

Molecular Characterization of a Phage-Encoded Resistance System in *Lactococcus lactis*

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A specific fragment of the genome of Tuc2009, a temperate lactococcal bacteriophage, was shown to contain several open reading frames, whose deduced protein products exhibited similarities to proteins known to be involved in DNA replication and modification. In this way, a putative single-stranded binding protein, replisome organizer protein, topoisomerase I, and a methylase were identified. When the genetic information coding for the putative replisome organizer protein of Tuc2009, Rep₂₀₀₉, was supplied on a high-copy-number plasmid vector, it was shown to confer a phage-encoded resistance (Per) phenotype on its lactococcal host UC509.9. The presence of this recombinant plasmid was shown to cause a marked reduction in Tuc2009 DNA replication, suggesting that the observed phage resistance was due to titration of a factor, or factors, required for Tuc2009 DNA replication. Further experiments delineated the phage resistance-conferring region to a 160-bp fragment rich in direct repeats. Gel retardation experiments, which indicated a protein-DNA interaction between this 160-bp fragment and the Rep₂₀₀₉ protein, were performed. UC509.9 strains harboring plasmids with randomly mutated versions of this fragment were shown to display a variable phage resistance phenotype, depending on the position of the mutations.

Lactococcus lactis strains are widely used as starter cultures for many dairy fermentations. Since lactococcal bacteriophages were first identified as a major cause of fermentation failure (46), much research effort has been directed at the development of bacteriophage-resistant starter strains for industrial use (15, 24). Spontaneous bacteriophage-insensitive mutants can be isolated following infection of a bacterial population with a specific phage at a high titer. However, these mutants are only of limited value as starter cultures since their spectrum of resistance tends to be very narrow, while their growth characteristics may also be altered (15, 19).

Naturally occurring phage resistance mechanisms have been identified in wild-type lactococcal strains, and these are often encoded on native conjugative plasmids, which has facilitated the generation of novel resistant starter strains through food grade techniques (5, 39). These resistance mechanisms have been divided into four main groups on the basis of their mode of action (5, 10, 13, 15, 23): adsorption interference, which prevents the adsorption of phage particles to the cell surface; DNA injection blocking, which prevents phages that have successfully attached to the cell surface from injecting their DNA in the cell cytoplasm; restriction-modification, causing intracellular degradation of incoming DNA molecules; and abortive infection, which encompasses a range of mechanisms, any of which may interfere with phage development at any time after the injection of intact phage DNA into the cell up to release of progeny phage from an infected cell.

The advancement of molecular techniques and the increased knowledge of lactococcal phage biology have enabled the development of artificial or "intelligent" phage resistance mechanisms, and a number of these have been described to date.

One such mechanism involves the use of antisense mRNA

(21, 22). It was found that plasmid vectors harboring certain conserved phage genes that had been cloned in the reverse orientation behind a strong promoter conferred an abortive infection-type resistance on host cells against a specific phage. It is thought that this resistance system acts through the formation of a nontranslatable double-stranded mRNA hybrid between a sense and its antisense mRNA which is subject to rapid degradation. Another more recently developed resistance mechanism involves the use of a phage-inducible promoter in combination with the *LlaI* restriction-modification system from the lactococcal plasmid pTR2030 (12). The middle phage-inducible promoter ($\phi 31p$) from bacteriophage $\phi 31$ was cloned upstream of the *LlaIR*⁺ restriction gene so that, upon $\phi 31$ infection of a cell harboring this plasmid, the lethal gene product of *LlaIR*⁺ is produced, resulting in death of the host cell before the infecting phage has a chance to reproduce itself.

The first of these intelligent systems to be described was termed Per 50, for phage-encoded resistance, and conferred resistance against the small isometric-headed phage, $\phi 50$, on *L. lactis* NCK203 (16). The Per 50 system consists of a piece of $\phi 50$ DNA containing the presumptive origin of replication (*ori50*) cloned into a high-copy-number *Streptococcus-Escherichia coli* shuttle vector. Following $\phi 50$ infection, this *ori50*-containing plasmid was shown to interfere with intracellular phage DNA replication in NCK203, while the concentration of intracellular plasmid DNA was seen to increase concurrently. This suggested that *ori50* supplied on the plasmid was responsible for the depletion of an essential DNA replication function, or functions, and was capable of enhancing plasmid replication. In a later study, it was found that the Per phenotype was directly dependent on copy number of the plasmid carrier, while it was also shown that the Per principle can be applied to other phage-host systems (35).

In this report, we have identified a putative replisome organizer protein from the temperate lactococcal phage Tuc2009, designated Rep₂₀₀₉. A region rich in direct repeats which is contained within the *rep*₂₀₀₉ gene, designated *ori*₂₀₀₉, was shown

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TABLE 1. List of bacteriophages, bacterial strains and plasmids used in this study

Phage, bacterial strain, or plasmid	Relevant feature(s)	Source and/or reference
Phage Tuc2009	Isolated following induction of <i>L. lactis</i> subsp. <i>cremoris</i> UC509	2
<i>E. coli</i> XL1-Blue	Transformation host	Stratagene
<i>L. lactis</i> UC509.9	Prophage-cured derivative of <i>L. lactis</i> subsp. <i>cremoris</i> UC509; host for Tuc2009	4
NZ9800	$\Delta nisA$	9
Plasmids		
pBluescript	Cloning vector Ap ^r	Stratagene
pNZ8048	<i>L. lactis</i> expression vector; PnisA Cm ^r (Per ⁻)	9
pSK220	pBluescript + 6.9-kb Tuc2009 genomic insert; Ap ^r	43
pNZRep-1	pNZ8048 + 783-bp Tuc2009 ORF16; Cm ^r (Per ⁺)	This study
pNZRep-3	pNZ8048 + 783-bp Tuc2009 ORF16, in reverse orientation to pNZRep-1; Cm ^r (Per ⁺)	This study
pNZ101	pNZ8048 + 1.5-kb randomly selected Tuc2009 genomic insert; Cm ^r (Per ⁻)	This study
pNZRep-A	pNZ8048 + 283-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁻)	This study
pNZRep-B	pNZ8048 + 526-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁺)	This study
pNZRep-C	pNZ8048 + 295-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁺)	This study
pNZRep-Ci	pNZ8048 + 112-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁻)	This study
pNZRep-Cii	pNZ8048 + 159-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁺)	This study
pNZRep-Ciii	pNZ8048 + 219-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁺)	This study
pNZRep-Civ	pNZ8048 + 187-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁺) ^a	This study
pNZRep-Cv	pNZ8048 + 158-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁺) ^a	This study
pNZRep-D	pNZ8048 + 553-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁺)	This study
pNZRep-E	pNZ8048 + 323-bp Tuc2009 ORF16 subclone; Cm ^r (Per ⁻)	This study

^a Slightly reduced Per effect.

to confer a Tuc2009-resistant phenotype on its lactococcal host, UC509.9. The possible role of *ori*₂₀₀₉ in Tuc2009 replication was further investigated by DNA mutagenesis and protein-DNA interactions between Rep₂₀₀₉ and *ori*₂₀₀₉.

