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DNA-Binding Activity of the *Streptococcus thermophilus* Phage Sfi21 Repressor

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The cloned *Streptococcus thermophilus* phage Sfi21 repressor open reading frame (orf) 127 gp protects a cell against superinfection with the homologous temperate, but not against virulent phages. As demonstrated by DNase protection assay and gel shift experiments, the repressor binds to a 25-bp operator site located upstream of the repressor gene. A second sequence-related operator was identified 265 bp apart at the 3′-end of orf 75, the topological equivalent of a *cro* repressor gene. The replacement of a bp at the middle or at the right side of the operator decreased substantially the affinity of the repressor for the operator. In gel shift assays, the 75 gp did not bind DNA from the genetic switch region. However, when increasing amounts of orf 75 gp containing cell extracts were added to orf 127 gp containing cell extracts, the repressor could no longer bind its operator site.

INTRODUCTION

Phage λ research has provided molecular biology with a number of influential paradigms. One of them, the genetic switch concept, concerns the decision between lysogeny-oriented or lytic infection-oriented transcription. The λ CI and Cro proteins are dimeric repressors that control RNA synthesis from promoters p_L and p_R by binding the operators o_L and o_R. Both operators consist of three suboperators, each with imperfect two-fold rotational symmetry, creating a total of six related but not identical sequences for each operator. The o_R operator is located between the divergently transcribed *cI* and *cro* genes. Both CI and Cro bind to all three suboperators of o_R and thereby control the overlapping promoters p_R and p_{RM}. The nonidentity of the binding sites allows CI and Cro to work differently, because the sites do not bind the proteins with equal strength (Gussin *et al.,* 1983).

Temperate *Siphoviridae* from low GC content Gram-positive bacteria show a remarkably λ-like organization of the late gene cluster (Brüssow, 2001; Brüssow and Desiere, 2001). However, the genetic organization of their lysogeny modules differs clearly from that of phage λ (Lucchini *et al.,* 1999a). These phages possess a genetic switch structure with lysogeny-related genes encoding a CI-like repressor, a zinc-binding protein, a superinfection exclusion protein, and an integrase (in this order) and divergently transcribed genes encoding a "Cro-like" repressor, an anti-repressor, an excisionase, and a series of DNA replication proteins (again in this order). With respect to the mode of phage DNA recognition, different types of CI- and Cro-like repressors were observed. *Lactobacillus* phages A2 (Garcia *et al.,* 1999; Ladero *et al.,* 1998, 1999) and phig 1e (Kakikawa *et al.,* 1998, 2000a, 2000b) showed an o_R-like operator that consisted of three and five related but nonidentical suboperators, respectively. CI and Cro repressors showed distinct binding affinities for the suboperators. CI from phage A2, when bound to operators O1 and O2 overlapping the *cro* promoter, enhanced the positioning of the RNA polymerase with respect to the *cI* promoter. A2 CI exerted a negative effect on the *in vitro* transcription of *cro.* In contrast, *Bacillus subtilis* phage φ105 possessed five suboperators upstream of the repressor gene, but remarkably the 14-bp operator sequence did not exhibit two-fold rotational symmetry (Van Kaer *et al.,* 1987, 1988, 1989). Since λ-like CI repressors bind DNA as dimers exploiting the dyad symmetry of the operator DNA, it is not clear how the φ105 repressor binds an operator lacking this symmetry element. A further interesting case is *Lactococcus* phage TP901-1. Upon transformation with a plasmid containing both the *cI*- and the *cro*-like genes and their cognate promoters, clonal variation was observed: in each transformant either one or the other promoter was open (Madsen *et al.,* 1999). Apparently, the relative amounts of the two repressor proteins determined the decision between lytic and lysogenic life cycle. To explain their data, the authors suggested a protein complex consisting of CI and Cro.

Here we demonstrate that the CI-like repressor from *Streptococcus thermophilus* phage Sfi21 (Bruttin *et al.,* 1997a; Lucchini *et al.,* 1999b) binds a 25-bp DNA site in

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the intergenic region between *cI*- and *cro*-like genes and a related site at the 3′-end of the *cro*-like gene. The Sfi21 Cro-like repressor lacked binding activity to DNA from the genetic switch region, but could prevent DNA binding of the Sfi21 CI-like repressor in a dose-dependent manner.

RESULTS

Cloning and biological activity of the two phage repressors

Phage repressor proteins were predicted for the two divergently transcribed open reading frames (orf) 127 and orf 75 located in the lysogeny module of phage Sfi21. The closest database matches for orf 127 gp were repressor proteins from prophages of *Streptococcus pyogenes, Staphylococcus aureus,* and *Lactococcus lactis* (58 to 48% aa identity), followed by matches with *S. thermophilus* phage TPJ-34 and the biochemically characterized CI-like repressor from *Lactobacillus casei* phage A2 (44% aa identity). For orf 75 gp related proteins were identified in the same prophages (50 to 41% aa identity) and it matched the Cro-like repressor from phage A2 (44% aa identity). Both Sfi21 phage genes were individually cloned into the vector pNZ124, resulting in plasmids pSFcI and pSFcro, respectively. The *S. thermophilus* indicator cell Sfi1 was transformed separately with the two plasmids. Both phage genes were expressed from their respective plasmids (Ventura *et al.,* 2002). When the genetic switch region with the divergently oriented repressor genes was cloned, only the cI-like gene was transcribed (Ventura *et al.,* 2002). The presence of pSFcro had no effect on the multiplication of phage Sfi21 or any of 30 distinct virulent *S. thermophilus* phages that infect the Sfi1 indicator cell. Plasmid pSFcI, in contrast, prevented the multiplication of the homologous phage Sfi21 (<10 pfu/ml on Sfi1[pSFcI]), compared with 10^8 pfu/ml on the vector control Sfi1[pNZ124]), while virulent phages were not inhibited. One notable exception was the virulent phage S3, which is a deletion derivative of phage SFi21 that had lost the phage DNA between the *attP* site and the middle of the *cI* gene (Bruttin *et al.,* 1997b).

