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Genetic and biological characterization of nine *Streptococcus salivarius* subsp *thermophilus* bacteriophages

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Summary — Nine bacteriophages of *Streptococcus salivarius* subsp *thermophilus* isolated from different fermentation accidents, with different geographic origins, were compared according to their genomic structures and growth characteristics. Their genomes are linear, double-stranded DNA, with complementary cohesive ends. They belong to one group and are classed into 2 closely related strains. The phages of the same strain show the same restriction pattern with *Bam*HI, *Eco*RI, *Hind*III and *Pvu*II. The bacteriophages ϕ B1.2 and ϕ A1.1 representative of the strain 1 and strain 2 respectively were characterized and compared according to their morphology, growth characteristics, restriction maps and DNA homology. They belong to Bradley's group B. The optimum concentration of 25 mM was determined for phage infections requiring calcium ions. One-step growth studies show that the latent period is 25 min for both ϕ A1.1 and ϕ B1.2; their burst size is 88 and 56 particles per infected cell, respectively. Their genomes are 36.2 ± 0.5 and 35.6 ± 0.5 kbp in length, respectively. The restriction maps of these genomes are constructed. Cross-hybridization experiments showed that the 2 bacteriophages are closely related. Using Southern blot hybridization, no homology was detected between the DNA of various bacterial strains, *ie*, strains sensitive or resistant to phage infections, and the ϕ B1.2 DNA, indicating that these bacteria are not lysogenic for these phages.

bacteriophage / *Streptococcus salivarius* subsp *thermophilus* / restriction map / DNA homology / growth characteristic / morphology

Résumé — Caractérisation génétique et biologique de 9 bactériophages de *Streptococcus salivarius* subsp *thermophilus*. L'analyse des profils de restriction de 9 bactériophages issus d'accidents de fabrication et d'origines géographiques différentes a montré une parenté entre ces phages. Leurs génomes sont linéaires avec des extrémités cohésives. Ils ont été classés en 1 seul groupe contenant 2 souches, chaque souche étant caractérisée par un même profil de restriction obtenu avec les endonucléases *Bam*HI, *Eco*RI, *Hind*III et *Pvu*II. Les bactériophages ϕ B1.2 et ϕ A1.1 correspondant respectivement à la souche 1 et 2 ont été étudiés au niveau morphologique, biologique et génétique. Ils appartiennent au groupe B de Bradley. Leurs caractéristiques de croissance ont été définies. Ils nécessitent la présence d'ions Ca^{2+} , la concentration optimale de $CaCl_2$ étant de 25 mM. La durée de leur cycle de multiplication est de 25 min. La taille de la récolte est de 88 particules par cellule infectée pour ϕ B1.2 et de 56 particules par cellule infectée pour ϕ A1.1. La taille du génome de ϕ A1.1 est de $36,2 \pm 0,5$ kb et celle de ϕ B1.2 est de $35,6 \pm 0,5$ kb. La cartographie de ces génomes montre une grande ressemblance, qui a été confirmée par hybridation ADN-ADN. Des expériences d'hybridation utilisant comme sonde l'ADN de ϕ B1.2 ne révèlent aucune homologie de séquence avec les génomes des bactéries industrielles, sensibles et résistantes, ce qui indique que ces bactéries ne sont pas lysogènes pour les bactériophages étudiés.

bactériophage / streptococcus salivarius subsp thermophilus / carte de restriction / homologie d'ADN / caractéristique de croissance / morphologie

* Correspondence and reprints

INTRODUCTION

Bacteriophage attack is one of the primary causes of slow acid production by lactic acid bacteria during industrial fermentation. Thus, extensive investigations have been carried out on phages and phage-resistance mechanisms, especially for the mesophilic N streptococci (Daly, Fitzgerald, 1987; Klaenhammer, 1987; Teuber, Loof, 1987; Mata, Ritzenthaler, 1988; Sanders, 1988). Conversely, little is known about phages and phage-resistance mechanisms in *S salivarius* subsp *thermophilus*. Studies based on the host range, structural proteins, serological and morphological properties were carried out (Kivi *et al*, 1987). The effect of incubation temperature on the development of *S salivarius* subsp *thermophilus* and its phages was studied (Sozzi *et al*, 1978). Their morphology has been investigated in several works (Accolas, Spillmann, 1979; Reinbold, Reddy, 1982; Krusch *et al*, 1987). On the basis of DNA homology and electrophoretic patterns of the structural proteins, 12 virulent phages of *S salivarius* subsp *thermophilus* were classified in the single phage species (Mata, Ritzenthaler, 1988). A DNA homology between virulent phages of *S salivarius* subsp *thermophilus* was also demonstrated by Benbadis *et al* (1987) and Neve *et al* (1989).