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. Bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1.

Media and growth conditions. *L. lactis* strains were cultured at 30°C in M17 broth (Difco Laboratories, Detroit, Mich.) (42) containing 0.5% glucose (GM17), or in GSB, a modified version of lactic streptococcus broth (LSB) which contains glucose instead of lactose (2). Medium for plaque assays was prepared as described by Lillehaug (26). Briefly, M17 double-layer agar plates were prepared; these plates contained 5 g of glucose/liter, 5 g of glycine/liter, and 10 mM CaCl₂, and 1 and 0.4% agar concentrations were used for the bottom and top layers, respectively. The medium was sterilized by boiling for 5 min in a microwave oven. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C (37). Chloramphenicol at 10 µg/ml and ampicillin at 100 µg/ml were used where appropriate.

Propagation of Tuc2009. Tuc2009 was propagated on a prophage-free derivative of *L. lactis* subsp. *cremoris* UC509, designated UC509.9. UC509.9 was grown to early log phase in GSB, at which point CaCl₂ was added to a final concentration of 10 mM prior to the addition of Tuc2009 phage. Incubation was subsequently continued until lysis occurred. Phage particles were collected from this lysate by centrifugation and concentrated by cesium chloride density gradient centrifugation, as described by Sambrook et al. (37).

Plasmid and phage DNA isolation and molecular cloning. Isolation of *E. coli* plasmid DNA was accomplished by using the QIAprep spin plasmid miniprep kit (Qiagen, Inc., Chatsworth, Calif.) as described in the manufacturers' instructions. Phage genomic DNA was isolated from a concentrated phage solution by the method described by Stanley et al. (41). Restriction enzymes and T4 DNA ligase were purchased from Boehringer GmbH (Mannheim, Germany) and used in accordance with the manufacturer's instructions.

PCR methods. PCR amplifications were carried out by using *Taq* DNA polymerase (Boehringer), as described by the manufacturer with a Gene Amp PCR system 2400 thermal cycler (Perkin-Elmer Cetus, Norwalk, Conn.). Suitable restriction sites were incorporated into the 5' end of synthetic primers when PCR-generated products were used for cloning. Random mutations were introduced into Per-conferring fragments by a method described by Dartois et al. (6),

which involves PCR amplification of DNA fragments with limiting amounts of one of the nucleotides.

DNA sequence analysis. DNA sequence determination of the Tuc2009 genomic subclone in pSK220 was performed with an Applied Biosystems (Foster City, Calif.) 373A automated DNA sequencer using synthetic oligonucleotides (Oligo 1000M; Beckman Instruments) as primers. Assembly of sequences was performed by using the Seqman program of the DNASTAR software package. Database searches were performed by using the BLASTP, BLASTN, and TBLASTN (1) programs with sequences present in the latest releases of the nonredundant databases found at the National Centers for Biotechnology Information website (33a). Sequence alignments were performed by using the Clustal method of the MEGALIGN 3.06 program of the DNASTAR 1996 release software package.

Electroporation procedure. Electrotransformation of plasmid DNA into *E. coli* was performed essentially as described by Sambrook et al. (37), whereas electrotransformation of plasmid DNA into *L. lactis* was performed as described by Wells et al. (45), with the following minor modifications. *L. lactis* cells were grown in GM17 broth containing 2% (wt/vol) glycine. Plasmid DNA for electrotransformation was prepared by precipitating the DNA from a single miniprep prepared from 2.5 ml of *E. coli* cells by using the QIAprep spin plasmid miniprep kit (Qiagen), prior to resuspension in 10 µl of storage buffer (10% glycerol, 0.5 M sucrose) and addition to 40 µl of electrocompetent cells.

Bacteriophage assays. Plaque assays were performed as described by Lillehaug (26). Lysis-in-broth experiments were performed as follows. Strains were grown in GSB (10 ml) to an optical density at 600 nm (OD₆₀₀) of approximately 0.2, followed by the addition of CaCl₂ (final concentration, 10 mM) and 10 µl of a solution containing the desired concentration of Tuc2009 particles, after which the OD₆₀₀ was monitored over a period of time.

Visualization of intracellular phage DNA replication. The procedure described by Hill et al. (16) was used to isolate phage DNA at various time points following infection. Phage DNA was run on an agarose gel and transferred to a Hybond N+ nucleic acid transfer membrane (Amersham Corp., Amersham, United Kingdom) by the procedure described by Southern (40). The enhanced chemiluminescence kit (ECL; Amersham) was used to label DNA for use as a probe according to conditions specified by the supplier.

Cloning and overexpression of Rep₂₀₀₉. A 783-bp fragment exactly encompassing the *rep*₂₀₀₉ gene was cloned into the *Nco*I and *Xba*I sites of the expression plasmid pNZ8048 in order to place it under the control of the nisin-inducible promoter contained on the vector. Restriction sites were incorporated into the *rep*₂₀₀₉ DNA fragment by using PCR with suitably designed synthetic primers to facilitate cloning, such that the original start codon of the *rep*₂₀₀₉ gene, GTG, was changed to ATG. This cloning was performed in *E. coli*, and the integrity of the

