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A Short Noncoding Viral DNA Element Showing Characteristics of a Replication Origin Confers Bacteriophage Resistance to *Streptococcus thermophilus*

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A 302-bp noncoding DNA fragment from the DNA replication module of phage ϕSfi21 was shown to protect the *Streptococcus thermophilus* strain Sfi1 from infection by 17 of 25 phages. The phage-inhibitory DNA possesses two determinants, each of which individually mediated phage resistance. The phage-inhibitory activity was copy number dependent and operates by blocking the accumulation of phage DNA. Furthermore, when cloned on a plasmid, the ϕSfi21 DNA acts as an origin of replication driven by phage infection. Protein or proteins in the ϕSfi21-infected cells were shown to interact with this phage-inhibitory DNA fragment, forming a retarded protein–DNA complex in gel retardation assays. A model in which phage proteins interact with the inhibitory DNA such that they are no longer available for phage propagation can be used to explain the observed bacteriophage resistance. Genome analysis of ϕSfi19, a phage that is insensitive to the inhibitory activity of the ϕSfi21-derived DNA, led to the characterisation of a variant putative phage replication origin that differed in 14 of 302 nucleotides from that of ϕSfi21. The variant origin was cloned and exhibited an inhibitory activity toward phages that were insensitive to the ϕSfi21-derived DNA.

INTRODUCTION

After virus adsorption and DNA injection, DNA replication is a critical step in the virus life cycle and is frequently targeted in strategies developed to block viral infection. Bacteria possess several native defence mechanisms that protect against bacteriophage infection. During the past decade, molecular approaches have provided a number of genetically defined resistance mechanisms directed against mesophilic lactococcal phages (reviewed by Garvey *et al.,* 1995b). Many of the resistance mechanisms are based on defence strategies native to *Lactococcus* and are frequently encoded by plasmids, the majority of which are self-transmissible. The resistance mechanisms characterised to date can be grouped into four main categories based on their mode of action: adsorption inhibition, DNA penetration blocking, restriction/modification (R/M), and abortive infection (Abi). A number of the Abi systems characterised from the lactic acid bacteria *Lactococcus* operate either directly or indirectly by blocking phage DNA replication (e.g., *abi*A, Hill *et al.,* 1991; *abi*F, Garvey *et al.,* 1995a). However, the exact mechanisms by which the phage DNA replication is inhibited remain to be determined (for a review, see Garvey *et al.,* 1995b). Hill *et al.* (1990) successfully exploited lactococcal bacteriophage ϕ50 DNA in the development of an abortive type of phage resistance. The protective DNA included a 500-bp region rich in secondary structure, which represented the phage origin of replication (*ori*).

Our laboratory is interested in bacteriophages of *Streptococcus thermophilus*, a thermophilic lactic acid bacterium. Little information is available concerning antiphage mechanisms in this species (Larbi *et al.,* 1992). However, Moineau *et al.* (1995) successfully protected several industrial *S. thermophilus* strains from phage infection by the introduction of an R/M determinant from a lactococcal plasmid. The fact that few phage defence mechanisms have been reported or developed for *S. thermophilus* reflects not only the fact that the molecular characterisation of *S. thermophilus* and its phages has started relatively recently but also that, in contrast to *Lactococcus*, plasmids are conspicuously scarce in *S. thermophilus* (Mercenier *et al.,* 1990).

The increasing amount of genome sequence information available for *S. thermophilus* phages should facilitate the development of bacteriophage resistance mechanisms for the protection of industrial starter cultures. Sequence analysis of the *S. thermophilus* ϕSfi21 genome, combined with Southern blot hybridisation and analysis of restriction profiles, led to the definition of a DNA replication module of ~5 kb (Desiere *et al.,* 1997). Based on DNA hybridisation experiments, this DNA module is present in >70% of *S. thermophilus* phages (Brüssow *et al.,* 1994). DNA sequence analysis of this region from ϕSfi21 identified several open reading frames (orfs) putatively coding for enzymes, including primase and helicase, implicated in the initiation of phage DNA rep-

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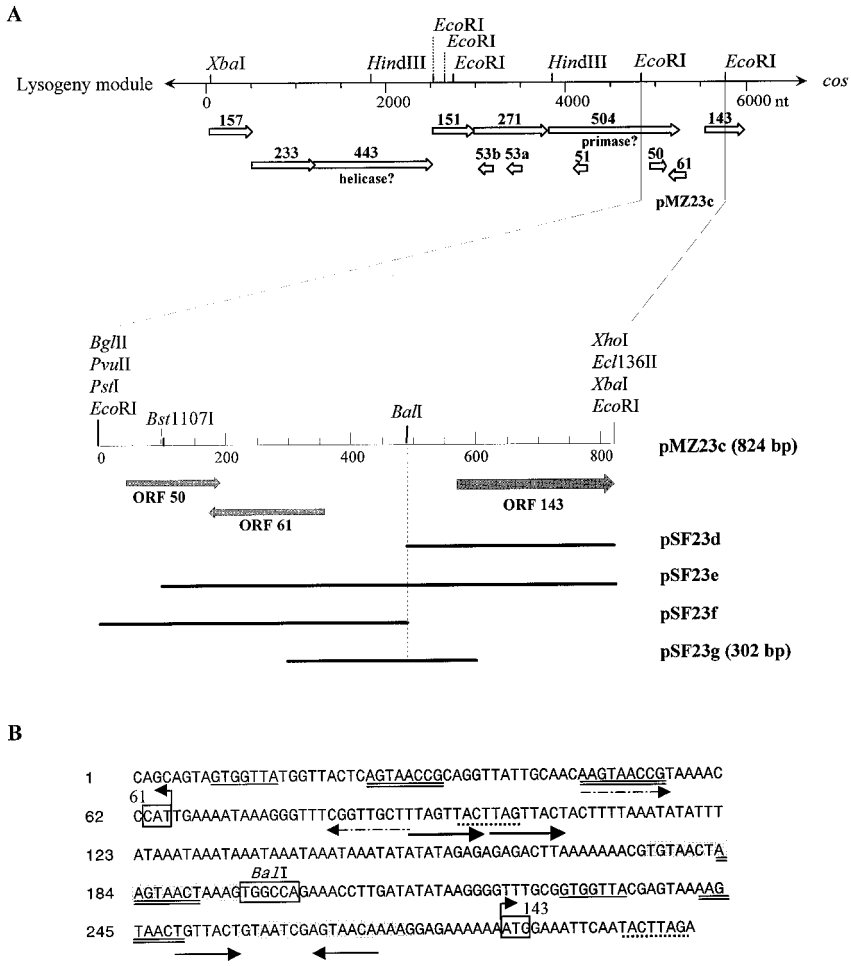

