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# Sequence analysis and molecular characterization of the temperate lactococcal bacteriophage r1t

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## Summary

**The temperate lactococcal bacteriophage r1t was isolated from its lysogenic host and its genome was subjected to nucleotide sequence analysis. The linear r1t genome is composed of 33 350 bp and was shown to possess 3′ staggered cohesive ends. Fifty open reading frames (ORFs) were identified which are, probably, organized in a life-cycle-specific manner. Nucleotide sequence comparisons, N-terminal amino acid sequencing and functional analyses enabled the assignment of possible functions to a number of DNA sequences and ORFs. In this way, ORFs specifying regulatory proteins, proteins involved in DNA replication, structural proteins, a holin, a lysin, an integrase, and a dUTPase were putatively identified. One ORF seems to be contained within a self-splicing group I intron. In addition, the bacteriophage *att* site required for site-specific integration into the host chromosome was determined.**

## Introduction

A group of organisms that is increasingly receiving more scientific attention is that of the lactic acid bacteria. Of these, the species *Lactococcus lactis* is the best studied (for an extensive overview, see Gasson and de Vos, 1994); it represents an economically extremely important group of microorganisms as it is used throughout the world in the dairy industry. From a biotechnological perspective *L. lactis* serves as a model organism for the application in the high-tech fermentation industry. From a scientific point of view, the species is highly interesting for its genetic manageability and flexibility. Genetic reorganization by conjugation, transduction and transposition has been described, and a panoply of IS elements and plasmids found in any one strain further contributes to the characteristic genetic instability of this species (Gasson and Fitzgerald, 1988; 1994). Although this genetic variability has been shown in some instances to result in the loss of important fermentation traits (McKay, 1983), by far the most disturbing problem leading to fermentation failure is the omnipotence of bacteriophages that infect *L. lactis* (first described by Whitehead and Cox, 1935). Both lytic and temperate phages have been identified and classified on the basis of criteria such as morphology, immunoresponse, as well as protein profiles and DNA hybridizations (see Jarvis *et al.*, 1991).

Nearly 50 years after their first description (Reiter, 1949), it is now generally accepted that many strains of *L. lactis* are lysogenic. From one to several prophages may be present in the chromosome of a lactococcal strain (for reviews see Davidson *et al.*, 1990; Klaenhammer and Fitzgerald, 1994). This widespread incidence of lysogeny, and the resulting superinfection immunity may have hindered the identification of suitable indicator strains, and it was not until 1980 that Gasson and Davies (1980) provided formal proof of classical lysogeny in *L. lactis*.

One of the main goals of our research group has been the development of basic genetic tools for the study and manipulation of lactic acid bacteria. This has resulted in the construction of the first cloning vectors for these organisms as well as plasmids for the isolation of promoters and terminators (Kok *et al.*, 1984; van der Vossen *et al.*, 1985; 1987). The isolated promoters have been employed for the construction of the first line of gene expression vectors for *L. lactis* (van de Guchte *et al.*, 1989). One of the reasons for analysing the temperate lactococcal bacteriophage r1t was to identify a simple genetic switch, similar to that of phage lambda (Ptashne, 1986), and to exploit this switch for regulated gene expression in lactic acid bacteria. Phage r1t, which was first detected in *L. lactis* subsp. *cremoris* R1 (Lowrie, 1974), belongs to the Siphoviridae, morphotype B1 (Ackerman and DuBow, 1987), and is characterized by a small isometric head with a diameter of 53 nm and a non-contractile tail of 126 nm (Jarvis, 1977). According to the classification of lactococcal bacteriophages as advanced by Jarvis *et al.* (1991), it is grouped

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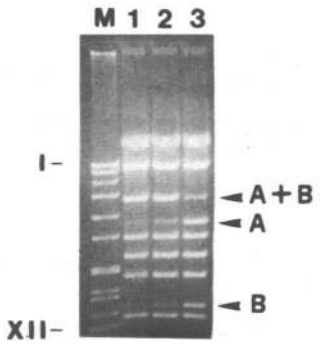


**Fig. 1.** Agarose (0.8%) gel electrophoresis of r1t DNA. Lanes: 1, r1t DNA treated with T4 DNA ligase before digestion with *Eco*RI and heated for 5 min at 60°C immediately before electrophoresis; 2, r1t DNA digested with *Eco*RI; 3, r1t DNA digested with *Eco*RI and heated for 5 min at 60°C immediately before electrophoresis; M, molecular size marker SPP1 DNA digested with *Eco*RI. Sizes (in bp) of fragments I–XII: 8545, 7425, 6150, 4965, 3590, 2745, 1970, 1910, 1560, 1450, 1160, and 990, respectively. Fragments indicated by the arrows are discussed in the text.

in species P335. Two independent reports have described the isolation of cured derivatives of strain R1 (Georghiou *et al.*, 1981; Gasson and Davies, 1980).

Similar to all lactococcal phages characterized thus far, the r1t genome is a double-stranded linear DNA molecule. Here, we report on the entire nucleotide sequence of the genome of r1t; the identification of *cos* and *att*; the description of its open reading frames (ORFs); and the assignment, on the basis of sequence similarities, N-terminal amino acid sequence data and functional analyses, of possible functions to a number of these ORFs.

## Results

### *The r1t genome possesses 3′ single-stranded cohesive termini*

Agarose gel electrophoresis of r1t DNA digested with *Eco*RI revealed the presence of three submolar DNA fragments (Fig. 1; lane 2). Heating this digest to 60°C for 5 min immediately prior to loading on the agarose gel resulted in decreasing amounts of the largest submolar fragment (A+B), while the intensity of the two smaller submolar fragments (A and B) increased (Fig. 1; lane 3). In contrast, treating the r1t DNA with T4 DNA ligase before *Eco*RI restriction caused fragment A+B to be resistant to heat dissociation (Fig. 1; lane 1). The presence of the heat-dissociable fragment in the *Eco*RI digest indicated that the r1t genome possesses cohesive ends representing a cleavage site that is recognized by the phage packaging apparatus (Murialdo, 1991). In order to determine the DNA sequence of the cohesive ends of the r1t genome, the largest submolar *Eco*RI fragment (A+B) containing the r1t *cos* site was subcloned and subjected to nucleotide sequence analysis. The obtained nucleotide sequence allowed the design of primers A and B complementary to sequences at either side of the presumed *cos* site and oriented such that their 3′ ends were directed towards *cos*. These primers were subsequently employed for sequencing the cloned A+B fragment (Fig. 2; lanes 2), and for sequencing-runoff experiments using: (i) phage genomic DNA (Fig. 2; lanes 1) or (ii) each of the submolar *Eco*RI restriction fragments A and B, isolated from an agarose gel (Fig. 2; lanes 3A and 3B, respectively), as templates. Comparing the nucleotide sequence of the *cos* region obtained from sequencing the cloned *Eco*RI A+B fragment with the runoff sequences revealed that r1t possessed a *cos* site with an 11 nucleotide (nt) 3′ overhang (Fig. 2). This sequence is identical to that of the *cos* site of the lactococcal phage φLC3 (Lillehaug *et al.*, 1991). As is clear from the runoff experiments using complete genomic DNA (Fig. 2; lanes 1), a significant portion of the r1t genomes possess termini which apparently extend much further than *cos*.

