APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1996, p. 1847–1851 Vol. 62, No. 5
0099-2240/96/$04.00+0
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Defective Site-Specific Integration Elements Are Present in the Genome of Virulent Bacteriophage LL-H of *Lactobacillus delbrueckii*

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Received 25 July 1995/Accepted 22 February 1996

The phage attachment site, *attP*, and the integrase-encoding gene, *int*, are sufficient to promote site-specific integration of the temperate phage mv4 genome into the chromosome of the *Lactobacillus delbrueckii* host (L. Dupont, B. Boizet-Bonhoure, M. Coddeville, F. Auvray, and P. Ritzenthaler, J. Bacteriol. 177:586–595, 1995). The mv4 genome region containing these elements was compared at the nucleotide and amino acid levels with that of the closely related virulent phage LL-H. Complex DNA rearrangements were identified; a truncated integrase gene and two sites homologous to the mv4 *attP* site were detected in the genome of the virulent phage LL-H. These observations suggest that the two phages derive from a common temperate ancestor.

Lactic acid bacteria, especially *Lactobacillus* and *Lactococcus* species, are important microorganisms in many biotechnological processes in the food and feed industries. Bacteriophage contaminations of lactic acid bacteria have been reported since the 1930s (38), and they are known to be the main cause of fermentation failures in the dairy industry. Phages can enter the processes from outside, and they can survive the pasteurization of milk or remain in factory environments for prolonged periods (1). Other possible sources of virulent phages are lysogenic bacteria and the prophages they harbor (11, 17).

The possible temperate origin of new virulent phages has been subject to speculation for years (11). It has been suggested that temperate phages do not contribute significantly to the emergence of new virulent phages, because virulent and temperate *Lactococcus* phages are not significantly homologous (11, 17). In contrast, there have been several observations of homology between virulent and temperate *Lactobacillus* phages (20, 21). There have been only a few examples in the literature of temperate phages losing their lysogenization ability and becoming virulent (11). Probably the best documented case of the emergence of a new virulent phage from a temperate one is the *Lactobacillus casei* phage øFSV (32). The virulent phage øFSV originated from the temperate phage øFSW, present in lysogenic *Lactobacillus casei* S-1, by acquisition of a new insertion sequence, ISL*1* (31–33).

To improve our understanding of the origins, evolution, and relationships of virulent and temperate phages of lactic acid bacteria, the genome region containing the recently characterized integration elements of the temperate phage mv4 (13) was compared with the corresponding genome region of the related virulent phage LL-H.

The virulent phage LL-H and the temperate phage mv4 are closely related phages of *Lactobacillus delbrueckii*. Phage LL-H is a virulent phage of *L. delbrueckii* subsp. *lactis* isolated in Finland in 1972 (3), and mv4 is a temperate phage present as a prophage in lysogenic *L. delbrueckii* subsp. *bulgaricus* LT4 isolated in France in 1963 (10). They are closely related (21). Morphologically, LL-H and mv4 are similar and are typical group B1 phages in the classification of Ackermann and DuBow (1), with small isometric heads and long noncontractile tails (10, 15). They are *pac*-type phages that have circularly permuted and terminally redundant double-stranded DNA genomes (15, 19, 34). The structural-protein-encoding genes (22, 35), terminase-encoding genes (23), and phage lysin-encoding gene (36) of LL-H have been characterized at the nucleotide and amino acid sequence levels. This continuous segment covers about 67% of the 34.6-kb LL-H genome (15). Two segments covering about 23% of the 36.0-kb mv4 genome (19) have been sequenced. They contain some mv4 structural-protein-encoding genes (35), genes involved in cell lysis (unpublished results) (GenBank accession no. Z26590), and elements required for site-specific integration of mv4 DNA into the host chromosome (13).

Most, but not all, of the restriction fragments of LL-H are homologous to mv4 DNA and vice versa (2, 21). The genes encoding the major capsid proteins of LL-H and mv4 have 91% identity at the nucleotide level and 96% identity at the amino acid level, and the other genes in the same regions have amino acid identities ranging from 78 to 87% (22, 35). The phage lysin-encoding genes have 80% identity at the nucleotide level and 85% identity at the amino acid level (36) (GenBank accession no. Z26590). Some of the genome regions that are unique may reflect the differences which make phages either virulent or temperate and perhaps contain the functions needed in the lysogenic cycle.

