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Taxonomic Differentiation of 101 Lactococcal Bacteriophages and Characterization of Bacteriophages with Unusually Large Genomes

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Sixty-three virulent bacteriophages of *Lactococcus lactis* were differentiated by DNA-DNA hybridization. The results, including those of a previous classification of 38 phages of the same bacterial species (P. Relano, M. Mata, M. Bonneau, and P. Ritzenthaler, J. Gen. Microbiol. 133:3053–3063, 1987) show that 48% of the phages analyzed belong to a unique DNA homology group (group III). Phages of this most abundant group had small isometric heads. Group I comprised 29% of the phages analyzed and was characterized by a small phage genome (19 to 22 kilobases) and a particular morphology with a prolate head. Like group III, this group contained representative phages of other classifications. Group II (21%) included virulent and temperate phages with small isometric heads. Two large isometric-headed phages, φ109 and φ111, were not related to the three DNA homology groups I, II, and III. The genome of φ111 was unusually large (134 kilobases) and revealed partial DNA homology with another large isometric phage, 1289, described by Jarvis (type e) (A. W. Jarvis, Appl. Environ. Microbiol. 47:343–349, 1984). The protein compositions of φ111 and 1289 were similar (three common major proteins of 21, 28, and 32 kilodaltons).

Lactic acid bacteria widely used in various food fermentation processes for production of dairy products are susceptible to bacteriophage infection. Determination of phylogenetic relationships between lactic acid bacterial phages should contribute to a better understanding of genetic exchanges and interactions between phages; these studies, combined with data on phage resistance mechanisms, should also provide a basis for genetic construction of bacteriophage-resistant strains useful in fermentations using these bacteria. Some lactococcal phages have previously been differentiated into phage species according to morphology, serological type, protein composition, or DNA homology (2–5, 7, 8, 20, 24, 28). The most valid criterion of relatedness between phages is their DNA homology (17). Thirty-eight virulent and temperate phages of *Lactococcus lactis* were compared by Relano et al. (24); three DNA homology groups designated I, II, and III were described. In the present study, we have characterized 63 new lactococcal phages by DNA homology. Most of them were isolated in cheese factories between 1985 and 1987, have different geographical origins (United States, France, and New Zealand), and originally propagated on different hosts. In order to correlate our classification to others previously defined, representative phages were used as references.

MATERIALS AND METHODS

Bacterial strains and phages. All bacterial strains were *L. lactis*. The reference phages were φ30 (group I); φT187 and φT189 (group II); φ40 (group III) (24); P001 and P109 (type P001) (2); c6A (21); c2 (1); 1289 (group e); and 1358 (group c) (8); these phages have been described previously. The 63 other phages included in this study were φ47, φ48, φ106 to φ113, φ122, φ125 to φ140, φ142 to φ145, φ147, φ149 to φ157, φ162, φ164, φ167, φ168, φ170 to φ172, φ174 to φ179, φ182 to φ184, φ190, φ191, and φ193 to φ196 and were provided by a private company.

Medium. All the strains were grown in M17 broth (27) at 30°C. For phage stocks, M17 agar plates were supplemented with 10 mM $CaCl_2$.

Phage production and purification. Phage production and purification were performed as described earlier (30).

DNA-DNA hybridization. Purified phage DNAs were digested with restriction endonucleases and electrophoresed on a 1% (wt/vol) or 0.7% agarose gel. The resulting fragments were transfered to nylon filter sheets (Hybond-N, pore size, 0.45 μm; Amersham Corp.) as described by Southern (26) and modified by Reed and Mann (23) (vacuum blot technique). The filter sheets were then incubated at 65°C for 24 h in hybridization buffer containing ^{32}P-labeled reference DNA obtained by nick translation (16, 25). Hybridization buffer is 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–0.05% Blotto (3 ml of 20× SSC–100 μl of 10% SDS–100 μl of 5% nonfat dry milk, for a 10-ml final volume). After hybridization, the strips were sequentially washed in (i) 2× SSC–0.1% SDS at room temperature for 30 min (two times) and (ii) 0.2× SSC–0.1% SDS at 68°C for 45 min (two times), (10); the filter was dried after the final wash and exposed to Kodak XAR film.