FIG. 1. (A) Schematic representation of the previously defined ϕSfi21 DNA replication module (Desiere *et al.,* 1997) and the subcloning of the phage-inhibitory clone pMZ23c. pMZ23c encodes only the 81 N-terminal amino acids of orf 143. Size of DNA inserts is indicated in brackets. (B) Nucleotide sequence of the 302-bp phage-inhibitory element present on pSF23g. Inverted and tandem repeats are indicated by arrows. Direct repeats are underlined or shaded. The start codons of orf 61 and orf 143 are boxed, as is the *Bal*I restriction site.

lication. An area of particular interest was the 231-nucleotide (nt) noncoding region located between orf 504 and orf 143, which revealed an 80% identity to the putative single-stranded origin (*sso*) of replication of the *S. thermophilus* plasmid pST1 (Janzen *et al.,* 1992). This, together with its location immediately downstream of orf 504, which demonstrated similarity to the helicase domain of P4 primase, and its richness in direct and inverted repeat sequences led to the speculation that the 231-bp noncoding sequence is the origin of ϕSfi21 DNA replication.

In the present communication, we report on the closer examination of the intergenic region and associate it with a biological activity, that of bacteriophage resistance. This study demonstrates that when present on a plasmid, this region can act as an origin of DNA replication. Genome analysis of a phage that was not inhibited by this DNA element led to the characterisation of a variant putative phage *ori.*

RESULTS AND DISCUSSION

Identification of bacteriophage resistance determinant

The clone pMZ23c, consisting of an 824-bp *Eco*RI fragment of ϕSfi21 cloned in the shuttle vector pNZ124 (Fig. 1A), protected the plasmid-free laboratory strain *S. thermophilus* Sfi1 from infection by a large number of phages (Table 1). Of 25 phages that are capable of propagating on Sfi1, 17 are sensitive to the presence of pMZ23c, as demonstrated by a dramatic reduction in the efficiency of plaquing (EOP). In fact, for all 17 phages, no plaques could be detected. This is equivalent to a $>$6-log reduction in phage titres, which compares favourably with the 4-log reduction observed for the phage-encoded resistance (Per) mechanism derived from a lactococcal phage (O'Sullivan *et al.,* 1993). When introduced in the *S. thermophilus* strain Sfi9, pMZ23c also protects against infection by the lytic group II phages ϕSfi11, ϕSfi9, ϕSt12, and ϕSt2 (data not shown).

TABLE 1

Titration of *S. thermophilus* Phages on Sfi1 Transformed with the Indicated Plasmids

Phage	pNZ124[a] (pfu/ml)	pMZ23c (pfu/ml)	pSF23g (pfu/ml)
Sfi21	9.7×10^7	$<10^{2b}$	$<10^2$
S89	1.7×10^8	$<10^2$	$<10^2$
S69	1×10^8	$<10^2$	$<10^2$
S3	2.3×10^7	$<10^2$	$<10^2$
Sfi2	3×10^9	$<10^2$	$<10^2$
Sfi3J	7.5×10^8	$<10^2$	$<10^2$
St3	2.6×10^8	$<10^2$	$<10^2$
St44A	3.2×10^8	$<10^2$	$<10^2$
S19	1×10^8	$<10^2$	$<10^2$
S96	6×10^6	$<10^2$	$<10^2$
St44	6×10^8	$<10^2$	$<10^2$
St42	8×10^8	$<10^2$	$<10^2$
M4-31	4.8×10^8	$<10^2$	$<10^2$
M4-15	2.7×10^6	$<10^2$	$<10^2$
St40	2×10^5	$\leq 10^3$	n.d.[c]
Sfi18	6×10^8	$<10^2$	$<10^2$
St20	3×10^8	$<10^2$	$<10^2$
Sfi19	2.8×10^9	1×10^8	1.4×10^8
St25	2.5×10^8	3×10^7	3×10^8
St33	3×10^8	1×10^6	7×10^6
St28	9×10^8	1×10^7	8×10^6
St17	6×10^8	6×10^7	5×10^8
St159	1×10^9	2×10^8	2×10^8
S17	4×10^7	2×10^7	4×10^7
St30	3×10^9	1×10^9	3×10^9

[a] Phage-sensitive vector control.
[b] $<10^2$ indicates that no plaques were detected on a lawn of bacterial growth.
[c] n.d., not determined.

To define further the DNA sequence responsible for mediating the observed bacteriophage resistance, the *Eco*RI insert fragment of pMZ23c was subcloned (Fig. 1A), and the various subclones were tested for their ability to protect the strain Sfi1 from phage infection. The insert fragment of pMZ23c contains three orfs: orf 61, the oppositely oriented orf 143, and orf 50 (Fig. 1A). The three subclones pSF23d (deletion of orf 50 and orf 61), pSF23e (deletion of orf 50), and pSF23f (deletion of orf 143) protected Sfi1 from infection by ϕSfi21, as determined by the inability to detect plaques (Table 2). Therefore, all three orfs present in pMZ23c could be deleted without loss of the resistance phenotype, suggesting that the observed resistance is not mediated by a phage protein.

