L promoter but none from the p_R promoter (Fig. 3, lanes 1). On the other hand, when the p_L promoter is in the repressed state as in the clonal variant PM94 containing the same plasmid (pPM127) as PM93, transcription from p_R but not from p_L is observed (lanes 2). For comparison, we investigated the primer extension results from the strain containing the *orf4* mutant plasmid, pPM131. Since the promoter fusions showed that ORF4 represses transcription from both promoters, we would expect to find transcriptional start sites from both promoters in this strain. As shown in lanes 3, this was also observed. The transcripts from p_R are 4 bp longer due to filling in of the *Spe*I site, which is located between the primer used and the promoter. Therefore, the same transcriptional start site is used for expression from p_R in strains PM94 and PM80, just as in the case of p_L expression in both PM93 and PM80. The results for strains containing pPM127 (*orf4* and *orf5* present) demonstrate that the two different phenotypes which can be obtained with this plasmid result from a strain where p_L is open and p_R is simultaneously closed, and vice versa: a strain where p_L is closed and p_R is open. However, as judged from the enzyme measurements in Fig. 1, p_R is only partially derepressed in this situation.

Effect of ORF4 and ORF5 on phage immunity. The effect of ORF4 and ORF5 on the plating capacity of phage TP901-1 was investigated after transformation of the relevant plasmids into the phage indicator strain *L. lactis* subsp. *cremoris* 3107. We tested the presence of ORF4 alone by using pPM126 (p_L fusion) and, as a control, the corresponding *orf4* mutant plasmid pPM138. A strain containing *orf4* (LKH51) showed full resistance to TP901-1 and an EOP of less than 10^{-6}, compared to the EOP of 1 for both the *orf4* mutant strain (PM97) and the indicator strain 3107 (Fig. 4). The effect of the presence of both *orf4* and *orf5* was tested with pPM127 (p_L fusion). Use of this plasmid also gave rise to clonal variation in the indicator strain 3107. LKH48 and LKH49 are representatives of clones containing repressed and derepressed p_L promoters, respectively. No plaques were detected with LKH48, while a few pinpoint plaques were obtained with LKH49. For comparison, we also used PM99 containing the *orf4* mutant plasmid pPM131, which should produce ORF5 from p_L since *orf5* is located downstream of p_L, which in this strain has a high activity (plasmid pPM131 in strain PM80 [Fig. 1]). As reported in Fig. 4, the presence of this plasmid is inhibitory to the plaque-forming capacity of TP901-1, giving an EOP of 10^{-4}. This is only about fivefold higher than the EOP on LKH49, indicating that the inhibitory effect in LKH49 is mainly due to the production of ORF5.

Induction by mitomycin C is dependent on RecA and ORF5. For bacteriophage λ, it is known that during the maintenance of lysogeny the cI repressor is bound to the p_R promoter, preventing transcription of the genes involved in lytic development. In the presence of UV light or mitomycin C, which both induce the SOS response, the cI repressor is inactivated by autocleavage induced by the RecA protein (17). To investigate whether transcription can be induced from the clone containing the lytic p_L promoter of TP901-1 by treatment with mitomycin C, β-galactosidase activity was monitored after addition of mitomycin C to strain PM94, carrying both *orf4* and *orf5*. Strain PM94 is the clonal variant carrying the p_L promoter in the repressed state. As seen in Fig. 5, the β-galactosidase activity was induced 28-fold by mitomycin C. Furthermore, mitomycin C induction of the p_L promoter fusion was found to be dependent on a functional *recA* allele, since no induction was seen in a *recA* mutant (LKH68 in Fig. 5). However when PM75, the *recA*$^+$ strain containing the p_L fusion plasmid pPM126 with only *orf4*, was exposed to mitomycin C, no induction of β-galactosidase activity was observed (data not shown).