DNA binding of the Sfi21 phage repressor in gel shift experiments

We then determined the DNA-binding specificity of the orf 127 repressor. To this purpose a series of 23 overlapping PCR products that covered the postulated genetic switch region were synthesized. DNA binding of the repressor was tested in gel retardation assays. The phage repressor was added as a cell extract of *S. thermophilus* Sfi1 containing plasmid pSFcI. Cell extracts of Sfi1[pNZ124] were used as the negative control and all experiments were conducted in the presence of excess heterologous DNA. The results of these gel shift assays are summarized in Fig. 1; a representative assay is shown in Fig. 1, insert A. Two distinct regions of phage DNA experienced a gel shift when incubated with the phage repressor extract. The minimal overlap of retarded PCR fragments allowed us to locate one binding site between bp positions 27,298 and 27,332 (numbering according to AF115103). This binding region was localized in the intergenic region between orf 127 and orf 75. Further limitations were set by PCR fragment H ending at bp position 27,315, which showed only a very weak shift, and PCR fragment N starting at bp position 27,319, which showed no shift (data not shown). A second binding site was placed by gel shift experiments between bp positions 27,595 and 27,610, i.e., at the very 3′ end of orf 75 (Figs. 1 and 2D). The specificity of repressor binding to both sites was demonstrated by a dose-dependent competition by homologous unlabeled DNA (Fig. 1, insert B and data not shown).

A comparison of the two binding sites revealed a region that shared 15 identical bp over 23 bp (Fig. 2A). Identical bps were found in two blocks separated by a 6-bp nonconserved region. The sites showed only a weak dyad symmetry. BS1 overlapped the putative promoter −35 box from orf 75 (Fig. 2D; Ventura *et al.,* 2002). BS2 showed an inverted orientation with respect to BS1, suggesting a distinct geometry for repressor binding. We screened the phage Sfi21 genome for DNA segments related to the BS1/BS2 consensus sequence. Five such sequences were identified (Fig. 2A). Interestingly, one of these sequence ("BS3") overlapped the putative promoter −35 box from the orf 127 repressor gene. However, neither PCR product L (Fig. 1), which covered the BS3 sequence entirely, nor a synthetic double-stranded DNA segment corresponding to this region (bp 27,237 to 27,261) were retarded by the Sfi1[pSFcI] cell extract (Fig. 3C). In addition, we failed to observe two retarded DNA fragments when the labeled PCR products B, D, E, and G (Fig. 1), each containing both the BS1 and the BS3 sequence, were incubated with a wide concentration range of Sfi1[pSFcI] cell extracts (Fig. 3A and data not shown).

Two further sites with sequence similarity to the BS1/BS2 consensus are notable since they were localized upstream of the early genes orf 140a (located in the putative lysogenic conversion region; Canchaya *et al.,* 2002) and orf 170 (located in the putative transcriptional regulation module; Ventura *et al.,* 2002). Notably, orf 170 is a candidate for a transcriptional regulator needed for middle and late transcription in phage Sfi21 (Ventura *et al.,* 2002). Appropriate PCR products covering these DNA sequences were synthesized (Fig. 2A), but none experienced gel retardation by the repressor cell extract (data not shown).