The intergenic region between the divergently oriented orf 61 and orf 143 was then tested. A 302-bp DNA fragment, covering the ϕSfi21 sequence showing similarity to the putative pST1 *sso* of replication, was PCR amplified and cloned in pNZ124, generating the construct pSF23g (Fig. 1A). The presence of pSF23g in *S. thermophilus* Sfi1 protected the bacteria from phage infection and was just as effective as the parent clone pMZ23c in terms of reduction in the EOP and range of phages inhibited (Table 1). From these observations, we can conclude that the observed bacteriophage resistance in pMZ23c is mediated by a noncoding DNA sequence. Furthermore, because the noncoding sequences in pSF23g and pMZ23c are in opposite orientations with respect to each other, the inhibitory effect of the 302-bp noncoding sequence must be independent of transcription originating in the vector.

Interestingly, both of the clones pSF23d and pSF23f, in which all of the DNA to the left and right, respectively, of the *Bal*I site is deleted, mediate resistance against phages ϕSfi21, ϕS69, and ϕS89 (Table 2). This implies that pMZ23c contains, in fact, two genetic elements located on either side of the *Bal*I site, each of which independently is capable of protecting the host cell from bacteriophage infection. A closer examination of the nucleotide sequence of pSF23g highlighted a number of sequences repeated to the left and right of the *Bal*I site (Fig. 1B). It should, however, be noted that the protection provided by pSF23g is much stronger than that of pSF23d or pSF23f in terms of the reduction in EOP, and/or the range of phages affected (Table 2). These

TABLE 2

Analysis of pMZ23c Subclones for Bacteriophage Resistance

Phage	pNZ124 (pfu/ml)	pSF23g (pfu/ml)	pSF23d (pfu/ml)	pSF23e (pfu/ml)	pSF23f (pfu/ml)
Sfi21	9.7×10^7	$<10^{2a}$	$<10^2$	$<10^2$	$<10^2$
S89	1.7×10^8	$<10^2$	$<10^2$	$<10^2$	$<10^2$
S69	9.9×10^7	$<10^2$	$<10^2$	n.d.[b]	$<10^2$
S3	2.3×10^7	$<10^2$	$<10^2$	n.d.	8×10^4
ST3	2.6×10^8	$<10^2$	8.8×10^7	n.d.	6×10^7
ST44A	3.2×10^8	$<10^2$	2.3×10^3	n.d.	1.4×10^3
S96	6×10^6	$<10^2$	2.6×10^6	n.d.	10^2

Note. All of the above plasmids were tested in strain Sfi1.
[a] $<10^2$ indicates that no plaques were detected on the lawn of bacterial growth.
[b] n.d., not determined.

differences probably reflect different binding requirements of the corresponding phage proteins.

Effect of copy number on bacteriophage resistance phenotype

pSF28 is a nonreplicative integration vector for *S. thermophilus* based on the integration functions of the phage φSfi21 and the *cat* gene from pC194 as a selectable marker (Bruttin *et al.,* 1997b). To examine whether the 824-nt φSfi21 fragment from pMZ23c is capable of interfering with phage infection when present as a single copy in the bacterial cell, it was cloned as an *Xba*I–*Pst*I fragment in pSF28. This cloning was performed in *Lactococcus lactis* ssp. *lactis* MG1363 because the phage integrase gene present in pSF28 is toxic in *Escherichia coli. S. thermophilus* Sfi1 was subsequently transformed with the resulting construct, pSF20, and the integrants obtained were confirmed by PCR analysis. The bacteriophage sensitivity of this integrant Sfi1::pSF20 was tested by plaque assays. For φSfi21, a 100-fold reduction in the EOP was observed together with a reduced plaque size. However, no reduction in the EOP or plaque size was observed for the two phages φS69 and φS89.

The 824-nt φSfi21 fragment from pMZ23c was subsequently cloned in the low-copy-number vector pNZ121, yielding the construct pSF24. Of the four phages (φSfi21, φS69, φS89, and φSfi3J) tested by plaque assays, pSF24 protected Sfi1 from infection by φSfi21 only (i.e., no plaques observed). This clearly indicates that to be effective against a larger range of phages, the φSfi21-derived DNA fragment must be provided at a high copy number.

Effect of bacteriophage resistance determinant on phage DNA replication

The relative amount of φSfi21 DNA was determined throughout the phage lytic cycle by comparing the total DNA isolated from phage-infected cells harvested at different times after infection. Using DNA probes specific for the phage or the vector pNZ124, it was possible to simultaneously monitor both phage and plasmid DNA. The high-copy-number clone pMZ23c had a dramatic effect on φSfi21 as demonstrated by the amount of φDNA accumulated in the strain Sfi1(pNZ124) compared with strain Sfi1(pMZ23c) after phage infection. In the phage-sensitive strain Sfi1(pNZ124), φSfi21 DNA was detectable 20 min after infection, increasing to a maximum at 40 min and followed by a reduction at 60 and 80 min (Fig. 2B). For Sfi1 containing the pMZ23c clone, φDNA was detected 20 min after infection, but in contrast to Sfi1(pNZ124), no further accumulation of φDNA was observed (Fig. 2B). A similar pattern was observed for Sfi1(pSF23g) (data not shown). When φDNA was monitored in Sfi1(pSF24), a pattern similar to the phage-sensitive control Sfi1(pNZ124) was observed, but the amount of DNA accumulated 40 min after phage infection was reduced in Sfi1(pSF24) compared with Sfi1(pNZ124) (Fig. 2A).

Plasmid replication driven by bacteriophage infection

To examine the effect, if any, of phage infection on the replication of a plasmid carrying the φSfi21-derived DNA fragment, Sfi1 containing the low-copy-number clone pSF24 was chosen. A significant increase in the pSF24 copy number was observed when the quantity of pSF24 DNA present in uninfected Sfi1(pSF24) was compared with that of φSfi21-infected cells, 40 min after infection (Fig. 3). Although φSfi21 infection apparently boosted pSF24 plasmid DNA replication, infection with the heterologous phage φS69 had no observable effect on pSF24 copy number (Fig. 3). This correlates with the plaque assay results that indicated the high-copy-number clone pMZ23c had an inhibitory effect on a large number of phages, including φSfi21 and φS69, whereas the low-copy-number clone pSF24 was effective against only φSfi21.