### *Determination of the* attP, attL, attR *and* attB *sites*

To locate the site of integration of phage r1t into the host chromosome, various restriction-enzyme digests of chromosomal DNA of *L. lactis* R1 were used for Southern hybridizations using r1t genomic DNA as a probe, and compared to corresponding restriction patterns of r1t genomic DNA. When compared to the hybridization pattern of r1t genomic DNA, an r1t fragment of 5.7 kbp was absent in the *L. lactis* R1 chromosomal *Sau*3A digest. The pattern contained two additional DNA fragments, which were the products of the integrative recombination event. It appeared that a 3500 bp chromosomal *Sau*3A DNA fragment contained *attL* and a 2800 bp chromosomal *Sau*3A DNA fragment *attR* (data not shown). In order to determine the bacteriophage/chromosomal DNA junctions in the lysogenized strain, primers were designed (with the aid of the r1t nucleotide sequence) to perform inverse polymerase chain reaction (PCR) using R1 chromosomal DNA, which had been cut with *Sau*3A and religated under dilute conditions. The resulting (junction) DNA fragments of 1300 and 1000 bp containing *attL* and *attR*, respectively, were used for direct sequencing (results not shown). The obtained sequences were used to design two new primers to amplify and sequence a chromosomal DNA fragment of 252 bp (EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries Accession number U38905) encompassing the *attB* site from strain R1K10, a prophage-cured derivative of R1. Comparing the obtained sequences from the DNA regions containing *attL*, *attR*, *attP* and *attB* revealed that they all shared a 9 bp sequence, TTCTTCATG, representing the site of recombination between r1t and the host chromosome. Identical sequences were found to be involved in site-specific integration of the

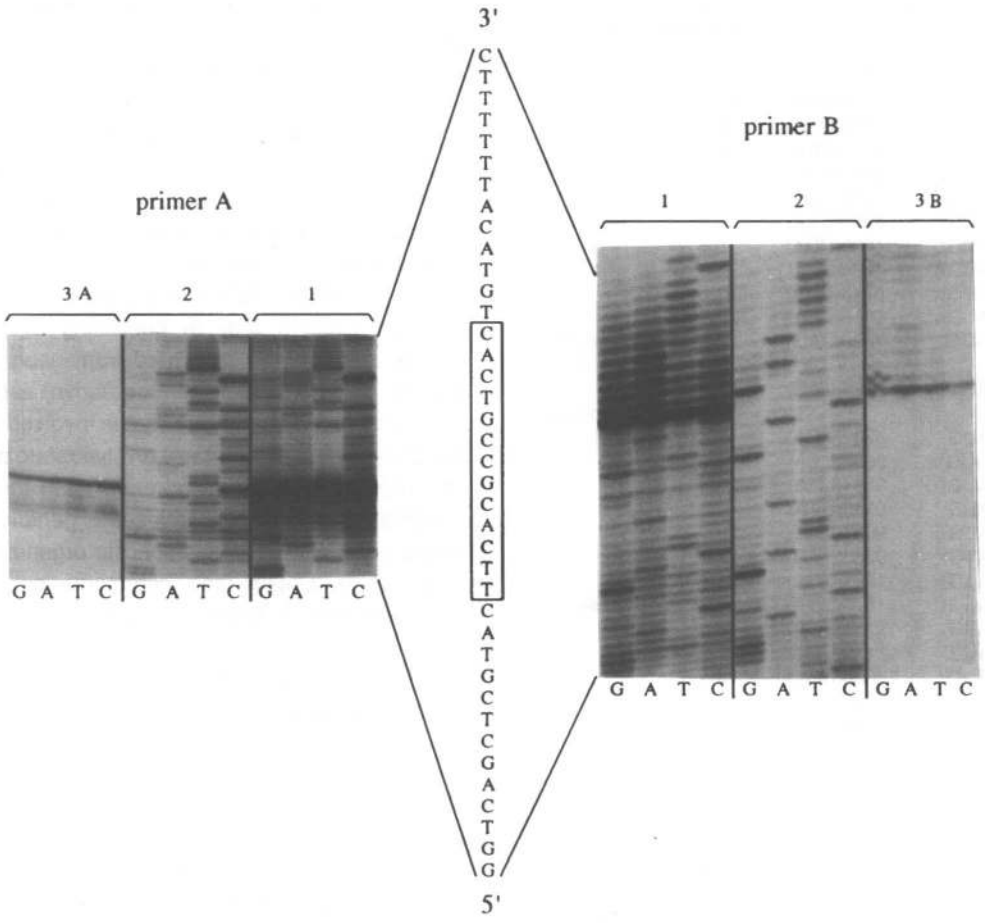


**Fig. 2.** Determination of the cohesive ends of the r1t genome. Cycle sequencing reactions were performed to produce runoff products using primers A (3′-GTGAAGCAAGAAGAACCAAAGG-5′; left panel) or B (3′-CATAGCGCATTTCTGCTCTTCG-5′; right panel) and Vent DNA polymerase (exo$^+$) in combination with the following templates: lanes 1, phage r1t genomic DNA; lanes 2, cloned submolar fragment A+B; lane 3A, purified submolar fragment A; lane 3B, purified submolar fragment B. The autoradiograph in the right panel is turned inside out and upside down to have it corresponding to the sequence depicted in the middle. The boxed sequence indicates the *cos* site.

temperate lactococcal phages ϕLC3 (Lillehaug *et al.*, 1993) and Tuc2009 (van de Guchte *et al.*, 1994a).