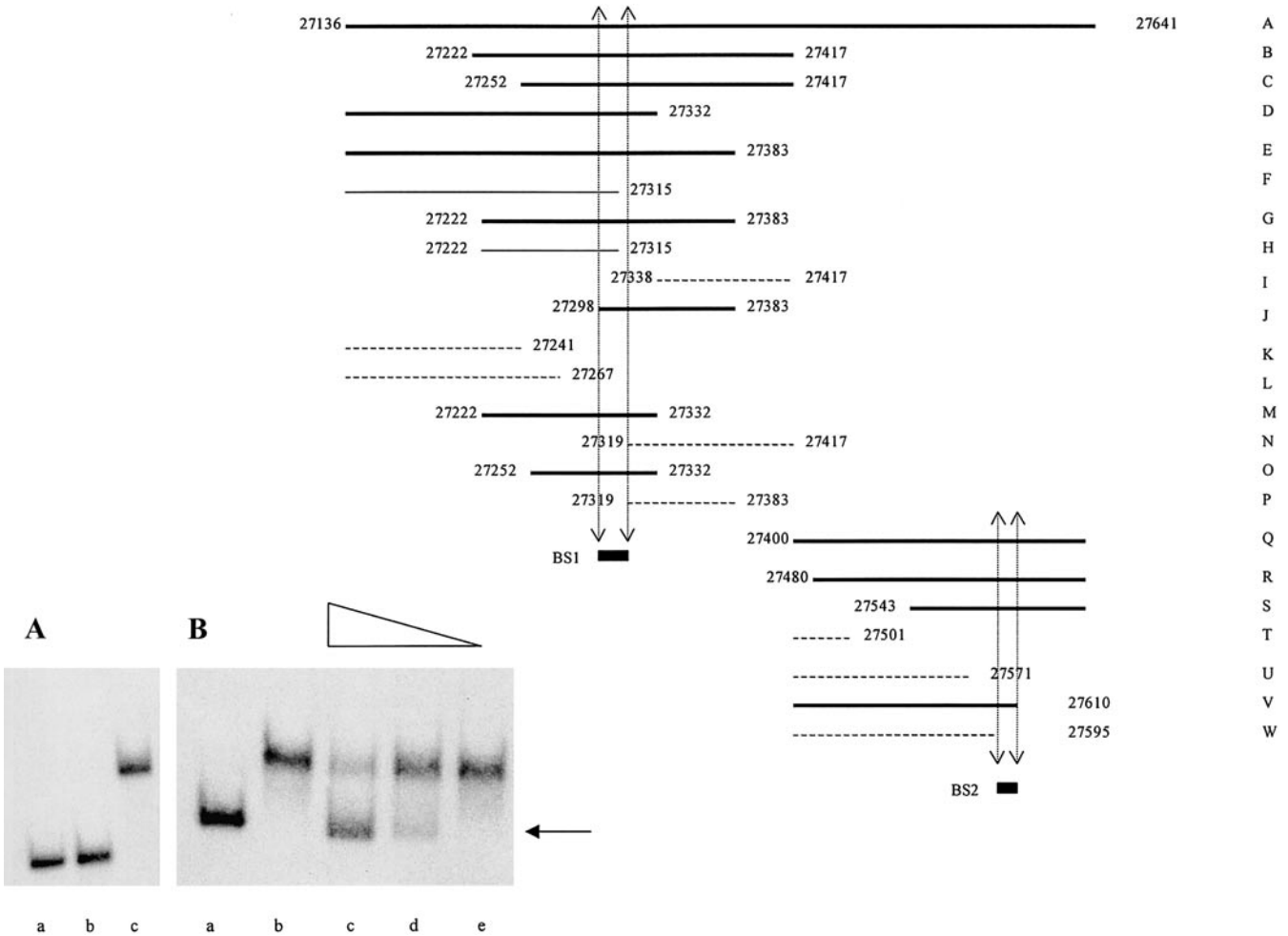

FIG. 1. Location of the repressor binding sites. Summary of gel shift assays using Sfi1[pSFcl] cell extracts and DNA from the genetic switch region represented by 23 PCR products, A to W. The PCR products are marked with their first and last bp position with respect to the phage Sfi21 genome sequence according to Accession No. AF115103. Bound PCR fragments are drawn as thick lines; non-bound DNA are dashed lines; the weak binders F and H are drawn as thin lines. The locations of the binding sites BS1 and BS2 were deduced from the comparison of all gel shift assays (and other evidence; see Figs. 2 and 3) and are indicated by vertical arrows and black rectangles. (Insert A) Gel shift analysis using the radiolabeled PCR fragment O incubated with the buffer control (lane a), Sfi1[pNZ124] control extract (lane b), and Sfi1[pSFcl] test extract (lane c). (Insert B) Gel shift analysis using the radiolabeled PCR fragment R incubated with a buffer control (lane a) and Sfi1[pSFcl] cell extract (lanes b to e) in the absence (lane b) or the presence of two-fold decreasing amounts of unlabeled homologous DNA (lanes c to e). The samples were separated on a nondenaturing polyacrylamide gel. The position of unbound DNA is marked with an arrow.

DNase protection assay and binding to synthetic DNA segments

Two further experiments confirmed the location of the repressor binding site BS1. First, a DNase I footprinting assay demonstrated the protection against nuclease attack of bp positions 27,297 to 27,321 by the repressor extract (Fig. 3B). In a second step two complementary 25-nt oligonucleotides corresponding to the protected DNA segment were synthesized and annealed to yield a synthetic binding site BS1. In gel shift experiments this synthetic double-stranded DNA segment was retarded by the repressor cell extract (Fig. 3C). A synthetic double-stranded 25-bp BS2 fragment was obtained in a similar approach. As in the case of the synthetic BS1 site, the synthetic BS2 site was bound and retarded by the repressor cell extract (Fig. 3c).

The binding of the repressor to annealed synthetic oligonucleotides offered the possibility of assessing the importance of individual bp positions for repressor binding. To this purpose binding sites that differed from BS1 in one or two bp positions were synthesized. To limit the number of mutated binding sites, a consensus sequence was derived for the BS1 and BS2 binding sites. The nonbinder orf 170 DNA sequence differed from the BS1/BS2 consensus sequence in three bp positions (Fig. 2A). Variant BS1 sites were synthesized in which each of these three positions was changed to the bp found in the nonbinder orf 170 sequence. The $T_{27,297} \rightarrow C$ change in BS1.1 did not affect the binding, while a $G_{27,306} \rightarrow C$ in

