Sequence Diversity and Functional Conservation of the Origin of Replication in Lactococcal Prolate Phages

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Prolate or c2-like phages are a large homologous group of viruses that infect the bacterium *Lactococcus lactis*. In a collection of 122 prolate phages, three distinct, non-cross-hybridizing groups of origins of DNA replication were found. The nonconserved sequence was confined to the template for an untranslated transcript, P_E1-T, 300 to 400 nucleotides in length, while the flanking sequences were conserved. All three origin types, despite the low sequence homology, have the same functional characteristics: they express abundant P_E1-T transcripts and can function as origins of plasmid replication in the absence of phage proteins. Using chimeric constructs, we showed that hybrids of two nonhomologous origin sequences failed to function as replication origins, suggesting that preservation of a particular secondary structure of the P_E1-T transcript is required for replication. This is the first systematic survey of the sequence and function of origins of replication in a group of lactococcal phages.

Lactococcus lactis is widely used in the production of fermented products by the dairy and food industries. Large numbers of bacteriophages infect this bacterium, causing the lysis of the host and fermentation failure. The most frequently encountered are two species of small phages with isometric heads and B1 morphology, named 936 and P335, and one species of phages with prolate heads and a B2 morphology, named c2 (32). Each of the species comprises numerous homologous phages.

A number of host defense mechanisms against lactococcal phages have been discovered (reviewed in reference 12). Collectively, these mechanisms affect every step of the phage life cycle. Individual mechanisms may have a narrow range, affecting just a few strains of a single phage species, or broad range, affecting strains of several phage species. Under the pressure of the host defense mechanisms, phages may mutate or recombine with the chromosome or resident plasmids to produce resistant progeny (5, 15, 20, 36, 51). The interplay between the host defense mechanisms and phage evasion strategies is most likely a strong evolutionary driving force in the evolution of both.

Although the adsorption proteins are usually the most divergent part of the genome within a group of interbreeding (recombining) phage, variation of the DNA replication origin has also been described (7, 14, 30, 45, 51). In the lactococcal phage P335 species, it has been shown that under the selective pressure of the host defense mechanisms AbiC or AbiK, recombination can lead to the replacement of the origin of replication by a dissimilar prophage-derived sequence from the

bacterial chromosome (5, 15). Recombination occurred via sequences of high identity flanking the divergent regions (5). This kind of module acquisition or shuffling is well noted in the bacteriophage world. A canonic example is the lambdoid phage family, in which the modules consisting of origin of replication, integration, lysogeny, and immunity control vary among the members. Despite variation in sequences, the functional organization of the whole module is preserved (7).

Prolate phages have the smallest genome among the lactococcal phages. The 20- to 22-kbp double-strand DNA genomes code for two blocks of divergently oriented open reading frames (ORFs), separated by a noncoding region. In phage c2, this intergenic fragment containing the early promoter 1 (P_E1) and downstream 307 bp that serve as a template for an untranslated transcript (P_E1-T) is sufficient to support plasmid replication in *L. lactis* in the absence of phage proteins (53). The P_E1-T template sequence was confirmed as the origin of c2 replication by two-dimensional agarose gel electrophoresis of the replicative intermediates (6).

Two examples of transcript-mediated initiation of replication are phage T4 and plasmid ColE1. In the phage T4 middle origin, it was proposed that transcription from a middle-mode promoter followed by the formation of an RNA-DNA hybrid liberates the nontemplate DNA strand for primosome assembly and initiation of replication (9). In this case, phage proteins are only required for modification of RNA polymerase to enable transcription from the middle-mode phage promoter (9). In ColE1 and a few other plasmids in which replication is independent of plasmid-encoded initiator proteins, high homology of the sequences (80 to 90%) is confined to the template of a transcript at the origin of replication, called RNA II, required for initiation of replication (49). During transcription, the nascent ColE1 transcript forms an obligatory DNA-RNA hybrid that is processed by RNase H to form the mature RNA II. RNA II serves as a primer for polymerase I (PolI), which initiates replication (23). Particular secondary structure of the

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TABLE 1. Strains, plasmids, and bacteriophages used in this study

Strain, phage, or plasmid	Description[a]	Reference(s) and/or source
Strains		
E. coli ER2206	*endA1 thi1 supE44 mcr67* (*mrcA*) (*mrcBC-hsdRMS-mrr*) *114::*IS*10* (*lac*)*U169/F′ proAB laqI*�q *ZM15* Tn*10*	New England Biolabs
L. lactis		
MG1363	Plasmid-free strain and prophage-cured derivative of NCDO 712	17
Uvs62	MG1363*polA*	16
IL1403	Plasmid-free strain	11
c6		41
112		NZ dairy plant isolate
AM1		NZ dairy plant isolate
2282		NZ dairy plant isolate
Bacteriophages		
c2	MG1363 and IL1403	25, 35, 39
c6A	c6 and IL1403	25, 41
bIL67	IL1403 and c6 (at 37°C only)	47
923	112, AM1, MG1363 (at 37°C only), IL1403 (at 37°C only)	NZ dairy plant isolate (24)
943	AM1, 112, MG1363 (at 37°C only)	NZ dairy plant isolate, 1995
5440	2282	NZ dairy plant isolate, 1995
5447	2282	NZ dairy plant isolate, 1995
5469	2282, MG1363, IL1403 (at 37°C only), 112, AM1	NZ dairy plant isolate, 1995
Plasmids		
pGEM-3Zf	Amp^r; T7 and SP6 promoters	Promega
pGEMc2	Amp^r; c2 *ori* fragment cloned into pGEM-3Zf	This study
pGEMbIL	Amp^r; bIL67 *ori* fragment cloned into pGEM-3Zf	This study
pGEM923	Amp^r; 923 *ori* fragment cloned into pGEM-3Zf	This study
pUCΩKm-2	pUC carrying the *Streptococcus faecalis* Ω element	38
pTRKL2	Ery^r shuttle vector	37
pVAΩ	pVA891 carrying the transcriptional terminator of the *Streptococcus faecalis* Ω element	This study
pVA891	Ery^r Cm^r; low-copy-number vector in *E. coli;* Ery^r is expressed in gram-positive bacteria	31
pLP203	Ery^r; 369-bp *ori* fragment from c2 cloned into pVA891	This study
pO923	Ery^r; 434-bp *ori* fragment from 923 cloned into pVA891	This study
pObIL	Ery^r; 444-bp *ori* fragment from bIL67 followed by the terminator from the Ω element cloned into pVA891	This study
pSc2-923	Ery^r; 418-bp chimeric *ori*-fragment cloned into pVA891	This study
pS923-c2	Ery^r; 415-bp chimeric *ori*-fragment cloned into pVA891	This study
pMc2-923	Ery^r; 434-bp chimeric *ori*-fragment cloned into pVA891	This study
pUC19-ori	Amp^r; 369-bp *ori* fragment from c2 cloned into pUC19 vector	53

[a] For strains, description concerns phenotypes. For bacteriophages, description concerns host specificity. For plasmids, description concerns characteristics or further modifications.

transcript is necessary for the ColE1 RNA-DNA persistent hybrid to form (33, 34).