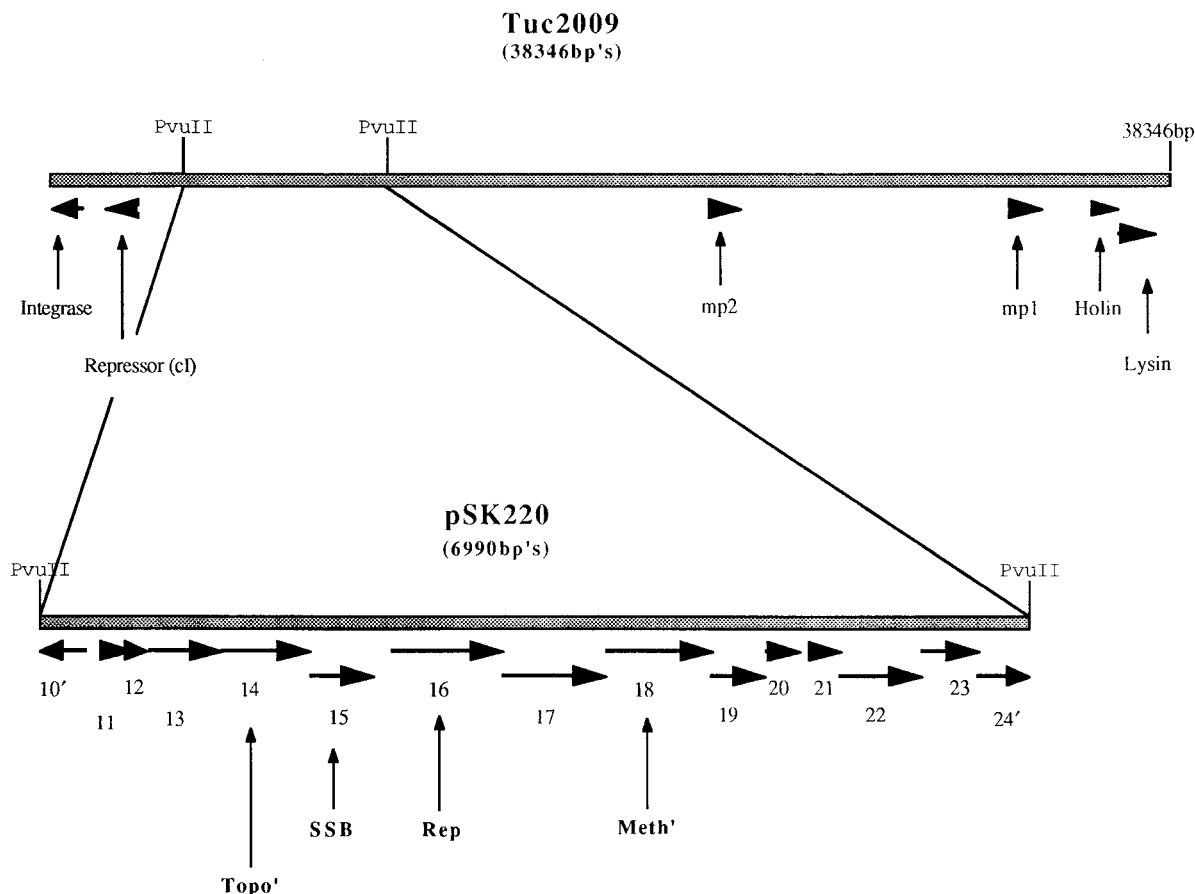


FIG. 1. Schematic representation of the Tuc2009 genome showing putative ORFs, depicted as arrows, to which functions have been assigned. The direction of the arrow is the same as the direction of the ORF. cI, repressor; mp1 and mp2, major structural proteins. The enlarged section represents all identified ORFs contained within the pSK220 subclone. SSB, putative single-stranded-DNA-binding protein; Rep, putative replisome organizer (Rep₂₀₀₉); Meth', putative methylase; Topo', putative topoisomerase I.

insert was confirmed by sequencing. The resulting plasmid, called pNZRep-1, was then introduced into *L. lactis* NZ9800, and Rep₂₀₀₉ was overexpressed as outlined by de Ruyter et al. (9).

Gel retardation assays. To prepare cell extracts, cells from 50-ml cultures of *L. lactis* were collected by centrifugation, resuspended in 500 μ l of breaking buffer (20 mM Tris [pH 8.0], 1 mM dithiothreitol), and ruptured by sonication (five consecutive sonication pulses of 30 s; samples placed on ice for 30 s between sonication pulses). Samples were then centrifuged at $15,000 \times g$ for 5 min in a bench top centrifuge to remove cell debris. Protein concentrations of the sample supernatants were determined with a protein assay kit (Bio-Rad Laboratories GmbH, Munich, Germany), with bovine serum albumin as the standard. Gel retardation assays were performed as outlined below. Probes were labelled with polynucleotide kinase by using [γ -³²P]ATP. DNA binding was carried out in 20- μ l reaction volumes containing 50 mM Tris (pH 8.0), 10% (vol/vol) glycerol, 1 mM EDTA, 5 mM MgCl₂, 500 mM KCl, 2 mM dithiothreitol, 50 μ g of bovine serum albumin/ml, 75 μ g of poly(dI-dC)/ml, probe (approximately 0.3 ng), and 2.5 μ g of total protein from cell lysate. Incubation was for 15 min at room temperature, followed by the addition of 5 μ l of 50% glycerol, after which the samples were loaded onto a 4% polyacrylamide gel containing 2.5% glycerol. Gels were run in TAE buffer (0.04 M Tris-acetate [pH 7.5], 2 mM EDTA) at 120 V for 3 h, dried, and exposed overnight at -70°C to X-Omat film (Kodak).

RESULTS

Nucleotide sequence determination and identification of ORFs. The sequence of the complete genome of Tuc2009 has been determined, and 58 putative open reading frames (ORFs) have been identified (44a). An ORF coding for the putative integrase of Tuc2009 has been designated ORF1, with subsequent ORFs numbered accordingly. Analysis of a *PvuII* frag-

ment derived from the genome of Tuc2009 and contained within plasmid pSK220 revealed the presence of 13 complete and 2 incomplete putative ORFs (Fig. 1). These were identified on the basis of the adopted criteria that an ORF consists of at least 30 codons preceded by a potential Shine-Dalgarno sequence at an appropriate distance (6 to 15 bp) from one of the commonly used initiation codons (AUG, UUG, GUG) (13, 14, 30). The nucleotide sequences and the deduced amino acid sequences of the putative ORFs were compared to the contents of the available databases. Eleven of the 15 deduced proteins showed significant similarity to sequences in the databanks (see Table 2 for percent similarity). These ORFs are discussed below.

(i) **ORF11.** The protein specified by ORF11 shows a high degree of similarity to the ORF71 protein product from ϕ 31 (11). This protein has no known function.

(ii) **ORF14.** The deduced protein product of ORF14 shows a high degree of similarity to the protein specified by ORF11 of the temperate lactococcal bacteriophage TP901-1, which in turn shows a low degree of similarity to topoisomerase I from two different *Mycoplasma* species (27). Topoisomerase I enzymes play a role in the relaxing of supercoiled DNA. The deduced ORF14 protein product also shows significant similarity to two other proteins of unknown function from the databases: ORF35 of the lytic lactococcal phage sk1 (3) and ORF14 of another lytic phage, bil170 (accession no AF009630).

