Close Relationship of Virulent Bacteriophages of *Streptococcus salivarius* subsp. *thermophilus* at Both the Protein and the DNA Level

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Comparisons of 23 virulent phages of *Streptococcus salivarius* subsp. *thermophilus* were made based on morphology, host-range, structural protein analysis, DNA restriction patterns and DNA homology. All the phages had isometric heads (diameters 55–58 nm) and striated tails (lengths 221–275 nm). The genome sizes ranged from 37 to 43 kb. The electrophoretic profiles of the structural proteins were related, with at least two major bands of about 23 and 29 kDa in common for all the phages except three, $\phi 80$, $\phi 96$ and $\phi 99$. These latter phages, which had a particular protein composition (main proteins of 29 and 40 kDa) and high DNA homology between each other, showed only a low DNA homology with the other phages. Extensive DNA homologies were demonstrated by Southern hybridization for all the other phages. All these results suggest a close evolutionary relationship between the phages.

INTRODUCTION

One of the primary causes of slow acid production by lactic acid bacteria during fermentations in the dairy industry remains lysis by bacteriophages. Physical and genetic studies of lactococcal phages have yielded useful information which, when combined with data on phage resistance mechanisms (Daly & Fitzgerald, 1987; Klaenhammer, 1987), may result in improved ability to limit phage infection. Similar analysis of phages of *Streptococcus salivarius* subsp. *thermophilus* (formerly *S. thermophilus*), which have been poorly studied thus far, may contribute to limiting phage infections in fermentations using these bacteria.

The morphology of some bacteriophages of *Streptococcus salivarius* subsp. *thermophilus* has been described. All the phages examined can be classified in group B of Bradley (1967) and appear to be very similar, with isometric heads (50–70 nm in diameter) and tails 200–300 nm long with small base plates (Reinbold *et al.*, 1982). Variants with shorter tails have also been isolated (Accolas & Spillman, 1979). Collar-like structures have not been detected, but a single thin tail fibre is often observed (Teuber *et al.*, 1987).

In the present study, we have compared 23 virulent phages of *S. salivarius* subsp. *thermophilus* in respect of their host-range, morphology, protein composition, DNA restriction patterns and DNA homology.

METHODS

Bacterial strains and phages. All bacteria (S1 to S35) were *Streptococcus salivarius* subsp. *thermophilus* strains used in the dairy industry. The 23 phages ($\phi 80$ to $\phi 102$) were isolated in various French factories from yoghurt or cheese whey samples collected from abnormal fermentations over a period of several years. They all behaved as virulent phages.

Culture medium. All the *S. salivarius* subsp. *thermophilus* strains were grown at 40 °C in M17 broth (Terzaghi & Sandine, 1975).

Determination of host specificity of bacteriophages. The host range of each phage was determined by spot tests of phage dilutions: 10 μ l of lysate or of appropriate dilutions was spotted onto M17 Ca²⁺ (5×10^{-3} M-CaCl₂) agar plates previously poured in a double layer, the top layer having been inoculated with cultures of the strain tested.

Phage purification and preparation of phage DNA. This was done as described earlier for *Lactobacillus lactis* bacteriophage 11h (Trautwetter *et al.*, 1986).

DNA-DNA hybridization. Phage DNA used as probe was labelled by nick-translation with 5'-[α - 32 P]dCTP as described by Rigby *et al.* (1977) and Maniatis *et al.* (1982). Unlabelled phage DNA was digested with restriction endonucleases and the resulting fragments were separated by electrophoresis on a 1% (w/v) agarose gel. The Southern blot technique (Southern, 1975) was used to transfer restriction fragments onto BA-85 nitrocellulose filter sheets (Schleicher and Schuell). Hybridization was done at 65 °C for 40 h in Denhardt reagent (Denhardt, 1966) as previously described (Mata *et al.*, 1986). After hybridization, the filter was sequentially washed in: (1) 0.1% SDS/salmon sperm DNA [50 μ g ml⁻¹ in 1 $\times$ SSC (1 $\times$ SSC is 0.15 M-NaCl plus 0.015 M-sodium citrate)]/1 $\times$ Denhardt solution at 65 °C for 1 min (four times) and for 45 min (three times); (2) salmon sperm DNA (50 mg ml⁻¹)/0.1% SDS/0.1 $\times$ SSC for 45 min at 65 °C (three times); and (3) 3 $\times$ SSC for 10 min at room temperature (twice).