The simultaneous monitoring of phage (Fig. 2) and plasmid (Fig. 3) DNA indicated that although the accumulation of phage DNA was inhibited, the replication of a plasmid containing the φSfi21-derived insert was stimulated on phage infection. Apparently, the cloned φSfi21 DNA serves as an *ori* on the plasmid driven by phage-encoded proteins. The experimental data therefore suggest a working hypothesis wherein this noncoding φSfi21-derived DNA serves as a binding site(s) for phage proteins which, when multiple copies are present, are capable of titrating essential phage proteins such that they are no longer available for phage DNA replication.

Bacteriophages have adopted a plethora of replication strategies that differ mainly in the method of priming and the dependence on host- and phage-encoded proteins. Among the phages of gram-positive bacteria, only the *Bacillus* phages φSPP1 (Pedré *et al.,* 1994) and φ29 (which uses protein-primed replication; Salas *et al.,* 1995) have been extensively characterised in terms of their replication functions. In contrast, although considerable sequence data exist for phages of lactic acid bacteria, replication origins have been identified only for the lactococcal phages φsk1 (Chandry *et al.,* 1997), φc2 (Waterfield *et al.,* 1996), and φ50 (Hill *et al.,* 1990; O'Sullivan *et al.,* 1993). The putative *ori* of the lactococcal φ31 has been functionally identified, although no sequence data are available (O'Sullivan *et al.,* 1993). As yet, no information is available concerning the general mechanisms of replication or the phage and host proteins required. All of the putative origins of lactic acid bacteria phages are rich in direct repeat sequences, but there is no apparent correlation in the size, relative location, or sequence of the repeated elements between lactococcal phages and those of *S. thermophilus*. In this report, we describe a

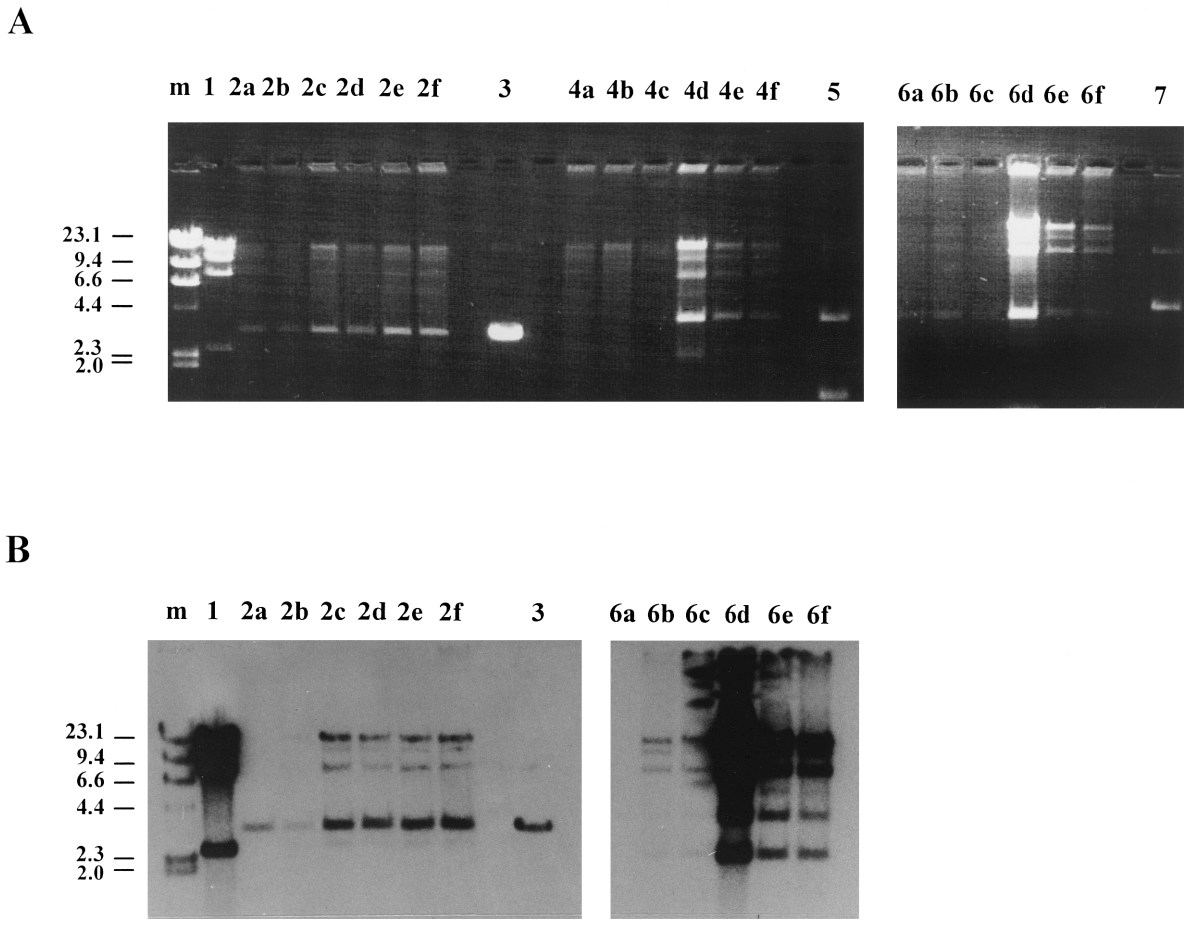

FIG. 2. (A) Agarose gel electrophoresis of total DNA isolated at various time points during phage ϕSfi21 infection. The cultures tested were Sfi1 containing pNZ124 (lanes 6a–f), pMZ23c (lanes 2a–f), and pSF24 (lanes 4a–f). a–f represent total DNA isolated before phage infection, immediately after infection, and 20, 40, 60, and 80 min after infection, respectively. Lane 1 contains ϕSfi21 DNA. Lanes 3, 5, and 7 contain pMZ23c, pSF24, and pNZ124 plasmid DNA, respectively. All DNA samples were digested with *Xba*I. The size of the λ-*Hin*dIII DNA molecular weight marker (lane m) is indicated in kilobases. (B) Hybridisation of the samples indicated in panel A using labeled λ and ϕSfi21 DNA as probes.