TABLE 2. General features of putative ORFs contained on the pSK220 subclone of Tuc2009 and comparison to sequences from databases

ORF	Start position (codon)	Stop position (codon)	Size of protein (kDa)	Function or similarity	P^a	Match (%) ^b	Accession no. (reference)
10	4935 (ATG)	4660 (TAG)	9.9	Unknown			
11	5038 (ATG)	5253 (TAG)	8.4	ORF71 from ϕ 31	$1e^{-35}$	98 (71)	U51128 (11)
12	5255 (ATG)	5353 (TGA)	3.7	Unknown			
13	5369 (ATG)	5887 (TGA)	20.1	Unknown			
14	5896 (ATG)	6519 (TAA)	24.0	ORF11 from TP901-1 Gene e12 from bil170	$1e^{-109}$ $2e^{-60}$	92 (207) 58 (203)	Y14232 (27) AF009630 (direct submission)
				ORF35 from sk1	$3e^{-62}$	61 (203)	AFO11378 (3)
15	6519 (ATG)	6971 (TAA)	16.6	Single-stranded binding protein from: <i>B. subtilis</i> ϕ PVL <i>H. influenzae</i> <i>E. coli</i>	$1e^{-37}$ $1e^{-35}$ $6e^{-14}$ $7e^{-12}$	67 (110) 51 (157) 37 (105) 41 (117)	P37455 (34) AB009866 (20) U04997 (18) g70816 (38)
16	7096 (GTG)	7878 (TAA)	29.3	ORF11 from r1t	$4e^{-10}$	30 (156)	U38906 (44)
17	7878 (ATG)	8603 (TGA)	27.0	Unknown			
18	8600 (ATG)	9358 (TAA)	29.4	Methyltransferase from: <i>Bacillus stearothermophilus</i> <i>Streptococcus pneumoniae</i> <i>Escherichia coli</i>	$5e^{-15}$ $9e^{-10}$ $4e^{-10}$	28 (231) 25 (259) 46 (65)	X97069 (7) P09358 (8) U48806 (31)
19	9348 (TTG)	9737 (TAA)	15.8	ORF14 from r1t	$2e^{-70}$	97 (128)	U38906 (44)
20	9741 (ATG)	9980 (TAA)	9.4	ORF16 from r1t	$2e^{-37}$	91 (79)	U38906 (44)
21	10092 (ATG)	10271 (TAA)	6.9	ORF17 from r1t	$7e^{-28}$	100 (59)	U38906 (44)
22	10261 (ATG)	10830 (TAG)	22.4	ORF18 from r1t	$3e^{-85}$	83 (189)	U38906 (44)
23	10841 (ATG)	11236 (TAA)	15.5	ORF115 from ϕ g1e	$2e^{-04}$	31 (124)	X98106 (25)
24	11229 (ATG)	11774 (TGA)	21.0	ORF1 from <i>L. lactis</i> ORF19 from r1t	$3e^{-18}$ $7e^{-06}$	37 (193) 34 (121)	Y11901 (36) U38906 (44)

^a Derived from BLASTP score; P , probability that the observed similarity had occurred by chance. Low P values indicate a high probability of two proteins being structurally related.

^b The number of amino acids over which the percentage match was determined is shown in parentheses.

(iii) **ORF15.** The protein specified by ORF15 shows significant similarity to single-stranded-DNA-binding (SSB) proteins from a wide variety of sources; a number of examples are given in Table 2. SSB proteins, also called helix-destabilizing proteins, play a number of roles in DNA replication, repair, and recombination. One of their main functions is to stabilize the transiently formed single strands of DNA that are generated as the replication fork moves through the DNA during replication (29, 30).

(iv) **ORF16.** The deduced protein product of ORF16, designated here as Rep₂₀₀₉, shows similarity to the protein specified by ORF11 of r1t (44) (30% identity), which in turn shows similarity to G38P of the *Bacillus subtilis* phage SPP1 (47) (26.7% identity). G38P has been demonstrated to function as a replisome organizer in SPP1 DNA replication. Replisome organizer proteins play a key role in the initiation event of DNA replication which occurs at a specific point on the genome, termed the origin of replication (*ori*), via a protein-DNA interaction (29). It has been demonstrated that a sequence containing several direct repeats (*oriL*) within *gene38* of SPP1 functions as an *ori*. Sequences containing direct repeats can also be identified within ORF11 of r1t and ORF16 of Tuc2009.

It is on this basis that we putatively identify the direct repeat-containing sequence within *rep*₂₀₀₉ as an *ori* for Tuc2009, designated *ori*₂₀₀₉. The deduced protein product of *rep*₂₀₀₉ shows a higher degree of similarity in the N-terminal region to the protein specified by ORF11 of r1t, whereas a higher degree of similarity is observed in the C-terminal region between this protein and G38P of SPP1. This may represent a modular-type conservation of the genetic material required to initiate DNA replication in the evolution of these phages.

(v) **ORF18.** The protein specified by ORF18 shows similarity to type II methyltransferases from a variety of organisms, several examples of which are given in Table 2. This type of methyltransferase methylates exocyclic amino nitrogens on the DNA to form N_6 -methyladenine or N_4 -methylcytosine (28). The protein product specified by this ORF may be involved in the methylation of specific sites on newly replicated Tuc2009 DNA, thus protecting it from degradation by host-encoded endonucleases which recognize those sites. A similar methylase enzyme has previously been identified in ϕ 50 (17). It has been suggested that ϕ 50 acquired the structural methylase gene, *LlaI*, via an in vivo genetic exchange event between the bacteriophage resistance-conferring lactococcal plasmid pTR2030

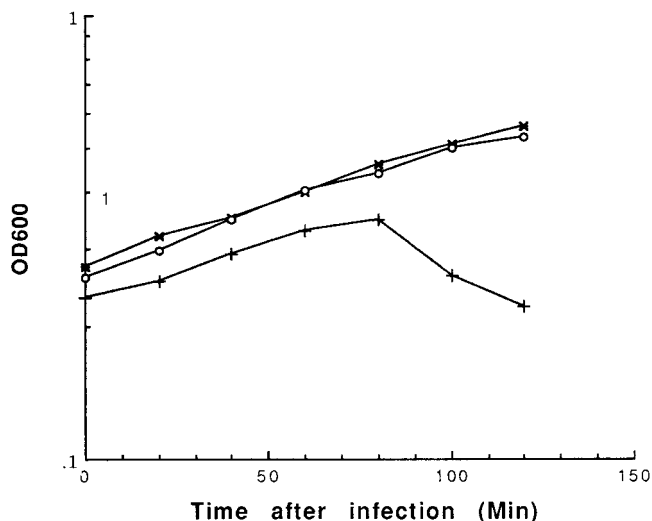


FIG. 2. Lysis-in-broth experiment. Phage was added at $T = 0$ at a multiplicity of infection of approximately 0.5, and the OD_{600} was measured over time. Increasing levels of Tuc2009 infection eventually caused lysis of UC509.9 harboring pNZRep-1 (data not shown). Symbols: \times , UC509.9(pNZRep-1) uninfected; \circ , UC509.9(pNZRep-1) plus Tuc2009; $+$, UC509.9(pNZ8048) plus Tuc2009.

and the $\phi 50$ genome. The acquisition of this functional methylase thus confers a selective advantage on $\phi 50$.

(vi) **ORF19 to -22.** The deduced protein products of ORF19 to -22 show high degrees of similarity to the proteins specified by ORF14, -16, -17, and -18 of r1t, respectively (44). These proteins have no assigned function.