In dot hybridization experiments, 0.2 μg of unlabeled whole DNA from each phage was denaturated by heating for 10 min at 100°C and quickly chilling at 4°C. The denaturated DNA was then dotted on a nylon filter sheet, and further processing was as described above.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (12), and the gels were silver stained by the method of Oakley et al. (19).

Electron microscopy of phages. Samples were applied to carbon-coated grids (3.05 mm, 400 mesh) which had been rendered hydrophilic by glow discharge. Phages were negatively stained with 2% phosphotungstic acid and examined under a JEOL 1200 EX transmission electron microscope at an accelerating voltage of 60 kV and at a magnification of ×60,000.

Pulsed-field gel electrophoresis. Phage DNAs for pulsed-

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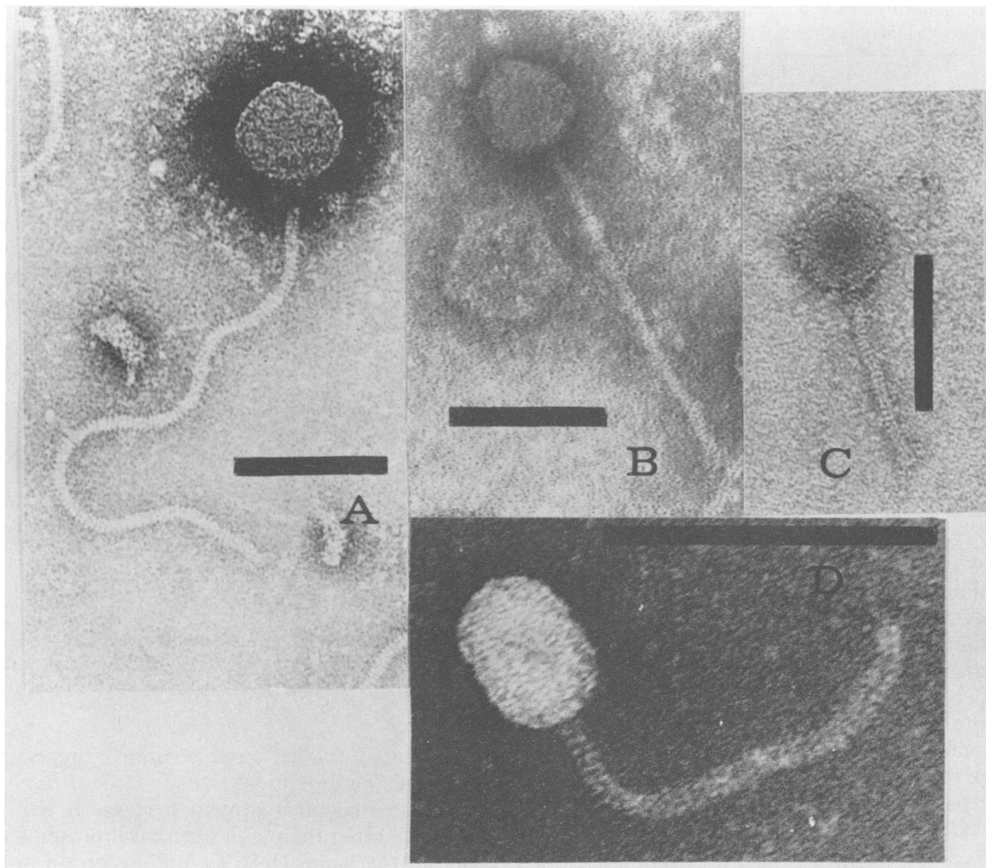

FIG. 1. Electron micrographs of bacteriophages. (A) φ111; (B) φ109; (C) φ110 (group II); (D) φ171 (group I). Bars, 100 nm.

field gel electrophoresis were prepared by the method of McClelland et al (18). DNA samples were incubated overnight with 25 U of restriction enzyme. Oligomers of lambda phage DNA were used as molecular markers as described by Waterbury and Lane (31). Electrophoresis of the DNA was done on a contour-clamped homogeneous electric field system (Pulsaphor Plus; LKB-Pharmacia) for 7 to 14 h in TBE (50 mM Tris base, 50 mM boric acid, 1 mM EDTA).