The nucleotide sequences and gene organizations of the *att* integration genome regions of virulent phage LL-H and temperate phage mv4. The genetic elements required for the integration of the phage mv4 genome into the bacterial chromosome have recently been characterized (13). mv4 DNA integration occurs by a site-specific recombination process between the phage attachment site, *attP*, and the bacterial attachment site, *attB*, located in a $tRNA^{Ser}$ gene. This recombination event is catalyzed by a phage integrase encoded by the *int* gene, which is located adjacent to the *attP* site and is transcribed convergently toward it (13). These genetic elements are located in a 2.2-kb *Bam*HI-*Sal*I fragment downstream of the phage region encoding cell lysis and separated from it by a

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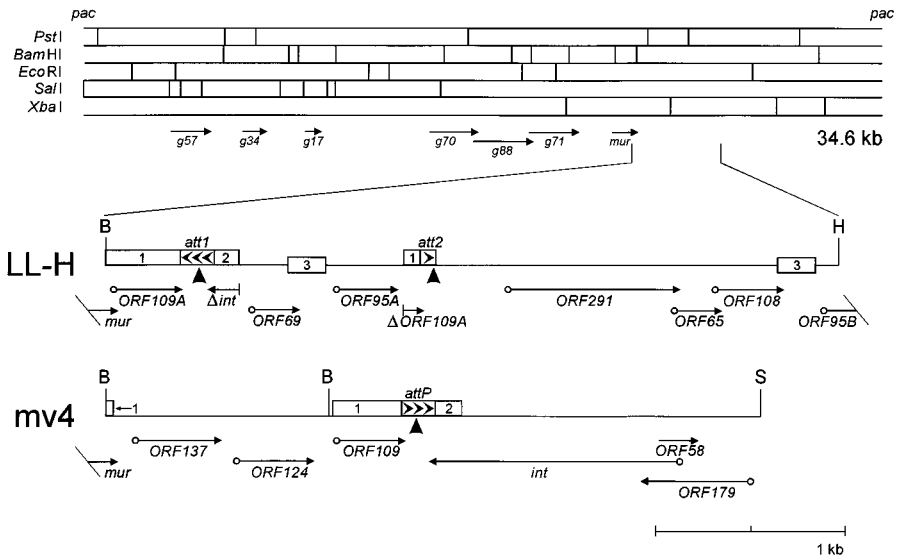

FIG. 1. Comparison of the integration regions of two related *L. delbrueckii* phages, virulent phage LL-H and temperate phage mv4. In the phage LL-H restriction map (15), the genome is shown linearized at its packaging site (*pac*). The previously characterized structural protein genes (*g57*, *g34*, *g17*, *g70*, *g88*, and *g71*) (22, 35) and the lysin gene, *mur* (36), of phage LL-H are indicated by arrows under the map. Under the restriction map are also shown more detailed representations of the 3.9-kb *Bam*HI-*Hin*dIII fragment and the corresponding region of phage mv4 (a 3.5-kb *Bam*HI-*Sal*I fragment) (13) (GenBank accession no. Z26590). Numbered boxes show the locations and extents of the nucleotide and amino acid sequence homologies found in these two phage fragments. *attP*, *att1*, and *att2* are indicated by boxes in which horizontal arrowheads indicate the direction of the homologous segment. Vertical arrowheads represent *att* core sequences. Horizontal arrows show the directions, positions, and lengths of the putative ORFs, with their names given beneath them. At the beginning of an arrow, an open circle denotes a ribosome binding site (24) and a vertical line means that the ORF has been partially deleted; the ORFs that extend beyond the sequence presented here have been cut off with diagonal lines. Dupont et al. (13) called mv4 ORF109, ORF179, and ORF58 by the designations ORF11, ORF12, and ORF13, respectively. B, *Bam*HI; H, *Hin*dIII; S, *Sma*I.

1.1-kb *Bam*HI fragment (Fig. 1). The nucleotide sequence of this 1.1-kb fragment of mv4 (GenBank accession no. Z26590) contains the 3′ end of the lysin-encoding gene, *mur* (bases 1 to 32), and two open reading frames (ORFs) of unknown function, ORF137 (bases 168 to 578) and ORF124 (bases 699 to 1070).