### *Nucleotide sequence determination of the r1t genome*

Both DNA strands of the entire r1t genome were sequenced (the r1t DNA sequence has been assigned Accession Number U38906 in the EMBL/GenBank/DDJB Nucleotide Sequence Data Libraries). The sequences were assembled using the strategy outlined in the *Experimental procedures.* A linear DNA sequence of 35 550 bp was obtained with each base pair sequenced, with a redundancy of approximately four. The predicted restriction maps and those obtained experimentally were in full agreement with each other, indicating that no obvious assembly errors had occurred. The total G+C content of the r1t phage genome is 35.5%, which is similar to that of the lactococcal host (Kilpper-Bälz *et al.*, 1982) and of the recently sequenced virulent lactococcal bacteriophage bIL67 (Schouler *et al.*, 1994).

### *Identification of r1t ORFs and genome organization*

Fifty putative protein-coding regions were identified in r1t, by applying the ANALYSEQ program of Staden (1986) and making use of available codon-usage information of known lactococcal phage genes (Klaenhammer and Fitzgerald, 1994). All of these ORFs except ORF3, ORF26 and ORF42 were preceded by potential Shine–Dalgarno (SD) sequences complementary to the 3′ end of the 16S rRNA of *L. lactis* (Ludwig *et al.*, 1985) and at an appropriate distance (6 to 15 bp) from one of the commonly used initiation codons (AUG, UUG or GUG). ORF3 encodes the putative repressor of r1t and its translation may depend on signals different from the conventional ribosome binding site (RBS), a situation that would be similar to that for

**Table 1.** General features of the identified r1t ORFs.

| ORF | Start | End | kDa | Comments |
|---|---|---|---|---|
| 1 | 1218(AUG) | 94(UAA) | 43.3 | Integrase |
| 2 | 1860(AUG) | 1339(UAA) | 18.7 | |
| 3 | 2757(AUG) | 1921(UAA) | 31.5 | Repressor |
| 4 | 2987(AUG) | 3229(UAG) | 9.1 | cro-like |
| 5 | 3241(AUG) | 4038(UAG) | 30.1 | |
| 6 | 4051(AUG) | 4383(UAA) | 12.9 | |
| 7 | 4385(AUG) | 4633(UGA) | 9.5 | |
| 8 | 4630(AUG) | 4806(UAA) | 6.8 | |
| 9 | 4910(AUG) | 5743(UGA) | 31.6 | |
| 10 | 5740(AUG) | 6666(UAA) | 34.5 | |
| 11 | 6895(GUG) | 7629(UAG) | 28.5 | Initiation of replication |
| 12 | 7639(AUG) | 8529(UAG) | 34.3 | DnaC homologue |
| 13 | 8538(AUG) | 8702(UGA) | 6.4 | |
| 14 | 8692(GUG) | 9087(UAA) | 16.0 | |
| 15 | 9090(AUG) | 9284(UAA) | 7.5 | |
| 16 | 9277(AUG) | 9516(UAA) | 9.5 | |
| 17 | 9628(AUG) | 9807(UAA) | 6.9 | |
| 18 | 9797(AUG) | 10366(UAG) | 22.4 | |
| 19 | 10420(AUG) | 11061(UGA) | 25.4 | |
| 20 | 11058(AUG) | 11477(UGA) | 15.2 | dUTPase |
| 21 | 11480(AUG) | 11731(UGA) | 9.4 | |
| 22 | 11728(AUG) | 12414(UGA) | 26.0 | |
| 23 | 12537(GUG) | 13028(UGA) | 19.4 | |
| 24 | 13399(AUG) | 13935(UAA) | 20.8 | |
| 25 | 14018(AUG) | 14449(UAA) | 17.3 | |
| 26 | 14575(AUA) | 14907(UAA) | 12.4 | |
| 27 | 15054(UUG) | 16283(UAA) | 45.8 | Structural protein C |
| 28 | 16283(AUG) | 17049(UAA) | 29.2 | |
| 29 | 17098(AUG) | 18507(UAG) | 53.2 | |
| 30 | 18580(UUG) | 19044(UAA) | 17.4 | |
| 31 | 19048(AUG) | 19944(UAA) | 32.3 | Structural protein A/B |
| 32 | 19959(AUG) | 20258(UAA) | 11.5 | |
| 33 | 20260(AUG) | 20658(UAA) | 14.7 | |
| 34 | 20636(AUG) | 20983(UAA) | 12.6 | |
| 35 | 20976(AUG) | 21212(UAA) | 8.7 | |
| 36 | 21213(AUG) | 21548(UAG) | 12.9 | |
| 37 | 21559(AUG) | 22116(UAA) | 19.9 | Structural protein H |
| 38 | 22194(AUG) | 22454(UAA) | 9.9 | |
| 39 | 22601(AUG) | 22843(UAA) | 9.3 | |
| 40 | 22843(AUG) | 23484(UAG) | 22.4 | |
| 41 | 23550(AUG) | 24110(UAA) | 21.6 | Endonuclease |
| 42[a] | 24329(?) | 26302(UAG) | 68.3 | |
| 43 | 26315(AUG) | 27211(UAA) | 33.2 | |
| 44 | 27216(AUG) | 28373(UGA) | 43.9 | |
| 45 | 28370(AUG) | 29416(UAG) | 38.2 | Structural protein D |
| 46 | 29429(AUG) | 29776(UAA) | 13.4 | |
| 47 | 29790(AUG) | 31793(UAA) | 72.0 | |
| 48 | 31818(AUG) | 32045(UAA) | 7.7 | Holin |
| 49 | 32046(AUG) | 32858(UAA) | 30.2 | Lysin |
| 50 | 33038(AUG) | 33223(UAA) | 6.8 | |

**a.** The precise start of this ORF is uncertain and is indicated by a question mark.

the CI repressor of bacteriophage lambda (see the accompanying paper by Nauta *et al.*, 1996; Shean and Gottesman, 1992). ORF26 is preceded by a strong SD sequence ($\Delta G = -24.8\,kcal\,mol^{-1}$ (Tinoko *et al.*, 1973)) followed by an AUA codon, and this combination may function as an unusual translation initiation signal as was shown previously in *Bacillus subtilis* (Peijnenburg *et al.*, 1990). The apparent absence of translation initiation signals for ORF42 may be explained by the finding that ORF42 is located just downstream of a sequence similar to group I introns (see below). This suggests that the primary transcript containing ORF42 is processed to form an mRNA molecule that supplies the required initiation signals for ORF42 translation.

The identified ORFs are organized in two oppositely oriented clusters (Fig. 3) consisting of three and 47 ORFs, respectively, separated from each other on one side by the attachment site (see above) and on the other side by an intergenic region that is involved in the genetic switch between the lysogenic and lytic state of r1t (Nauta *et al.*, 1996 — accompanying paper). On the basis of the observed sequence similarities, this genetic arrangement would suggest a life-cycle-specific organization (see the *Discussion*). Details and comments on the identified ORFs are given in Table 1. Codon usage of the proposed 50 protein-coding regions of r1t was shown not to be significantly different from that of the lactoccal host (data not shown; van de Guchte *et al.*, 1992).