The two prolate phage genomes (c2 and bIL67) that have been sequenced to date have an overall sequence identity of 80% (29, 47). Apart from several DNA insertions and/or deletions ("indels"), the highest diversity was detected in the putative adsorption protein gene (L10), minor coat protein gene (L16) and the 300 to 400 bp downstream of the P_E1 promoter within the origin (29). The nonconserved region of the origin begins at position +16 of the P_E1-T template and extends over most of its length, ending 25 nucleotides (nt) 5′ to the −35 box of the downstream early promoter, P_E2. The bIL67 P_E1-T template is longer by 72 bp than that of c2, and the nucleotide identity between the P_E1-T template sequences is only 19%.

We report here that there are three unrelated types of P_E1-T template sequences in a survey of 122 prolate phages. All three origin types are functionally analogous in that they serve as origins of replication in a plasmid model system in the absence of phage proteins and express abundant P_E1-T transcripts. Two chimeric origins were nonfunctional in the plasmid system, suggesting that the secondary structure of P_E1-T, rather than putative common sequence motifs, is essential for origin function. Based on searches of the GenBank database, we propose that diversity of the prolate phage origins was generated by horizontal transfer from other phage, plasmids or the lactococcal chromosome by recombination via microhomologous sequences.

MATERIALS AND METHODS

Strains, media, and culture conditions. Bacterial and phage strains used in this study are described in Table 1. *L. lactis* strains were grown in M17 medium supplemented with 0.5% (wt/vol) glucose at 30°C (50). For selection of *L. lactis* carrying plasmids pVA891, pTRKL2, and derivatives thereof, erythromycin (5

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′→3′)[a]	Specificity	Restriction site(s)
AS termin	TCG*GAATTC*GCTTGTAAACCGTTTTGTGAA	Ω element	*Eco*RI
JR120	CTTTGTTAATTGCTTTGATGTCGTC	Conserved	
JR124	CGATAAAATAACCGTTACAATTAGCC	Conserved	
JR139	CCTTGAGTTGTCTATGGTTGCTAA	bIL67	
JR166	GCTGACATTAWCCAATAAA	Conserved	
JR167	GATTATGGTATTATTATAGCA	Conserved	
JR169	CG*GGATCC*ATCAAAAATTTAACGACTGTTA	bIL67	*Bam*HI
JR170	CG*GGATCC*AACGTATGTTATAATATAAATA	923	*Bam*HI
JR171	C*GAATTC*GCTGACATTATCCAATAAA	Conserved	*Eco*RI
JR172	ACGCAAACGCAGTTTTTATCC	c2	
JR173	CTAAGGCTTGTCTGATGTCTT	c2	
JR174	GCCCTTGCCTTTTTGGTTAAG	bIL67	
JR177	GCGAGGCGAAAGCCTATGAA	923	
JR178	TCGGAATTC*CCATGG*CTTATGTTTTTGACCCTAA	bIL67	*Nco*I
JR179	TCG*GAATTC*TATGTTACTCTTTAATTACAA	923	*Eco*RI
JR180	TCGGAATTC*CCATGG*CTTGTGTTTTTTACCCT	923, c2	*Nco*I
JR184	TATTATAACATACGTTTT	Conserved	
JR185	AAAACGTATGTTATAATA	Conserved	
JR203	GTAGAGATTCTGATAAGGTAG	923	
JR204	CATG*CCATGG*AA*CTGCAG*TGGATGACCTTTTGAATGAACC	Ω element	*Nco*I/*Pst*I
JR205	AAAA*CTGCAG*TAGTTACTTTATTTTAGACAA	bIL67	*Pst*I
JR206	CAACCTGTCCACTTcttatttaactttgcc	923/c2 (chimeric)	
JR207	gttaaataagAAGTTGGACAGGTTGTGAG	c2/923 (chimeric)	
JR221	GTTGTGAGTaagttaaataagaagttac	923/c2 (chimeric)	
JR222	cttatttaacttACTCACAACCTGTCCAC	c2/923 (chimeric)	
JR223	CATG*CCATGG*TCGACCTTGTGTTTTTTACCCT	923	*Nco*I
Latedel	AAAG*AATTC*CTTGTATTTTTGACCCTG	c2	*Eco*RI
pUC19 −20[b]	GTAAAACGACGGCCAGT	M13/pUC	
ts/+1	CG*GGATCC*ATAAAAATTGAATACGCC	c2	*Bam*HI

[a] In chimeric oligonucleotides, uppercase and lowercase letters represent, respectively, 923 and c2 sequences. Restriction sites are shown in italics.
[b] From New England Biolabs.

μg/ml) was added to the medium. *Escherichia coli* strain ER2206 was propagated on Luria-Bertani (LB) broth or brain heart infusion broth at 37°C. Antibiotics ampicillin and chloramphenicol were added to LB at 200 and 25 μg/ml, respectively, and erythromycin was added to brain heart infusion broth at 150 μg/ml as required.

Bacteriophage propagation. Preparation of phage stocks was carried out in liquid medium or by plate lysis (24). When required, phages were concentrated by centrifugation at 40,000 × *g* for 2 h in a Sorvall centrifuge, or by precipitation with polyethylene glycol (24).

Recombinant DNA methods. Basic cloning procedures in *E. coli* were as described previously (44). For purification of PCR products and for isolation of DNA from agarose gels, commercial DNA purification kits from Roche Molecular Biochemicals, Qiagen and Invitrogen were used according to the manufacturer's instructions. *L. lactis* electrocompetent cells were prepared using the glycine method (22). Typically, *L. lactis* cells were electroporated with 500 ng of plasmid DNA dissolved in water (40). Plasmid DNA was isolated from *L. lactis* by alkaline lysis (3). The plasmids were resolved by agarose gel electrophoresis (0.8%). To reveal the pObIL bands from the IL1403 transformant, the Southern blot was carried out using the pObIL plasmid as a probe and the ECL detection system (Amersham-Pharmacia) according to the manufacturer's instructions. The DNA from polyethylene glycol-precipitated phages was isolated using the Qiagen λ phage DNA purification kit (Midi) according to the manufacturer's instructions.

Construction of plasmids. Plasmids that served as templates for synthesis of antisense probes were derivatives of pGEM-3Zf− (Promega). The inserts were amplified by PCR using appropriate primers and DNA from phage particles as templates. All primers (Table 2) contained recognition sites for appropriate restriction endonucleases: *Bam*HI (forward primers) and *Eco*RI (reverse primers). The forward primers were: ts/+1 (c2), JR169 (bIL67), and JR170 (923). The reverse primer for all three clones was JR171. Amplified fragments were cloned into *Eco*RI and *Bam*HI sites of the vector. The resulting template plasmids, named pGEMc2, pGEMbIL, and pGEM923, were used for synthesis of c2, bIL67, and 923 antisense probes, respectively, as described below.