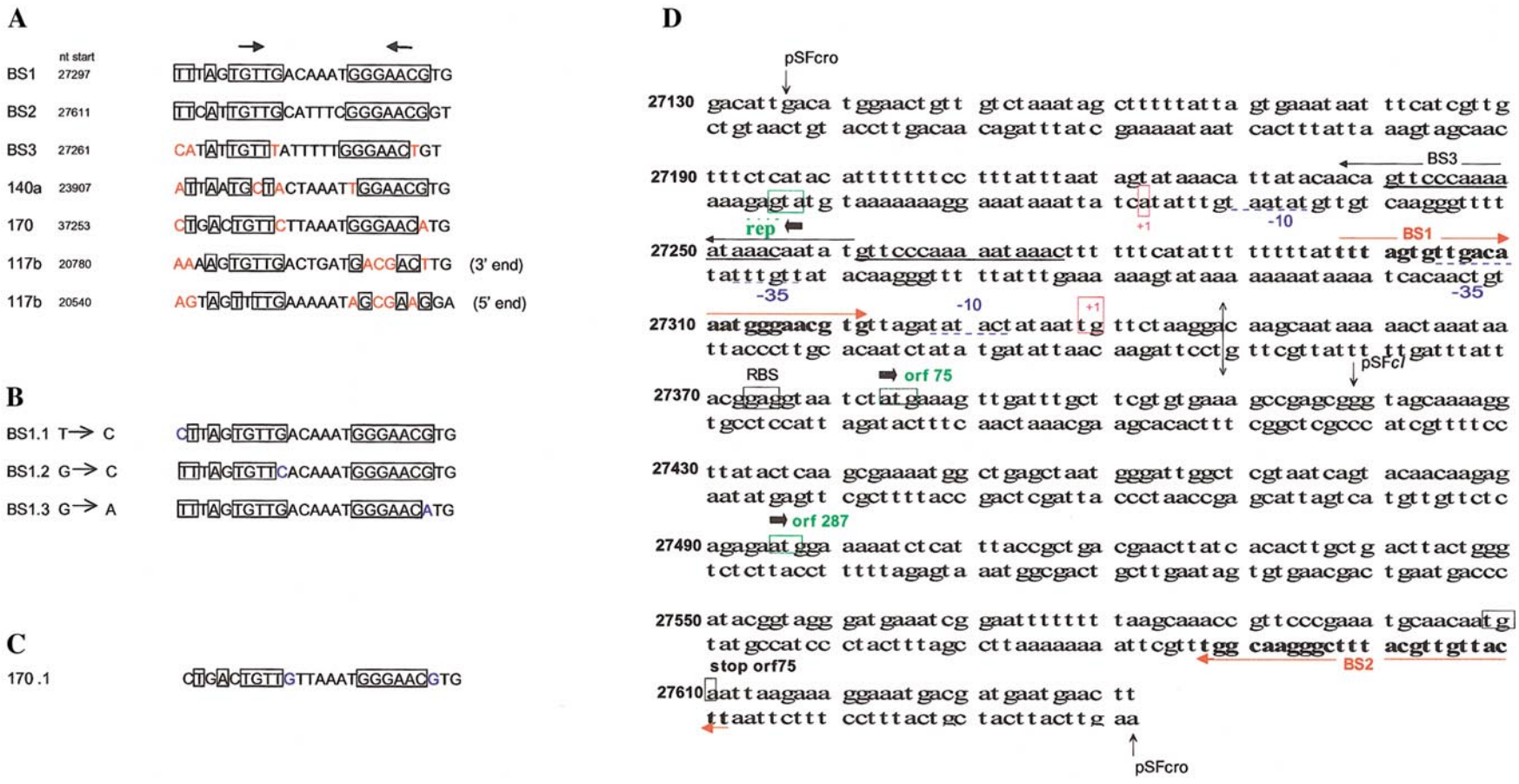

FIG. 2. Sequence of the genetic switch region and the repressor binding sites. (A) Alignment of BS1 and BS2 repressor binding sites with related sequences of phage Sfi21. Bp positions that are shared between BS1 and BS2 are boxed. Additional Sfi21 sequences that resemble either BS1 or BS2 are identified by their orf and nt position in the Sfi21 sequence. Bp positions shared with the BS1/BS2 consensus are boxed; bp positions that differ from the consensus are in red. The arrows indicate a weak dyad symmetry. (B) Modified BS1 sequences. The bp substitutions are indicated and highlighted in blue. (C) Modified 170 sequence. The 2-bp substitutions are highlighted in blue. (D) Annotated DNA sequence of the genetic switch region. Start and stop codons of the relevant orfs are boxed. The two operator sites bound by the Sfi21 repressor are in bold and annotated in red as BS1 and BS2. BS3, a sequence-related but unbound DNA segment, is indicated with a thin arrow. A 16-bp direct repeat upstream of the repressor gene is underlined. The start and/or end of cloned inserts in pSFcro and pSFcI are marked with vertical arrows. The −10 and −35 promoter regions as deduced from primer-extension experiments (Ventura *et al.,* 2002) are underlined with dashed lines. The ribosomal binding site (RBS) upstream of orf 75 is boxed. The start of the transcripts as determined by primer extension analysis are boxed and annotated with +1; since the experimentally determined 5′ end of the repressor transcript was downstream of the orf 127 start codon, the next in-frame start codon was marked (orf 116). The vertical double-arrowed line indicates a correction to the GenBank entry, the presence of an additional A.