To compare the genome region containing the integration elements of mv4 with the corresponding genome region of LL-H, the 3.9-kb *Bam*HI-*Hin*dIII fragment located immediately downstream of the LL-H lysin gene, *mur* (36), has been sequenced. The LL-H nucleotide sequence was determined independently from both strands by the dideoxy chain termination method (29). Most of the sequence was obtained with Sequenase version 2.0 (United States Biochemicals) from restriction fragments cloned into M13mp18 and M13pm19 vectors (39), which were propagated without helper phages in *Escherichia coli* JM101 (39) grown in 2× TY broth (28). The cycle sequencing technique, modified from the method of Murray (26), and TAQuence version 2.0 (United States Biochemicals) were used to confirm the LL-H nucleotide sequence by direct sequencing of the phage LL-H DNA. The sequences were separated on a standard 6% acrylamide–7 M urea gel (28). Computer analyses of sequences were performed by using the Genetics Computer Group sequence analysis software package version 8-UNIX (12), LKB DNASIS (version 7.0), and LKB PROSIS (version 6.02). Figure 1 shows the location and organization of the sequenced LL-H fragment (3,868 bp) and its comparison with the corresponding fragments of mv4 (3,462 bp).

The LL-H sequence contains six complete putative ORFs, ORF109A (bases 45 to 371), ORF69 (bases 775 to 981), ORF95A (bases 1230 to 1514), ORF291 (bases 2133 to 3005), ORF65 (bases 3021 to 3215), and ORF108 (bases 3231 to 3554), the 3′ end of the lysin-encoding gene, *mur* (bases 1 to 32), and the 5′ end of ORF95B (bases 3808 to 3868). All putative LL-H ORFs are preceded by a ribosome binding site showing good complementarity to the 3′ end of *L. delbrueckii* subsp. *lactis* 16S rRNA (24). These ORFs are located in the main coding strand, which also contains the previously characterized genes (22, 23, 35, 36).

Virulent phage LL-H has a partially deleted integrase gene. A complete integrase gene could not be located in the LL-H DNA. However, one region of the LL-H genome (Δ*int* [Fig. 1]) shows nucleotide similarity to the 3′ end of mv4 *int*. The 132 nucleotides forming LL-H Δ*int* are 69% identical to the last 10% of mv4 *int*. From Δ*int*, a 43-amino-acid protein could be translated. On the amino acid level, the mv4 integrase and the product of LL-H Δ*int* are very similar. Their amino acids, including the tyrosine residue conserved among integrases (5), are 77% identical (Fig. 2).

Interestingly, many of the nucleotide differences between the LL-H and mv4 integrases were such that they conserved the amino acid sequence. It is reasonable to assume that the two genes had already diverged prior to the event which caused the deletion of 90% of LL-H Δ*int*. Because of the deletion of the integrase gene, phage LL-H is unable to lysogenize host bacteria. Southern blotting experiments using the PCR-amplified 5′ end of mv4 *int* as a probe confirmed that no other part of the LL-H genome hybridized with the mv4 integrase gene (data not shown).

Phage LL-H has two regions similar to mv4 *attP*. The phage mv4 attachment site (*attP*; 192 bp) (19) is located in the intergenic region between the *int* gene and ORF109 (13). During phage integration, the strand exchange takes place in the 17-bp-long core sequence (13). mv4 *attP* is similar to two regions of the LL-H genome, *att1* and *att2*. *att1* (bases 369 to 569; Fig. 1 and 3) is located between ORF109A and Δ*int* and is opposite in orientation from mv4 *attP*. It has 72% nucleotide identity with mv4 *attP*. The *att1* core has been rather poorly conserved; only 11 of 17 nucleotides are identical. *att2* (bases 1642 to

