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Physical and Genetic Characterization of the Genome of *Lactobacillus lactis* Bacteriophage LL-H

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Bacteriophage LL-H is a virulent phage of *Lactobacillus lactis* LL23. A restriction map of the phage genome was constructed with various restriction endonucleases. This chromosome has a 34-kilobase size and seems to be circularly permuted. We used a bank of LL-H restriction fragments to study the expression of five of the seven main phage particle proteins. Immunoblotting experiments permitted the mapping on the chromosome of several genes coding for phage particle proteins. We also show that the gene of the main capsid protein is expressed from its own promoter in an *Escherichia coli* strain.

Lactic acid bacteria, such as *Streptococcus* and *Lactobacillus* spp., are widely used in the fermentation and dairy industries. Fermentation is based on acidification and features which delay or lead to low acidification imperil the quality and value of the end product. Such problems are principally due to bacteriophage contamination, either by virulent or temperate phages.

Strikingly, although lactic acid bacteria are widely used in industry (29), little information is available concerning lactic acid phages, particularly the *Lactobacillus* phages. The published data describe phage morphology (1, 33) or homologies based on serological (15, 16) and DNA (16, 17, 31) hybridization studies, but little is known about phage genome organization (18, 20, 31).

Because an improvement in the prevention of acidification could result from wider knowledge of phage biology, we have begun molecular biology studies of virulent phage LL-H of *Lactobacillus lactis* LL23. This phage was originally isolated in a Finnish dairy in 1972. Some data regarding the morphology and the protein composition of the phage particle have been published (3). LL-H phage DNA may be a linear molecule, as seen in electron microscopy (3).

We report here the results of restriction mapping of LL-H DNA and the construction of a library of LL-H fragments in *Escherichia coli* plasmid vectors. We assayed the expression of these hybrid plasmids in *E. coli* by the technique of immunoblotting with an antiserum raised against the whole phage particle. We show that five of the main LL-H particle proteins are synthesized in *E. coli*. These results permitted us to map structural gene organization on the LL-H genome.

MATERIALS AND METHODS

Strains, bacteriophage, and plasmids. Virulent phage LL-H (3) was grown on *L. lactis* LL23. *E. coli* HB101 (7) and plasmid vectors pBR322 (6) and pBR325 (5) were used in cloning experiments.

Culture media. Strain LL23 was grown on MRS broth (11), and strain HB101 was grown on Luria broth, as described by Miller (24). When needed, ampicillin, tetracycline, or chloramphenicol was added at a concentration of 40, 10, or 50 μg/ml, respectively, to Luria broth. MRS agar plates were supplemented with 10 mM Ca^{++} when they were used for phage titration.

Chemicals and enzymes. MRS medium was purchased from Institut Pasteur Production. Restriction endonucleases, DNA polymerase I, DNA polymerase I Klenow fragment, and radioactive deoxyribonucleotides [α-^{32}P]dCTP and [α-^{32}P]dATP were purchased from Amersham International, Buckinghamshire, England.

Preparation of phage lysates and phage purification. Lysates were prepared as previously described (3), and phage titrations were performed by the agar layer method (2). Phage were concentrated by precipitation with 10% polyethylene glycol 6000 by the method of Yamamoto and Alberts (41), and phage purification was performed stepwise and by equilibrium CsCl gradient centrifugation, as described by Maniatis et al. (22).

Phage DNA purification. Phage DNA purification was performed as for bacteriophage λ (22) by heating the phage suspension for 15 min at 65°C in the presence of 20 mM EDTA (pH 8)–50 μg of proteinase K per ml–1% sodium dodecyl sulfate (SDS). Further extractions were made with buffer-saturated phenol. Residual phenol was eliminated with chloroform and ether extractions. Purified DNA was precipitated at −60°C with absolute ethanol in the presence of 0.3 M sodium acetate.

Plasmid DNA purification. Purified plasmid DNA was obtained from overnight cultures of HB101 in the presence of the appropriate antibiotic by the alkaline extraction procedure described by Birnboim and Doly (4). Further purification of DNA was done by centrifugation in CsCl density gradients containing ethidium bromide (26).