(vii) **ORF23.** The protein specified by ORF23 shows similarity to the deduced protein product of ORF115 of a temperate lactobacillus phage, $\phi g1e$. This protein is of no known function.

(viii) **ORF24.** The deduced protein product of ORF24 shows similarity to ORF19 of r1t (44) and ORF1 of *Lactococcus lactis* subsp. *cremoris* S114 (36). No function has been ascribed to these proteins.

rep₂₀₀₉ confers a Per phenotype. It has been suggested previously that phage origins of replication, when supplied in *trans*, confer on the host a phage-resistant phenotype against specific phages (16, 35). To determine whether the putative origin of replication of Tuc2009, *ori₂₀₀₉*, had the ability to confer a Tuc2009-resistant phenotype on UC509.9, the following experiment was performed. The complete *rep₂₀₀₉* gene was cloned on the high-copy-number plasmid pNZ8048. The resulting recombinant plasmid, pNZRep-1, was introduced into UC509.9. Plaque assay and lysis-in-broth experiments were then performed to determine if pNZRep-1 conferred a phage-resistant phenotype.

When present in UC509.9, pNZRep-1 strongly inhibited the ability of Tuc2009 to form plaques, the size of which was reduced from 1 to 1.5 mm to pinpoint. The efficiency of plaquing was determined to be 0.1 on UC509.9 harboring pNZRep-1. Lysis-in-broth experiments indicated that UC509.9 carrying pNZRep-1 exhibited an increased resistance to lysis by Tuc2009 at levels of infection that lysed control strains (Fig. 2).

To verify that the observed Per phenotype was specifically due to the presence of the *ori₂₀₀₉*-containing DNA fragment and not just a general phenomenon displayed by phage fragments distributed throughout the Tuc2009 genome, and also to determine if the orientation of *ori₂₀₀₉* had any effect on Per, two additional plasmids were constructed. (i) A 1.5-kb frag-

ment of Tuc2009 DNA was selected randomly and cloned in pNZ8048 to create pNZ101. When UC509.9 harboring pNZ101 was challenged with Tuc2009, no difference in plaque size, efficiency of plaquing, or resistance in broth was observed when compared with these characteristics in UC509.9 harboring the control plasmid pNZ8048, indicating that the observed Per phenotype is specifically due to the *rep₂₀₀₉* gene on pNZRep-1 (data not shown). (ii) The *rep₂₀₀₉* gene was cloned in the reverse orientation in pNZ8048, to create pNZRep-3. When UC509.9 containing pNZRep-3 was challenged with Tuc2009, an phenotype identical to that of UC509.9 containing pNZRep-1 was observed, indicating that the phage resistance conferred by *ori₂₀₀₉* is orientation independent (data not shown).

The presence of pNZRep-1 affects intracellular Tuc2009 DNA replication. It has been shown for $\phi 31$ and $\phi 50$ that Per-conferring DNA fragments interfere with intracellular phage DNA replication (16, 35). To determine if this was the case for Tuc2009, total DNA was isolated at 20-min time intervals from two separate phage-infected UC509.9 cultures, one harboring pNZRep-1 and the other harboring the control plasmid pNZ8048. Samples of these DNA preparations were then subjected to agarose gel electrophoresis, followed by Southern blot analysis using a 1.9-kb Tuc2009 DNA PCR-generated fragment, containing ORF11 to -15, as a probe. As can be seen from Fig. 3, following infection of the control strain (time $[T] = 0$), Tuc2009 DNA replication commences at $T = 40$ min, followed by a dramatic increase until cell lysis arrests

1 2 3 4 5 6 7 8 9 10 11 12 13 14

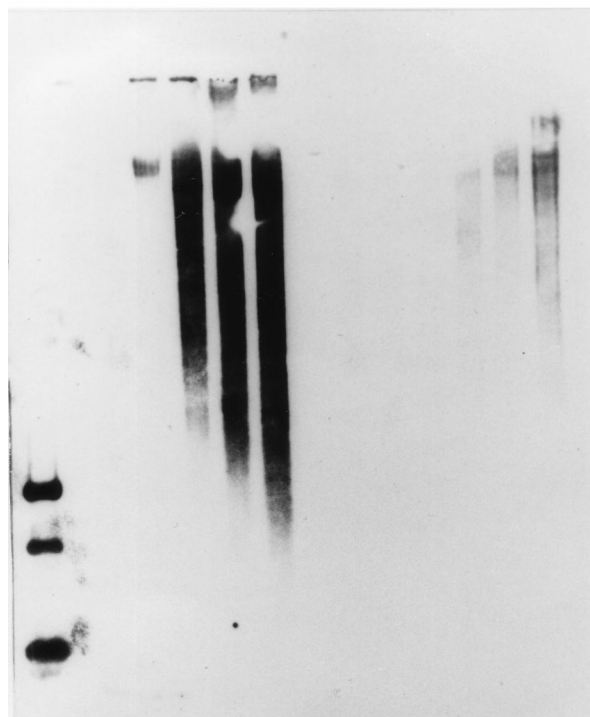


FIG. 3. Southern blot analysis showing the effect of pNZRep-1 on intracellular Tuc2009 DNA replication. Lanes: 1, positive control (*RsaI* digest of Tuc2009 genomic DNA); 2 to 7, total cellular DNA, isolated at 20-min intervals, from Tuc2009-infected UC509.9 harboring pNZ8048 (lane 2, $T = 0$); 8, blank; 9 to 14, total cellular DNA, isolated at 20-min intervals, from Tuc2009-infected UC509.9 harboring pNZRep-1 (lane 9, $T = 0$). Tuc2009 was added at a multiplicity of infection of approximately 0.1. The blot was probed with a 1.9-kb PCR-generated Tuc2009 DNA fragment.