sequence and functional symmetry for the ϕSfi21-derived origin fragment. The observed symmetry is, however, very different than that of the 500-bp ϕ50 origin of replication identified by Hill *et al.,* (1990), which consists of a 236-bp direct repeat separated by 28 bp and for which only one phage-inhibitory determinant was described.

S. thermophilus plasmid pST1 does not mediate bacteriophage resistance

The selection pressure maintaining cryptic plasmids in lactic acid bacteria has not been defined. Because the ϕSfi21-derived DNA described above shows 80% identity to the putative *single-stranded* origin of the cryptic *S. thermophilus* plasmid pST1 (Janzen *et al.,* 1992), it may be interesting to examine whether a phage-inhibitory activity could be associated with the pST1 *sso.* For this purpose, the *sso* region of pST1 was PCR amplified and cloned in pNZ124. The presence of the cloned putative *sso* from pST1 in *S. thermophilus* Sfi1 did not protect the host from infection by phages in our collection or those in Kiel, from which the pST1-containing *S. thermophilus* strain was obtained (H. Neve, personal communication).

Binding of phage-specific complex to putative ϕSfi21 replication origin

The model described above for phage inhibition depends on a specific interaction between a phage-encoded protein(s) and the putative *ori* of ϕSfi21. To test this, gel retardation assays were performed using a labeled 302-bp DNA fragment corresponding to the phage-inhibitory DNA from ϕSfi21 and crude cell extracts prepared from ϕSfi21-infected and uninfected Sfi1 cells. ϕSfi21 infection resulted in a DNA fragment of lower mobility in polyacrylamide gels (Fig. 4). The specificity of the protein–DNA interaction was demonstrated by competition experiments in which the presence of a 500-fold excess of the unlabeled DNA fragment abolished the retardation (data not shown). Interestingly, when the same experiment was repeated using crude cell extracts

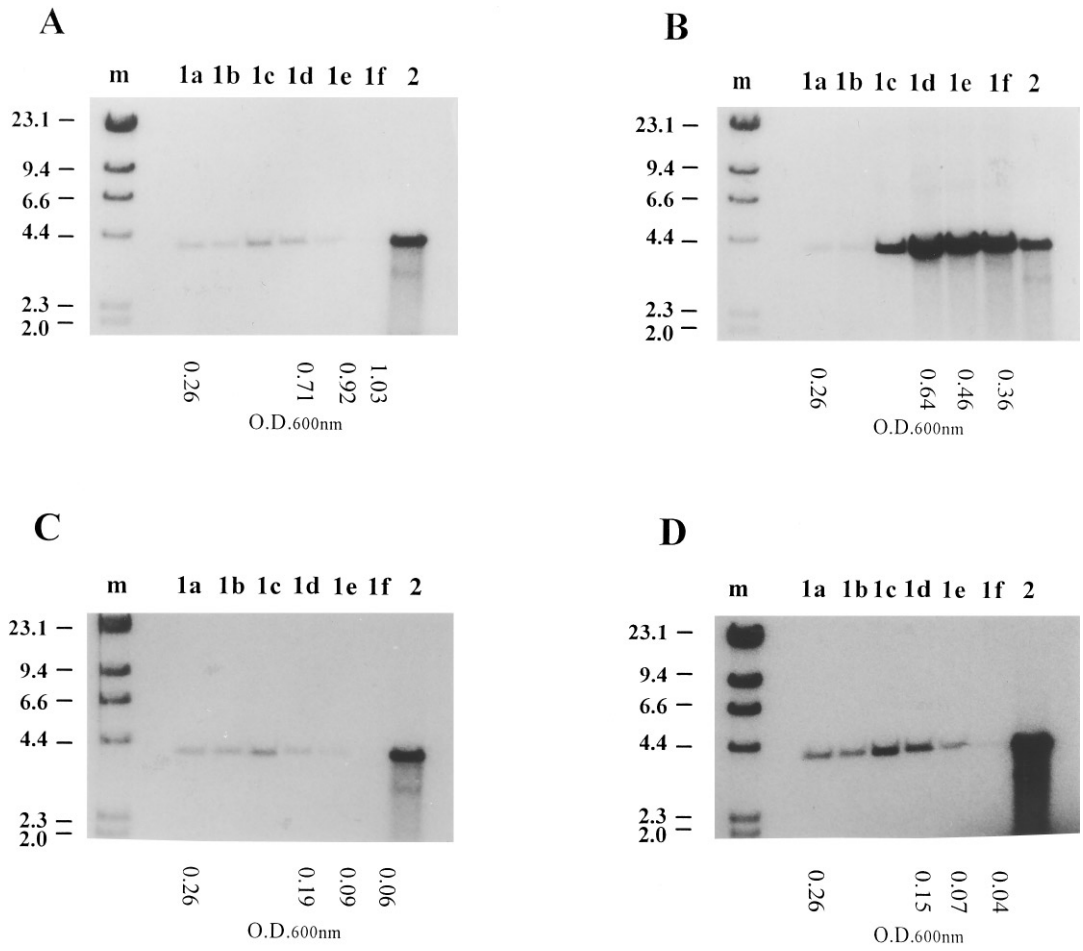

FIG. 3. Hybridisation of *Xba*I-digested total DNA isolated from Sfi1(pSF24) without phage infection (A) and infected with ϕSfi21 (B), ϕS69 (C), and ϕSfi19 (D). The probe consisted of labeled λ and pNZ121 DNA. 1a–1f represent total DNA isolated at various time points: before phage infection, immediately after infection, and 20, 40, 60, and 80 min after infection, respectively. Lane 2 contains *Xba*I-digested pSF24 plasmid DNA. The size of the λ-*Hin*dIII DNA molecular weight marker (lane m) is indicated in kilobases. Measurements obtained of OD_{600nm} at various time points are also indicated.

prepared from ϕSfi19-infected Sfi1, no retarded protein–DNA complex was observed (Fig. 4). This observation is significant because the ϕSfi21-derived DNA has no inhibitory effect on infection by ϕSfi19.