To assay the origin function of c2, bIL67, and 923 P$_E$1 transcripts, *ori* sequences containing the P$_E$1 promoter starting from position −62 and down-

stream template sequence extending nearly to the −35 box of the subsequent downstream early promoter, P$_E$2, were cloned into the origin probe vector pVA891 (31). The ends of the template fragments were 20 nt (c2 and bIL67) and 9 nt (923) upstream of the −35 box of P$_E$2 and therefore did not carry any promoter sequences of P$_E$2. The c2 *ori* fragment was amplified using primers pUC19 −20 (New England Biolabs) and Latedel and plasmid pUC-ori (53) as template. The resulting fragment was cleaved with *Eco*RI and cloned into the *Eco*RI site of pVA891, and the plasmid was named pLP203. The 923 *ori* fragment was amplified using the primers JR180, JR179, and 923 phage as a template. The amplified PCR product was cloned into the *Nco*I and *Eco*RI sites of pVA891, and the plasmid was named pO923.

The bIL67 *ori* fragment was cloned into the modified pVA891, named pVAΩ, which carried the transcriptional and translational terminator from the *Streptococcus faecalis* Ω element cloned between the *Nco*I and *Eco*RI sites. A unique *Pst*I site was engineered, so that the inserts could be directionally cloned into *Pst*I/*Nco*I-cut vector upstream of the terminator, which ends the transcription and prevents read-through into the vector. The terminator was on a 130 bp insert created by PCR, using primers JR204, AS termin, and plasmid pUC4Ωkm-2 (38) as template. The bIL67 *ori* fragment was amplified using primers JR178, JR205, and bIL67 phage as template, and was inserted into the *Nco*I and *Pst*I sites of vector pVAΩ. The resulting plasmid was named pObIL.

Chimeric *ori* inserts for cloning into pVA891 were composed of portions of the *ori* regions of c2 and 923 phage. They were constructed by overlap extension PCR, carried out in two steps. In the first step, origin halves, each from different phage, were amplified separately. The reverse primer for amplification of the 5′ half and the forward primer for amplification of the 3′ half were chimeric and complementary to the other. Thus, the PCR products amplified using the chimeric primers carried complementary ends. In the second step, an overlap extension PCR was used to join the two halves into a chimeric *ori*. The equimolar amounts of products of the first round of PCR served as templates. The primers corresponded to 5′ and 3′ ends of the chimera and included the restriction sites, *Eco*RI and *Nco*I, respectively, to allow cloning into the *Eco*RI and *Nco*I sites of the vector pVA891. Three chimeric *ori* constructs were made. The first construct, pSc2-923, carried the c2/923 chimeric *ori*, composed of the P$_E$1 promoter and 5′ half of c2 *ori* (62 + 172 bp) and 3′ half of the 923 *ori* (184 bp). Primers used for

amplification of the c2 fragment were JR180 and JR206, using c2 phage as template. For amplification of the 923 phage fragment, primers JR207, JR179, and 923 phage were used as templates. The second round of PCR was carried out using primers JR180 and JR179 and the two products of the first round of PCR as templates. The second construct, pS923-c2, carried a 923/c2 chimeric *ori* consisting of the P$_E$1 promoter and 5′ half of 923 *ori* (62 + 200 bp) and the 3′ half of the c2 *ori* (153 bp). Primers used for amplification of the 923 fragment were JR223 and JR222 and the template was 923 phage. For amplification of the c2 fragment, primers were JR221 and pUC19 −20 and the template was pUC-ori plasmid (53). The chimeric insert was amplified in the second round of PCR, using primers JR223 and pUC19 −20, and the two products of the first round of PCR as templates. In the third chimeric construct, pMc2-923, the junction of the chimera was at the P$_E$1 promoter. Thus, although the *ori* was chimeric, the entire template sequence of the P$_E$1-T template sequence was derived from a single phage. The P$_E$1 promoter from position −62 to −27 was from c2, followed by identity of 20 bp, and a portion of 923 from −7 to +367. The c2 portion of the insert was amplified using primers JR180, JR184 and c2 phage as template. The 923 portion of insert was amplified using primers JR185, JR179, and 923 phage as templates. The chimeric insert was amplified in the second round of PCR, using primers JR180, JR179, and the two products of the first round of PCR as templates.

Sequencing of the phage origins of replication. Origin fragments equivalent to the genomic c2 sequence from coordinates 6353 to 7685 (29) were sequenced in 6 prolate phage: c6A, 923, 943, 5440, 5447, and 5469. The *ori* fragments were first amplified by PCR, using primers JR120, JR124 and DNA from phage particles as templates. To minimize the error rate, proof-reading polymerase *Pwo* (Roche Molecular Biochemicals) was used. As an additional precaution, each preparative PCR was divided in four PCR tubes prior to cycling. After the PCR, the four reactions were combined for use in the subsequent purification and sequencing. The sequencing was carried out using the BigDye mix (Applied Biosystems) at Alan Wilson Centre Genome Service (Massey University). Both strands were sequenced by primer walking, each with at least twofold redundancy. Sequences were assembled and analyzed using the GeneWorks program (Oxford Molecular-Accelrys) and BioEdit (Tom Hall, North Carolina State University, Raleigh). The GenBank accession numbers for the phage origin sequences are AF522295 for 923, AY129505 for 943, AY129506 for 5440, AY129507 for 5447, AY129508 for 5469 and AY129509 for c6A.

Survey of the *ori* regions from industrial prolate phage isolates. *ori* regions from 116 prolate phage isolates from the Fonterra Research Centre (formerly New Zealand Dairy Research Institute) collection were amplified using the primers complementary to the conserved sequences flanking the divergent P$_E$1-transcript template sequence, JR167, JR166 and DNA from phage particles as templates. The PCR products were arrayed in triplicate on a Nitrocellulose filter using Multi-Blot replicator (V&P Scientific, Inc.) (see Fig. 3), or separated by agarose gel electrophoresis and transferred to the Nitrocellulose membrane (not shown). The types of origin were distinguished using type-specific probes derived from the nonconserved P$_E$1-T template region by PCR. The c2 probe was amplified using primers JR173 and JR172 with c2 phage as a template; the bIL67 probe was amplified using primers JR174 and JR139 with bIL67 phage as a template; and 923 probe was amplified using primers JR177 and JR203 with 923 phage as a template. The hybridization was detected using the ECL system (Amersham-Pharmacia).

RNA purification and analysis. For the phage RNA analyses, cells were infected at an optical density of 0.1 and a multiplicity of infection of 5 and harvested 15 min after infection, the time point at which the P$_E$1 transcript accumulates to a high level (28). RNA isolation was carried out as described by Lubbers et al. (28), with the following modifications: instead of the hot phenol treatment, the cells were broken in cold phenol (4°C) using acid-washed glass beads (0.6 mm), at 4°C with continual vortexing for 10 min (2). The mixture was centrifuged at 4°C and the clear water phase was collected, extracted with chloroform, precipitated and resuspended in TE (10 mM Tris, 1 mM EDTA), pH 7.5. The samples were then treated with DNase I (Roche) to eliminate the remaining DNA, and subjected to another round of phenol-chloroform and precipitation as described (28). The concentration of RNA samples was determined spectrophotometrically and the quality of preparation examined by agarose electrophoresis (44).