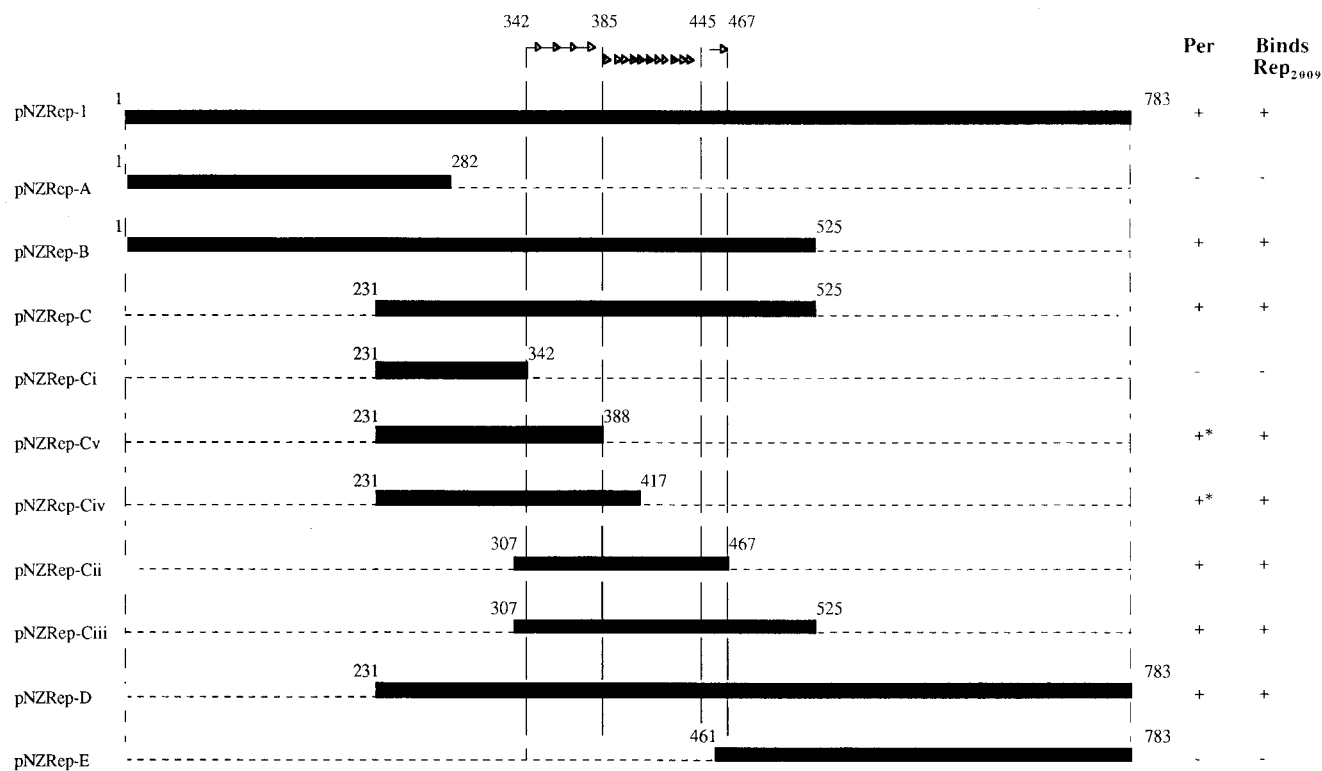


FIG. 4. Diagram showing the experiment performed to determine the minimum DNA fragment from *rep*₂₀₀₉ that confers a Per phenotype on UC509.9. Synthetic oligonucleotide primers were designed to generate subfragments spanning the *rep*₂₀₀₉ gene by PCR. These fragments were then cloned in pNZ8048 and introduced into UC509.9. Plaque assay and lysis-in-broth experiments were performed to determine which fragments conferred a Per phenotype, which is indicated in the column labelled Per. Radioactive probes, made from these *rep*₂₀₀₉ subfragments, were used in gel retardation experiments to determine if the Rep₂₀₀₉ protein was capable of binding to the fragments (column labelled Binds Rep₂₀₀₉). Arrows indicate the positions of the repeated sequences contained within *ori*₂₀₀₉. *, the Per effect conferred by this DNA fragment was not as strong as that conferred by other Per⁺ DNA fragments.

further replication at $T = 60$ to 80 min (lanes 4 to 7). In contrast, Tuc2009 DNA replication in strain UC509.9 harboring pNZRep-1 is not apparent until $T = 60$ min and remains at a level that is severely reduced in comparison to that of the control strain with no apparent lysis (lanes 12 to 14).

Delineation of the Per phenotype-conferring region. To determine the smallest DNA fragment responsible for conferring the Tuc2009 resistance phenotype, DNA fragments generated by PCR, each spanning a different portion of the *rep*₂₀₀₉ gene, were cloned separately into pNZ8048 and examined for their Per effect. These experiments indicated that the resistance-conferring region, located within *rep*₂₀₀₉, could be assigned to an internal 160-bp fragment rich in direct repeats, essentially the region that had been previously designated *ori*₂₀₀₉ (Fig. 4).

Rep₂₀₀₉ specifically interacts with *ori*₂₀₀₉. Initiation of DNA replication in SPP1 requires the interaction of G38P with *oriL*, which is located within gene 38 on the SPP1 genome (32, 47). To determine if a similar DNA-protein interaction between the Rep₂₀₀₉ protein and *ori*₂₀₀₉ occurs, a series of gel retardation experiments was performed. Crude cell extracts from *L. lactis* NZ9800 expressing Rep₂₀₀₉ and a γ -³²P-labelled 160-bp DNA fragment representing *ori*₂₀₀₉ were used for these experiments. As can be seen in Fig. 5, the presence of Rep₂₀₀₉ in the cell extract decreases the electrophoretic mobility of the *ori*₂₀₀₉ DNA fragment in a polyacrylamide gel, indicative of a protein-DNA interaction. To determine if this protein-DNA interaction was specific, increasing amounts of unlabelled *ori*₂₀₀₉ DNA fragments or of a nonspecific DNA fragment of approximately the same size were incorporated into the binding reaction mixture. As can be seen in Fig. 5 (lanes 4

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

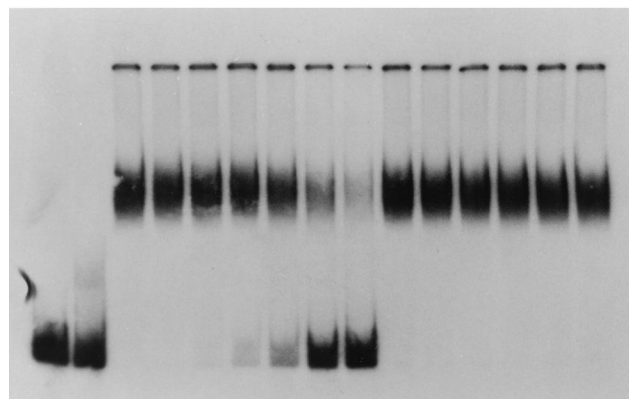


FIG. 5. Gel retardation assay showing that the binding between the Rep₂₀₀₉ protein and *ori*₂₀₀₉ is the result of a specific interaction. Gel retardation was performed as described in Materials and Methods. An 0.3-ng amount of ³²P-labelled *ori*₂₀₀₉ DNA fragment (160-bp Per-conferring region) was used as a probe. Lanes: 1, probe only; 2, probe plus cell extract (2.5 µg of total protein) from *L. lactis* NZ9800 harboring pNZ8048 (negative control); 3 to 15, probe plus cell extract (2.5 µg of total protein) from *L. lactis* NZ9800 containing overexpressed Rep₂₀₀₉ protein (approximate Rep₂₀₀₉ protein molecular mass, 29 kDa). Lanes 4 to 9 represent binding assays performed with the labelled *ori*₂₀₀₉ probe, cell extract, and 1.2, 2.4, 4.8, 9.6, 19.2, and 38.4 ng of unlabelled *ori*₂₀₀₉ DNA fragment, respectively. Lanes 10 to 15 represent binding assays performed with the labelled *ori*₂₀₀₉ probe, cell extract, and 1.2, 2.4, 4.8, 9.6, 19.2, and 38.4 ng of an unlabelled nonspecific DNA fragment of approximately the same size as the labelled DNA fragment, respectively.