Sequence comparison of the ϕSfi19 and ϕSfi21 DNA replication modules

Although pMZ23c is effective against a range of phages, eight of the phages tested in this study, including ϕSfi19, are insensitive to the ϕSfi21-derived DNA (Table 1). Comparative sequencing of a 17-kb region of ϕSfi21 and ϕSfi19 encompassing the lytic cassette and the structural gene cluster revealed that these two phages are similarly organised (Desiere *et al.,* 1998). Therefore, it was of interest to extend this comparison to the region encompassing the DNA replication module (previously defined for ϕSfi21 by Desiere *et al.,* 1997) because it may facilitate the identification of the genetic determinant or determinants responsible for the insensitivity of ϕSfi19 to the ϕSfi21-derived DNA element. ϕSfi19 revealed an identical organisation of orfs and a high degree of sequence similarity to ϕSfi21 (Table 3). An interesting observation was the near absence of silent point mutations (Table 3).

A sequence alignment of the inhibitory ϕSfi21 DNA element with the corresponding intergenic region of ϕSfi19 revealed an overall nucleotide identity of 95% (i.e., 14-nt differences over a stretch of 302 nt; Fig. 5). When this comparison was extended to eight additional phages, a clear division emerged in which phages sensitive or insensitive to the ϕSfi21-derived DNA yielded a sequence resembling that of ϕSfi21 and ϕSfi19, respectively (Fig. 5). Exclusion of the polymorphisms within the ϕSfi21 group identified nine nucleotide differences between the ϕSfi21- and ϕSfi19-type sequences. An extension of this sequence comparison to a broader range of phages may lead to the identification of the critical nucleotides determining the

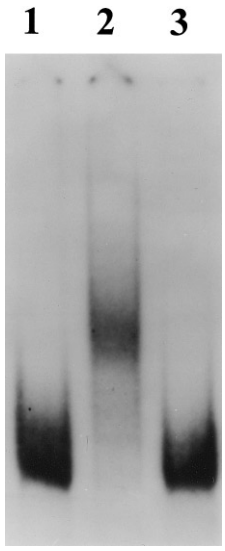

FIG. 4. Gel retardation assay using the ^{32}P-labeled 302-bp phage-inhibitory DNA fragment from φSfi21. Crude cell extracts were prepared from *S. thermophilus* Sfi1 infected with φSfi21 (lane 2) and φSfi19 (lane 3). Lane 1 is a control lane in which a crude cell extract prepared from uninfected Sfi1 cells was used.

specificity of the protein–DNA interactions at the phage putative replication origin.

The differences between the putative origins could reflect the molecular basis for the insensitivity of φSfi19 to the φSfi21 origin. To test this, the noncoding DNA region of φSfi19, located downstream of orf 504, was PCR amplified and cloned in pNZ124, resulting in the construct pSF191. Sfi1(pSF191) was found to be resistant to φSfi19 infection. Sfi1(pSF191) was then tested against the remaining seven phages, which were insensitive to the resistance mechanism provided by pMZ23c and was found to be resistant (i.e., no plaques) to all but two phages: φS17 and φST30. It is interesting to note that φS17 and φST30, which are insensitive to the φSfi21 and φSfi19 origin-mediated phage resistance, do not possess the highly conserved 2.2-kb *Eco*RI DNA fragment present in >70% of *S. thermophilus* phages (data not shown; Brüssow *et al.,* 1994).

Paradoxically, the φSfi19-derived fragment in pSF191 protects Sfi1 from infection by φSfi21 and the other phages inhibited by the φSfi21 DNA element, whereas in contrast, the φSfi21-derived fragment does not protect against φSfi19. Apparently, a φSfi21 protein is capable of interacting with both types of origin, whereas the equivalent φSfi19 protein is origin specific. In phages, DNA sequences targeted by proteins are frequently found directly in the vicinity of the gene coding for the protein in question. The genes preceding orf 504 in the DNA replication module are unlikely to code for the origin-binding protein because all of the amino acid replacements in φSfi19 proteins (compared with φSfi21) were also detected in proteins predicted for φSfi11 or φSfi18 (Table 3), both of which are susceptible to the φSfi21-derived inhibitory activity. Interestingly, the orf 504 gp of φSfi21 and φSfi19 differ in only one amino acid position in which a C-terminal lysine residue is substituted by a glutamic acid residue in φSfi19. The difference between a basic and acidic residue may have consequences for protein-folding. To explain the complex inhibitory activity of the different origins will require a sequence comparison (both nucleotide and amino acids) of a broader range of phages together with the elucidation of the specific DNA–protein interaction(s) and the identification of the components involved.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and media

The *E. coli* strain XL1-Blue was propagated in LB broth or on LB broth solidified with 1.5% (w/v) agar (Sambrook

TABLE 3

Comparison of the Predicted Gene Products within the DNA Replication Modules of φSfi21 and φSfi19

orf gp of φSfi21	Size (aa) of equivalent orf gp in φSfi19	No. of aa (nt) changes between φSfi21/φSfi19	No. of aa (nt) changes between φSfi21/φSfi11	No. of aa changes specific to φSfi21/φSfi19[a]
157	157	2 (7)	2 (7)	0
233	233	1 (1)	1 (1)	0
443	443	0 (1)	0	0
151	151	6 (6)	8 (8)	0
271	271	4 (5)	2 (3)	0
504[b]	504[b]	1 (2)	0	1[c]
143	143	0 (0)	0	0

[a] φSfi21 predicted gene products were compared to those of φSfi11 and φSfi18 in order to identify the amino acid (aa) replacements specific to the φSfi21/φSfi19 comparison.