### *Similarities of the r1t nucleotide sequence and deduced proteins to known sequences*

The r1t nucleotide sequence as well as the deduced amino acid sequences specified by the identified r1t ORFs were screened for similarities with sequences present in the available databases (see the *Experimental procedures*). In addition to the *att* and *cos* sites (see above), significant similarities were observed for twelve protein-coding regions or DNA sequences on the r1t genome and will be discussed below.

*ORF1.* The deduced product of ORF1 as well as the DNA sequences upstream and downstream of ORF1 were almost identical to the integrase genes and their flanking regions of the temperate lactococcal phages ϕLC3 (Lillehaug and Birkeland, 1993) and Tuc2009 (van de Guchte *et al.*, 1994a). As is described above, all three phages recognize identical *attB* sites to integrate into their respective hosts.

*ORF3.* The protein encoded by ORF3 resembles lambdoid repressors, especially in the region that undergoes RecA-mediated cleavage (Sauer *et al.*, 1982), and regulator proteins involved in SOS induction. The ORF3 product also shares extended similarity with the putative repressor of the temperate lactococcal phage Tuc2009 (van de Guchte *et al.*, 1994b). This similarity is restricted to the C-terminal half of the ORF3 product and does not include the putative DNA-binding region which is thought

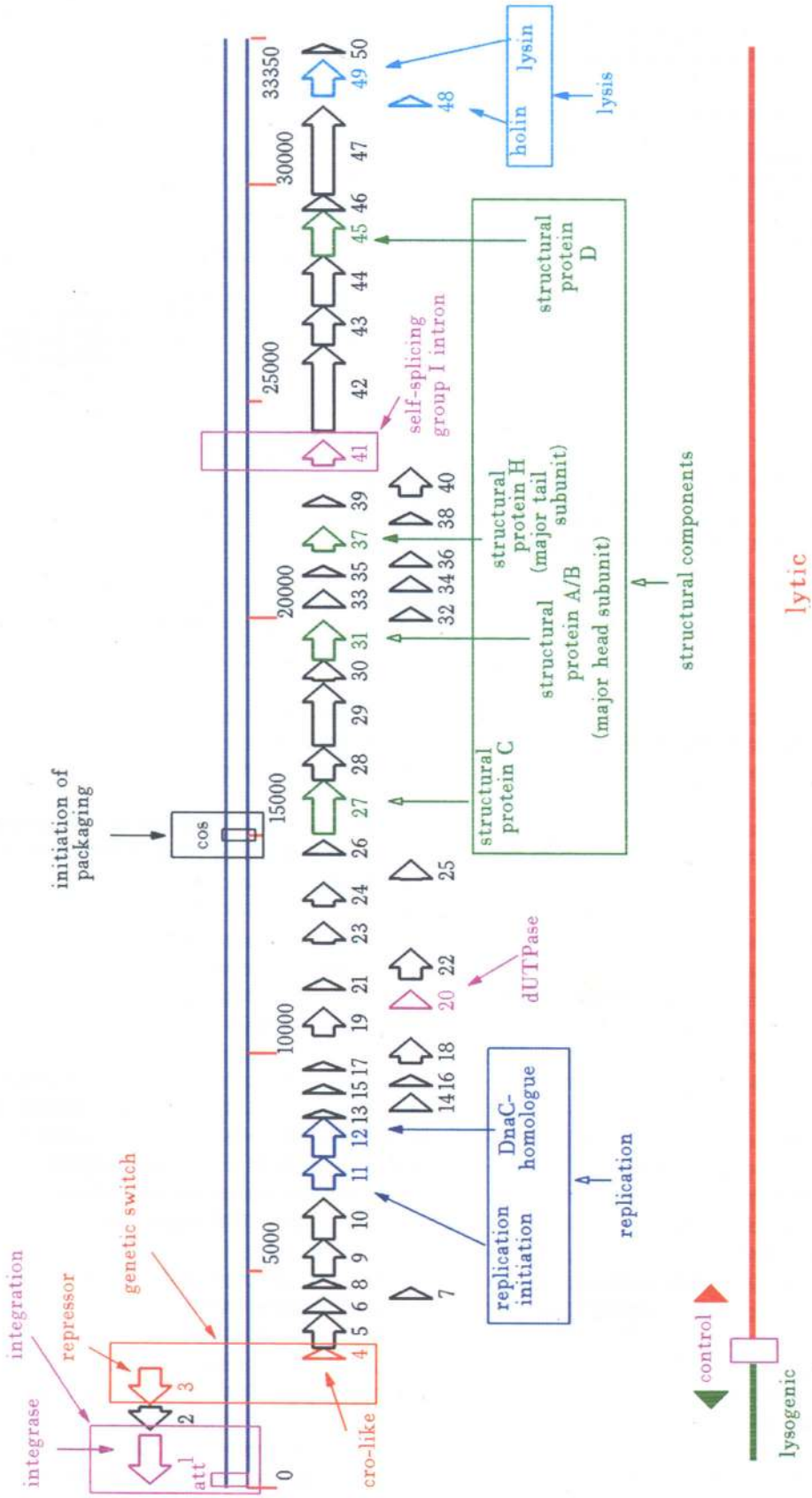


**Fig. 3.** Schematic representation of the r1t genome with its identified ORFs and features. The numbered ORFs are depicted as arrows or arrowheads above or below the r1t genome (double blue line) depending on their orientation. Putative or demonstrated functions of ORFs or sequences are indicated. Multiple genetic determinants constituting a general phage function are boxed and depicted by similar colours. The green and red bar at the bottom of the Figure corresponds to r1t ORFs which are required for the lysogenic and lytic life cycle, respectively (see Nauta *et al.*, 1996 — accompanying paper). A more detailed description of the various ORFs is given in the text.