SDS-PAGE. Purified phage particles containing approximately 0.05 mg protein were suspended in a solution containing 0.06 M-Tris/HCl (pH 6.8), 5% (v/v) β -mercaptoethanol, and 2% (w/v) SDS, disrupted by boiling at 100 °C for 8 min, electrophoresed as described by Laemmli (1970) and silver stained by the method of Oakley *et al.* (1980).

Electron microscopy of phages. Copper grids (3.05 mm, 400 mesh) were coated with carbon film. The phages were absorbed to the coated grids and examined in a transmission electron microscope (Jeol 1200 EX) at an accelerating voltage of 60 kV. Phages were photographed at 60000 $\times$ magnification.

RESULTS

Host-range determination

The 23 phages (ϕ 80 to ϕ 102) were each purified by two successive single-plaque isolations on a lawn of a sensitive strain, and the resulting lysates were used to determine their host-range using 26 strains of *S. salivarius* subsp. *thermophilus*. From the data presented in Table 1, the

Table 1. *Lytic pattern of the phages on some representative strains*

Strain	Efficiency of plating of the following phages*																			
	ϕ 81	ϕ 82	ϕ 83	ϕ 84	ϕ 85	ϕ 86	ϕ 88	ϕ 89	ϕ 90	ϕ 91	ϕ 93	ϕ 94	ϕ 95	ϕ 80	ϕ 96	ϕ 97	ϕ 98	ϕ 99	ϕ 100	ϕ 102
S1	+	+	-	-	-	(+)	+	-	+	-	-	-	+	-	+	-	-	-	-	-
S2	+	+	+	+	+	+	+	+	(+)	-	-	-	+	-	+	-	-	-	-	-
S4	+	+	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-
S7	+	+	+	-	+	+	+	+	+	(+)	+	-	+	-	-	-	-	-	-	-
S12	+	+	+	+	+	+	+	-	+	-	-	+	+	-	-	-	-	-	-	-
S15	+	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-
S19	+	+	(+)	+	-	+	-	-	-	-	(+)	-	+	-	-	-	(+)	-	-	-
S23	+	+	-	+	-	+	+	-	+	-	-	-	+	-	-	-	+	+	-	+
S28	+	+	+	+	+	+	+	-	+	-	-	-	+	-	-	-	+	-	-	+
S29	+	+	-	-	+	+	+	+	-	+	+	-	+	-	-	-	-	-	-	+
S30	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	-	-	+	-	-
S32	+	-	+	-	+	+	+	-	-	+	+	+	+	-	-	-	+	-	-	-
S33	+	+	+	-	+	+	+	-	-	+	+	-	-	-	-	-	+	-	-	-
S34	+	+	+	-	+	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-
S35	+	+	+	-	+	+	+	-	+	+	-	-	+	-	-	-	+	-	-	-
S5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
S6	-	-	-	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-
S16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	(+)	-	-
S17	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	(+)
S18	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+
S21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
S24	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-
S25	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-
S26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	-	-	-
S27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-
S31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-

* -, Absence of plaque formation; (+), efficiency of plating $< 10^{-4}$; +, efficiency of plating $> 10^{-2}$ and < 10 . The efficiency of plating of a phage on a given strain is the ratio of the p.f.u. ml⁻¹ on this strain to that obtained on the propagating strain. Phages ϕ 87, ϕ 92 and ϕ 101, which were able to form plaques only on their propagating host, are not included in the table.