RNase protection was carried out as previously described (44). Radioactively labeled antisense probes were generated in vitro using the Promega T7 riboprobe kit and [α-^{32}P]CTP (Amersham-Pharmacia) according to the manufacturer's instructions. Templates for synthesis of antisense probes used to detect c2, bIL67, and 923 P$_E$1 transcripts were pGEMc2, pGEMbIL67, and pGEM923, respectively. The antisense probes were complementary to the P$_E$1-T template starting from +1 position (c2 and bIL67) or −20 position (923), comprising the variable portion and extending 93 nt into the downstream conserved region. Thus, the probes detected the full length of the nonconserved region of P$_E$1 transcripts and 93 nt of downstream conserved sequence. In addition to the phage sequences, all three probes carried at the 3′ end residual vector sequence, retained after restriction enzyme cleavage to linearize the template prior to the in vitro transcription.

RNase-protected fragments of radioactively labeled probes were separated by denaturing polyacrylamide gel electrophoresis on a large gel (40 cm). Size standards were used to allow accurate determination of the length of the RNA fragments: end-labeled low-molecular-weight RNA standard (Invitrogen) and a sequencing reaction generated using an end-labeled primer.

For Northern blots, RNA was separated using agarose-formamide gels (44), and detected using PCR-generated DNA probes and the ECL system (Amersham-Pharmacia) according to the manufacturer's instructions. The probes used for the Northern blots were the same as ones used for the Southern blot survey of the origins of replication of prolate phage isolates.

Modeling of the RNA secondary structures was performed using the program ALIFOLD (http://rna.tbi.univie.ac.at/cgi-bin/alifold.cgi [21]).

RESULTS

Sequence diversity of the origin of replication in prolate phage. To determine the extent of variation of the P$_E$1-transcript template segment and flanking sequences in prolate phages, the sequence of ca. 1,300 bp of that region was determined in six phage isolates (Fig. 1A). Five phages were isolates from New Zealand dairy plants: 923, 943 (both in 1975), 5440, 5447, and 5469 (all three in 1995). The sixth phage, c6A, was a 1960s isolate from Australia and has been an object of several publications (41–43). Alignment of the newly and previously sequenced phage origins revealed three groups of P$_E$1-T templates, sharing only 13% identity. In contrast, the flanking sequences were conserved in all phages (Fig. 1A and B). The P$_E$1-T template sequences of c2, c6A, and 5440 belonged to one group (c2 type *ori*); bIL67, 5447, and 5469 belonged to the second (bIL67 type *ori*); and 923 and 943 belonged to the third (923 type *ori*; Fig. 1C). Despite the sharp divergence in the P$_E$1-T templates among the three types, within a group they were highly conserved, even more so than the flanking sequences. For example, overall identity of the 1,300-bp fragment within the c2 *ori* group phages (c2, c6A, and 5440) was 89%, while P$_E$1-T template identity was 95%.

To examine whether prolate phages carry yet other types of *ori*, we surveyed origins of replication of the entire collection of prolate phage isolates of Fonterra Research Centre, which consists of 116 isolates collected systematically over the last 55 years from all major dairy plants in New Zealand. Identity of all prolate phages is verified by electron microscopy (L. Ward and A. Jarvis, unpublished data). Origin fragments containing the nonconserved P$_E$1-T sequences were amplified by PCR, using primers complementary to the flanking conserved regions, and DNA from phage particles as template. The PCR-amplified fragments were analyzed by hybridization with three *ori* type-specific probes derived from the nonconserved P$_E$1-T templates of representative phage, c2, bIL67, and 923 (Fig. 2). All PCR-amplified origins from prolate phage hybridized with one and only one of the three P$_E$1-T probes, showing that there were only three types of *ori* present in the New Zealand prolate phages surveyed, and that there were no hybrid origins (composed of parts of two origin types). The c2 type *ori* was the most frequent (72 isolates), followed by bIL67 type (25 isolates) and 923 (19 isolates).
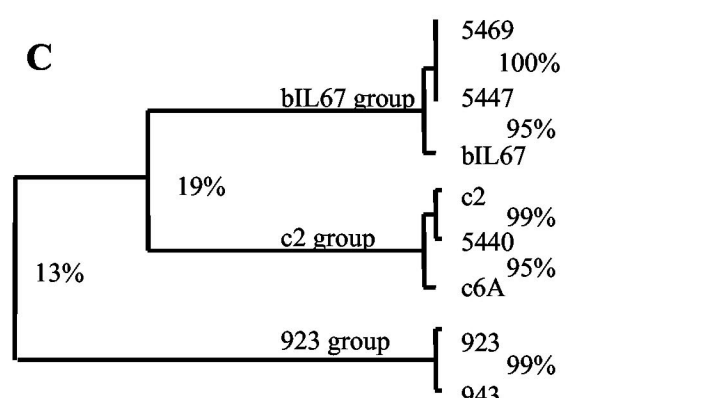
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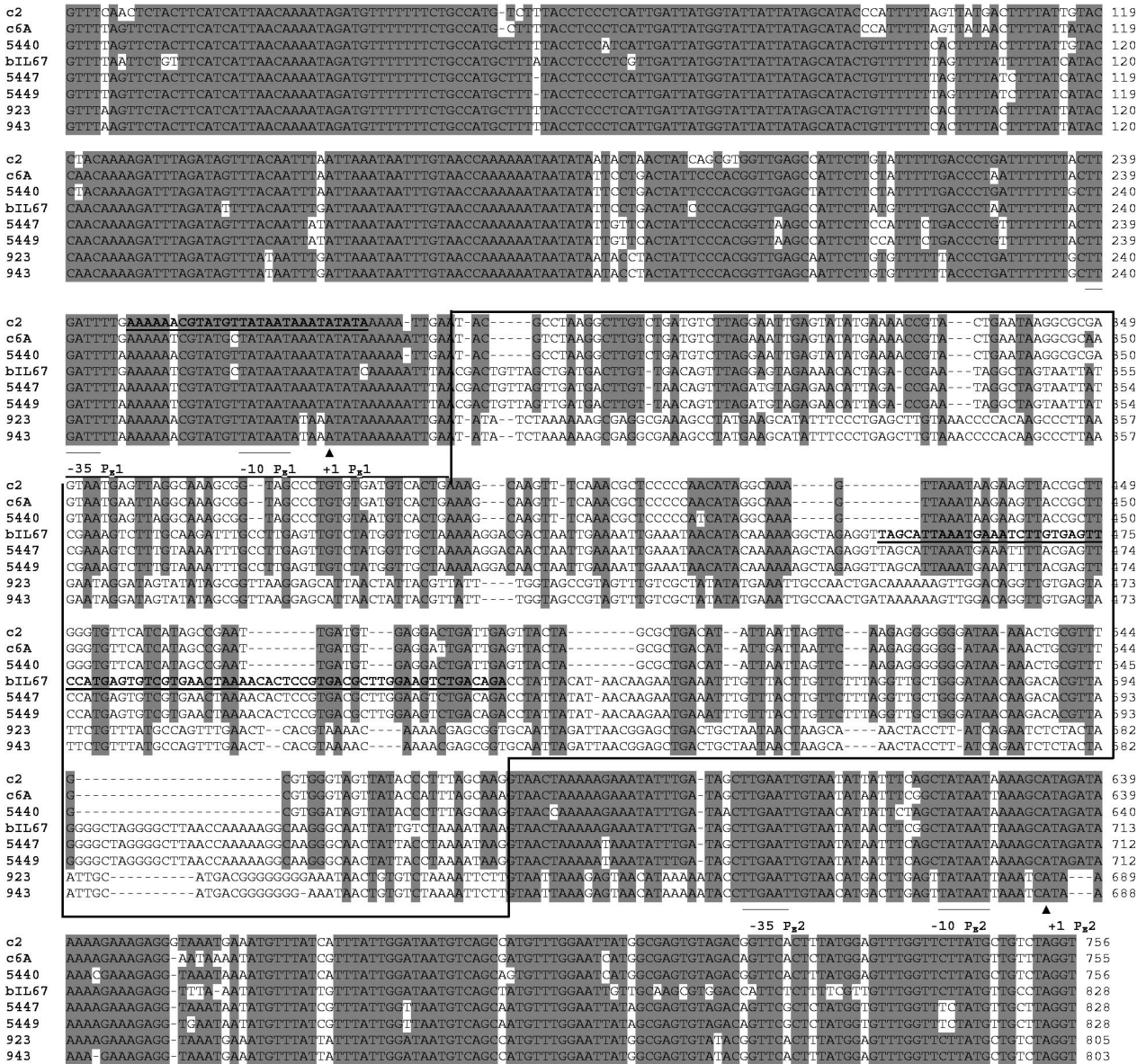
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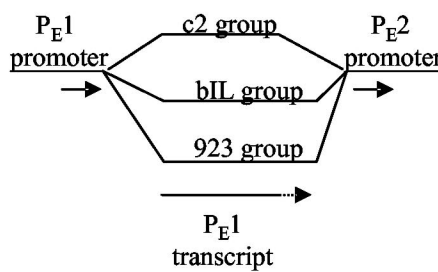
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FIG. 1. (A) Alignment of P_E1 transcript template and flanking sequences. Sequences were aligned using the ClustalW multiple alignments program (52) within the BioEdit sequence analysis package (Tom Hall, North Carolina State University, Raleigh). The aligned sequences start at position 7294 and end at position 6540 of the c2 genomic sequence. The −35 and −10 boxes of P_E1 and P_E2 promoters are underlined, and the + 1 residues labeled with the symbol ▲ (28). The nonconserved portion of the alignment is boxed. The two highlighted sequences (in boldface type
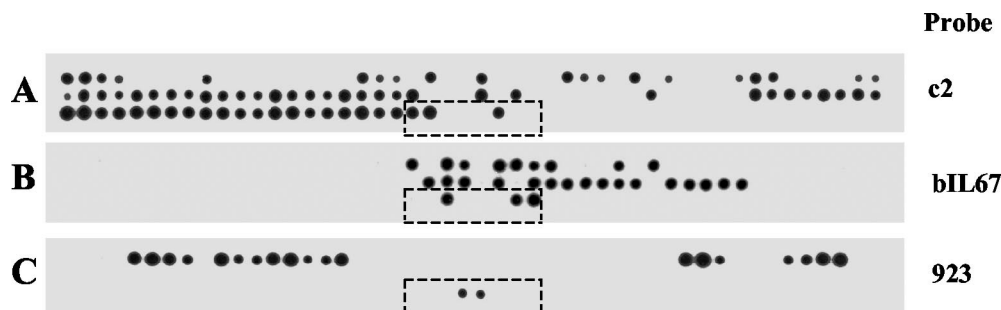
Probe