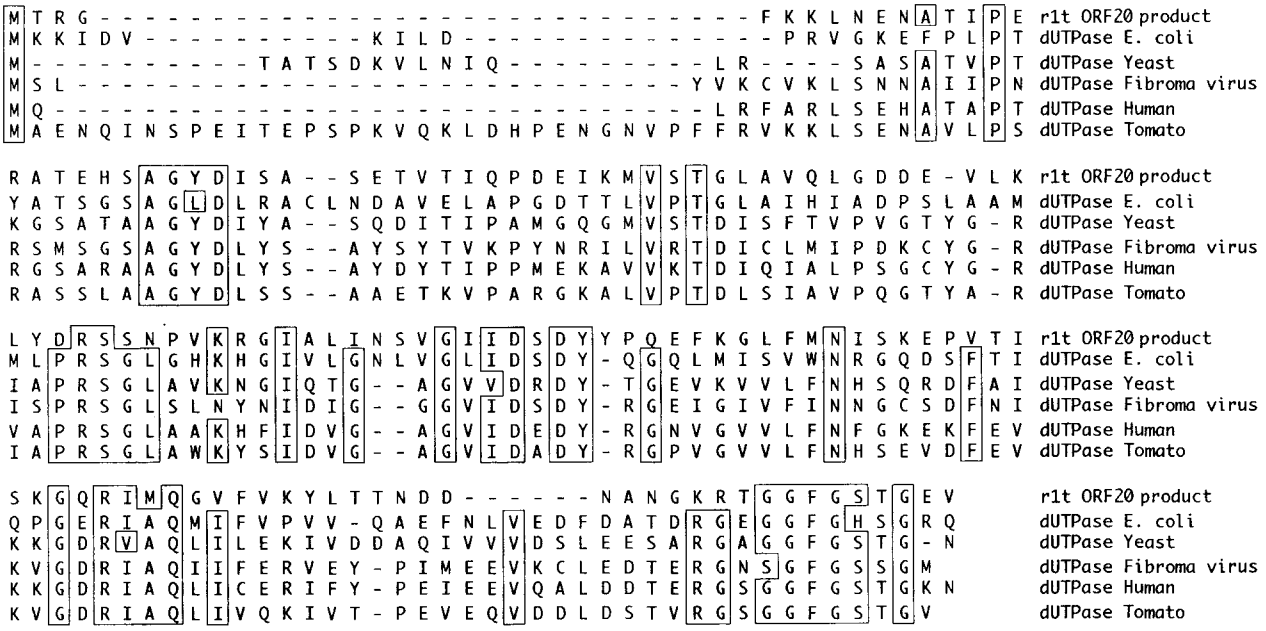


**Fig. 4.** Multiple sequence alignment of the deduced product of r1t ORF20 and various dUTPases. Amino acids are boxed if more than four of the depicted proteins contain identical residues at corresponding positions. The dUTPases shown are from *E. coli* (Accession Number P06968), yeast (Accession Number S38189), Fibroma virus (Accession Number P32208), human (Accession Number P33316), and tomato (Accession Number S40549).

to be involved in target recognition (van de Guchte *et al.*, 1994b). Experimental evidence that ORF3 indeed encodes a repressor is presented in the accompanying paper by Nauta *et al.* (1996). This protein, together with the putative λ Cro analogue encoded by ORF4, probably governs the genetic switch of phage r1t.

*ORF11 and ORF12.* The derived protein products of ORF11 and ORF12 are probably required for the initiation of DNA replication of r1t. The product of ORF11 is significantly similar (26.7% overall identity) to G38P, a DNA-binding protein that has been implicated as being the replisome organizer in replication initiation of the *B. subtilis* bacteriophage SPP1 (Pedré *et al.*, 1994). The *Escherichia coli* DnaA protein (Fuller *et al.*, 1984), of which SPP1 G38P is the functional analogue, initiates chromosomal DNA replication by binding to the replication origin *oriC* to form the initiation complex. Following delivery of the DNA helicase DnaB to the initiation complex via the action of DnaC, the DNA template starts to unwind in the presence of single-stranded DNA-binding protein (SSB) in order to facilitate subsequent priming and elongation (van der Ende *et al.*, 1985). Although phages SPP1, P22 and λ require many host-encoded proteins for replication, they all contain a genomic region which specifies a DnaA analogue for sequence-specific initiation and one or more components (*E. coli* DnaB, DnaC or SSB equivalents) of the initiation complex (Pedré *et al.*, 1994). In agreement with this genomic organization is the finding that r1t ORF11, the proposed DnaA analogue, is followed by ORF12, which specifies a protein with significant similarity (18.6% overall identity) to *E. coli* DnaC.

*ORF20.* The protein specified by ORF20 shows significant similarity to a large family of dUTPases (EC 3.6.1.23) of bacterial, viral and eukaryotic origin (Fig. 4). The function of this universal enzyme is to convert dUTP to dUMP, which is subsequently used for the synthesis of dTTP. Its action keeps intracellular dUTP levels low, thus preventing the synthesis of mutagenic uracil-substituted DNA (Brynoff *et al.*, 1978; Grafstrom *et al.*, 1978).

*ORF27.* The deduced amino acid sequence of ORF27, which specifies one of the structural proteins (see below), shows very high similarity (98.3% overall identity) to the product of an ORF immediately downstream of the *cos* site of the temperate lactococcal phage ϕLC3 (Birkeland *et al.*, 1993; NBRF-PIR, Accession Number X57797).

*ORF37.* The deduced protein of ORF37, identified as a structural component of the r1t phage (see below), was found to exhibit significant similarity (35.6% overall identity) to a structural protein (27.2 kDa) of the actinophage VWB (Anné *et al.*, 1990; Accession Number S34250).

*ORF41.* ORF41 resembles several entries in the databases of phage sequences (Fig. 5) shown to be contained within self-splicing group I introns. For two of these ORFs,