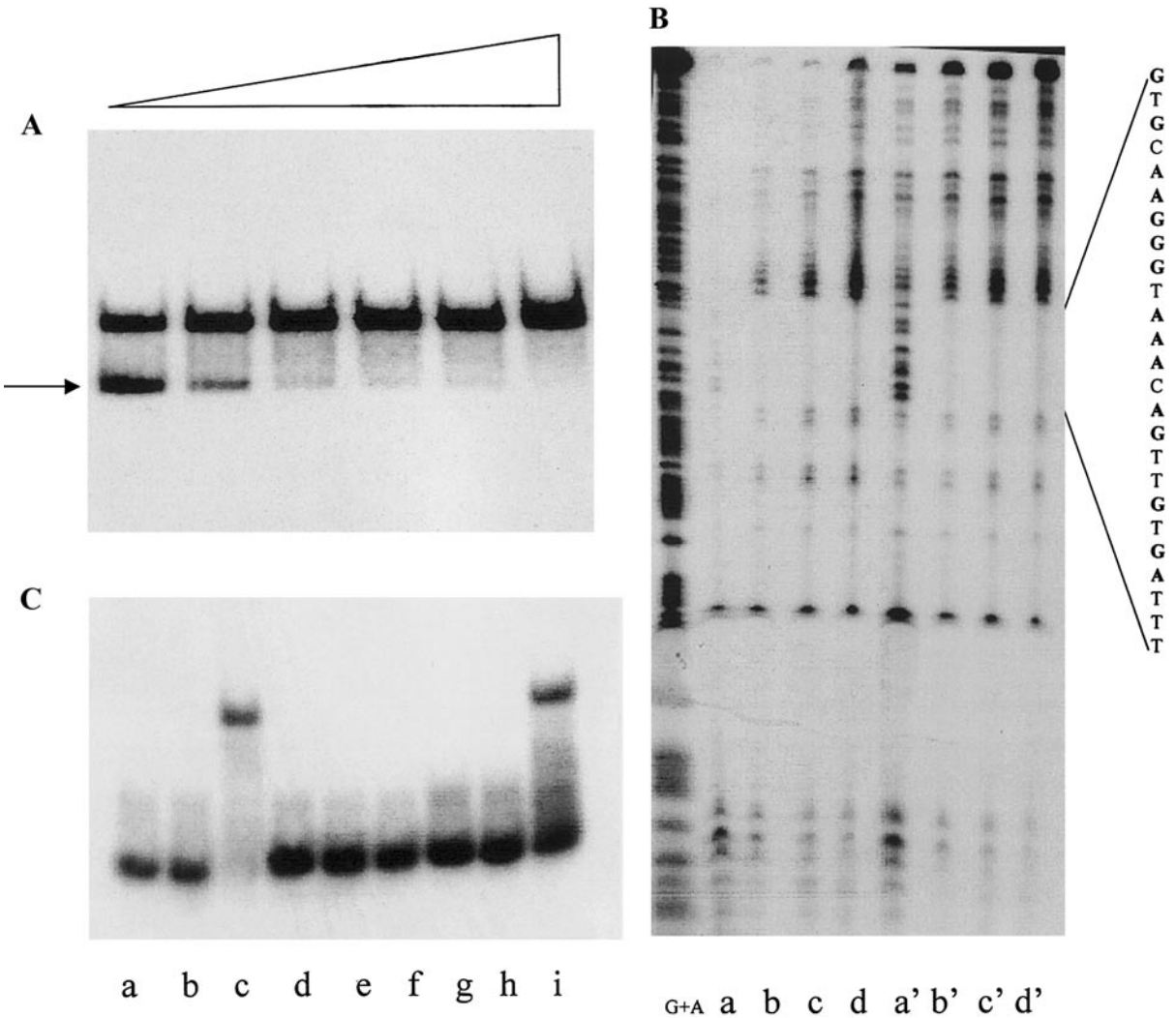

FIG. 3. Biochemistry of repressor–phage DNA interaction. (A) The radiolabeled PCR fragment G (see Fig. 2 for location) covering the intergenic region between orf 127 and orf 75 was incubated with two-fold increasing amounts of Sfi1[pSFcI] cell extract (starting concentration was 0.2 mg protein/ml). The arrow indicates the position of unbound DNA. (B) DNase I footprinting analysis of the repressor–DNA complex. PCR product G was incubated without cell extract (lane a, a') and with 4, 2, and 1 μl of the Sfi1[pSFcI] cell extract (lanes b, b' to d, d'). The operator site is indicated with the sequence at the right side deduced from a G + A sequencing ladder. Lanes a–d, 9 U of DNase I; lanes a'–d'; 3 U of DNase I. (C) Electrophoretic mobility shift assays with synthetic 25-bp DNA segments. Labeled sites BS1 (lanes a to c), BS2 (lanes g to i), and BS3 (lanes d to f) were incubated without cell extract (lanes a, d, and g), Sfi1[pNZ124] control extract (lanes b, e, and h), and Sfi1[pSFcI] test extract (lanes c, f, and i).

BS1.2 or a $G_{27,319} \rightarrow A$ change in BS1.3 substantially decreased the binding affinity by the repressor cell extract, suggesting the involvement of these two positions in repressor binding (Fig. 4). Interestingly, the introduction of the BS1-specific nucleotides at these two positions into the nonbinding synthetic orf 170-specific DNA segment (170.1 sequence, Figs. 2A and 2C) caused a weak binding of the modified DNA fragment by the repressor extract, thus confirming the importance of these two nucleotide positions (data not shown). Finally, synthetic 13-bp DNA fragments corresponding to the left or right halves of the BS1 site were not retarded by orf 127 gp in gel shift assays (data not shown). This experiment excludes the possibility that the BS1 site is composed of two adjacent smaller target sites.

Sfi21 phage repressor orf 75 gp

The Sfi1[pSFcro] cell extract caused no electrophoretic shift of BS1 or BS2 under conditions in which the Sfi1[pSFcI] cell extract caused a gel shift of both DNA fragments (Fig. 5A and data not shown). The Sfi1[pSFcro] cell extract also failed to retard the BS3 fragment or any other DNA fragments from the noncoding region between orf 127 and orf 75 or the coding sequences flanking the genetic switch region (data not shown). However, when the Sfi1[pSFcro] extract was added in increasing amounts to a fixed concentration of the Sfi1[pSFcI] extract, a dose-dependent loss of retardation of the BS1 DNA was observed (Fig. 5B). The inhibitory effect was specific to the Sfi1[pSFcro] cell extract since no inhibition

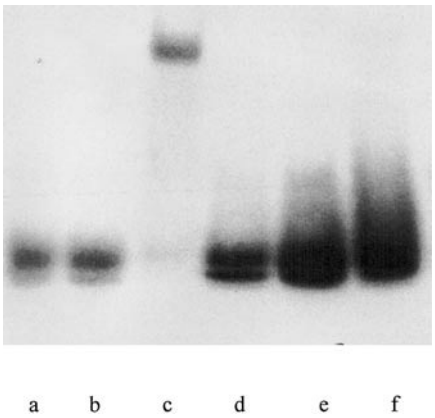

FIG. 4. Determination of critical bp positions for the repressor binding reaction. Effect of base-pair substitutions in BS1 for repressor binding. Labeled synthetic sites BS1.1 (lanes a–c) and BS 1.2 (lanes d–f) were incubated without cell extract (lanes a and d), with Sfi1[pNZ124] control extract (lanes b and e), and with Sfi1[pSFcI] test extract (lanes c and f).

of BS1 retardation was observed when comparable amounts of Sfi1[pNZ124] cell extract were used (Fig. 5B).