RESULTS

DNA-DNA hybridizations. Three phages representative of the three DNA homology groups proposed by Relano et al. (24) were chosen as reference phages: φ30 (group I), φT187 (group II), and φ40 (group III). DNAs from these phages were used as probes to hybridize to each entire phage DNA by dot blotting under high-stringency conditions of hybridization. A very high level of homology was observed between phage φ30 and 14 phages of the 63 phages tested (φ125, φ127, φ147, φ151 to φ153, φ155, φ157, φ162, φ171, φ172, and φ182 to φ184). Fourteen other phages (φ47, φ48, φ110, φ126, φ128, φ129, φ131, φ132, φ144, φ145, φ154, and φ176 to φ178) showed strong homology with phage φT187, which belongs to group II. The third probe (φ40, group III) was able to hybridize to 33 phage DNAs (φ106 to φ108, φ112, φ113, φ122, φ130, φ133 to φ140, φ142, φ143, φ149, φ150, φ156, φ164, φ167, φ168, φ170, φ174, φ175, φ179, φ190, φ191, and φ193 to φ196). No detectable DNA homology was observed between the two remaining phages of the collection (φ109 and φ111) and any of the three probes. All the other phages were included in one of the three DNA homology groups according to their responses in the DNA-DNA hybridization experiments.

Phages c2, c6A, P001, and P109, which are representative of phage types in other classifications, all revealed strong DNA homology with phage φ30 (group I).

Ultrastructure and host range. At least two phages of each of the three DNA homology groups were taken at random, and their morphologies were examined, as were those of the two atypical phages, φ109 and φ111 (Fig. 1; Table 1). As expected, phages φ152 and φ171 (group I) had prolate heads. Phages of group II (φ48, φT187, and φ110) and phages of group III (φ108, φ112, and φ113) had similar morphologies, with small isometric heads and tail lengths ranging from 118 to 141 nm. Phages φ109 and φ111 had larger isometric heads and larger tails than the other phages examined. The tail of φ111 was particularly long (470 nm); the structure of this phage seemed to be unstable, since heads separated from tails were frequently observed.

Phages φ109 and φ111, which seemed to be significantly different from all the other phages of the collection, were examined in more detail.

The host range of these two phages on five *L. lactis* strains (S3, S13, S14, S17, and 205) chosen for their high phage sensitivity was determined. Phage φ111 was unable to lyse any of these strains, whereas φ109 was able to propagate on one strain (S17). It is noteworthy that high-titered stock suspensions (10^8 to 10^9 PFU/ml) were hard to obtain for both the φ109 and the φ111 phages and that the titer dramatically

TABLE 1. Ultrastructures of some representative phages of each DNA homology group

Phage	DNA homology group	Head		Tail length (nm)[a]
		Morphological type	Size (nm)[a]	
φ152	I	Prolate	41 by 52	100
φ171	I	Prolate	40 by 52	102
φ110	II	Small isometric	58	126
φT187	II	Small isometric	53	128
φ48	II	Small isometric	52	118
φ108	III	Small isometric	55	137
φ112	III	Small isometric	52	141
φ113	III	Small isometric	52	141
φ109	IV	Large isometric	65	245
φ111	V	Large isometric	70	470

[a] Student *t* test confidence intervals were 99%.

decreased in a few days. This observation can be related to the frequent spontaneous disruption of φ111, which resulted in capsids separated from tails.

Restriction endonuclease patterns of φ109 and φ111 and DNA-DNA hybridizations. The two phages φ109 and φ111 had double-stranded DNA molecules. The entire genomes of φ109 and φ111 were analyzed by pulsed-field gel electrophoresis, and the chromosome sizes were estimated to be 53 and 134 kilobases (kb), respectively. φ111 appeared to be unusually large for a lactococcal phage. In almost all the cases, the genome length, calculated as the sum of the lengths of fragments generated by digestion with the different endonucleases used (*Pst*I, *Hpa*II, and *Eco*RV for φ109 and *Bgl*I, *Sal*I, and *Xho*I for φ111), was correlated with that determined by pulsed-field gel electrophoresis of the entire genome. In a few cases (*Eco*RI and *Bam*HI digests of φ109), the genome size estimated from the restriction fragments was slightly less than that determined from the entire genome, probably because small-sized bands or doublets were not detected.