Fig. 1. Electron micrograph of phage $\phi 81$. Bar, 100 nm.

phages can be classified into two distinct groups. Lytic group I contains 13 phages ($\phi 81$ to $\phi 86$, $\phi 88$ to $\phi 91$, and $\phi 93$ to $\phi 95$) which were unable to attack strains S5, S6, S16, S17, S18, S21, S24, S25, S26, S27 and S31 but showed some similarities in their host-range on the other strains. Lytic group II is composed of phages $\phi 80$, $\phi 96$, $\phi 97$, $\phi 98$, $\phi 99$, $\phi 100$ and $\phi 102$, which were able to propagate on at least three of the above-mentioned strains. In the two lytic groups, some phages have a narrow host-range ($\phi 80$, $\phi 89$, $\phi 94$, $\phi 100$) and others have a wide host-range ($\phi 81$, $\phi 82$, $\phi 85$, $\phi 86$, $\phi 88$, $\phi 95$); nevertheless each phage, except $\phi 81$ and $\phi 86$, has a specific host-range. Phages $\phi 87$, $\phi 92$ and $\phi 101$, which were able to attack only their propagating host, were not classified.

Ultrastructure

The morphology of the virulent phages was very similar to that described for other phages of *S. salivarius* subsp. *thermophilus*. All the phages had an isometric head (55–58 nm in diameter) and a striated tail (221–275 nm long and 8 nm wide) (Fig. 1). Occasionally, a thin fibre seemed to be attached to the terminal plate. As described by Reinbold *et al.* (1982), the phages showed a tendency to form phage clusters.

Protein composition

The structural proteins of purified phages were analysed by SDS-PAGE. Preparations of all the phages showed at least two intense bands, probably corresponding to the major proteins of the tail and the capsid, and other minor bands (Fig. 2). For each phage except $\phi 80$, $\phi 96$ and $\phi 99$, two intense protein bands approximating 23 and 29 kDa could be detected; in addition to these major proteins, two common minor bands of 43 and 58 kDa were also observed in some profiles. In the pattern of $\phi 80$, $\phi 96$ and $\phi 99$ the major protein of 23 kDa was present but that of 29 kDa was replaced by a 40 kDa protein; the minor proteins were also specific to these phages. An additional intense band corresponding to a major protein of 26.5 kDa could be detected in the pattern of $\phi 81$, $\phi 84$ and $\phi 85$; at the same position a band of weaker intensity also appeared in the protein patterns of $\phi 86$ and $\phi 95$. Whether the minor bands observed represent phage protein present in a low amount or were derived from the host bacterium is unknown, but the second hypothesis seems unlikely since the phages were purified by two successive caesium chloride gradients.

Fig. 2. Patterns of structural proteins of some of the phages by SDS-PAGE on 12% (w/v) acrylamide gel. Lanes: A, $\phi 81$; B, $\phi 82$; C, $\phi 88$; D, $\phi 95$; E, $\phi 84$; F, $\phi 85$; G, $\phi 86$; H, $\phi 99$; I, $\phi 96$; J, $\phi 98$; K, $\phi 80$; L, $\phi 100$. Molecular mass markers (kDa): chymotrypsinogen A (25); hen egg white albumin (45); and bovine serum albumin (68).

Comparison of restriction endonuclease patterns of the phage DNAs

All the phages contained a double-stranded DNA molecule; the genome size of each phage was determined by summing the molecular sizes of restriction fragments generated by digestion with at least two endonucleases (*EcoRI* and *EcoRV*). The estimated size of the different phage genomes ranged from 37 to 43 kb. In *EcoRV* digestion (Fig. 3, Table 2), an identical restriction pattern with 12 DNA fragments was obtained for phages $\phi 81$ to $\phi 95$ except for phages $\phi 83$, $\phi 86$ and $\phi 89$, in which a fragment of 4 kb was absent and was replaced by a 3.4 kb fragment. All these phages belong to lytic group I ($\phi 81$ to $\phi 95$). Six fragments (14, 7, 2.2, 1.65, 1.3 and 0.75 kb) were detected when DNAs from these same phages were digested with *EcoRI* (Fig. 4, Table 3); an additional fragment of 12.2 kb appeared in the digests of all these phages except $\phi 83$, $\phi 86$ and $\phi 89$. The restriction pattern of these last three phages showed two additional fragments of 10.6 and 1.6 kb whose sum corresponds to the size of the 12.2 kb restriction fragment of the other group I phages. This result can be explained by the presence of an additional *EcoRI* site located in the 12.2 kb fragment. All the other phages (lytic group II) showed individual *EcoRV* and *EcoRI* restriction patterns.