In this report, 9 bacteriophages isolated from abnormal fermentations, with various geographic origins, were classed into 1 group containing 2 closely related strains according to their restriction patterns. The phages ϕ A1.1 and ϕ B1.2 corresponding to the 2 different strains were further studied at both biological and genetic levels.

MATERIALS AND METHODS

Phage and bacterial strains

The *streptococcus salivarius* subsp *thermophilus* strains used in this study are listed in table I. The strains were grown on M17 broth (Terzaghi and Sandine, 1975) at 42 °C from 1% subcultures in reconstituted milk (10%). M17 solid media (1.5% agar) and soft agar (0.5% agar) were used. The phages used in this study and their geographic origins are listed in table I. They were isolated from abnormal fermentations. The lysates were prepared by propagating these phages on the NST3 strain.

Host range determination

The phage host range was determined by plating up to 10^8 PFU (plaque forming units) on a lawn of the tested bacterial strain (table I) at 42 °C in the presence of 25 mM CaCl_2 .

Determination of CaCl_2 optimum concentration for infection

Plaque forming units (PFU) of the phage lysates were determined in the presence of different concentrations of CaCl_2 according to the technique described by Accolas and Spillmann (1979).

Preparation of phage lysates

The bacterial strains were grown in M17 broth and harvested in early growth logarithmic phase. The culture sample was then incubated with the phage at a multiplicity of infection (MOI) of ≈ 0.3 in the presence of 25 mM CaCl_2 at 37 °C for 15 min. The mixture was then diluted (1/10) by the addition of 9 vol of prewarmed M17 broth and incubated at 42 °C until lysis oc-

curred. The phage lysate was then filtered (Millipore, pore size 0.45 μm) and treated by DNase (Sigma) at a final concentration of 5 $\mu\text{g/ml}$, RNase (Sigma) (10 $\mu\text{g/ml}$), and lysozyme (Boehringer Mannheim) (50 $\mu\text{g/ml}$), at 37 °C for 30 min. The determination of plaque forming units (PFU) of phage lysate was carried out by using the double agar layer technique.

Table 1. Bacterial strains and bacteriophages used in this study and their origins. ATCC : American Type Culture Collection; CNRZ : Centre National de la Recherche Zootechnique; IP : Institut Pasteur.
Bactéries et bactériophages utilisés dans cette étude.

Bacterial strain	Origin
NST1	Industrial strain
NST3	Industrial strain
NST5	Industrial strain
NST9	Industrial strain
ATCC19258	ATCC
ATCC14485	ATCC
ATCC19987	ATCC
CNRZ302	CNRZ
CNRZ368	CNRZ
CNRZ455	CNRZ
CNRZ388	CNRZ
CNRZ391	CNRZ
CNRZ7	CNRZ
CNRZ307	CNRZ
CNRZ385	CNRZ
CNRZ308	CNRZ
CNRZ160	CNRZ
CNRZ445	CNRZ
IP6631	IP
Phage	Geographical origin
ϕ A1.1	France
ϕ B1.2	Egypt
ϕ T12	Ireland
ϕ T17	France
ϕ T58	Portugal
ϕ T9	Portugal
ϕ D1	Argentina
ϕ T21	France
ϕ T33	Argentina

Determination of burst size

A phage suspension was added to *Streptococcus salivarius* subsp *thermophilus* strain NST3 so that the MOI was \approx 0.01 in a total vol of 400 μl . Adsorption was allowed to continue to completion at 37 °C for 15 min. Cells were then immediately pelleted by centrifugation at 8 000 *g* for 2 min. The number of free phage particles in the supernatant was determined by titration. The cells were washed twice with prewarmed M17 broth and resuspended in 40 ml of the same medium. The lysis of culture at 42 °C was controlled by plating 0.1 ml of the mixture at 10-min intervals by using the double-layer technique. This experiment was repeated 3 times.