DNA from φ111 was highly refractory to restriction by *Eco*RI, *Bam*HI, *Pst*I, *Hpa*II, *Apa*I, *Sac*II, *Sma*I, *Bgl*II, and *Mlu*I endonucleases. In the *Hin*dIII digests of the two phages, no comigrating fragments could be detected. DNAs from φ109 and φ111 were used as probes to hybridize to DNAs from φ30 (group I), φT187 (group II), φ40 (group III), phage 1358 (group c of Jarvis [8]), and φ111 digested with *Eco*RV and *Hin*dIII. No homology was detected between these phage DNAs except in the control experiments. Conversely, by using entire genomes from φ30, φT187, φ40, and 1358 as probes to hybridize to restriction fragments of φ109 and φ111, no homology was found. These results confirm that φ109 and φ111 do not belong to group I, II, III, or c and that they are not genetically related.

The morphology of φ111 was very similar to that of the large isometric-headed phages described by Jarvis and classified in group e (8). The DNA from phage 1289, which represents group e, was used to detect possible DNA homology with φ111. The φ111 probe was able to hybridize to some *Hin*dIII fragments of the phage 1289 genome. The signal was strong with three fragments of 5.1, 4.5, and 4 kb and weak with at least 13 other bands (Fig. 2). These results show that φ111 and phages of group e can be classified in a unique DNA homology group. In pulsed-field gel electrophoresis of the entire genomes of φ111 and 1289, the two bands comigrated to the same position, suggesting that the two genomes had identical sizes.

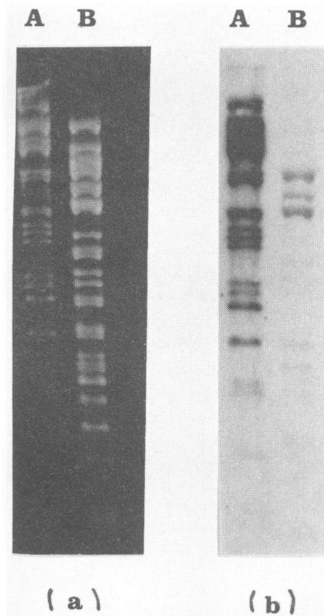

FIG. 2. Agarose gel electrophoresis of the *Hin*dIII-generated fragments of phage DNAs (a) and the corresponding blots hybridized with ^{32}P-labeled DNA from φ111 (b). Lanes: A, φ111; B, 1289.

Phage structural protein profiles. It has previously been observed that the major structural proteins of phages classified in the same DNA homology group are especially well conserved (24). To confirm the unrelatedness of φ109 and φ111, the structural protein composition of these phages was analyzed by SDS-polyacrylamide gel electrophoresis and compared to that of the reference phages of groups I, II, and III and that of phage 1358. The profiles of the large isometric-headed phages φ109 and φ111 were completely different from those of the prolate-headed phage φ30 (group I) and the small isometric-headed phages φT187 (group II), φ40 (group III), and 1358. The profile of φ109 showed a unique major protein band with a molecular weight of 33,000, whereas in the profile of φ111, three major proteins with molecular weights of 21,000, 29,000, and 32,000 and two minor proteins with molecular weights of 61,000 and 90,000 were detected (Fig. 3 and 4). The three major proteins of φ111 were also detected in the profile of 1289 (group e of Jarvis) (Fig. 4). The phage protein composition analysis confirms that φ111 and φ109 cannot be classified in group I, II, or III and that φ111 is related to phage 1289.