to 9), increasing amounts of unlabelled *ori*₂₀₀₉ DNA fragment resulted in the retardation of decreased amounts of the labelled *ori*₂₀₀₉ DNA fragment to the lower-mobility position, presumably because of competitive binding of the Rep₂₀₀₉ protein to the unlabelled *ori*₂₀₀₉ DNA fragment. In contrast, addition of comparable amounts of the unlabelled nonspecific DNA fragment did not cause changes in the mobility profile of the labelled *ori*₂₀₀₉ DNA fragment. These experiments clearly demonstrate that the binding of Rep₂₀₀₉ to *ori*₂₀₀₉ is the result of a highly specific interaction. Interestingly, the ability of a fragment to confer a Per phenotype always coincided with its ability to interact with the Rep₂₀₀₉ protein. Likewise, fragments that do not confer a Per phenotype were shown to not interact with Rep₂₀₀₉ (Fig. 4).

Random mutations in *ori*₂₀₀₉ can affect Per but do not eliminate the Rep₂₀₀₉-*ori*₂₀₀₉ interaction. To identify the individual nucleotides within *ori*₂₀₀₉ which are responsible for conferring the Tuc2009 resistance phenotype, and also to determine if the Per effect is a direct consequence of the Rep₂₀₀₉-*ori*₂₀₀₉ interaction, random mutations were introduced into *ori*₂₀₀₉ DNA fragments. A PCR protocol with conditions such that point mutations or deletions occurred with a relatively high frequency was employed (see Materials and Methods). DNA fragments generated in this way were cloned in pNZ8048, introduced into UC509.9, and then tested for their ability to confer a Per phenotype. A number of clones that conferred a weakened level of Tuc2009 resistance on UC509.9 relative to that of the wild-type *ori*₂₀₀₉ DNA fragment were identified. These clones along with other randomly selected clones that displayed no loss of phage resistance were subjected to DNA sequence analysis. A number of individual clones containing one or more point mutations or deletions that caused a reduction in the efficiency of the Tuc2009-resistant phenotype conferred were identified. In total, 23 clones containing mutations were identified (Fig. 6). Mutations that had the greatest effect on Per were localized around a series of four direct repeats, designated here as region I. The main feature of region I is the presence of two imperfect repeated sequences followed by two perfect repeated sequences. A third copy of the latter sequence is located at the 3' end of the *ori*₂₀₀₉ DNA fragment and is designated here as region Ia. One mutation was identified in this region, and this had no effect on Per. Mutations in the series of 11 imperfect direct repeats of 4 nucleotides, designated here as region II, appear to have had little or no effect on the Per phenotype, unless they were accompanied by other mutations in region I. Gel retardation experiments using crude cell lysates from *L. lactis* NZ9800 expressing Rep₂₀₀₉ and a number of probes representing the randomly mutated versions of the *ori*₂₀₀₉ DNA fragment were performed. All mutated fragments that completely lost the ability to confer a Per phenotype along with a number of randomly selected mutated fragments that displayed only a slight reduction or no reduction in the Per phenotype conferred were examined in this way. Surprisingly, Rep₂₀₀₉ was found to bind all fragments examined, with an apparent similar affinity. This suggests that the observed Per phenotype of UC509.9 cells harboring pNZRep-1 is not solely a consequence of limitation of Rep₂₀₀₉ binding to *ori*₂₀₀₉ and that at least one other interaction must take place at *ori*₂₀₀₉.

DISCUSSION

Initiation of DNA replication requires a specific starting point where opening of the double-stranded double helix takes place prior to the recruitment of the replication machinery. The interaction of a sequence-specific duplex DNA-binding