DNA manipulation. Cleavages with restriction endonucleases were performed according to the instructions of the manufacturer. Construction, cloning, restriction endonuclease cleavages, gel analysis of recombinant plasmids (9, 22, 30), and transformation of *E. coli* with plasmid DNA (21) have all been previously described. All the LL-H DNA restriction fragments with 5′ protruding ends were 3′ end labeled with *E. coli* DNA polymerase I large fragment (1 U) in 66 μl of 100 mM Tris (pH 8.5)–50 mM $MgCl_2$ in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP and of cold dGTP

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TABLE 1. Size of fragments generated after digestion of LL-H DNA with various restriction endonucleases

Restriction enzyme	Minimal no. of sites	Size (kb) of fragment:																
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
*Sal*I	6	19.5	4.5	3.6	3.3	1.2	1.17	0.9										
*Eco*RI	6	8.55[a]	7.3	5.9	1.9	1.5	0.9											
*Pst*I	6	9.0	8.0	5.4	4.8	3.75	1.65	1.35										
*Bam*HI	10	8.25	8.0	4.35	3.3	2.85	1.95	1.7	1.6	0.9	0.6	0.45						
*Hin*dIII	17	6.3	2.76	2.73	2.6	2.55	2.5	2.45	2.4	1.87	1.73	1.5	1.3	0.8	0.7	0.52[a]	0.4	0.25
*Pvu*II	11	7.2	6.6	3.75	3.45	2.7	2.5	1.95[a]	1.42[a]	0.6[a]								
*Hpa*I	8	6.9[a]	6.5	2.9[a]	2.4	2.3	1.8	1.5										
*Sma*I	2	18.3	14.0	1.5														

[a] Two fragments are believed to migrate in this position.

and dTTP (22). The reaction was conducted at 37°C for 1 h. Labeled fragments were fractionated on 1% agarose and 8% polyacrylamide gels and visualized after autoradiography. The two largest *Sal*I fragments were electroeluted from the gel and precipitated by ethanol; they were then rinsed with 70% ethanol and dried. The two fragments were then digested with *Eco*RI for 3 h at 37°C, 3′ end labeled with polymerase Klenow fragment as before, and analyzed on a 1% agarose gel.

DNA-DNA hybridizations on filters. The DNA probe was labeled by nick translation with *E. coli* DNA polymerase I and [α-^{32}P]dCTP as previously described (23, 28). Unlabeled DNA was always LL-H DNA fragments generated by various restriction endonucleases. The transfer of unlabeled DNA fragments from agarose gels onto 0.45-μm-pore-size nitrocellulose membranes (BA 85; Schleicher and Schüll, GmbH, Dassel, Federal Republic of Germany) was as described by Southern (32). Hybridization between the ^{32}P-labeled DNA probe and the membrane-immobilized LL-H DNA fragments was performed at 65°C for 40 h in Denhardt solution (0.2% polyvinylpyrrolidone 360, 0.2% Ficoll 400 [Pharmacia Fine Chemicals, Piscataway, N.J.], 0.2% bovine serum albumin) made up in 0.45 M NaCl–0.045 M sodium citrate (pH 7.7)–50 μg of sheared denatured salmon sperm DNA per ml–0.1% SDS.

Phage and cell extract protein analysis and immunoblotting technique. LL-H phage preparations or *E. coli* cells were heated for 3 min at 100°C in the presence of 2.3% SDS and 5% β-mercaptoethanol, and proteins were fractionated on a 12.5% SDS-polyacrylamide gel (19). Gels were stained with 0.25% Coomassie brilliant blue R-250. Recombinant plasmid-encoded phage proteins were detected in *E. coli* cell extracts by the immunoblotting procedure of Towbin et al. (36). LL-H phage, LL-H ghost, and cell extracts of HB101 strains harboring the plasmid vectors pBR322 or pBR325 constituted the controls. The proteins separated on acrylamide gel were transferred onto nitrocellulose sheets. Immunological detection of phage proteins on nitrocellulose was performed with rabbit anti-LL-H antibodies, and immunological complexes were located with goat anti-rabbit antibodies conjugated with horseradish peroxidase (Nordic Immunology). The color reaction was performed with 2-chloro-1-naphthol and H_2O_2.

The synthesis in *E. coli* of LL-H-encoded enzyme(s) responsible for the lysis of strain LL23 was detected as follows. *E. coli* strains harboring various recombinant plasmids were simultaneously streaked onto plates containing Luria broth medium and incubated for 3 to 4 h at 37°C. Soft agar MRS (3 ml) containing 0.5 ml of a late-exponential culture of strain LL23 was then poured on, allowed to solidify, and incubated overnight at 42°C. In the absence of lytic activity, strain LL23 grew as a continuous dense lawn around the *E. coli* clones. When lytic activity was produced by an *E. coli* clone, a clear halo of lysis of LL23 appeared around the clone. The halo diameter increased with further incubation even when the LL23 strain had reached the stationary-growth phase.