Since the pSFcro plasmid contained the BS1 binding site, the plasmid DNA in the cell extract could theoretically act as a competitor DNA in the gel shift assay. The pSFcro plasmid was isolated from the cell extract by two different protocols (QIAprep and phenol extraction/ethanol precipitation) and added as supercoiled or linearized plasmid DNA into the binding assay. Plasmid concentrations corresponding to the DNA amounts in the highest extract concentrations used in the shift assays did not inhibit the CI-induced BS1 gel shift (data not shown). In fact, in all assays the molar concentration of the synthetic DNA segment BS1 was in excess of the plasmid DNA molar concentration. In a next step, the Sfi1[pSFcro] extract was digested with trypsin. Trypsin activity was then terminated by either a heat treatment or addition of soybean trypsin inhibitor. When these trypsin-pretreated extracts were added in a 10:1 ratio to the Sfi1[pSFcI] extract, a CI-induced gel shift of BS1 was again observed (data not shown).

DISCUSSION

Orf 127 gp from phage Sfi21 is a DNA-binding protein that recognizes phage DNA in a sequence-specific manner. The sequence similarity with the CI-like repressor from *Lactobacillus* phage A2 suggests a phage repressor function for orf 127 gp. In fact, orf 127 is one of the few phage genes transcribed from the Sfi21 prophage and it is only weakly detectable in cells lytically infected with phage Sfi21 (Ventura *et al.,* 2002; Ventura *et al.,* in press). Both observation are compatible with repressor functions. However, the Sfi21 repressor is substantially shorter than the A2 repressor (127 vs 224 aa) and matches only the N-terminal half of the A2 repressor. Interestingly, orf 127 showed a complementary protection pattern to orf 203, located upstream of the integrase gene in Sfi21. Orf 203 encodes a superinfection exclusion function that was highly effective against many virulent phages, but ineffective against phage Sfi21 (Bruttin *et al.,* 1997a). In analogy with the λ system one might speculate that in the Sfi21 phage system orf 127 gp plays the role of the λ CI repressor and prevents vegetative development of the prophage as well as infection with the homologous temperate phage. Orf 203 might play the functional but not the mechanistic role of the *rex* and *sie*B genes in λ that exclude the superinfection of lysogen with virulent phages (Court and Oppenheim, 1983). Orf 203 is the most prominent phage transcript in the lysogenic cell (Ventura *et al.,* in press). An analogous protein from *L. lactis* phage Tuc2009 associates with the cell

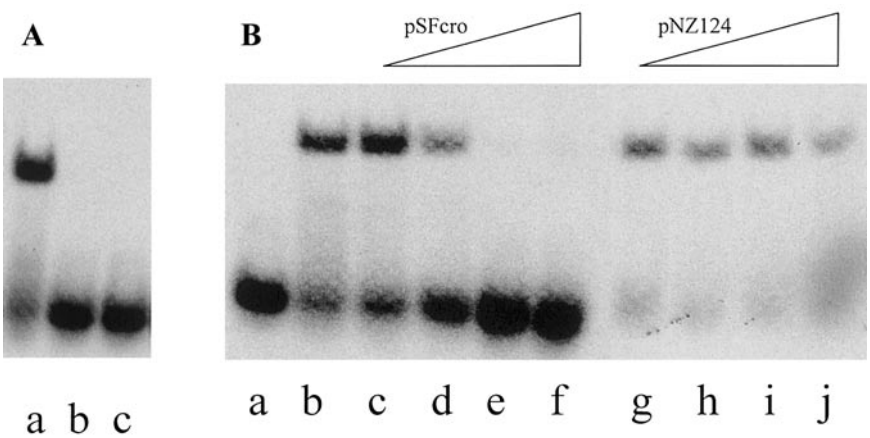

FIG. 5. The effect of the Sfi1[pSFcro] cell extract on the BS1 binding by orf 127 gp. (A) Lack of BS1 binding by orf 75 gp. Labeled BS1 was incubated with extracts from Sfi1[pSFcI] (lane a), Sfi1[pNZ124] (lane b), and Sfi1[pSFcro] (lane c). (B) Labeled BS1 was incubated with buffer (lane a), Sfi1[pSFcI] extract (lane b), a mix of Sfi1[pSFcI] and Sfi1[pSFcro] extracts in the ratios 1:1, 1:2, 1:5, and 1:10 (lanes c to f), and Sfi1[pSFcI] and Sfi1[pNZ124] extracts in the ratios 1:1, 1:2, 1:5, and 1:10 (lanes g to j).

membrane and blocks injection of the phage DNA (McGrath *et al.,* 2002).

The repressors from A2 and all lambdoid coliphages recognize an operator consisting of three operator subsites (Carlson and Little, 1993; Garcia *et al.,* 1999; Ogawa *et al.,* 1988a, 1988b). The Sfi21 repressor, in contrast, recognizes two operators, but each operator consists of a single 25-bp binding site that could not be further divided into suboperators. The first site (BS1) was located in the noncoding region between the divergently transcribed orfs 127 and 75. The second site (BS2) was located 265 bp apart at the 3′ end of orf 75. The two operators showed different but related sequences and both demonstrated only weak dyad symmetry.

The possession of a single binding site in the noncoding region is unusual also for dairy phages. Those that have been investigated showed at least two (*Lactococcus* phage r1t; Nauta *et al.,* 1996) and up to five DNA-binding sites (*Lactobacillus* phage phig1e; Kakikawa *et al.,* 2000b) between the *cI*- and *cro*-like genes. A second independent operator within the coding region of the *cro*-like gene was also found in several dairy phages (e.g., r1t and phig1e; Nauta *et al.,* 1996; Kakikawa *et al.,* 2000b). An operator consisting of a single DNA-binding site, characteristic for repressors from the bacterial host, has not yet been reported for repressors from phages infecting lactic acid bacteria.