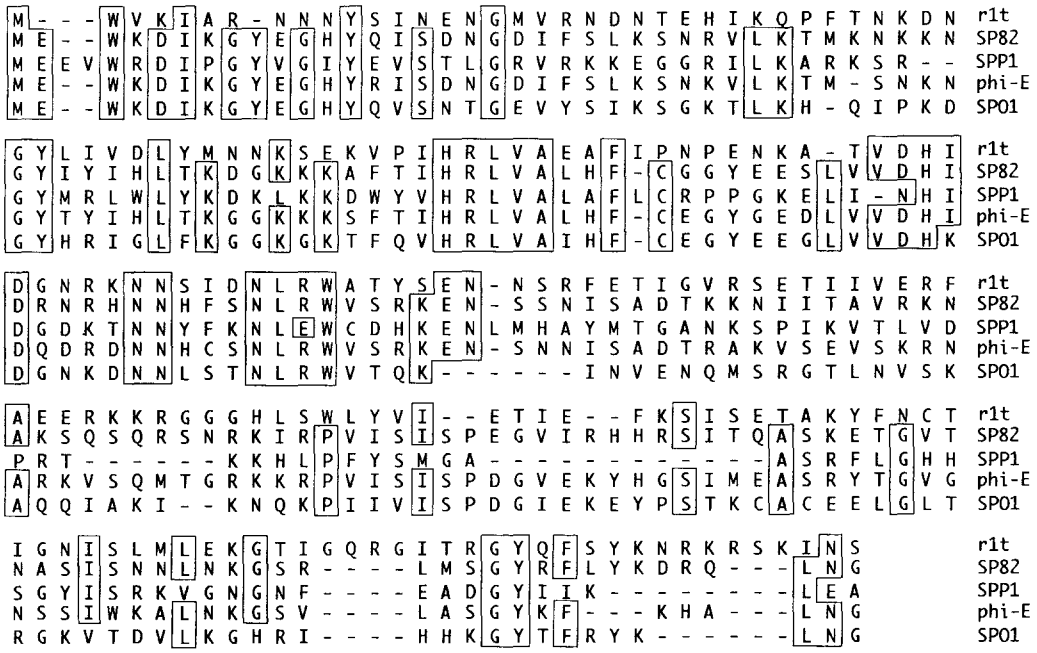


**Fig. 5.** Multiple sequence alignment of the deduced amino acid sequences of r1t ORF41 and various phage intron ORFs. Amino acids are boxed if four or five of the depicted proteins contain identical residues at corresponding positions. Sequences are from the following phages: SP82 (Accession Number U04812), SPP1 (ORF36.1; Accession Number X67865), phi-E (Accession Number U04813) and SPO1 (Accession Number P34081). See the text for further explanations of the similarity.

SPO1 and SP82, it has been shown that they encode endonucleases (Goodrich-Blair *et al.*, 1994). It is likely that ORF41 is also present on a group I intron. Group I introns contain conserved DNA sequence elements which are involved in the folding of the corresponding mRNA to facilitate RNA processing (Cech, 1988). Since the two most conserved sequence elements (S and R) can also be identified in the DNA region surrounding ORF41 (data not shown) it is tempting to speculate that r1t contains a group I intron, and that from a large mRNA transcribed from this region, the ORF40 and ORF42 transcripts will be joined to form one contiguous message. The latter could account for the apparent lack of signals required for translation initiation of ORF42.

*ORF48 and ORF49.* Specific portions of the deduced amino acid sequence of ORF49 exhibit similarities to various lytic enzymes, reflecting a modular design common to many lysins (García *et al.*, 1990; Birkeland, 1994). The N-terminal part of the ORF49 product is similar to the N-terminal domain of a family of chromosomally or phage-encoded lytic enzymes (García *et al.*, 1990). As the N-terminal domain of this class of lysins has been shown to contain the active site (Sanz *et al.*, 1992), it is conceivable that ORF49 specifies a protein with *N*-acetylmuramoyl-L-alanine amidase activity. The C-terminal portion of the ORF49 product shows considerable similarity to the corresponding region of lysins encoded by three closely related prolate-headed lactococcal phages c2 (Ward *et al.*, 1993), bIL67 (Schouler *et al.*, 1994), and vML3 (Shearman *et al.*, 1989). ORF48, situated immediately upstream of ORF49, specifies a protein with structural features similar to so-called holins: two hydrophobic membrane-spanning domains separated by a short beta-turn region, a hydrophobic N-terminus and a highly charged C-terminus (Young, 1992). Holins are proteins required for phage-induced lysis and their presumed function is to effect lesions in the bacterial membrane, thus allowing the lysin to gain access to its substrate. The involvement of holins in phage lysis of Gram-positive bacteria has previously been established in *B. subtilis* (Steiner *et al.*, 1993) and implicated in *L. lactis* (Birkeland, 1994). On the basis of the observed similarities, it is likely that ORF48 and ORF49 constitute the r1t lysis casette. Indeed, we have recently shown that the protein specified by ORF49 exhibits lytic activity and that this lytic enzyme requires the action of the product of ORF48 to cause cell lysis (A. Nauta, D. van Sinderen, H. Karsens, G. Venema and J. Kok, unpublished).

*Additional homologous DNA sequences.* In addition to the protein similarities discussed above, two short DNA sequences deposited in the EMBL data library were almost identical to sequences present in the r1t genome. A stretch of 49 bp located immediately downstream of the transcription start site of the Pg2 promoter of the

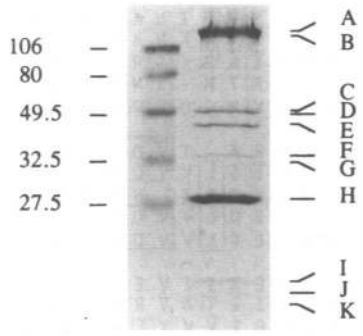


**Fig. 6.** Protein profile of phage r1t particles after separation through a 12.5% SDS–PAGE gel (right lane). The identified proteins are marked by upper-case letters (see the text). A protein size marker, of which the sizes are indicated in kDa in the left margin, was run in the left lane.

temperate lactococcal phage BK5-T (Lakshmidevi *et al.*, 1990) was identical to a non-coding DNA sequence located between ORF22 and ORF23 (coordinates 12411–12459 of the r1t sequence). A sequence located downstream from the 3′ end of the *Lla*PI gene of the lytic lactococcal phage φ50 (coordinates 1319–1481 in the sequence published by Hill *et al.* (1991)) is nearly identical to the DNA region of r1t encompassing the 3′ end of ORF18 and the 5′ end of ORF19 (coordinates 10236–10434). The *Lla*PI gene, which encodes a methylase, was shown to be acquired by φ50 through genetic exchange between the phage genome and the lactococcal plasmid pTR2030 (Hill *et al.*, 1991). The DNA region of φ50 that is homologous to r1t only encompasses sequences downstream from this inserted methylase gene and starts at the point where the φ50 and pTR2030 sequences begin to diverge. This would suggest that the insertion of the *Lla*PI gene into the φ50 genome has caused replacement or deletion of parts of the r1t ORF18 homologue of φ50, and that the product of this ORF is non-essential for φ50 propagation.