FIG. 2. Survey of prolate phage origin types. The PCRs of the origin fragments of 116 prolate phage isolates from Fonterra Research Centre collection and 8 phage from which the *ori* sequence has been determined were arrayed in triplicate and each array was probed with a type-specific probe. (A) c2 probe; (B) bIL67 probe; (C) 923 probe. The PCRs of the sequenced origins are boxed. Type-specific probes were PCR fragments derived from the nonconserved P_E1-T template sequences.

All *ori* types express P_E1-T transcripts. It has previously been shown that the c2 P_E1 promoter expresses several short and abundant noncoding P_E1-T transcripts, 250 to 360 nt in length (28). We compared the abundance and length of P_E1-T transcripts in representative phage of all three *ori* types by Northern hybridization and RNase protection analyses of RNA isolated from phage-infected cells (Fig. 3). Northern hybridization revealed abundant short P_E1 RNAs (260 to 500 nt) in all (c2, bIL67, and 923) phage-infected cells (Fig. 3A). In addition, one (c2) and two (bIL67 and 923) longer and less abundant RNA species were detected. Of those, 1.35- and 1.9-knt transcripts were common to bIL67 and 923, while the corresponding c2 transcript was 1.8 knt.

To determine the composition and length of the short P_E1 transcript bands, RNase protection was carried out with two sets of probes: one longer probe set (described in the Materials and Methods section), extending beyond the nonconserved region (Fig. 3B), and a set of shorter probes (not shown). The products were resolved by polyacrylamide gel electrophoresis on high-resolution sequencing gels (Fig. 3B). The most striking feature was the presence of multiple bands, consistent with the appearance of a smear on Northern blots after the agarose gel electrophoresis (Fig. 3A) (27). The major protected bands were of different lengths in each phage, consistent with the diversity of the P_E1 template sequences (Fig. 3B). The longest bands, nearly as long as the full-length probes (excluding the vector-derived and upstream sequences), were protected by long transcripts that extended beyond the end of the antisense probe. This is consistent with the presence of longer transcripts detected by Northern blotting (Fig. 3A).

The RNase protection data indicated that the estimated lengths of the following major P_E1 RNAs from this experiment were as follows: c2, 260, 265, 280, 295, and >419 nt; bIL67, 330, 360, 400, and >489 nt; 923, 280, 325, 330, 440, and >466 nt. The dominant bIL67 transcript was longer than the probe,

indicating that its 3′ end corresponds to the downstream conserved sequence.

All three types of P_E1 RNA templates are replication origins in the absence of phage proteins. It has recently been shown that the intergenic region of c2 phage (611 bp), carrying the promoter and nonconserved P_E1-T template sequence, is sufficient to drive replication of the origin probe plasmid pVA891 in the absence of phage proteins (53). To examine whether the analogous regions of bIL67 and 923 could function as origins of plasmid replication, appropriate DNA fragments from the two phage were inserted into the origin-screening plasmid pVA891 (31), which lacks an origin function in *L. lactis*. Each insert started at the −62 position of P_E1 and ended at the point of reestablishment of homology: +307 in c2, +382 in bIL67 and + 372 in 923 (Fig. 1). In the 923 type of *ori*, identity resumes 1 nt 5′ to the −35 box of the downstream promoter, P_E2. In bIL67, the homology to c2 phage starts 26 nt 5′ to the −35 box of P_E2 promoter (Fig. 1A).