Although the presence of low-affinity binding sites cannot be ruled out, it should be noted that in Sfi1[pSFcI] cells the repressor gene was expressed from a high copy number plasmid under the control of its own promoter. The repressor concentrations were high enough to cause a strong suppression of Sfi21 phage growth on Sfi1[pSFcI] cells. Furthermore, in *Lactobacillus* phages, the change in occupancy from high- to low-affinity sites occurred over small repressor concentration changes. A two- to four-fold increase in the phig1e repressor concentration caused a change from weak binding of the high-affinity site to strong binding to both high- and low-affinity sites (Kakikawa *et al.,* 2000a, b). In the A2 system, both high- and low-affinity sites were bound even by low repressor concentrations (Garcia *et al.,* 1999). In contrast, no difference in DNA occupancy was observed when Sfi21 phage DNA was incubated with Sfi1[pSFcI] extract concentrations that differed by a factor of 400. A theoretical limitation of our experimental system is the exclusive use of linear phage DNA for the binding assays. However, no conformation-dependent DNA binding was seen for repressors of *Lactobacillus* phages (Kakikawa *et al.,* 2000a).

The present work is the first study with dairy phages that looks at the contribution of critical nucleotides in operator sequences to repressor binding. Two nucleotide positions were identified that made major contributions to the repressor binding. Changing bp positions 27,307 or 27,319 within BS1 dramatically reduced repressor binding. Conversely, changes in the equivalent positions of a nonbinder synthetic DNA fragment allowed weak binding of this modified DNA fragment by the Sfi21 repressor.

In phage Sfi21 the topological equivalent of *cro* predicts a DNA-binding protein with high sequence similarity to the *Lactobacillus* phage A2 Cro repressor. The A2 Cro protein competes with CI for binding to the same operator subsites (Ladero *et al.,* 1999). No DNA-binding activity could be demonstrated with the Sfi1[pSFcro] extract. However, the presence of orf 75 gp competed with phage DNA binding by orf 127 gp in a dose-dependent manner. The effect of the trypsin digestion showed that the inhibitor of the CI-BS1 binding reaction in the Sfi1[pSFcro] extract is of proteinaceous nature. The data suggest but do not prove the formation of a protein–protein complex between orf 127 gp and orf 75 gp resulting in a decreased affinity of orf 127 gp for BS1. Such a mechanism is not without precedence. In the *Salmonella* phage P22 the Ant protein associates with the P22 CI repressor homologue C2, leading to a decreased binding specificity (Susskind and Youderian, 1983). There are also indirect indications for repressor–repressor interactions at the protein level in *Lactococcus* phage TP901-1 (Madsen *et al.,* 1999). In this phage the relative amounts of the divergently transcribed orf 4 and orf 5 gps determined the decision between lytic and lysogenic life cycle after phage infection. Mutant and deletion analysis combined with transcriptional studies suggested that a protein complex consisting of orf 4 and orf 5 gp was formed.

The genetic switch region of phage Sfi21 probably involves a further player. Orf 287 overlaps *cro* and predicts a two domain protein, with one representing a helix-turn-helix DNA-binding motif (Neve *et al.,* 1998). Comparable genes were found downstream of the topological complement of *cro* in a number of temperate dairy phages (Lucchini *et al.,* 1999c). Orf 287 gp was called an anti-repressor due to its sequence similarity with the anti-repressor of coliphage P1 over its C-terminal DNA-binding domain (Neve *et al.,* 1998). There is currently no indication that orf 287 from Sfi21 fulfills a function similar to that of the anti-repressor from phage P1. Preliminary experiments with orf 287 deletion mutants from phage Sfi21 indicated that the establishment of the lysogenic state was delayed in the mutants compared with the wild-type phage, while the lytic growth properties of the mutant were unaffected (A. Bruttin, unpublished results). It is thus possible that orf 287, like *cII,* its topological equivalent in **λ**, helps in the establishment of the early transcription of *cI.*

In lytic infections with phage Sfi21 neither orf 127 nor orf 75 give rise to early transcripts. At the promoter of orf 75 a prominent 2.1-kb middle transcript is initiated, covering orf 75 and orf 287 repressors and two further downstream genes. A weak middle transcript is initiated at the orf 127 promoter. Early transcripts are initiated in

four distinct phage genome regions, one covering the orf 170 repressor (Ventura *et al.,* 2002). Chloramphenicol inhibition experiments demonstrated that phage protein translation is necessary for middle gene transcription. Phage Sfi21 is therefore likely to deviate substantially from the phage λ genetic switch paradigm. In addition, different dairy phages have apparently distinct genetic switch designs (J. Blatny and D. van Sinderen, personal communication). Further investigations into repressor/corepressor systems in dairy phages could thus provide important insights into the evolution of transcriptional control systems in temperate phages from this branch of Gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and media

The *Escherichia coli* strain XL1-Blue was propagated in LB broth (with agitation) or on LB solidified with 1.5% (w/v) agar at 37°C. *S. thermophilus* strain Sfi1 and transformants thereof were routinely subcultured at 42°C in either LM17 (M17 supplemented with 0.5% lactose) or Belliker (Elliker plus 1% beef extract) media. Chloramphenicol was used when required at a final concentration of 3 and 20 μg/ml, respectively, for *S. thermophilus* and *E. coli.* The *S. thermophilus* phages used in this study were obtained from the Nestlé phage collection. The phages were propagated on their appropriate *S. thermophilus* strain in LM17 broth. Phage enumeration was achieved by plaque assay as described by Foley *et al.* (1998).