RESULTS

Restriction pattern of phage LL-H chromosome. A total of 10 restriction endonucleases were tested on phage LL-H DNA. The number and size of the fragments generated are reported in Table 1. The sizes of all fragments identified for each restriction enzyme were added, and the average length of LL-H DNA was 34 kilobases (kb). Enzymes *Xho*I and *Kpn*I had no activity on LL-H DNA under our test conditions.

Construction of a bank of LL-H DNA fragments in *E. coli*. To construct a library of LL-H genes, three restriction enzymes which produced a few well-separated fragments were chosen: *Eco*RI, *Pst*I, and *Sal*I. Each enzyme generated seven or more fragments; very small fragments may have escaped detection (Table 1). The *Eco*RI fragments were introduced into the *Eco*RI site of *E. coli* plasmid pBR325, whereas the *Pst*I and *Sal*I fragments were inserted into the *Pst*I or *Sal*I site, respectively, of plasmid pBR322. Despite repeated attempts, it was impossible in any of the three cases to isolate on the vectors all the restriction fragments generated by an enzyme. Thus, four, five, and five restriction fragments, respectively, were cloned from the *Eco*RI, *Pst*I, and *Sal*I LL-H DNA digests. The LL-H restriction fragments which were not cloned were the *Sal*I A and B fragments, the *Eco*RI A (one member of the doublet), B, and F fragments, and the *Pst*I A and D fragments.

Restriction map of the LL-H genome. The order of the restriction fragments of the four endonucleases was based on two complementary techniques. On the one hand, the restriction maps of the main cloned LL-H fragments were established and compared to detect eventual overlappings. On the other hand, DNA homology relationships were determined between the cloned LL-H fragments used as ^{32}P probes and various endonuclease digests of phage LL-H. The results of the hybridization experiments (data not shown), together with the data from the restriction map of the cloned fragments (Fig. 1), permitted the ordering of all fragments generated with the *Sal*I, *Pst*I, *Eco*RI, and *Bam*HI endonucleases. From these results, the order of the *Sal*I fragments was deduced to be BEFDGCA (using the designations of Table 1). The *Eco*RI, *Pst*I, and *Bam*HI fragments were ordered in the same way and are represented in Fig. 1.

The chromosome of phage LL-H was expected to be a linear double-stranded molecule, as was seen previously in electron microscopy (3); however, our results were not

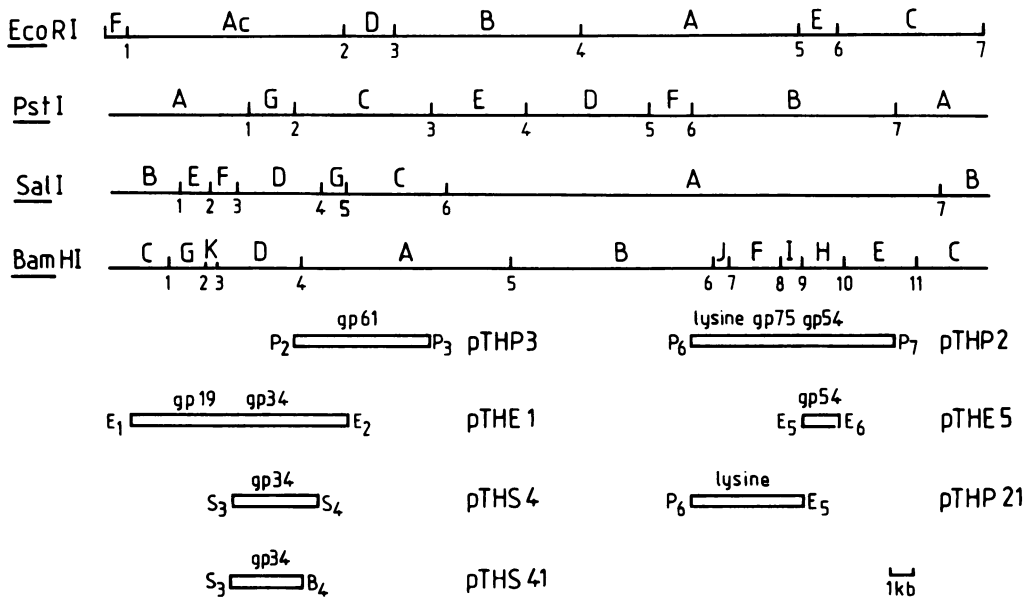

FIG. 1. Restriction map of LL-H chromosome and localization on the restriction fragments of five structural proteins and lysin genes. The restriction fragments generated by *Eco*RI, *Pst*I, *Sal*I, and *Bam*HI are designated by letters as indicated in Table 1; gp61 is the gene of the protein that has a molecular weight of 61,000. Abbreviations: P, *Pst*I; E, *Eco*RI; S, *Sal*I; B, *Bam*HI.