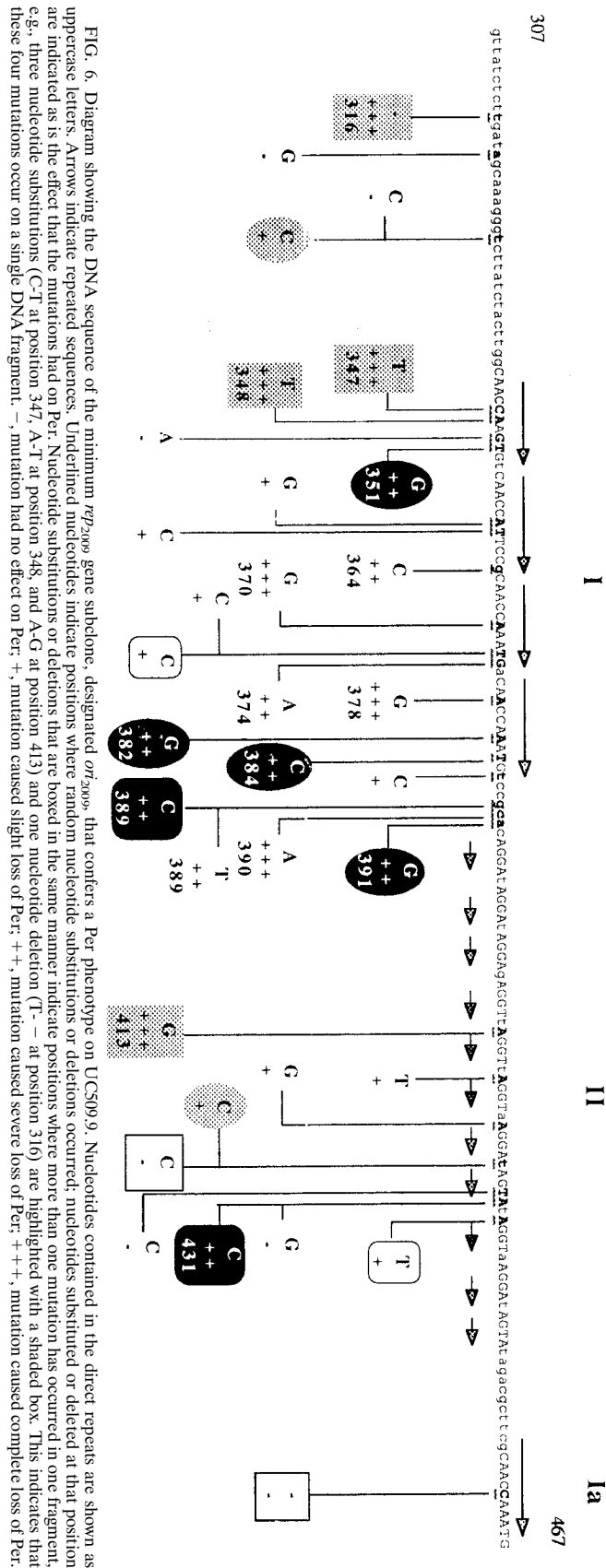


FIG. 6. Diagram showing the DNA sequence of the minimum *rep*₂₀₀₉ gene subclone, designated *ori*₂₀₀₉, that confers a Per phenotype on UC509.9. Nucleotides contained in the direct repeats are shown as uppercase letters. Arrows indicate repeated sequences. Underlined nucleotides indicate positions where random nucleotide substitutions or deletions occurred; nucleotides substituted or deleted at that position are indicated as is the effect that the mutations had on Per. Nucleotide substitutions or deletions that are boxed in the same manner indicate positions where more than one mutation has occurred in one fragment, e.g., three nucleotide substitutions (C-T at position 347, A-T at position 348, and A-G at position 413) and one nucleotide deletion (T- at position 316) are highlighted with a shaded box. This indicates that these four mutations occur on a single DNA fragment. -, mutation had no effect on Per; +, mutation caused slight loss of Per; ++, mutation caused severe loss of Per; +++, mutation caused complete loss of Per.

protein with a series of direct repeated sequences in DNA represents the primary event for the initiation of replication. These repeated sequences form the origin of replication (*ori*). The resulting nucleoprotein binding complexes generally consist of 150 to 250 bp of DNA and multimers of the DNA-binding proteins. Subsequently, an A+T-rich region of DNA directly adjacent to the *ori* becomes denatured and presumably coated with SSB protein. By so marking the origin region and providing available single-stranded DNA, the replication proteins can be recruited to the correct initiation point and nascent strand synthesis can ensue. Examples of this type of initiation of DNA replication include the binding of DnaA to *oriC* and of λ -O to *ori λ* in *E. coli* and λ , respectively (29).

A series of direct repeats was identified within the *rep*₂₀₀₉ of Tuc2009 as has been shown for *oriL*, which is contained within gene 38 of SPP1 (32, 47). The Rep₂₀₀₉ protein was putatively identified as a replisome organizer protein through sequence similarities with the deduced protein product of ORF11 of r1t and G38P of SPP1. It has been demonstrated that initiation of DNA replication in SPP1 requires the binding of G38P to *oriL*. When a piece of DNA encompassing *ori*₂₀₀₉ is supplied in trans on a high-copy-number plasmid vector, it confers an abortive infection-type resistance on UC509.9 against Tuc2009, similar to that which has been previously reported for ϕ 50 and ϕ 31 (16, 35). We have demonstrated that intracellular Tuc2009 DNA replication is severely inhibited in UC509.9 harboring these plasmids. We have also demonstrated a protein-DNA interaction between the Rep₂₀₀₉ protein and the specific portion of the *rep*₂₀₀₉ gene that confers Per, *ori*₂₀₀₉. These data would indicate that the observed phage resistance is due to the titration of Rep₂₀₀₉ proteins away from the true *ori* on the phage DNA and towards the *ori*₂₀₀₉ DNA fragments supplied on the plasmid vectors.

However, when DNA fragments representing versions of the *ori*₂₀₀₉ DNA fragment that no longer conferred a Per phenotype due to the introduction of random mutations in the DNA sequence were used as probes in gel retardation experiments, no decrease in the ability of Rep₂₀₀₉ binding was observed. Data from the mutational analysis of *ori*₂₀₀₉ lead us to conclude that the DNA region encompassing four direct repeats designated as region I is the most significant contributor to the Per phenotype (Fig. 6). This finding is in good agreement with experimental results which showed that fragments containing deleted versions of other repeated sequences from *ori*₂₀₀₉ confer a Per phenotype only slightly reduced relative to that of fragments containing all the repeat-containing regions (Fig. 4). Interestingly, gel retardation experiments displayed no discernible difference in the ability of the Rep₂₀₀₉ protein to bind to probes representing *ori*₂₀₀₉ or these two deleted fragments.

The finding that *ori*₂₀₀₉ and equivalent sequences in ORF11 of r1t and *oriL* for SPP1 are located within the genes coding for putative replisome organizer proteins suggests a modular-type evolution of the genetic material necessary for directing initiation of DNA replication in the respective phages. This feature may also represent a means of self-regulating the expression of these proteins, whereby the binding of the protein to the *ori* contained within the gene could arrest the progression of the RNA polymerase.

On the basis of the current information available, a detailed model for Tuc2009 DNA replication remains unclear. However, we can putatively assign functions to some of the ORFs identified. The protein specified by ORF14 of Tuc2009 shows a high degree of similarity to the deduced protein product of ORF11 of TP901-1; this protein in turn shows low similarity to topoisomerase I from two different *Mycoplasma* species. The protein product of ORF14 of Tuc2009 may represent a highly

diverged type of topoisomerase I enzyme. These enzymes are responsible for the unwinding of supercoiled DNA, thereby making the double helix accessible to the replication machinery.

The role of SSB proteins in DNA replication, as in DNA recombination and repair, is well understood. In DNA replication, they serve to stabilize the single strands of DNA that are transiently formed as the replication fork moves through the double helix (29). The deduced protein product of ORF15 shows a high degree of homology to these proteins, and therefore we assume that this protein does play the role of SSB proteins in Tuc2009 DNA replication.

The protein specified by ORF18 of Tuc2009 shows similarity to a range of type II methyltransferases; this protein most likely plays a role in methylating newly replicated DNA, thus protecting it from degradation by host-encoded endonucleases. It is possible, as has been suggested for ϕ 50 (17), that the incorporation of this methylase into the Tuc2009 genome is the result of a recombinogenic event between the host and phage genomic DNA.

We envisage that the Rep₂₀₀₉ protein plays the part of replisome organizer in Tuc2009 DNA replication. We propose that the series of direct repeats contained within the *rep*₂₀₀₉ gene sequence *ori*₂₀₀₉ represents a possible *ori* for Tuc2009 and that DNA replication in Tuc2009 is initiated by the interaction of Rep₂₀₀₉ with *ori*₂₀₀₉. The results from gel retardation experiments with the mutated *ori*₂₀₀₉ DNA fragments that no longer conferred a Per phenotype but retained the ability to bind Rep₂₀₀₉ indicate that *ori*₂₀₀₉ is the target for at least one other phage-encoded protein. The titration of this protein (or proteins) appears to be responsible for the Per phenotype, and future work will be aimed at the identification of this important replication factor.

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