### *Structural components of r1t phage particles*

Separation of phage particles by SDS–PAGE using various percentages of polyacrylamide (PAA) (ranging from 7.5–15%) revealed the presence of at least 11 protein bands (a typical protein profile of r1t phage particles obtained after separation through a 12.5% polyacrylamide gel is shown in Fig. 6). These include two protein species (A and B) that are larger than the predicted coding capacity of any of the identified r1t ORFs (see Table 1). N-terminal amino acid sequence analysis of these two proteins showed that both contain identical amino-terminal sequences (see Table 2) corresponding to the predicted product of ORF31, which has a calculated molecular mass of only 32.2 kDa (Table 1). This discrepancy between the observed and calculated molecular mass is similar to that observed for the head shell proteins of the unrelated mycobacteriophage L5 (Hatfull and Sarkis, 1993) and bacteriophage HK97 (Popa *et al.*, 1991). In the latter case it has been demonstrated that the head shell subunits are covalently cross-linked to each other. It is therefore conceivable that ORF31 specifies the major phage-head protein and that protein bands A and B represent covalently cross-linked hexameric and pentameric forms of the ORF31 product, respectively. N-terminal sequencing of other protein bands indicated that protein H is the product of ORF37. As protein H is the most abundant, it is a likely candidate for the major tail protein. Proteins C and D, which could only be resolved on a 7.5% PAA gel, are the products of ORF27 and ORF45, respectively (Table 2). The apparent discrepancy between the calculated and observed molecular masses of these proteins may be attributed to their physical properties. N-terminal sequencing of the remaining protein bands gave inconclusive results (double or no phenylthiohydrantoin (PTH) signals) although protein bands E, F and G, in addition to a non-identifiable protein component, appear to contain the ORF37 product (data not shown). The latter result may indicate that the product of ORF37 is covalently cross-linked to several other phage proteins.

## Discussion

r1t is the first small isometric-headed lactococcal bacteriophage for which the complete nucleotide sequence has

**Table 2.** Phage r1t structural proteins.

| Protein band | Amino acid sequence | Corresponding ORF | Calculated size (kDa) | Observed size (kDa) |
|---|---|---|---|---|
| A | VLNKGTLFDPTLVTDLISKV | 31 | 32.3 | 190 |
| B | VLNKGTLFDPTLVTDLISKV | 31 | 32.3 | 160 |
| C | TEKGIGYLRFKLSV | 27 | 45.8 | 50 |
| D | TQYSFPW | 45 | 38.2 | 48 |
| E | | | | 43 |
| F | | | | 7 |
| G | | | | 33 |
| H | AQVENVTTAKPKIDGAIY | 37 | 19.9 | 24 |
| I | | | | 16 |
| J | | | | 14 |
| K | | | | 11 |

been determined. Analysis of the r1t genome showed that it is organized into two divergent sets of coding regions consisting of three and 47 ORFs. Because two ORFs out of the cluster of three, i.e. the ORFs specifying the integrase and repressor, are probably required for the establishment and maintenance of the lysogenic state of r1t, it appears that the genome is arranged in a life-cycle-specific manner (Fig. 3). In agreement with this assumption is the finding that the intergenic region between the two clusters contains regulatory sequences most probably involved in the genetic switch of r1t (Nauta *et al.*, 1996). Unlike the situation in the prolate-headed phage bIL67 recently sequenced by Schouler *et al.* (1994), and the small isometric-headed phage sk1 (Chandry *et al.*, 1994b), which have differently oriented clusters of genes required for lytic growth, r1t appears to have organized its genetic determinants required for the lytic cycle into one large cluster.

The genome of r1t contains cohesive ends identical to those of φLC3 (Lillehaug *et al.*, 1991). The presence of cohesive termini has been established for several lactococcal phages and all appear to have 3′-extended ends (Lillehaug *et al.*, 1991; Chandry *et al.*, 1994a; Ermel *et al.*, 1994; Lubbers *et al.*, 1994). Direct sequencing of the r1t genome showed that a significant fraction of the r1t DNA molecules contained ends different from the sequenced *cos* termini. This observation may be explained by assuming that packaging of the r1t genome is accomplished by a combination of unit-length packaging (Murialdo, 1991) and packaging via the so-called headful mechanism (Casjens *et al.*, 1987). The former mechanism uses *cos* as a stop and start signal during each round of packaging. In contrast, packaging using the headful mechanism may or may not start at a specific site and terminates when the head of the prophage is filled (with more than a unit length of phage genomic DNA) before the next round of packaging is initiated. It is conceivable that r1t employs a packaging enzyme whose activity in determining the amount of DNA to be packaged may be partly dependent on the recognition of the *cos* site and partly dependent on physical parameters reflecting phage-head filling.

The genetic elements required for site-specific integration of r1t into the host chromosome are nearly identical to those found in φLC3 (Lillehaug and Birkeland, 1993) and Tuc2009 (van de Guchte *et al.*, 1994a). All three phages use the same core region within which strand exchange takes place. In accordance with this finding is that the identified r1t integrase is nearly identical to the integrases required for site-specific integration of Tuc2009 and φLC3. These results imply that r1t, φLC3 and Tuc2009 employ a similar integration system. Whether this integration system is widely distributed among temperate lactococcal phages remains to be established, although one distinctly different system has already been identified (Christiansen *et al.*, 1994).

The function of the majority of the deduced products of the identified r1t ORFs remains unclear, but possible roles of several ORFs have been assigned on the basis of observed similarities to sequences with known functions. The products of ORF11 and 12 are likely to be required for phage replication and their conserved genetic organization can be exploited to locate the origin of replication of r1t. The observed similarity of ORF41 to ORFs identified in self-splicing group I introns is of particular interest. This finding implies that this type of intron is widespread among phages infecting Gram-positive hosts. The group I introns of both Gram-negative and Gram-positive bacteriophages identified thus far, interrupt genes involved in DNA synthesis (Goodrich-Blair *et al.*, 1994). Whereas the introns of T4 and other T-even phages have been found in genes associated with the synthesis of DNA precursors, the introns of Gram-positive bacteriophages reside within their DNA polymerase genes. The presence of introns within genes involved in DNA synthesis prompted the speculation that they might be part of a regulatory mechanism controlling the pathway. In contrast, the putative r1t intron does not appear to be localized within a gene affecting DNA synthesis but, rather, within a gene affecting some unknown structural aspect of r1t. On the basis of the similarities to the products of the ORFs contained within the introns of SPO1 and SP82, and the fact that the a motif, which is found in group I intron endonucleases and a bacterial restriction endonuclease (Shub *et al.*, 1994), is also present in the deduced product of ORF41, this protein is also likely to be an endonuclease.

The two-component configuration of the r1t lysis system appears to be common to small isometric-headed lactococcal phages, even though the individual components may not always be related to each other (Platteeuw and de Vos, 1992; Arendt *et al.*, 1994; Birkeland, 1994). The modular design of the phage lysin seems to be a general aspect of cell wall-degrading enzymes and may facilitate rapid protein evolution (García *et al.*, 1990).