DISCUSSION

In a previous study (24), we characterized 38 virulent and temperate phages of *L. lactis*. These phages were classified into three groups according to protein composition, DNA homology, and serological type. In this work, 63 new phages were examined, and their DNA homologies were determined by using reference phages of the previous classification. Among the 101 phages analyzed, 29 phages could be assigned to group I, 21 phages to group II, and 48 phages to group III (Table 2). This last group appears to be the most abundant and geographically widespread, since 48% of the phages studied belong to this group. As previously noted (17), this phage species includes phage P008, which was

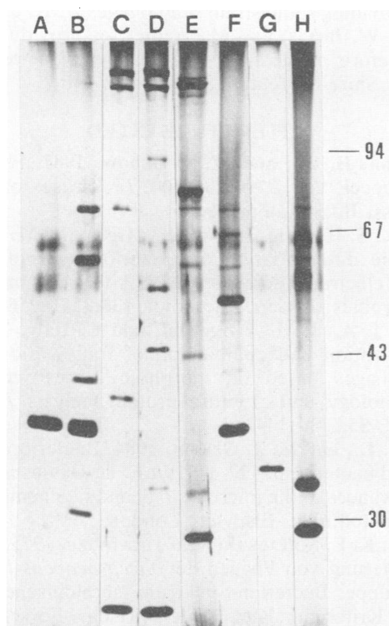

FIG. 3. Patterns of structural proteins of some representative phages obtained by SDS-polyacrylamide gel electrophoresis. Lanes: A, φ40 (group III); B, φ106 (group III); C, φT187 (group II); D, φT189 (group II); E, φ30 (group I); F, 1358 (group c of Jarvis); G, φ109; H, φ111. Molecular mass markers in kilodaltons are given on the right.

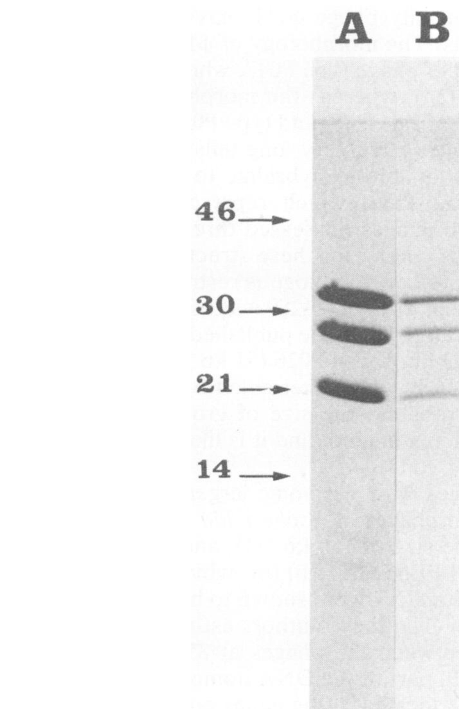

FIG. 4. Patterns of structural proteins of phages φ111 (lane A) and 1289 (lane B) by SDS-polyacrylamide gel electrophoresis. Molecular mass markers in kilodaltons are given on the left.

isolated in the Federal Republic of Germany (5, 15), group a-b of Jarvis (8), and phages uc1001 and uc1002, which were described by Coveney et al. (3). Four other phages (e8, hp, ml1, and sk1) were shown to possess the major characteristic protein (37 to 38 kilodaltons) of group III (20), and therefore these phages can also be included in group III. All these phages had similar morphologies, with small isometric heads.

In contrast, group I phages had prolate heads, as did other lactococcal phages such as uc3001 (3), c2 (1), c6A (21), P109 (14), the seven phages of group d in the Jarvis classification (8), and six phages (c6B, c6D, c10I, c10W, drc1, and 643) described by Powell et al. (20). Here, we have demonstrated DNA homology between phages of group I and c6A, c2, P001, and P109. It is now clear that all these prolate-headed phages belong to a unique DNA homology group because common phages have been used in the different classifications. Another characteristic of the prolate-headed phages is the smallness of their genomes (19 to 22 kb) compared with those of the other groups (Table 2).

Group II contains fewer phages than group I and III, but in our classification, it is the only group which includes virulent and temperate lactococcal phages. Some virulent and temperate phages have been classified in a single DNA homology group by other authors (2, 13). These groups cannot be related, since no common reference phages were used in these three classifications.