DNA-DNA hybridizations

DNA homologies were detected by Southern blotting experiments. DNAs from $\phi 81$ (lytic group I) and $\phi 99$ (lytic group II) were labelled with [α - 32 P]dCTP by nick-translation and were used as hybridization probes to each phage DNA digested with *EcoRI* or *EcoRV*. The two probes hybridized to some or to the majority of the restriction fragments of the different phages, depending on the phage tested; the intensity of the signals also varied from phage to phage.

Fig. 3. (a) Agarose gel electrophoresis of *EcoRV*-generated fragments of phage DNAs; (b) the corresponding Southern blots hybridized with ³²P-labelled DNA from phage φ81. Lanes: A, φ83; B, φ89; C, φ88; D, φ95; E, φ81; F, φ96; G, φ98; H, φ99; I, φ80; J, φ100.

Table 2. Homology between φ81 DNA probe and *EcoRV* restriction fragments of other phages

Phage	Size of restriction fragments (kb)*											
	9.2	8	6.6	4	3.7	2.2	1.2	1	0.75	0.7	0.65	0.62
φ81 to φ95 (except φ83, φ86, φ89)	+	+	+	+	+	+	+	+	+	+	+	+
φ83, φ86, φ89	9.2	8	6.6	3.7	3.4	2.2	1.2	1	0.75	0.7	0.65	0.62
	+	+	+	+	+	+	+	+	+	+	+	+
φ98	12	6.4	6.3	6.3	4.2	1.3	1.25					
	+	+	+	+	(+)	—	—					
φ100	13	6.6	6.1	4.2	3.4	2.8	1.2	1	0.65	0.62		
	+	+	+	+	(+)	+	+	+	+	+		
φ80	12	6.7	6.6	5.8	3.8	0.9	0.8	0.65				
	+	(+)	(+)	—	—	—	—	—				
φ96	15	10	3.7	3.6	3.2	1.8	1.7	1.25	1.1	0.73	0.62	
	(+)	—	(+)	(+)	—	—	—	—	—	—	—	
φ99	8	6.5	5	4	4	3.5	3.5	2.2	1.7			
	(+)	—	—	(+)	(+)	—	—	—	—			

* +, Strong hybridization; (+), weak hybridization; —, no hybridization.

Fig. 4. (a) Agarose gel electrophoresis of the *Eco*RI-generated fragments of phage DNAs; (b) the corresponding Southern blots hybridized with ³²P-labelled DNA from phage ϕ 99. Lanes: A, ϕ 83; B, ϕ 82; C, ϕ 86; D, ϕ 96; E, ϕ 98; F, ϕ 99; G, ϕ 80.

Table 3. Homology between ϕ 99 DNA probe and *Eco*RI restriction fragments of other phages

Phage	Size of restriction fragments (kb)*							
	14	12.2	7	2.2	1.65	1.3	0.75	
ϕ 81 to ϕ 95 (except ϕ 83, ϕ 86, ϕ 89)	(+)	(+)	(+)	—	—	—	—	
ϕ 83, ϕ 86, ϕ 89	(+)	(+)	(+)	—	—	—	—	0.75
ϕ 98	17	8	7.2	3	2			
	(+)	—	—	—	—			
ϕ 100	12.5	11.5	6.9	4.6	2.3			
	(+)	(+)	—	—	—			
ϕ 80	20	16	3.4	1.1				
	+	+	(+)	(+)				
ϕ 96	18	8	8	6.7				
	(+)	+	+	+				
ϕ 99	18	9	3.7	3.4	2.5	0.6		
	+	+	+	+	+	+		

* +, Strong hybridization; (+), weak hybridization; —, no hybridization.