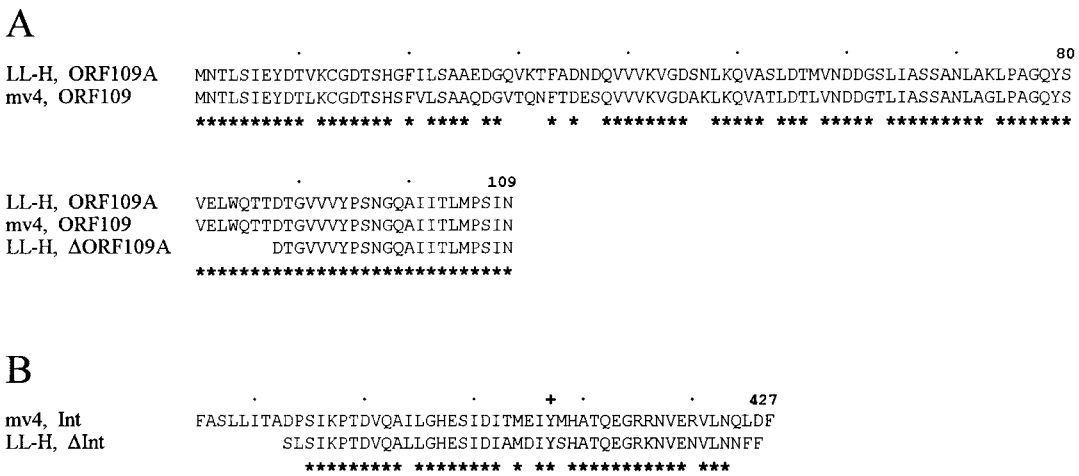

FIG. 2. Alignments of the amino acid sequences translated from the homologous ORFs found in the integration regions of phages LL-H and mv4. Amino acids identical in all sequences are indicated by asterisks. (A) Products of ORF109A (LL-H), ORF109 (mv4), and truncated ΔORF109A (LL-H). (B) C-terminal part of the mv4 integrase (Int) and the amino acid sequence translated from the truncated integrase gene (ΔInt) found in the LL-H genome. The highly conserved Tyr residue is indicated by a plus sign above the sequence.

1743) is adjacent to ΔORF109A (Fig. 1 and 3), about 1 kb downstream of *att1*, and it is in the same orientation as mv4 *attP*. *att2* is more similar to mv4 *attP* (88% identical nucleotides), and the *att2* core is identical to the mv4 *attP* core, except that the extreme 3′ nucleotide is T instead of G. However, the homology of *att1* covers the whole of mv4 *attP*, but the homology of *att2* covers only the left half of mv4 *attP* (sequences diverge after the *att* core sequences).

Both LL-H *att1* and *att2* contain perfect inverted repeats (16 and 12 bp, respectively). They are similar to a stem-loop structure in *attP* of phage mv4 (Fig. 3). Both LL-H inverted repeats could form stem-loop structures followed by a poly(T)-rich sequence. These features are characteristic for rho-independent terminators (16). Since these possible LL-H terminators are preceded by poly(A) sequences, they could equally well terminate transcription in both directions.

Highly similar and unique ORFs are present in these two integration regions. LL-H ORF109A and mv4 ORF109 show the highest degree of similarity, 84% identity at the amino acid level. This similarity covers the lengths of these ORFs and ends just after the stop codons. ΔORF109A is also very similar to LL-H ORF109A (92% nucleotide identity) and mv4 ORF109 (86% nucleotide identity). The last 36 amino acids of these ORFs (including the 22 amino acids of ΔORF109A) are completely identical (Fig. 2). These ORFs may code for some necessary function which must be preserved, since the majority of the nucleotide differences are silent mutations that conserve the amino acid sequence.

In virulent phage LL-H, the phage lysin gene, *mur* (36), and ORF109A are separated by 9 bp, but in temperate phage mv4, the corresponding genes, *mur* (GenBank accession no. Z26590) and ORF109, are separated by 1,186 bp containing two unique ORFs, ORF137 and ORF124 (Fig. 1). Southern blotting experiments using a PCR-amplified fragment spanning these two ORFs as a probe confirmed that no part of the LL-H genome hybridized with these ORFs (data not shown).

In the mv4 sequence, ORF137 and ORF124 are flanked by direct repeats of 10 nucleotides, which are formed by the end of *mur* (ATAaGAaGgAA) and the ribosome binding site of ORF109 (ATAgGA · GaAA) (the chemical point indicates a missing nucleotide). One copy of this sequence can be found in the LL-H sequence, covering the end of the *mur* gene and the ribosome binding site of ORF109A (ATAgGA · GaAA). Direct repeats are known to promote deletions (18). Accordingly, ORF137 and ORF124 have most probably been deleted from LL-H, but their insertion into the mv4 genome cannot be ruled out. In a recent study of *Lactococcus lactis* temperate phage BK5-T (8), a single crossover recombination event between direct repeats caused the deletion of *attP* and the 3′ end of the integrase-encoding gene, thus creating a lytic phage which had lost the ability to form lysogens but was still unable to infect strains lysogenic for BK5-T (8).

Databases were searched with the nucleotide and amino acid sequences of these two integration regions by using the programs FASTA (27) and BLAST (4). The sole similarity detected in these database searches was to an *L. delbrueckii* chromosomal fragment encoding a minor $tRNA^{Ser}$ (UCG) (40), part of which was similar to LL-H ORF109A and ΔORF109A and mv4 ORF109. In fact, this fragment could be the *attL* site of an *L. delbrueckii* strain lysogenic for a temperate phage closely related to LL-H and mv4 (13).

