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An Origin of DNA Replication from *Lactococcus lactis* Bacteriophage c2

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An origin of DNA relication was identified in the intergenic region between the early and late gene regions of prolate lactococcal phage c2. A DNA fragment containing this origin, designated *ori*, was shown to direct DNA replication in *Lactococcus lactis* but not in *Escherichia coli*. A comparison of *ori* with the corresponding regions of other prolate phages revealed strict conservation of the nucleotide sequence in one half of this intergenic region. This conserved region alone would not support DNA replication. No open reading frames were identified in the *ori* fragment, suggesting that host factors alone are sufficient to initiate DNA replication at *ori*. A novel class of lactococcal vectors and *E. coli-L. lactis* shuttle vectors based on *ori* have been constructed.

Bacteriophage c2 is a prolate-headed lytic phage of the industrially important bacterium *Lactococcus lactis*, belonging to the *Siphoviridae* family. Previous work has described a transcription map identifying early and late expressed regions of the genome (1). The 22,163-bp linear double-stranded genome has recently been fully sequenced and analyzed (GenBank accession number L48605) (6). The present work describes the identification of an origin of DNA replication of phage c2.

The genes of phage c2 are organized into two divergently orientated blocks corresponding to the early and late transcribed genes. Between the two blocks of genes is a 611-bp noncoding intergenic region. To study regulation of the late promoter, a 521-bp DNA fragment (*ori*) containing a late gene promoter (*LP1*) and the divergently transcribed constitutive early gene promoter (*EP1*) was amplified by PCR and ligated to a tetracycline resistance reporter gene (8). This construct was then ligated to the *Sma*I site of the broad-host-range plasmid pMIG1, which contains an origin of replication active in *L. lactis* (15), to create pMIG1-*tet-ori*. However, upon transformation in *L. lactis* MG1363, a structural instability not seen in *Escherichia coli* MC1022 was observed. This instability typically represented deletions encompassing either c2 *ori* or the pMIG1 rolling circle origin (2), as determined by restriction analysis. Deletions involving the pMIG1 origins of replication always resulted in the loss of both the single-stranded and double-stranded origins (13). The ability of pMIG1-*tet-ori* to replicate despite deletion of the pMIG1 vector origin suggested that the c2 *ori* fragment contained an origin of replication and that the instability arose from the presence of two active origins on one molecule in the lactococcal cytoplasm. A similar instability was noted by Schouler et al. when they inadvertently coinsertionally cloned a fragment which appears to contain the corresponding genomic region of lactococcal prolate phage ϕ197 into the theta-replication plasmid pIL253 (pOA18) (11). To test if the c2 *ori* fragment could function as an origin of replication in *L. lactis*, it was cloned in the origin probe vector pVA891 (7), creating pVA891-*ori*, and in pUC19 containing a selectable chloramphenicol resistance gene (*cat*), creating pUC19-*ori-cat*. pVA891 and pUC19 do not contain lactococcal origins of replication and do not replicate in *L. lactis*. They do, however, replicate in *E. coli* because of the presence of an *E. coli* origin of replication from pACYC184 (pVA891) or pMB1 (pUC19). Both plasmids containing the *ori* fragment were cloned in *E. coli* MC1022 and then electroporated into *L. lactis* MG1363. pVA891-*ori* and pUC19-*ori-cat* were recovered from these transformants and shown to contain a single copy of the *ori* fragment by restriction analysis, Southern hybridization, and DNA sequencing. These data indicate that the *ori* fragment can function as a plasmid origin of replication.