DNA techniques

Phage purification, DNA extraction and purification, agarose gel electrophoresis, Southern blot hybridization, and DNA labeling were done as described previously (Bruttin *et al.,* 1997c). General DNA techniques were performed as described by Sambrook *et al.* (1989). The Qiaprep plasmid kit (Qiagen) and the Jetstar Plasmid Maxi-kit (Genomed) were used for the rapid isolation of plasmid DNA from *E. coli.* Restriction enzymes and T4 DNA ligase were obtained from Boehringer–Mannheim and used according to the supplier's instructions. *E. coli* and *S. thermophilus* were electrotransformed as described in the Bio-Rad instruction manual and by Slos *et al.* (1991), respectively.

PCR

DNA samples were amplified in a Perkin–Elmer thermal cycler programmed for 30 cycles, each consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. Synthetic primers were designed according to the established phage Sfi21 DNA sequence (Accession No. NC_000872) and used together with the relevant DNA template and *Taq* polymerase Fermentas. PCR products were gel-purified using Ultrafree-MC Centrifugal Filter Units (Millipore) and by following the manufacturer's instructions.

Construction of plasmids

The cloning vector used in this study was the high copy number *E. coli*/lactococcal/streptococcal shuttle vector pNZ124 (Platteeuw *et al.,* 1994). A 2534-bp *Xba*I fragment from the lysogeny module of the *S. thermophilus* phage Sfi21 was cloned in the *Xba*I site of pUC19, generating the construct pX6. The putative cI-like repressor gene, orf 127, was cloned independently of orf 75, the putative Cro repressor gene, by inserting the 664-bp *Bsr*BI/*Bam*HI fragment of pX6 in the *Ec*/136II/*Bam*HI sites of pNZ124, generating the construct pSFcI. pSFcro consists of a 517-bp *Bam*HI/*Xba*I PCR fragment (containing the putative genetic switch region, orf 75, and the 5′ ends of orf 127 and orf 287) cloned in the respective sites of pNZ124. The PCR fragment was generated using phage Sfi21 DNA as the template DNA and primer pair 1F/R (5′-GGC TGC AGGATCC GAC ATG GAA CTG TTG TC-3′ and 5′-GC TCTAGA AAG TTC ATT CAT CGT CA-3′, containing *Bam*HI and *Xba*I restriction sites, respectively).

Gel retardation assay

Overnight cultures of the relevant *S. thermophilus* strains were inoculated (2%) in Belliker broth and grown to an O.D. (600 nm) of 0.5. The cells were harvested by centrifugation and washed, and the pellets were resuspended in 750 μl Tris buffer (10 mM, pH 8, 4°C). The crude cell extracts were prepared using a cell disrupter and glass beads (Sigma; 106 μm) as described by Foley *et al.* (1996).

DNA fragments generated by PCR using phage Sfi21 as the template were gel-purified and subsequently digested with the relevant restriction enzyme. The digested fragments were then end-labeled with the Klenow fragment of DNA polymerase I (New England Biolabs) in the appropriate buffer and in the presence of 30 μCi α-[^{32}P]dATP. The labeled DNA was phenol-extracted and ethanol-precipitated. The binding conditions used were as described by Foley *et al.* (1996) and included 4 μl of crude cell extract (~5 μg total protein). After 10 min incubation at room temperature, 5 μl of 50% glycerol was added to each reaction prior to immediate loading on a 4% nondenaturing polyacrylamide gel containing 2.6% glycerol. Electrophoresis was performed at room temperature for 4 h at 10 V/cm. The gel was vacuum-dried onto 3 MM Whatmann paper and autoradiographed for 36 h.

To generate 25-bp synthetic double-stranded DNA fragments for gel retardation assays, two complementary oligonucleotides were used. One oligonucleotide was end-labeled with polynucleotide kinase (Promega) in the appropriate buffer for 1 h at 37°C using 30 μCi γ-[^{32}P]-ATP (Amersham). Labeled oligonucleotide was collected

on a Bio-Gel A 1.5-m column (Bio-Rad) and further purified by phenol–chloroform extraction. After ethanol precipitation in the presence of 1 μg poly(dI–dC) and 1 mM $MgCl_2$, the labeled oligonucleotide was annealed with its cold synthetic complementary oligonucleotide as described by Nauta *et al.* (1996).

DNase I footprinting assay

DNA fragment G was generated by PCR using phage Sfi21 DNA as a template and the primer pair 2F/2R (5′-GC GGATCC GTA TAA ACA TTA TAC AAC AG-3′ and 5′-GC GAATTC GAT TAC CTC CGT TTA TTT AG-3′ containing *Bam*HI and *Eco*RI sites, respectively). The PCR-generated DNA fragment was gel-purified, digested with *Bam*HI, and end-labeled with Klenow fragment of DNA polymerase I and α-[^{32}P]dATP. The labeled DNA fragment was then used for DNase I footprinting using the SureTrack Footprinting Kit (Pharmacia Biotech). The manufacturer's instructions were followed incorporating the following modifications: unless otherwise indicated in the text 1 μl of crude cell extract of *S. thermophilus* Sfi1 [pSFcI] and 3 units of DNase I were used. The binding conditions used were identical to those optimized for gel retardation assays (see above). Following the footprinting reaction, the reactions were ethanol-precipitated and the pellets resuspended in 10 μl of loading dye, of which 3.5 μl was loaded on an 8% acrylamide/7 M urea denaturing polyacrylamide gel. The same labeled DNA fragment was used in purine (G + A) Maxam and Gilbert sequencing reactions following the protocols provided by Pharmacia Biotech and were run in parallel to the DNase I footprinting reactions as a marker ladder.

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