Intergenic regions of LL-H contain truncated ORFs. In the LL-H sequence, the distances between ORFs are relatively high, ranging from 248 to 618 bp. This gene arrangement differs from that of the LL-H structural protein genes, which are typically separated by only a few nucleotides (22, 35). These intergenic regions might be noncoding sequences. Nevertheless, truncated ORFs could be detected in some of these regions. These included the 5′-truncated Δ*int* gene (132 bp) and ΔORF109A (66 bp), located between ORF109A and ORF69 and between ORF95A and ORF291, respectively (Fig. 1). A 3′-truncated ORF was duplicated inside two direct repeats (185 bp; 72% identity) located in the intergenic regions between ORF69 and ORF95A and between ORF108 and ORF95B (boxes 3 [Fig. 1]). Both copies (positions 987 to 1079 and 3572 to 3664) are preceded by ribosome binding sites and code for 31-amino-acid peptides of 84% identity.

Virulent phage LL-H and temperate phage mv4 could have a common temperate ancestor. The genes encoding the late functions (structural proteins and cell lysis) of these two phages are highly similar (3, 22, 35, 36) (GenBank accession no. Z26590). In contrast, the genome regions containing the integration elements (early functions) have major differences. The LL-H sequences which are highly or moderately similar to

FIG. 3. Alignment of *att1* and *att2* of LL-H and *attP* of mv4. The numbering of the mv4 sequence starts from the leftmost *Bam*HI site in Fig. 1. The *att* core sequence is boxed. The inverted repeats in LL-H *att1* and *att2* homologous to those in mv4 *attP* are indicated by arrows. ORF109A and Δ*int* in LL-H *att1*, ORF109 and *int* in mv4 *attP*, and ΔORF109A in LL-H *att2* are in bold.

those of mv4 (Δ*int* and *attP*) are sprinkled around in a very complex pattern, and they probably represent vestiges of site-specific integration elements. It is not surprising to observe major differences in these regions of the two phages, since it is well-known that the regions containing the integration elements of temperate phages are common targets for rearrangements (6, 37).

It seems very improbable that either LL-H or mv4 evolved directly from the other. Most likely, they have a common temperate ancestor. It is impossible to deduce the exact series of events that have led to the present situation in the LL-H genome. No single event can account for all the observed differences. The creation of the present condition in the phage LL-H genome could have included any combination of mechanisms such as aberrant excision of a prophage, insertion(s), deletion(s), and recombination between two related phages, a phage and a (defective) prophage, or a phage and a bacterial chromosome.

The events responsible for the rearrangements found in this part of the phage LL-H genome seem to be quite recent. When truncated ORFs, e.g., Δ*int* and ΔORF109A, are compared with their complete counterparts, the nucleotide substitutions have not been random but have been restricted to those conserving the amino acid sequence.

The theory of the modular evolution of bacteriophages (7) proposes that phages evolve as a population of interchangeable modules, each of which codes for some specific function. Exchange of a given module for another of the same biological function occurs by homologous recombination among the phages that form an interbreeding population. Phage recombination is known to exist in nature, for example, among the lambdoid phages (9). Interestingly, in a recent study of *Lactococcus lactis* phages, recombination between virulent phage ul36 and a defective prophage in the host genome gave rise to a new type of virulent phage, ul37 (25).

DNA homology group a contains 4 temperate and 17 virulent phages, including LL-H and mv4 (2, 14, 15, 20, 21). All of these phages are able to propagate on *L. delbrueckii* subsp. *lactis* LKT (15, 20, 21). Thus, strain LKT could provide for these phages an appropriate environment in which recombination and genome rearrangement can take place.

This study suggests that temperate phages are one possible source of virulent phages. It is difficult to estimate the extent of their contribution to the outbreaks of new virulent phages observed in industrial processes. Prophages are frequently present in *Lactobacillus* strains (11, 17, 30). Curing industrially used bacterial strains from prophages would be a solution, but prophages also protect lysogenic strains from related phages by the superinfection immunity mechanism.

Nucleotide sequence accession numbers. The nucleotide sequences described here have been deposited in the GenBank database under accession no. L42315 for phage LL-H and Z26590 and U15564 for phage mv4.

We thank Michèle Coddeville and Anne Karjalainen for excellent technical assistance.

This study was supported by grants from the Ministry of Agriculture, the Foundation for Biotechnical and Industrial Fermentation Research, the Academy of Finland (project SA 1051317), the Région Midi-Pyrénées, and the CEC BIOTECH G-project (BIOT-CT94-3055) on the biotechnology of lactic acid bacteria.

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