To examine the activity of the *ori* fragment in the absence of any plasmid sequences, apart from a selectable marker, a small (approximately 2.05-kb) novel plasmid, pLPE+, was created by the ligation of c2 *ori* to a fragment containing an erythromycin resistance gene and a multiple cloning site. This plasmid was maintained in *L. lactis* MG1363, and large amounts of pLPE+ DNA were recovered from transformed *L. lactis* cells. However, electrophoresis of this DNA revealed only a large amount of high-molecular-weight DNA in the sample well. Restriction analysis suggested the presence of a range of molecules containing multiple tandem duplications of the c2 *ori*. Similar high-molecular-weight DNA was observed with pMIG1-*tet-ori* and was also associated with the deletion products obtained by attempts at cloning c2 *ori* into the *L. lactis-E. coli* shuttle vector pNZ272 (9). However, high-molecular-weight DNA was not observed with pVA891-*ori* or pUC19-*ori-cat*. It is possible that large multimeric forms of the plasmid are produced during replication and that recombination within and between these forms results in the observed molecules containing duplications of *ori*. The production of large replication intermediates may be an important functional feature of this phage origin. Powell et al. (10) have suggested that the genome of lactococcal prolate phage c6A, which is closely related to phage c2 (4), is synthesized in the form of large replicative intermediates, which are possibly membrane associated (3). The presence of a highly recombinogenic resolution site, common in *E. coli* plasmids (14), may be responsible for maintaining the monomeric forms observed for pVA891-*ori* and pUC19-*ori-cat*.

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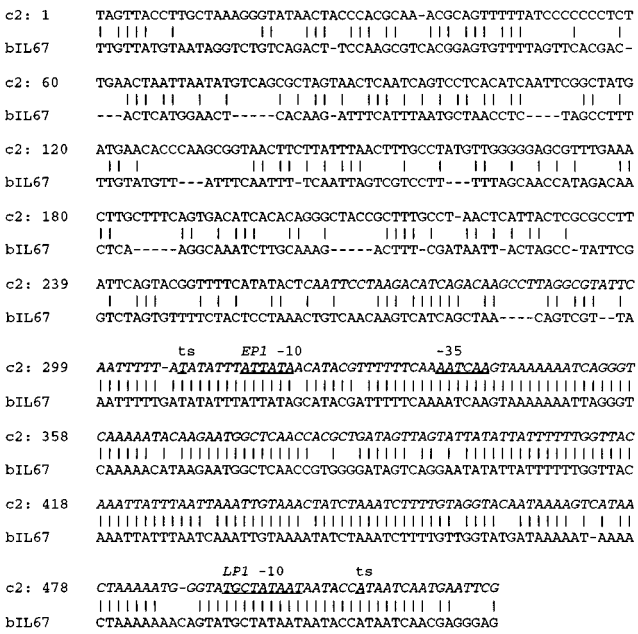

FIG. 1. Alignment of the nucleotide sequences of the c2 *ori* PCR fragment and the homologous region from related phage bIL67. The transcription start points (ts) and conserved hexamer motifs (−10 and −35) of the late promoter, *LP1*, and divergently orientated early promoter, *EP1*, are underlined. Vertical bars indicate sequence identity, and gaps have been introduced to maximize the alignment. The map positions of the *ori* fragment are given. The 260-bp subfragment unable to support replication alone is highlighted in italics.

The nucleotide sequence of the 521-bp *ori* fragment, together with the homologous region of related phage bIL67, is presented in Fig. 1. An AT-rich region containing repeat sequences is characteristic of DNA origins of replication (5). Analysis of the c2 *ori* sequence revealed a very AT-rich region (78% A+T) between bases 294 and 521 and several small perfect and imperfect inverted and direct repeats. Almost all of the inverted repeats and several direct repeats were located in the AT-rich region. This region of the *ori* fragment was also highly conserved in prolate lactococcal phages bIL67 (12) and ϕ197 (11), suggesting that it is important for origin function. Both the intact *ori* fragment and a 261-bp PCR-generated proximal subfragment (map positions 261 to 521 bp) were successfully cloned in a pUC12 derivative (which contains a macrolide-lincosamide-streptogramin B resistance gene for selection in lactococci) in *E. coli* MC1022. Unlike the intact *ori* fragment, the conserved 260-bp subfragment did not support replication in *L. lactis* MG1363. These data indicate that the sequence between positions 1 and 261 bp is necessary for *ori* function. Interestingly, this region is not conserved in the more distantly related prolate phage bIL67 (Fig. 1).