DISCUSSION

By primer extension studies and use of transcriptional fusions, the early transcribed region of phage TP901-1 has been shown to contain two divergently orientated promoters. The

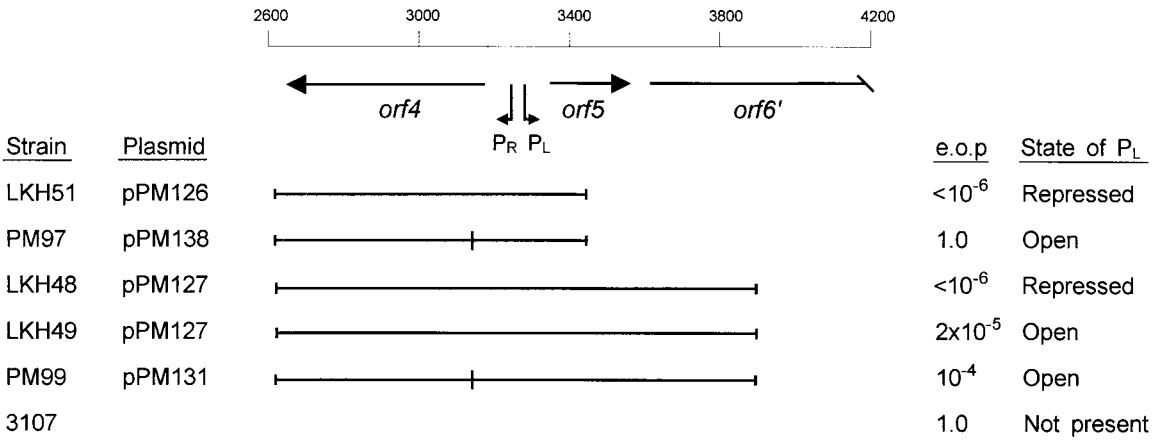

FIG. 4. Effect of *orf4* and *orf5* on phage TP901-1 immunity. The lines indicate fragments cloned upstream of the *lacLM* genes of the pAK80 vector. In all cases, the *lacLM* genes are located downstream of the p_L promoter, and the state of p_L is indicated as either repressed or open, as judged from the color of the strains on plates containing X-Gal. A small horizontal line indicates a frameshift mutation in *orf4*. For each strain, the transformed pAK80 plasmid derivative and the EOP are noted. For details, see Materials and Methods.

untranscribed DNA between the mRNA start sites located at positions 3212 and 3314 has a length of 101 bp. Both promoters have close to consensus sequences with 17 bp between the −10 and −35 regions; furthermore, p_L has the TG motif 1 bp upstream of the −10 region. By deletion of the −10 region of the p_R, promoter it was shown that the efficiency of p_L is independent of the activity of p_R. Furthermore, it was shown that both promoters are active in the absence of expressed phage TP901-1-encoded genes.

In this study, we investigated the functions of ORF4 and ORF5 that are located topologically equivalent to the cI and Cro proteins of phage λ. The transcriptional fusions demonstrated that ORF4 is necessary for negative regulation of both p_R and p_L, and the primer extensions (Fig. 3) strongly indicate that the negative effect of ORF4 is at the level of transcription initiation. As shown by the phenotype of the strains containing plasmids pPM126 and pPM137, ORF4 alone is furthermore sufficient for repression of both promoters (Fig. 1). This repression is also seen when ORF4 is donated in *trans* to the promoter fusions (Table 3). The role of ORF4 as the putative phage repressor was further substantiated by proving that the *orf4*-containing plasmids conferred immunity to TP901-1 when transferred to the phage indicator strain 3107, while introduction of a frameshift mutation in *orf4* resulted in abolishment of the immunity of strain 3107 (Fig. 4). The experiments furthermore showed that large amounts of ORF5 are inhibitory to formation of plaques.