The three pVA891 derivatives, pLP203, pObIL, and pO923, were transformed into *L. lactis*, and their ability to replicate was examined. Because the major transcript of bIL67 P_E1 extends beyond the end of the insert, the bIL67 construct carried a transcriptional terminator between the 3′ end of the insert and the vector to prevent transcriptional read-through into the vector sequence. As each of the three phages plate on a distinct cognate host strain, and it was possible that diversity of origins was important for host-specificity, each of the three hosts (MG1363, IL1403, and 112) was electroporated with all three plasmids. The transformation efficiencies were compared with a control plasmid, pTRKL2, which carries pAMβ1 origin of replication that is functional in a broad range of Gram-positive bacteria (37). All plasmids conferred Ery resistance to all strains, suggesting that they replicated in all strains. The IL1403 transformants that carried pLP203 and pO923 grew much faster than those carrying pObIL (24 h to form a colony

and underlined) showed significant identities with sequences from the NCBI nucleotide database: nt 247 to 275, 96% identity to the *L. lactis* IL1403 chromosome; nt 449 to 525, 88% identity to the small isometric phage bIL170; and nt 470 to 525 (contained within the preceding sequence), 92% identity to the plasmids pAW601 and pIL103. (B) Schematic representation of origin divergence. Single lines represent the conserved sequences and branched lines the divergent region; short arrows, promoters; long arrow, P_E1 transcript template. Note that the c2 group P_E1-T template is shorter than that of the bIL67 and 923 groups. The dotted portion of the arrow symbolizes the length difference. (C) Rectangular cladogram based on the ClustalW alignment of P_E1 templates only (nonconserved portion of sequences, boxed in A). Numbers represent % identities between the phage and phage groups.
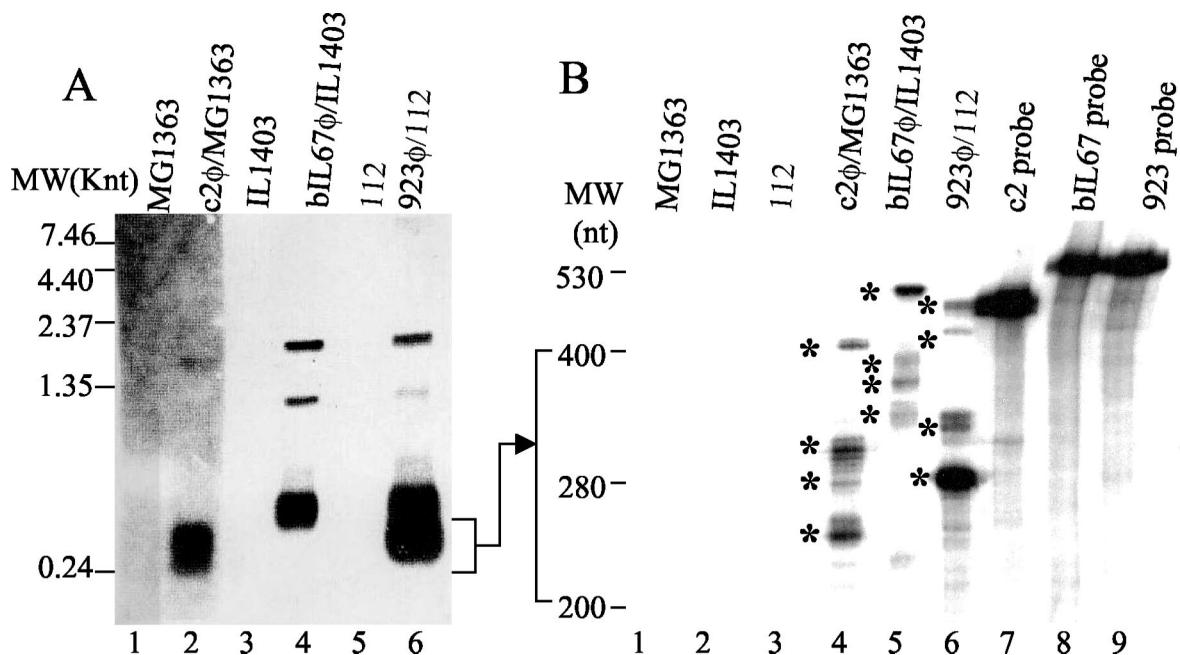
FIG. 3. P_E1 transcripts of c2, bIL67 and 923 phage. (A) Northern blot of RNA resolved by denaturing agarose gel electrophoresis. Each lane was loaded with 20 μg RNA. Lanes 1 (MG1363) and 2 (c2 phage-infected MG1363) were probed with a c2 probe; lanes 3 (IL1403) and 4 (bIL67 phage-infected IL1403) with a bIL67 probe; lanes 5 (112) and 6 (923 phage-infected 112), with a 923 probe. The probes were *ori*-specific PCR fragments derived from nonconserved P_E1 template sequences. (B) RNase protection of P_E1 transcripts, resolved by high resolution acrylamide gel-electrophoresis. Lanes 1 to 6, RNase protection reactions of the RNA from MG1363 (lane 1), IL1403 (lane 2), 112 (lane 3), c2 phage-infected MG1363 (lane 4), bIL67 phage-infected IL1403 (lane 5), and 923 phage-infected 112 (lane 6); lanes 1 and 4, reactions with the c2 probe; lanes 2 and 5, the bIL67 probe; lanes 3 and 6, 923 probe. Lanes 7, 8, and 9 contain probes only (lane 7, c2; lane 8, bIL67; lane 9, 923). Lanes 1, 2, 4, and 5 contain 26 μg of RNA per reaction. Lanes 3 and 6 contain 13 μg of RNA per reaction. Asterisks indicate major P_E1 transcripts. c2, bIL67, and 923 ori-specific probes were synthesized by in vitro transcription using plasmids pGEMc2, pGEMbIL, and pGEM923 (described in Materials and Methods) as templates.

on solid media compared to 72 h for pObIL transformants). When transformants were analyzed for plasmid content (Fig. 4), the relatively small amount of plasmid recovered per cell from pObIL transformants compared to the other *ori* plasmids. The pObil plasmid bands isolated from the IL1403 transformant were detectable only by Southern blotting (Fig. 4, lane 12). Probe, the full-length pObIL plasmid, did not hybridize to the chromosomal band, indicating that the plasmid was not integrated into the chromosome. Therefore, the low yield was most likely due to relatively inefficient replication of this plasmid rather then from integration into the chromosome. The low efficiency of pObil replication in IL1403 might be due to the truncation of the bIL67 P_E1-T in pObIL, since the major bIL67 transcript in the phage extends into the downstream conserved region (Fig. 3B). However, the longer fragment proved recalcitrant to cloning and hence could not be used in this experiment.

No ribosome-binding sites or ORFs longer than 36 bp were encoded by P_E1-T template of c2 (28), bIL67 (47), and the six prolate phages sequenced in this study. Thus, it may be inferred that the prolate phage origins are functional in the absence of phage proteins. Accordingly, the prolate origins of replication do not exhibit the phage resistance phenotype (*per*), even when carried on high copy number plasmids (J. Rakonjac and A. Schiemann, unpublished data).