consistent with a unique linear configuration and suggest that the DNA molecules were circularly permuted. In support of this, restriction fragments considered to be at the two extremities of the phage genome did not show independent hybridization patterns. For example, the *Eco*RI Ac and *Eco*RI C fragments both hybridized with the *Pst*I A, *Sal*I B, and *Bam*HI C fragments (Fig. 1). This feature is not a result of the sequence homologies between the *Eco*RI Ac and *Eco*RI C fragments, because the *Eco*RI Ac probe did not hybridize with the *Eco*RI C fragment (and vice versa).

To confirm the *Eco*RI restriction map of the LL-H genome, we mapped the *Eco*RI sites on the *Sal*I A and *Sal*I B fragments. The size of the *Eco*RI fragments generated from the *Sal*I A and *Sal*I B fragments were 8.55, 5.4, 3.7, and 1.5 kb and 2.1, 1.7, and 0.9 kb, respectively. The 8.55-, 1.5-, and 0.9-kb sizes represented the lengths of the *Eco*RI A, *Eco*RI E, and *Eco*RI F fragments, respectively (Table 1). The distance between *Eco*RI-6 and *Sal*I-7 was 3.7 kb, and the distance between *Sal*I-7 and *Eco*RI-7 was 2.1 kb; this was because the *Eco*RI C fragment was cleaved into two fragments by the *Sal*I enzyme. Analysis of the *Eco*RI Ac restriction map confirmed that the 1.7-kb fragment was the *Eco*RI-1–*Sal*I-1 segment. These results demonstrated that the *Sal*I A and *Sal*I B fragments were contiguous on the phage chromosome and suggest a circularly permuted configuration of the genome.

LL-H gene expression in *E. coli*. Structural proteins of the LL-H phage were previously analyzed by SDS-polyacrylamide gel electrophoresis (3). Disrupted purified LL-H particles gave two major bands with estimated molecular sizes of 19 and 34 kilodalton (kDa) and at least five other bands (54, 58, 61, 75, and 89 kDa) (Table 2). All the proteins were able to react with a serum raised against the phage.

To identify the LL-H structural protein genes encoded by the recombinant plasmids, we assayed for the expression of these genes in *E. coli* by the immunoblotting method of Towbin et al. (36). We observed that LL-H proteins were expressed by some *E. coli* strains bearing recombinant plasmids. Thus, of the seven main LL-H structural proteins, five were expressed (Fig. 1, Table 3). No proteins were found in control cells. The 34-kDa protein presented the highest expression level in the *E. coli* extracts.

The *Sal*I D fragment expressed the 34-kDa protein when cloned in both orientations in the pBR322 vector. This result

TABLE 2. Characterization of main proteins of LL-H particle

Mol size of proteins (kDa)	Relative band intensity with[a]:		Probable antigenicity	Presence in ghost
	Coomassie brilliant blue staining	Immunostaining		
89	Strong	Medium	Medium	Yes
75	Strong	Medium	Medium	Yes
61	Strong	Strong	High	Yes
58	Strong	Strong	High	Yes
54	Variable medium	Variable strong	High	No
34	Strongest	Strongest	Medium	Yes[b]
19	Strongest	Weak	Low	Yes[c]

[a] Comparisons of band intensities of purified LL-H phage preparation after SDS-polyacrylamide gel electrophoresis were made with Coomassie brilliant blue staining and after immunoblotting with anti-LL-H and immunoperoxidase staining by 2-chloro-1-naphthol and H_2O_2.

[b] Probably the main capsid protein.

[c] Probably the main tail protein.

TABLE 3. Expression of LL-H cloned fragments in *E. coli* HB101[a]

Recombinant plasmid	Cloned fragments (kb)	Mol size (kDa) of protein(s) detected by immunoblotting
pTHE5	*Eco*RI-E (1.5)	54
PTHE1	*Eco*RI-Ac (8.55)	19, 34
pTHS4	*Sal*I-D (3.3)	34
pTHP3	*Pst*I-C (5.4)	61
pTHP2	*Pst*I-B (8.0)	54, 75

[a] Crude extracts of the strains harboring hybrid plasmids or pBR322 or pBR325 and a sample of the LL-H phage were denaturated and loaded onto a 12.5% polyacrylamide gel in the presence of SDS. After electrophoresis the proteins were transferred onto nitrocellulose sheets and brought into contact with rabbit anti-LL-H antibodies. The immunological complexes were detected with peroxidase-conjugated goat immunoglobulins to rabbit immunoglobulins.

suggests that the promoter implicated in the expression of this gene is a phage promoter. Moreover, the plasmid pTHS41 (Fig. 1) containing the 2.4-kb *Sal*I-3–*Bam*HI-4 fragment also expressed the 34-kDa protein.