Latent period determination

The NST3 strain was infected by phages with a MOI of 3 in the liquid medium (M17) at 37 °C in the presence of 25 mM CaCl_2 . Bacterial kinetic growth was determined by reading optical density at 650 nm until lysis occurred.

Phage purification and electron microscopy

Phages were concentrated by polyethylene glycol precipitation (Yamamoto *et al*, 1970), resuspended in TMG buffer (Tris 10 mM Tris-HCl, 10 mM MgSO_4 , 0.01% gelatin, pH 7.4) and were purified by preformed caesium chloride gradient (Maniatis *et al*, 1982) which were over-layered with 50% glycerol. The CsCl was removed by extensive dialysis against TMG buffer containing 0.5 M NaCl. The phages were negatively stained with 2% uranyl acetate (pH 4.5) and examined under a Siemens 102 electron microscope.

Phage and bacterial DNA purification

Phages were concentrated by centrifugation of lysate at 45 000 *g* for 2.5 h. Purified phages in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0) were heated for 15 min at 65 °C in the presence of 5 mM EDTA and 0.1% sodium dodecyl

sulfate (SDS). Then proteinase K (Boehringer) was added at the final concentration of 200 µg/ml and the mixture was incubated for 30 min at 37 °C. Subsequently, protein extraction was carried out once with Tris-HCl-saturated phenol (pH 7.5) and once with chloroform-isoamyl-alcohol (24/1, V/V). DNA was precipitated with isopropanol at -20 °C in the presence of 0.3 M sodium acetate. Bacterial DNA was purified as previously described (Marmur, 1961).

Preparation of [³²P] labelled phage DNA and DNA-DNA hybridization

Phage DNA was purified by the GeneClean process (Bio 101), and then labelled with [α -³²P]-dCPT by using a nick-translation kit (Amersham) (Rigby *et al*, 1977).

Bacterial DNA (3 µg) was digested by restriction endonucleases, the resulting fragments were separated by electrophoresis on a 0.8% agarose gel and transferred to a hybond-N membrane (Amersham) according to the Southern method (Southern, 1975). DNA fragments were covalently linked to the membrane by exposure to UV light for 5 min. Membranes were then prehybridized at 42 °C for 4 h, in 6 x SSC (1 x SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 5 x Denhart (Ficoll 0.1%, bovine serum albumin 0.1%, polyvinylpyrrolidone 0.1%), 0.5 x SDS, 50% formamide and 200 µg/ml denatured salmon sperm DNA. The ³²P-labelled phage DNA (8 x 10⁶ cpm) was then added. The hybridization was allowed for 14 h at 42 °C. The membrane was then washed; (i), twice in 2 x SSC, 0.1% SDS at room temperature for 30 min; (ii), 3 times at 60 °C for 40 min in 0.1 x SSC, 0.1% SDS; (iii), twice in 0.1 x SSC at room temperature. Filters were then exposed to X-ray Hyperfilm (Amersham) with an intensifying screen. Hybridization between phage DNA (0.5 µg) was assured in the same conditions.

Restriction analysis of phage DNA

Cleavages with restriction endonucleases were carried out according to suppliers' instructions (Boehringer Mannheim, Appligène). Digests were heated at 70 °C for 10 min, held in ice and loaded onto 0.5-1% agarose horizontal slab

gels in TAE buffer (0.04 M Tris acetate, 2 mM EDTA, pH 8.0), and electrophoresis was performed for 16 h at 1 V/cm. A *Hind*III digest of lambda DNA was used as a molecular weight standard.

RESULTS

Host range

The ability of the 9 bacteriophages to propagate on 19 strains (table I) was tested. Identical lytic patterns were observed for the 9 phages. All phages were able to propagate only on the NST3 and NST5 strains. Thus, the other strains were inappropriate hosts to propagate these phages.

Comparison of restriction patterns and structure of phage genomes

The phage DNA were digested by *Bam*HI, *Eco*RI, *Hind*III, *Pvu*II and analysed by agarose gel electrophoresis. The phages ϕ B1.2, ϕ T9, ϕ T21, ϕ T33, ϕ T58 and ϕ D1 have the same patterns of their genomes and were classed in the same phage strain: strain 1, while the phages ϕ A1.1, ϕ T12, and ϕ T17 corresponded to a second strain: strain 2. In order to determine whether the phage DNA has cohesive ends, it was digested with various endonucleases and subjected to heat treatment (70 °C for 10 min) and immediately loaded on agarose gel in the presence of unheated digests. In each digest, a minor band gave 2 fragments after heat-treatment. We concluded that the heat dependent bands were end fragments. The phage genomes were linear with single stranded cohesive ends. This was confirmed by pretreating phage DNA with T4 DNA ligase prior to the digest (data not shown).