Chimeric P_E1-T template sequences do not function as origins of replication. The small number (three) of P_E1-T vari-
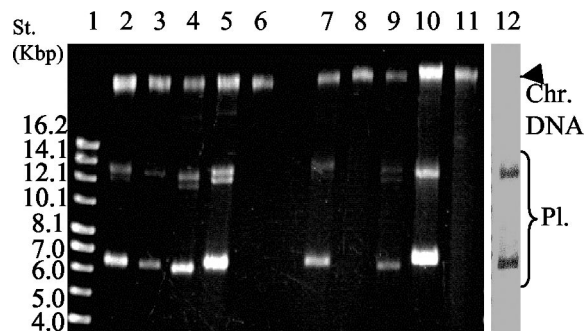

FIG. 4. *ori* plasmid preparation from *L. lactis* strains MG1363 and IL1403. *ori* constructs are derivatives of the origin-less plasmid pVA891 (31), each harboring a prolate phage promoter P_E1 (61 bp) and downstream P_E1-T template sequence. Plasmid pLP203 carries the c2 phage *ori* (369 bp), pObIL carries the bIL67 *ori* (444 bp); pO923 carries the 923 *ori*, (434 bp). pTRKL2 is a lactococcal plasmid with pAMβ1 origin of replication (37). Lanes: 1, supercoiled DNA standard; 2 to 11, EtBr-stained agarose gel electrophoresis of the plasmid preparations: 2, pLP203; 3, pObIL; 4, pO923; 5, pTRKL2, all isolated from MG1363; 7, pLP203; 8, pObIL; 9, pO923; 10, pTRKL2, all isolated from IL1403. Lanes 6 and 11 are the preparations of the host strains MG1363 and IL1403 included to indicate residual chromosomal DNA in the samples. Lane 12, Southern blot of the pObIL plasmid preparation from IL1403 transformant. The blot was probed with the full-length pObIL plasmid. Each lane was loaded with 1/20 volume of a plasmid prep from 7 optical density units (at 600 nm) of late exponential culture. Abbreviations: Pl., plasmid DNA bands; Chr. DNA, chromosomal DNA band.
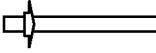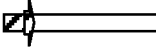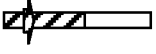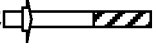
Construct	P_E1-T		Insert size (bp)	Transformation efficiency (c.f.u./µg DNA)
pLP203	c2		369	$2.83 \pm 0.19 \times 10^4$
pO923	923		434	$4.98 \pm 0.25 \times 10^4$
pMc2-923	923		434	$6.09 \pm 0.21 \times 10^4$
pSc2-923	c2/923		418	<2
pS923-c2	923/c2		415	<2

FIG. 5. Schematic representation of chimeric *ori* constructs and their ability to support plasmid replication. Diagonally hatched, c2 sequences; white, 923 sequences. Block arrow, P_E1 promoter. Transformation efficiency was tested using strain MG1363 as a recipient for electroporation.

ants found in prolate phage population, and the high conservation of P_E1-T templates within a single *ori* type, suggested that the sequence integrity of a P_E1-T transcript might be critical for origin function. Origin function might be dependent on P_E1-transcript secondary structure, primary sequence motifs, or both. To distinguish between these possibilities, we constructed two chimeric origins: one was composed of the promoter and 5′ half of the c2 P_E1-T template fused to the 3′ half of 923 P_E1-T template (pSc2-923), and the other was the reverse, promoter and 5′ half from 923 and 3′ half from c2 (pS923-c2). As a control, a chimera that contained the complete P_E1 coding sequence from c2, but the promoter from 923 phage (pMc2-923) was constructed (Fig. 5).

Neither construct with the chimeric P_E1-T template could drive replication of pVA891. In contrast, the control chimera was functional as a plasmid origin. This suggests that the integrity and perhaps conserved secondary structure of the P_E1 transcript is important for replication.

Secondary structures are conserved within each *ori* type. To assess the relationship between the secondary structures of the P_E1 transcripts, ClustalW alignments of each of the three types of *ori* P_E1 transcripts were subjected to the modeling of the secondary structures using the program ALIFOLD (http://rna.tbi.univie.ac.at/cgi-bin/alifold.cgi) (21). The predicted structures suggest that most of the nucleotide differences among the origins of the same type were conserved (Fig. 6A, B, and C). Also apparent was that the predicted secondary structures of the three types of P_E1 transcripts were different. When the alignment all sequenced origins was subjected to the ALIFOLD analysis, very few nucleotides showed conserved changes, confirming the absence of conservation of the secondary structure among the three origin types (Fig. 6D). The lack of conservation of secondary structure is consistent with the lack of functionality of the chimeric origins.

Host requirements for prolate phage replication. PolI is required for initiation of replication of ColE1 plasmid, where the origin transcript RNA II serves as a primer for this replicase. To determine whether the PolI is required for prolate phage replication, phage with c2 and 923 origins were plated on the *polA* mutant of MG1363 (16). These phages plated on

polA mutant with the 100% efficiency relative to the parent MG1363 showing that PolI is not required for the initiation of replication.

BLAST searches for sequences with high identities to P_E1-T templates and flanking sequences. Variants of the prolate phage P_E1-T templates could have been acquired during evolution by horizontal transfer, through recombination with other phage, plasmids or chromosome, via the short sequences of high identity. To look for sequence identities with other organisms, we carried out BLAST searches (1) using the P_E1-T template sequences of c2, bIL67, and 923 phage as queries. No sequences significantly similar to the c2 P_E1-T template were found in the GenBank nonredundant database, other than that of a prolate phage of the same origin type (46), nor were there any significant sequence identities to the 923 P_E1-T sequence. Interestingly, the bIL67 P_E1-T template shared a region of homology, 76 nt in length and 88% identity, with the intergenic sequence of a small isometric phage of the 936 species, bIL170 (positions 28949 to 29025) (13). Within this bIL67 sequence, a 57-nt stretch also shared homology to a sequence 480 nt upstream of the −35 box of the *repB* gene near the replication origin of plasmids pIL103 and pAW601 (92% identity) (18, 48; N. N. Matvienko, A. Madsen, and J. Josephsen, unpublished data [NCBI accession no. AJ132009]). Another BLAST search was carried out, with the sequence that included conserved sequences (60 nt on either side) flanking the divergent P_E1 template. This search detected identity of the P_E1 promoter of the c2 phage to *L. lactis* strain IL1403 genomic sequence (4), 28 nt long with 96% identity (nt 7399 to 7426; section 185). The identity is overlapping with the promoter of ORF coding for the putative phenolic acid decarboxylase (position −44 to −16). The prolate phage origin sequences with the above homologies were highlighted (bold and underlined) in Fig. 1A.

DISCUSSION

The prolate phage c2 origin of replication is functional in the absence of phage proteins (53). There have been no other reports of a lactococcal phage origin that drives plasmid replication in the complete absence of any phage protein, although the protein requirement is unclear for the phage sk1 (936 species) (10). The sk1 origin plasmid construct tested included an incomplete ORF, and the involvement of this ORF in replication has not been determined (10). We have now analyzed the requirements for replication of prolate phages other than c2 and showed that prolate phages have at least three types of P_E1 RNA template sequences, which we have named the c2, 923, and bIL67 types, after the phages representing each group. These sequences shared only 13% identity among eight prolate phages. An exhaustive survey of the entire prolate phage isolate collection from New Zealand dairy plants with different geographical and temporal histories showed that there were only three types of P_E1 RNA template sequences in New Zealand isolates over the last 50 years.