Because some LL-H genes were expressed and translated in *E. coli*, strain HB101, which contains various recombinant plasmids, was tested for the presence of lytic activity on strain LL23. Generally, late in the phage growth cycle, enzymes are made that possess lytic activity toward the cell wall of the bacterial host (12, 25, 27, 35).

The experiment described in Materials and Methods shows that the strain harboring recombinant plasmid pTHP2 (Fig. 1) was able to produce a halo of lysis on strain LL23. This halo can be interpreted as cell lysis of LL23 and not as growth inhibition, because the halo increased when plates were further incubated either at room temperature or at 42°C after the cells had reached the stationary-growth phase. Strain HB101, harboring plasmid vector pBR322, was unable to produce a halo. The lytic activity encoded by plasmid pTHP2 (Fig. 1) was also tested on strains other than LL23. All four *L. lactis* strains tested were sensitive, whereas three of six *L. helveticus* strains and four of ten *L. bulgaricus* strains were resistant to lysis, as were *L. casei* and *L. plantarum* strains. However, *Bacillus subtilis*, belonging to a bacterial group phylogenetically distant from the *Lactobacillus* group, was sensitive to this activity. By subcloning experiments from plasmid pTHP2, we mapped this activity more precisely on the 4.35-kb *Pst*I-6–*Eco*RI-5 fragment contained in pTHP21 (Fig. 1).

DISCUSSION

The LL-H phage of *L. lactis* may be placed into group B of the bacteriophage classification described by Bradley (8). In electron microscopy, an LL-H particle showed a hexagonal head, with a diameter of about 50 nm and a tail of about 180 nm (3). This morphology is typical of *Lactobacillus* bacteriophages. The purified LL-H DNA genome has a 34-kb molecular size, which is also approximately the size of other characterized *Lactobacillus* bacteriophages (33). The LL-H DNA phage is sensitive to numerous restriction endonucleases, contrary to other phage DNAs of lactic acid bacteria (20). It is therefore likely that the LL-H phage host, *L. lactis* LL23, has few if any modification systems.

To establish the structural organization of LL-H DNA, a study was undertaken with the following steps: (i) determination of the number and size of the fragments generated with various restriction endonucleases; (ii) cloning and restriction mapping of the *Eco*RI-, *Pst*I-, and *Sal*I-generated fragments; and (iii) restriction map of the entire phage, supported by data from the homology relationships between the different restriction fragments analyzed by the Southern blotting technique. From these results (Fig. 1) and results based on the linear appearance of LL-H DNA molecules when studied by electron microscopy (3), we propose that the LL-H genome is circularly permuted with a linear structure, as was observed for phage P22 (13, 14, 38, 39) and phage MX-8 (34).

Despite repeated experiments, cloning with the three endonucleases tested is still incomplete; this could be explained by the particular structure of LL-H extremities or by the presence on some LL-H restriction fragments of genes whose expression is lethal for the host cell. Nevertheless, five of the seven main structural LL-H protein genes were localized on the LL-H genome. These genes were clustered in one region, on the *Pst*I BAGC fragments (Fig. 1). The two structural proteins which were not detected in recombinant clones may be encoded by the host genome and not by viral genes.

Some genes were mapped more precisely by our subcloning experiments, which showed, for example, that the gene encoding the 34-kDa protein was borne by a 2.4-kb fragment, whereas the gene encoding the 54-kDa protein was contained within a 1.5-kb *Eco*RI fragment. The lytic activity was shown to be expressed from a 4.35-kb segment. These localizations were made possible by the expression of the LL-H-cloned genes in *E. coli*. The heterologous expression of gram-positive bacterial genes in gram-negative bacteria has already been reported, principally for *B. subtilis* genes (37) and also for *Streptococcus* and *Lactobacillus* genes (10, 40). The LL-H gene coding for the 34-kDa protein seems to be expressed from its own promoter in *E. coli*. Interestingly, this phage promoter should be strong, since in *L. lactis* it is responsible at least for the expression of the main phage structural protein. Probably the transcriptional and translational signals of *L. lactis* phage LL-H are recognized by *E. coli* RNA polymerase and ribosomes.

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