Although sequence comparisons have shown that the temperate, isometric-headed lactococcal phages r1t, Tuc2009 and φLC3 contain stretches of DNA that are virtually identical, they also contain DNA regions which are completely different (N. K. Birkeland, personal communication). Homologous DNA sequences, such as the 49 bp DNA fragment found in phages BK5-T and r1t (see the *Results*), may represent recombination hot spots to direct genetic exchange of DNA between members of this phage family. The availability of exchangeable modules specifying various essential functions, e.g. integration, replication and lysis, would give the phage a tremendous evolutionary potential, a concept which has already been implicated for other phages (Botstein, 1980). The finding

that the lytic phage φ50 and r1t contain homologous sequences indicates that lytic and lysogenic phage may be related to each other either through a common ancestor or through horizontal evolution.

## Experimental procedures

### *Bacterial strains, phage, plasmids, and media*

*L. lactis* subsp. *cremoris* R1 and its prophage-cured derivative R1K10 were grown in lactose M17 broth (Terzaghi and Sandine, 1975), or on lactose M17 1.5% (w/v) agar. *E. coli* strain XL1-Blue (Stratagene) was grown in TY broth (Rottlander and Trautner, 1970) or on TY broth solidified with 1.5% (w/v) agar. Ampicillin, IPTG and Xgal (all from Sigma Chemical Co.) were used at concentrations of 100 $\mu g\,ml^{-1}$, 1 mM and 0.002% (w/v), respectively.

### *DNA techniques*

General DNA techniques were performed as described by Sambrook *et al.* (1989). Plasmid DNA was isolated by the method of Birnboim and Doly (1979) and/or by using QIAGEN Midi-Plasmid isolation columns (Diagen Inc.). Restriction enzymes and T4 DNA ligase were obtained from Boehringer Mannheim and used according to the instructions of the supplier. Oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer (Applied Biosystems Inc.). PCR was performed using Vent polymerase (New England Biolabs Inc.). After heating of the samples to 94°C for 2 min, target DNA was amplified in 30 subsequent cycles under the following conditions: 94°C for 1 min; 50°C for 2 min; 73°C for 2 min. PCR fragments were purified using the QIAEX DNA Gel Extraction Kit or the QIAquick-spin PCR Purification Kit (Diagen Inc.). All plasmids were constructed in *E.coli*, which was transformed by the method of Mandel and Higa (1970).

### *Southern transfer and DNA hybridization*

After electrophoresis on a 0.8% agarose gel in TBE buffer (Sambrook *et al.*, 1989), DNA fragments were transferred to a GeneScreen Plus membrane (NEN Research Products) by the method of Southern, as modified by Chomczynski and Quasba (1984). The enhanced chemiluminescence (ECL) labelling and detection system and accompanying protocols (Amersham International) were used for labelling of the probes and hybridization.

### *Isolation of r1t phage particles and DNA*

An overnight culture of *L. lactis* subsp. *cremoris* R1 was diluted 100-fold in 500 ml fresh lactose M17 medium and grown until the culture reached an $OD_{600}$ of 0.8, at which point mitomycin C (Sigma) was added to a final concentration of 2.5 $\mu g\,ml^{-1}$. Incubation at 30°C was continued in the dark until lysis occurred (usually after 2–3 h). Cell debris was removed by centrifugation for 10 min at 6000 × *g*. Phage particles were precipitated by incubation with NaCl (0.5 M) and polyethylene glycol 6000 (10% w/v) for 3 h on ice, and purified by a CsCl step gradient as described by Sambrook *et al.* (1989). The bacteriophage r1t suspension was dialysed against several changes of 150 mM NaCl, 15 mM tri-sodium citrate. Phage DNA was obtained by extracting the suspension twice with phenol. The DNA solution was subsequently dialysed against 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

### *Determination and analysis of the nucleotide sequence of r1t*, attL, attR *and* cos

Most (approximately 90%) of the genome was cloned in pUC18 and pUC19 (Yannish-Perron *et al.*, 1985) as large fragments (ranging from 2–13 kbp) using various restriction enzymes. Subsequently, these large fragments were restricted with the restriction enzymes *Sau*3A, *Rsa*I, or *Alu*I and the generated DNA fragments were shotgun cloned in pUC19 (Yanish-Perron *et al.*, 1985) or pBluescript-II SK+ (Stratagene). This cloning strategy resulted in a plasmid bank containing small fragments of the r1t genome. Randomly selected clones of this bank were sequenced with the aid of an ALF automatic sequencer (Pharmacia AB) or by means of manual sequencing (Sanger *et al.*, 1977) using the T7 sequencing kit (Pharmacia AB). With the standard universal and reverse primers, approximately 70% of the double-stranded DNA sequence of r1t present in the various random subclones was thus obtained. Further sequence information (±25%) was obtained from available subclones using synthesized oligonucleotide primers based on previously obtained sequences. The nucleotide sequence of the remaining 5% of the r1t genome that proved to be refractive to cloning, that of *attL* and *attR*, and that of the *cos* site, were determined by means of cycle sequencing using the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit with Vent ($exo^-$) or Vent ($exo^+$) DNA Polymerase (New England Biolabs). Programs developed by Staden (1982) and the PC Gene (version 6.7) program ASSEMGEL (IntelliGenetics Inc.) were used to assemble the subclone sequences. Homology searches were performed using FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1990) with sequences present in the following data bases: SWISSPROT (release 30); NBRF-PIR™ (release 42); GenBank translated (release 86); GenBank™ (release 86) and EMBL (release 38). Sequence alignments were performed using the Clustal method of the MEGALIGN program of the DNASTAR software package (DNASTAR Inc.).

### *N-terminal amino acid sequence determinations*

Purified r1t particles (approximately $10^{15}$ pfu per ml) were diluted in loading buffer and boiled for 5 min before performing SDS–PAGE analysis (Laemmli, 1970) with the Protean II Minigel System (Bio-Rad). Electrophoresis buffer was supplemented with 0.1 mM thioglycolate (Artoni *et al.*, 1984). Prestained molecular weight marker was obtained from Bio-Rad Laboratories. Proteins were transferred to ProBlot membrane (Applied Biosystems Inc.) with 25 mM Tris-HCl, 192 mM glycine buffer, pH 8.3, in 15% methanol (Towbin *et al.*, 1979) as the transfer buffer. Proteins were visualized by staining with Coomassie brilliant blue. Protein bands were excised for N-terminal amino acid sequencing by Edman degradations

using an Applied Biosystems model 477A pulse-liquid sequenator, on-line connected to a Model 120A Applied Biosystems RP-HPLC unit for identification of the step-wise released PTH-amino acids.

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