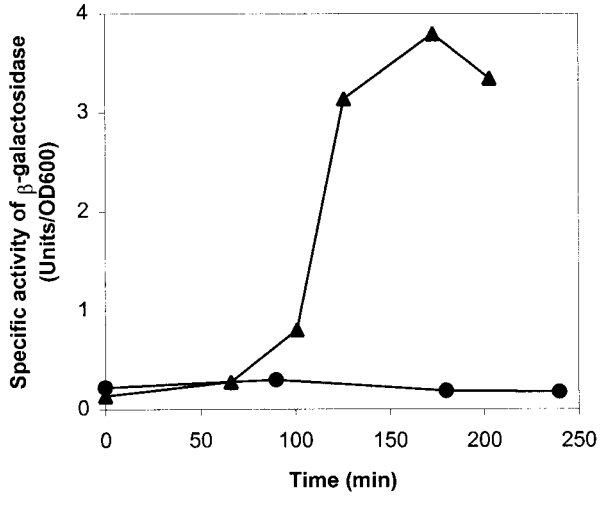

FIG. 5. Induction of p_L by mitomycin C in strains containing the p_L promoter fused to the *lacLM* genes of pAK80. At time 5 min, mitomycin C (2.5 μg/ml) was added. Triangles, strain PM94 containing pPM127 (*orf4 orf5* with repressed p_L); circles, strain LKH68 *recA* derivative containing pPM127 (*orf4 orf5* with repressed p_L).

We investigated the effect of the presence of both ORF4 and ORF5 on p_L and p_R promoter activities by analysis of plasmids pPM127 and pPM132 and found that both plasmids gave rise to clonal variation among the transformants. The presence of ORF5 was found to be necessary for the observed clonal variation, but ORF4 was still responsible for repression of the promoters. It was demonstrated that the p_L promoter was either open or closed in the obtained transformants and that the clonal variation was stably inherited. However, an open configuration of p_L could be changed to the closed configuration by introduction of additional amounts of ORF4 donated from a high-copy-number plasmid, while no change was seen when ORF4 was derived from a plasmid with moderate copy number (Table 3). Furthermore, a burst of ORF5 production from the nisin promoter was sufficient to alter the configuration of the p_L promoter from closed to open, and the phenotype was stably inherited when the cells were plated without nisin. These results show that the amount of the regulatory proteins ORF4 and ORF5 as well as the ratio determines the choice of the open or repressed state of the p_L promoter. We have also shown that ORF5 is able to inhibit the ORF4-mediated repression of p_L in the short fusion containing only 145 bp of the p_L transcript.

By primer extension analysis, we found that when p_L is open p_R is totally repressed, while p_R transcripts can be found when p_L is closed. However, the enzyme levels of the transcriptional p_R fusions demonstrated that p_R is only partially derepressed under these conditions, 0.5 Miller units in PM95 compared to 0.1 in PM81 (Fig. 1).

When the p_L promoter is open (43 Miller units in PM93), this results in a large production of p_L mRNA encoding ORF5 and therefore presumably rather high levels of ORF5 in the cell (Fig. 1). In these conditions p_R is repressed (0.1 Miller

units), but some ORF4 must be produced since ORF4 is necessary for the repression of p_R, as verified by the *orf4* mutation in strain PM92 (Fig. 1). The presence of the high level of ORF5 must somehow preferentially interfere with the binding of ORF4 to the p_L promoter but not with the binding of ORF4 to the p_R promoter. Thus, initiation of transcription from p_R is repressed by ORF4, whereas the presence of large amounts of ORF5 prevents repression of p_L by ORF4. This resembles the commitment to lytic growth where the p_L transcripts are needed (19).

In the situation where p_L is repressed, stimulating the lysogenic state, activity from the p_L promoter in PM94 is only 0.04 Miller units, and therefore only small amounts of ORF5 could be produced in these cells (Fig. 1). The p_R fusion in PM95 shows activity of 0.5 Miller units; thus, considerably more ORF4 is produced than when p_L is open. When both ORF4 and ORF5 are present in the cell, p_L is repressed 25-fold more than in the situation where only ORF4 is present, as seen by comparison of PM94 (0.04 Miller units) with PM75 (1.0 Miller units) in Fig. 1. This does not seem to be due to different levels of ORF4 produced in these cells since the output from p_R is the same under both conditions (compare PM86 [0.47 Miller units] with PM95 [0.48 Miller units] in Fig. 1). Therefore, we suggest that some ORF5 is produced when p_L is repressed and that ORF5 is able to enhance the repression of p_L in these cells. In summary, these results suggest that during commitment to lysogenic growth, sufficient ORF4 is produced from the p_R promoter to repress initiation of transcription from both p_R and p_L and that the repression of p_L is further enhanced by the presence of small amounts of ORF5.