The region of sequence divergence of the prolate phage origin extended from the +16 nucleotide of the P_E1-T template sequence to the −35 box of the next downstream early promoter, P_E2. The flanking regions, including the P_E1 and P_E2 promoters, were highly conserved. BLAST searches using the prolate phage P_E1-T template variable and the flanking
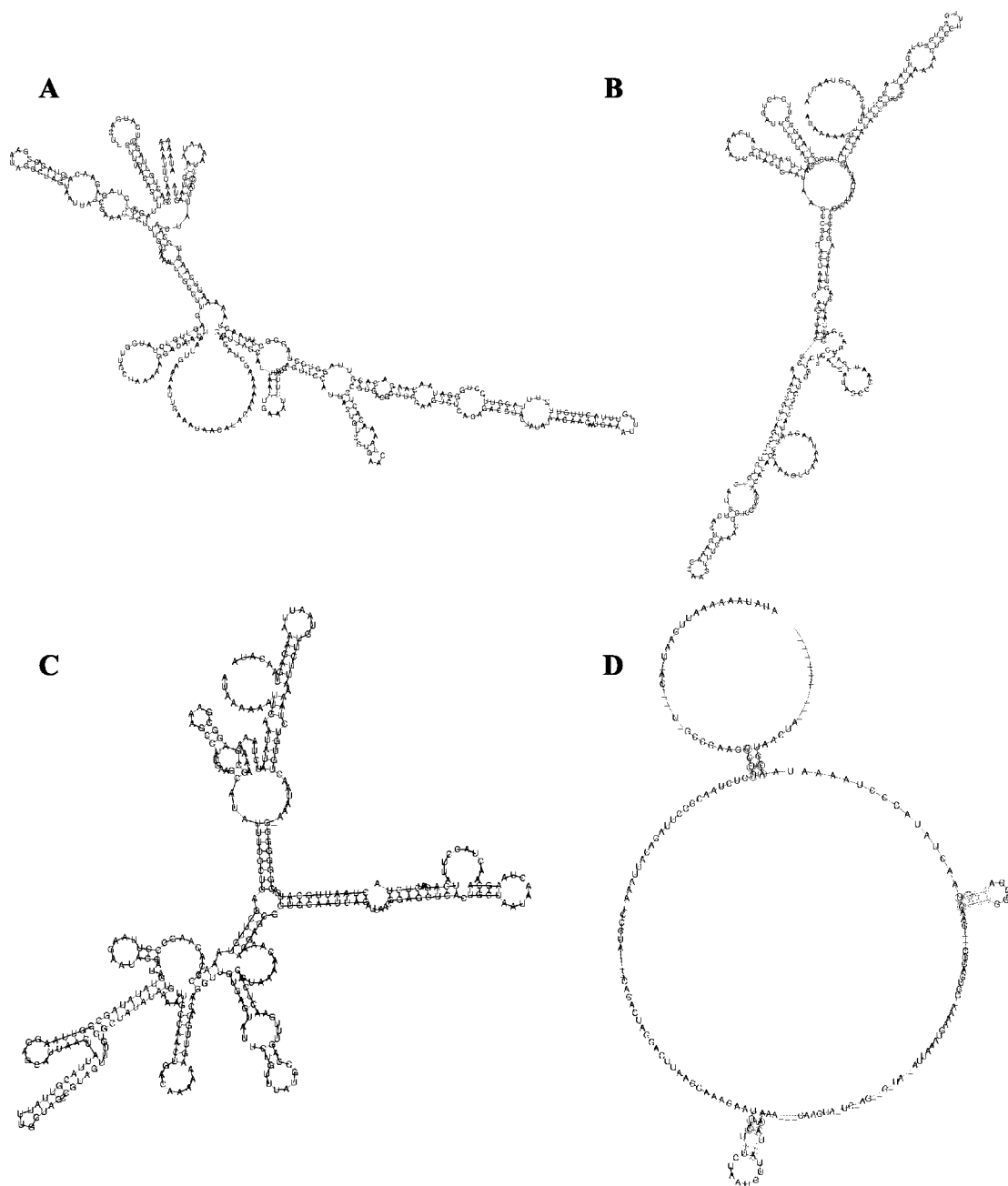
FIG. 6. Secondary structure predictions of the aligned P_E1 transcripts using the program ALIFOLD (http://rna.tbi.univie.ac.at/cgi-bin/alifold .cgi) (21). (A) c2 type; (B) bIL67 type, (C) 923 type, (D) all sequenced origins.

conserved sequences as queries detected short highly identical sequences in phage, plasmid and chromosome. Thus, it is possible that new P_E1-T templates in prolate phages could have been acquired by recombination from other lytic phages, resident plasmids or the lactococcal chromosome via the short highly homologous sequences (microhomologies), as proposed by Cambpell and Botstein for lambdoid phages (8). Similarly to previous reports of recombination in lactococcal phages of the P335 species (5, 15, 36), acquisition of a new origin in the prolate phages could have been selected for in hosts that carried a putative defense mechanism(s) targeting the origin of a

particular type. An extensive cross-plating matrix analysis would be required to detect such strains in our phage/host strain collection. A small-scale experiment, with six host strains and corresponding phages of which the *ori* sequences were determined in this study did not detect any such strain (data not shown).

Despite the lack of conservation of their sequences, all three types of minimal origins served as templates for transcription of untranslated P_E1 RNAs. The Northern blot and RNase protection experiments detected multiple P_E1 RNAs in cells infected with phages representing the three *ori* types, c2,

bIL67, and 923. Each type produced a unique pattern of P_E1 RNAs, in accordance with different lengths and divergent sequences of the P_E1-T templates. Sequence analysis did not detect appropriately positioned transcriptional terminators downstream of the 3′ ends of the P_E1 RNAs. Therefore, it is possible that the mature P_E1 RNAs are derived from longer precursor transcripts by host RNA-processing enzymes, such as RNase H, 3′-5′ exoribonucleases, RNase P, or RNase III (19, 26, 27).

Despite the lack of homology among the three types of P_E1 RNAs, it was possible that putative short sequence motifs that they shared might be required for replication. This was tested by constructing precise c2-923 and 923-c2 chimeric origins. The chimeric constructs contained complete origin sequences—thus, any potential functionally important shared sequences would have been present—but they failed to replicate. Since specific sequence motifs are not sufficient for replication, the proper secondary structure is most likely required for function of P_E1 transcripts. The transcript does not serve as a primer for PolI-dependent initiation of replication; thus, it is possible that, as in T4 phage, it serves to capture the coding strand and liberate the noncoding strand to allow assembly of the primosome and initiation of replication.

This study characterized RNAs expressed from replication origins in a family of homologous phages and suggested their role in replication. Further research will be required to determine the mechanism of processing, stabilization and initiation of replication by prolate phage P_E1 RNAs.

ACKNOWLEDGMENTS

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