Three phages were unrelated to groups I, II, and III: phages 1358, φ109, and φ111. Phage 1358 has a small isometric head and represents group c of Jarvis. The absence of DNA homology between phage 1358 and the 101 phages tested suggests that group c of Jarvis, which contains only two phages, is probably rare. Phages φ109 and φ111 have morphologies markedly different from that of the other

TABLE 2. Classification of 101 lactococcal phages and linkage with other classifications

DNA homology group	No. of phages[a]	Head morphology	Genome size (kb)	Molecular masses of major common proteins (kDa)[b]	Related phages from other classifications[c]
I	29 V	Prolate	19–22	27, 78, 115	uc3001, c2, c6A, group d of Jarvis, 6 Australian phages
II	16 V, 5 T	Small isometric	30–40	23, 113, 119	
III	48 V	Small isometric	30–35	37	uc1002, type P008, group a-b of Jarvis, 4 Australian phages?
	1 V	Small isometric		36, 51	Group c of Jarvis
IV	1 V	Large isometric	53	33	Type P087?
V	1 V	Large isometric	134	21, 32	Group e of Jarvis, type P026?

[a] V, Virulent; T, temperate.
[b] kDa, Kilodaltons.
[c] ? indicates a possible relationship, but DNA homology has not been determined. The prolate Australian phages were c6B, c6D, c10I, c10w, drc1, and 643; the small isometric Australian phages were hp, e8, ml1, and sk1 (20).

phages; both, especially phage ϕ111, have large isometric heads and long tails. The morphology of ϕ109 appears to be very similar to that of phage type P047, which was described by Lembke et al. (14), whereas the morphology of ϕ111 is like that of group e of Jarvis (8) and type P026 of Braun et al. (2). These last phages have very long tails (450 to 500 nm). DNA from ϕ111 was able to hybridize to three restriction fragments of phage 1289, which represents group e; in addition, these two phages possessed three common major proteins. The genes coding for these structural proteins are most probably located on homologous restriction fragments.

The genome size of ϕ111 and 1289 was estimated to be 134 kb, which is much larger than the published value for phages of group e (51 to 52 kb [8]) or P026 (51 kb [2]). The genome sizes of ϕ111 and 1289 were determined by pulsed field gel electrophoresis. Probably, the size of group e phages and P026 has been underestimated, and it is likely that their size is similar to that of ϕ111.

Only a few phages with a genome larger than 100 kb are known: the T-even phages of *Escherichia coli* (T2, T4, and T6), with a genome size of 160 kb (11), and the two phages SPO1 (150 kb) and PBS1 (280 kb) (6), which infect *Bacillus subtilis*. Phages T2 and T4 were shown to be homologous by Kim and Davidson (11); these authors estimated the overall DNA homology between the phages at 85%. As observed with ϕ111 and 1289, part of the DNA homology between the T-even phages was located in the genes coding for the viral structural proteins. The ϕ111 genome is large enough to contain more than a hundred genes; some of them could be involved in the DNA metabolism. In contrast to the other large-genome phages cited above, which contained contractile tails of 110 (phage T4) to 240 nm (phage PBS1) in length, phage ϕ111 had a much longer tail (470 nm) which seemed to be noncontractile.

Though ϕ109 was cut by various endonucleases, a marked paucity of restriction sites was observed for the ϕ111 genome. This phenomenon was also described for some lactococcal phages (c6A [22], P008 [15], and uc1001 and uc1002 [3]) and was emphasized by Relano et al. (24) and Jarvis and Meyer (9). In all but one of the cases, *Eco*RI was able to digest the lactococcal phage genomes; the exception was ϕ111, which, like the group e phages of Jarvis (8), was refractory to restriction by *Eco*RI. This phenomenon seems to be a characteristic of these large isometric-headed phages. One explanation of this observation is that phage ϕ111 might code for a methyltransferase that methylates specific nucleotides at the *Eco*RI site. An identical mechanism has been described by Trautner et al. (29) for the temperate phage SPβ of *B. subtilis*, which is not cleaved by the restriction endonucleases *Bsu*RI and *Hpa*II.

Another feature of ϕ111 was a rapid decrease (3 to 4 logarithmic units) in the titers of phage stocks in only a few days. This observation has also been noted for phages 1289 and P026 by Jarvis and Teuber (A. W. Jarvis and M. Teuber, personal communication). In contrast to most of the phages studied here, phage ϕ111 isolated from whey was not responsible for fermentation failures. This behavior is probably due to the apparent fragility of the ϕ111 structure and can explain why only a few large isometric phages have been isolated anywhere in the world.

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