[b] Several start codons are possible for the putative primase gene. The one with the most suitable ribosome binding site (RBS) is UUG at position 3758 giving an orf, orf 504 as was selected for φ01205 (Stanley *et al.,* 1997).

[c] Comparison of φSfi21 and φSfi19 orf 504 gp revealed a Lys to Glu transition (Fig. 5).

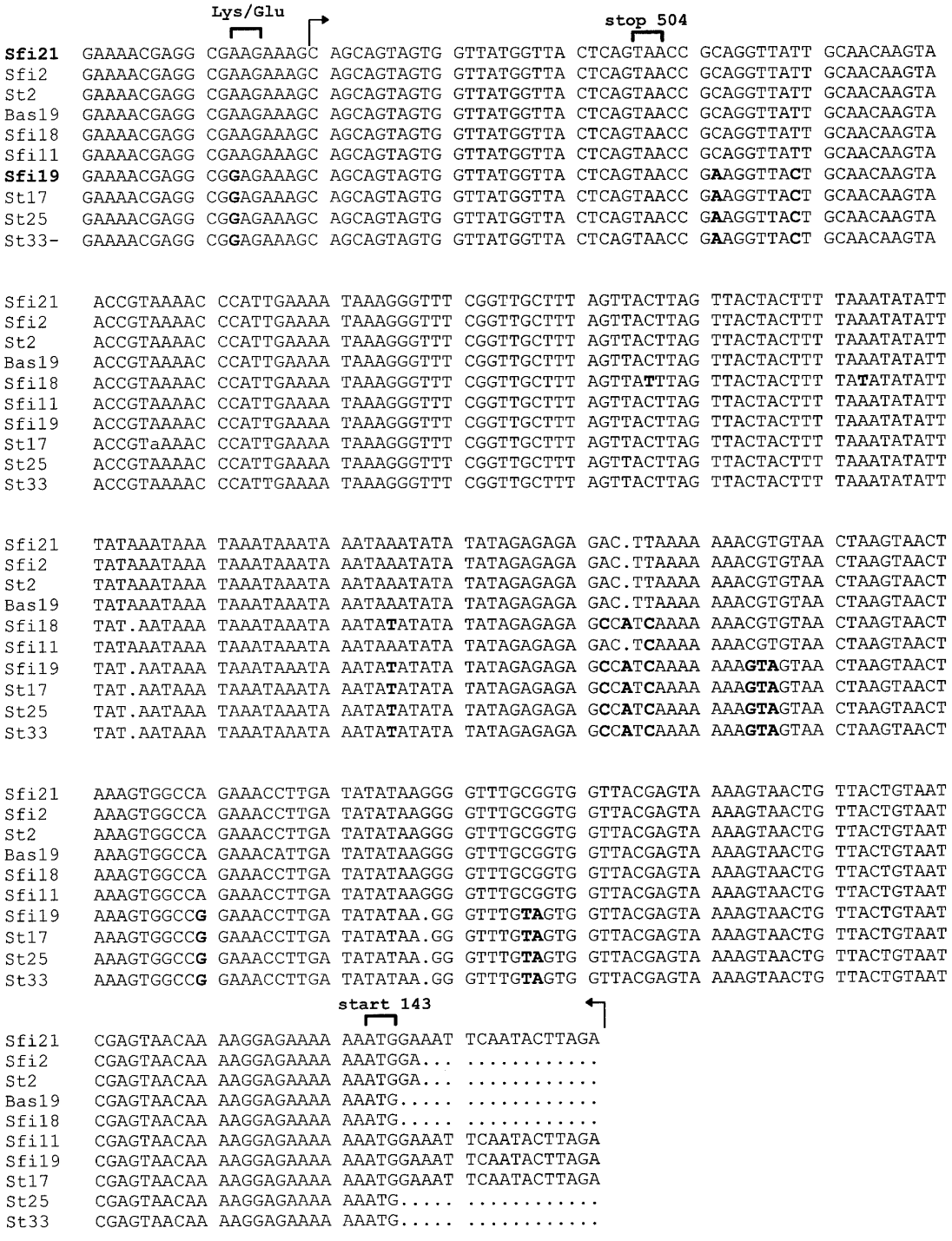

FIG. 5. Nucleotide sequence alignment of the phage-inhibitory sequence derived from ϕSfi21 and ϕSfi19 and comparison with the equivalent sequence from additional *S. thermophilus* phages. Sequence differences between the ϕSfi21- and ϕSfi19-type sequences are in bold. The single amino acid difference (Lys/Glu) in orf 504 gp is indicated. The region corresponding to the 302-bp phage-inhibitory fragment is indicated by arrows.

et al., 1989) at 37°C under agitation. *S. thermophilus* strain Sfi1 and transformants thereof were routinely subcultured at 42°C in either LM17 (M17 supplemented with 0.5% lactose) (Terzaghi and Sandine, 1975) or Belliker (Elliker plus 1% beef extract) media. *Lactococcus lactis* MG1363 was propagated in GM17 (M17 supplemented with 0.5% glucose). Chloramphenicol was used when required at a final concentration of 3, 5, and 20 μg/ml, respectively, for *S. thermophilus*, *Lactococcus*, 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 as described previously (Brüssow

and Bruttin, 1995; Bruttin and Brüssow, 1996). Phage enumeration was achieved by plaque assay using LM17 agar supplemented with 0.25% (w/v) glycine and 0.1% (w/v) skim milk, to enhance plaque formation, and MRS top agar.