The c2 *ori* region is closely linked with promoters *EP1* and *LP1*, and the location of the origin between the divergently oriented early and late genes means that replication would be codirectional with transcription. The absence of open reading frames in the *ori* fragment indicates that lactococcal host factors alone were used for the replication of pVA891-*ori* and pUC19-*ori-cat*. This is surprising since it is common for phage proteins to be required for replication of phage DNA (5). It is unknown if phage factors are required for replication of the phage c2 genome or whether the *ori* described here is the main or only origin of phage c2. However, given the location of *ori*, the size of the phage genome, and the sizes and amounts of the DNA molecules resulting from pLPE+ replication, it seems possible that this sequence represents the primary origin of replication. DNA replication in prolate phage c6A, which is closely related to phage c2 (4), was shown to begin between 5 and 6 min into phage infection and to increase at a constant rate throughout the cycle (10). Calculations by Powell et al. suggested that the bacterial host DNA replication apparatus could easily synthesize sufficient DNA from a single origin in the phage latent period to satisfy the synthesis rate requirement.

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REFERENCES

1. **Beresford, T. P. J., L. J. H. Ward, and A. W. Jarvis.** 1993. Temporally regulated transcriptional expression of the genomes of lactococcal bacteriophages c2 and sk1. Appl. Environ. Microbiol. **59:**3708–3712.
2. **De Vos, W. M.** 1986. Gene cloning in lactic streptococci. Neth. Milk Dairy J. **40:**141.
3. **Firshein, W.** 1989. Role of the DNA/membrane complex in prokaryotic DNA replication. Annu. Rev. Microbiol. **43:**89–120.
4. **Jarvis, A. W., G. F. Fitzgerald, M. Mata, A. Mercenier, H. Neve, I. B. Powell, C. Ronda, M. Saxelin, and M. Teuber.** 1991. Species and type phages of lactococcal bacteriophages. Intervirology **32:**2–9.
5. **Keppel, F., O. Fayet, and C. Georgopoulos.** 1988. Strategies of bacteriophage DNA relication, p. 145–264. *In* R. Calendar (ed.), The bacteriophages, vol. 2. Plenum Press, New York.
6. **Lubbers, M. W., N. R. Waterfield, T. P. J. Beresford, R. W. F. Le Page, and A. W. Jarvis.** 1995. Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. Appl. Environ. Microbiol. **61:**4348–4356.
7. **Macrina, F. L., P. R. Evans, A. J. Tobian, D. L. Hartley, D. B. Clewell, and K. R. Jones.** 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. Gene **25:**145–150.
8. **Mojumdar, M., and S. A. Khan.** 1988. Characterization of the tetracycline resistance gene of plasmid pT181 of *Staphylococcus aureus*. J. Bacteriol. **170:** 5522–5528.
9. **Platteeuw, C., G. Simons, and W. M. de Vos.** 1994. Use of the *Escherichia coli* β-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl. Environ. Microbiol. **60:**587–593.
10. **Powell, I. B., D. L. Tulloch, A. J. Hillier, and B. E. Davidson.** 1992. Phage DNA synthesis and host DNA degradation in the life cycle of *Lactococcus lactis* bacteriophage c6A. J. Gen. Microbiol. **138:**945–950.
11. **Schouler, C., C. Bouet, P. Ritzenthaler, X. Drouet, and M. Mata.** 1992. Characterization of *Lactococcus lactis* phage antigens. Appl. Environ. Microbiol. **58:**2479–2484.
12. **Schouler, C., D. S. Ehrlich, and M.-C. Chopin.** 1994. Sequence and organisation of the lactococcal prolate-headed bIL67 phage genome. Microbiology (Reading) **140:**3061–3069.
13. **Seegers, J. F. M. L.** 1994. Ph.D. thesis. Rijksuniversiteit Groningen, Groningen, The Netherlands.
14. **Summers, D. K., and D. J. Sherratt.** 1984. Multimerization of high copy number plasmids causes instability—ColE1 encodes a determinant essential for plasmid monomerization and stability. Cell **36:**1097–1103.
15. **Wells, J. M., P. W. Wilson, and R. W. F. Le Page.** 1993. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. J. Appl. Bacteriol. **74:**629–636.