The percentage of DNA homology between the probe and a given phage was calculated as the size of all the restriction fragments of the phage able to hybridize expressed as a proportion of the size of the entire phage DNA. This method gives an over-estimated value since partial hybridization between two fragments is sufficient to give a signal on the autoradiograms;

nevertheless it enables an estimation of the homology between the phage DNAs. When $\phi 81$ was used as reference phage, the DNA homology was 100% for phages of lytic group I and about 80–90% for phages of lytic group II, except for phages $\phi 80$, $\phi 96$ and $\phi 99$ (Table 2). Only one or two restriction fragments of these three phages gave a weak signal when hybridized to the $\phi 81$ probe (Fig. 3). Conversely, $\phi 99$ DNA revealed homology of about 88% and 56% with $\phi 80$ and $\phi 96$ DNAs respectively, but gave only a few, weak signals when used as a probe with the restriction fragments of all the other phages of lytic groups I and II. In these cases, only the largest fragments were labelled (Fig. 4, Table 3). No hybridization was observed when representative phages of other species of lactic acid bacteria were used as probes: phage mv4 of *Lactobacillus delbrueckii* subsp. *bulgaricus* (Mata *et al.*, 1986); $\phi T189$ and $\phi 936$ of *Lactococcus lactis* (Relano *et al.*, 1987).

DISCUSSION

The majority of the phages of *Streptococcus salivarius* subsp. *thermophilus* analysed in this study showed a close relationship, demonstrated by their protein composition and by DNA–DNA hybridization experiments: their DNAs were able to hybridize at high stringency. In contrast, the phages could be classified into two distinct lytic groups. Phages belonging to lytic group I were very similar: they had analogous *EcoRI* and *EcoRV* restriction patterns and possessed two identical major proteins. Lytic group II was more heterogeneous: phages of this group had individual restriction patterns. Within group II, phages $\phi 98$ and $\phi 100$ possessed the two characteristic major proteins of lytic group I and their DNA hybridized strongly with the lytic group I phage DNAs. All the phages except $\phi 80$, $\phi 96$ and $\phi 99$ formed a single DNA homology group and were closely related. The differences observed in the host-range of the various phages do not involve large changes in the phage genomes since this criterion reflects the expression of only a small part of the phage genome. In previous work (Relano *et al.*, 1987), it has been underlined that host range could not be correlated with molecular groups.

Phages $\phi 80$, $\phi 96$ and $\phi 99$ of lytic group II had two major proteins, only one of which was common to all the phages, the other being specific to these three phages. Their DNAs hybridized strongly with each other but showed weak homology with the other phage DNAs. Therefore they cannot be classified into a group completely separate from the main group, but they can be clearly differentiated into a subgroup.

Our results on restriction analysis contrast with those described in a preliminary report of Benbadis *et al.* (1987), in which 12 phages of *Streptococcus salivarius* subsp. *thermophilus* could be separated into two major groups with respect to their restriction patterns. In a study involving nine *S. salivarius* subsp. *thermophilus* phages isolated from Finnish cheese plants, Kivi *et al.* (1987) observed that the electrophoretic profiles of the structural proteins appeared nearly identical. The molecular masses of the four major proteins were estimated to be 63, 49, 42 and 11 kDa. These protein profiles differed from our results, suggesting that these Finnish phages were different from those described here. In contrast, the phages of *Streptococcus salivarius* subsp. *thermophilus* described by Neve *et al.* (1989) seemed to have a protein composition similar to that of the phages analysed in this study: phages P6 and P8, with main proteins of 39.8 and 24.0 kDa, could be related to phages $\phi 80$, $\phi 96$ and $\phi 99$; and phages PO, PSt, P55, P53, 71/45 and 71/St15, with two major proteins of 30.9 and 24.0 kDa, could be related to all the other phages of this study. The latter group seemed to be the most widespread. The techniques used to characterize *Streptococcus salivarius* subsp. *thermophilus* phages in this study and *Lactobacillus* and *Lactococcus* phages in previous studies (Jarvis, 1984; Mata *et al.*, 1986; Relano *et al.*, 1987; Coveney *et al.*, 1987; Lahbib-Mansais *et al.*, 1988) have proved very useful in determining the relatedness between phages and have facilitated their classification into groups. For each bacterial species, phages could be classified into a small number of groups according to their DNA homology; within a family, the genes coding for the structural proteins seemed to be well conserved since at least the major proteins were common to all the phages of the group (Mata & Ritzenthaler, 1988).

The presence of non-homologous sequences flanked by homologous sequences in the phage genome probably plays an important role in phage evolution by permitting block DNA exchanges by homologous recombination between phages (Botstein, 1980).

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