We assume that the ORF4 protein binds somewhere in the +10 to −45 region of the promoters in order to repress initiation of transcription. ORF5 may also be a DNA binding protein since it contains a helix-turn-helix motif (from Gln19 to Asn38) (19). In bacteriophage λ, the regulatory proteins cI and Cro regulates expression of the p_R and p_{RM} promoters by competition for the same operator sites in the region of the two promoters. But a similar regulation is not likely for TP901-1 since ORF5, in the presence of ORF4, both enhances (large amounts) and inhibits (small amounts) the level of initiation of transcription of the p_L promoter. In addition to this, ORF5 alone is not able to repress transcription from the TP901-1 early promoters. Instead, ORF5 may exert its action by binding to the phage DNA in a complex with ORF4 or just by binding to ORF4 and thereby somehow prevent repression of p_L by ORF4. The latter case would thus resemble the situation for the antirepressor Ant from *E. coli* phage P22 (for a review, see reference 22). In the first case, the high amount of ORF5 present during commitment to lytic growth may result in formation of an ORF4-ORF5 protein complex which is able to repress initiation of transcription of p_R and not that of p_L. On the other hand, during commitment to lysogenic growth ORF4 is free to bind to the target sites in both promoters, since no inhibition occurs from small amounts of ORF5. This could explain why the amounts of the regulatory proteins ORF4 and ORF5 as well as their relative abundance have a significant effect on the choice of the open or repressed state of the p_L promoter.

The expression of the p_L fusion present in pPM127 can be induced from the repressed state to the derepressed state by addition of mitomycin C. This induction was found to be dependent on the presence of a functional RecA protein. When the shorter fusion pPM126 was used, no mitomycin C induction was observed. This could indicate that ORF5 is needed for mitomycin C induction; however, we cannot completely rule out the possibility that mitomycin C acts on a separate promoter present in the extra DNA found in pPM127 between bp 3458 and 3900. RecA has been shown to act by stimulating repressor self-cleavage in the investigated cases of LexA and λ repressor and is therefore mechanistically a coprotease (17; for a review, see reference 18). A consensus AG autodigestion site has not been found in ORF4 (19), but ORF4 could belong to a different protein family than the repressors analyzed so far. Furthermore, the possibility exists that RecA acts on a protein complex consisting of both ORF4 and ORF5.

A homology search between ORF4 and ORF5 of TP901-1 identified a region of ORF4 (from Tyr59 to Asp76) that is similar to the proposed helix-turn-helix motif in ORF5 and thus might constitute a DNA binding motif. This notion is further supported by the very high similarity of this region of ORF4 to the suggested helix-turn-helix motif of ORF4 (from Leu64 to Asp82) of *Streptococcus thermophilus* phage φO1205 (25). A high degree of similarity on the level of protein sequences is found between ORF4 and ORF5 of TP901-1 and φO1205, the degrees of identity being 48% in 122 amino acids for ORF4 and 47% in 67 amino acids for ORF5. Interestingly, ORF4 of φO1205 is considerably shorter than ORF4 from TP901-1 (134 versus 180 amino acids) and also does not contain a suggested RecA binding site. Furthermore, ORF4 and ORF5 show extensive homology to the repressor (50% identity in 168 amino acids) and protein 31a (75% identity in 44 amino acids), respectively, of *Staphylococcus aureus* phage phiPVL (16). In addition to this, the intergenic region located between *orf4* and *orf5* of TP901-1 and the repressor and 31a genes of phiPVL show 62% identity at the nucleotide level in a 66-bp region. The similarity between the TP901-1, φO1205, and phiPVL phages in this region indicates that they may share a common mechanism for the switch determining lysis or lysogeny, but this has to be proven experimentally. Future studies may enable us to determine whether a protein complex consisting of ORF4 and ORF5 is responsible for the features of this new type of phage switch exemplified by TP901-1.

ACKNOWLEDGMENTS

We acknowledge Patrick Duwat for donation of the *recA* strain. We sincerely appreciate the expert technical assistance of Lise Sørensen and Lotte Bredahl.

This work is part of the FØTEK program supported by the Danish Dairy Research Foundation (Danish Dairy Board) and the Danish government through The Center of Advanced Food Studies. We also thank the Carlsberg Foundation for support.

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