DNA techniques

Phage purification, DNA extraction and purification, agarose gel electrophoresis, Southern blot hybridisation, and DNA labelling were done as described previously (Brüssow and Bruttin, 1995; Bruttin *et al.,* 1997a; Bruttin and Brüssow, 1996). 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* was electrotransformed as outlined in the BioRad instruction manual. *Lactococcus* and *S. thermophilus* were electrotransformed using the procedures described by Holo and Nes (1989) and 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 ϕSfi21 and ϕSfi19 DNA sequences 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 according to the manufacturer's instructions.

DNA sequencing and analysis

DNA was sequenced on both strands by the Sanger method of dideoxy-mediated chain termination using the *fmol*™ DNA Sequencing System of Promega (Madison, WI). The sequencing primers were end-labeled with [γ-^{33}P]ATP according to the manufacturer's instructions. The thermal cycler (Perkin-Elmer) was programmed at 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min. The sequence obtained was analysed as previously described by Desiere *et al.* (1998). The relevant accession numbers for ϕSfi21 and ϕSfi19 sequences are AF004379 and AF077306, respectively.

Construction of plasmids

The cloning vectors used in this study were as follows: the high-copy-number *E. coli*–lactococcal–streptococcal shuttle vector pNZ124 (Platteeuw *et al.,* 1994), the low-copy-number shuttle vector pNZ121 (de Vos and Simons, 1994), and the *S. thermophilus* integration vector pSF28 (Bruttin *et al.,* 1997b).

pMZ23c consists of an 824-bp *Eco*RI fragment from the ϕSfi21 DNA replication module cloned in pNZ124. The pMZ23c subclones pSF23d, pSF23e, and pSF23f were constructed by deleting the *Pvu*II–*Msc*I (isochizomer of *Bal*I), *Pvu*II–*Bst*1107I, and *Msc*I–*Ecl*136II fragments, respectively, from pMZ23c.

pSF23g consists of a 302-bp *Eco*RI–*Bgl*II PCR fragment cloned in the high-copy-number vector pNZ124. The PCR fragment was generated using pMZ23c as the template DNA and primers (5′-GCG AAT TCA GCA GTA GTG GTT ATG G-3′ and 5′-GGA GAT CTA AGT ATT GAA TTT CC-3′) containing *Eco*RI or *Bgl*II restriction sites (underlined).

The low-copy-number clone pSF24 was constructed by ligating the 876-bp *Bgl*II–*Xho*I fragment from pMZ23c to *Bgl*II–*Sal*I-digested pNZ121 DNA. For chromosomal integration, the 834-bp *Xba*I–*Pst*I fragment from pMZ23c was cloned in the equivalent sites of the *S. thermophilus* integration vector pSF28, thereby generating the construct pSF20.

pSF191 consisted of a 352-bp *Eco*RI–*Bgl*II-digested PCR fragment cloned in the equivalent sites of the high-copy-number vector pNZ124. The PCR fragment was generated using ϕSfi19 DNA as a template and primers (5′-GCG AAT TCG GGA CGA AAA CGA GGC GG-3′ and 5′-GCA GAT CTC ATT AGG TTC GTG TTC TTG-3′) containing *Eco*RI or *Bgl*II sites (underlined) to facilitate cloning.

Analysis of intracellular phage DNA

Bacterial cultures were grown at 40°C in Belliker broth to an $OD_{600\ nm}$ of 0.2. $CaCl_2$ was added to a final concentration of 10 mM, followed by the addition of the relevant bacteriophage at a multiplicity of infection (m.o.i.) of >2. Samples (1 ml) were removed at regular intervals: before infection, immediately after infection, and at 20, 40, 60, and 80 min after infection. The samples were rapidly centrifuged, and the pellets were frozen by immersion in ultracold ethanol (<-50°C). After thawing, the pellets were resuspended in 1 ml of lysis buffer (6.7% sucrose, 50 mM Tris, 1 mM EDTA, pH 8.0) containing 10 mg/ml lysozyme and incubated for 1 h on ice. SDS was added (125 μl of 10% stock solution) together with proteinase K (50 μl of 20 mg/ml stock) and the lysates were incubated at 65°C for 30 min. RNase A (50 μg/ml) was subsequently added, and incubation was continued at 65°C for an additional 30 min. Two phenol–chloroform extractions were performed on the resulting lysates, followed by an ethanol precipitation. The DNA pellet obtained was washed in 70% ethanol and finally resuspended in 50 μl of distilled water, of

which 25 μl was used for restriction analysis. Restricted DNA samples were electrophoresed, and by hybridisation of Southern blots, the amount of phage DNA was assessed at various time points during infection.

Gel retardation assay

An overnight culture of *S. thermophilus* Sfi1 was inoculated (2%) in Belliker broth and grown to an $OD_{600\ nm}$ of 0.2. $CaCl_2$ was then added (final concentration, 10 mM), and the culture was aliquoted in 50-ml volumes. The cultures were subsequently infected with the relevant phage at an m.o.i. of 2 and incubated at 40°C for 30 min. The cells were harvested by centrifugation and washed, and the pellets resuspended in 750 μl of Tris buffer (10 mM, pH 8, 4°C). The crude cell extracts were prepared as described by Foley *et al.* (1996).

A 302-bp *Bgl*II-digested PCR-generated DNA fragment corresponding to the phage DNA insert in pSF23g was end-labeled with the Klenow fragment of DNA polymerase I (New England Biolabs) in the appropriate buffer and in the presence of 30 μCi of [α-^{32}P]ATP. The labeled DNA was phenol extracted and ethanol precipitated. The binding conditions used were as described by Foley *et al.* (1996) and included 11 μl of crude cell extract ($\sim$5 μg of total protein). After a 10-min incubation at room temperature, 5 μl of 50% glycerol was added to each reaction before 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 3MM Whatman paper and autoradiographed for 36 h.

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