Restriction maps of ϕ A1.1 and ϕ B1.2

The phages ϕ B1.2 and ϕ A1.1 corresponding to the strain 1 and 2, respectively, were chosen for further studies. The DNA of each phage was digested by various endonucleases and the resulting fragments were separated on agarose gels. The phage DNA were not cleaved either by *KpnI* or *SmaI*. They were digested by *AvaI*, *BamHI*, *BglII*, *EcoRI*, *HindIII*, *SacI*, *SalI*, *PvuII* and *XhoI*. Their genome sizes were estimated from restriction digests and cal-

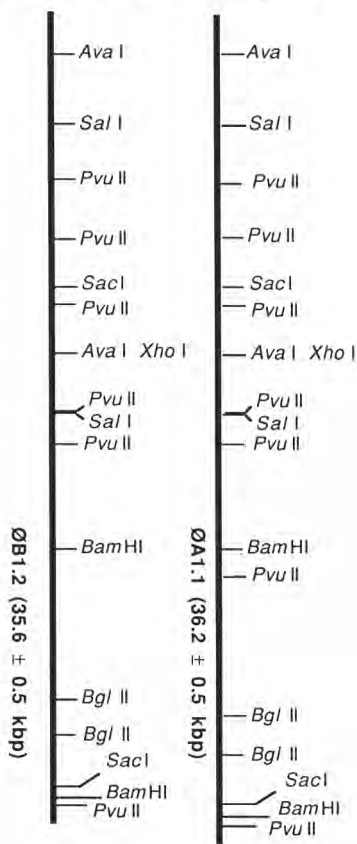


Fig 1. DNA restriction maps of ϕ A1.1 (36.2 ± 0.5 kbp) and ϕ B1.2 (35.6 ± 0.5 kbp) genomes. Cartes de restriction des génomes de ϕ A1.1 ($36,2 \pm 0,5$ kbp) et de ϕ B1.2 ($35,6 \pm 0,5$ kbp).

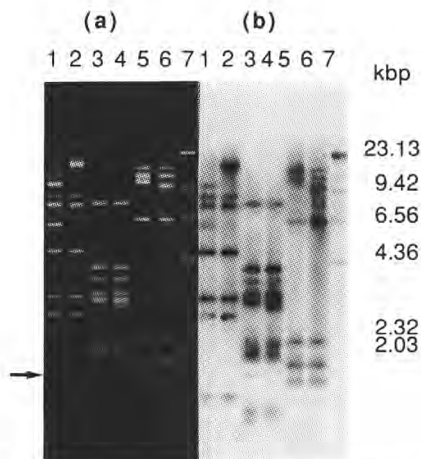


Fig 2. a), Digestion patterns of ϕ A1.1 and ϕ B1.2 DNA. Gel electrophoresis of *EcoRI*, *HindIII* and *PvuII* digests of ϕ A1.1 and ϕ B1.2 genomes. b), Hybridization between ϕ A1.1 and ϕ B1.2 DNA. Autoradiogram prepared after hybridization of ^{32}P -labeled DNA from phage ϕ B1.2 with phage DNA digests; lane 1, ϕ A1.1 DNA digested by *PvuII*; lane 2, ϕ B1.2 DNA digested by *PvuII*; lane 3, ϕ A1.1. DNA digested by *HindIII*; lane 4, ϕ B1.2 DNA digested by *HindIII*; lane 5, ϕ A1.1 DNA digested by *EcoRI*; lane 6, ϕ B1.2 DNA digested by *EcoRI*; lane 7, *HindIII* digest of Lambda DNA. DNA digests were heated for 10 min at 70°C and separated by electrophoresis on agarose gel (1%) for 16 h at 1 V/cm. A *HindIII* digest of lambda DNA was used as the molecular weight standard. The 1.5 kbp *Hind III* fragment is indicated by arrows.

a) Profils de restriction des ADN de ϕ A1.1 et de ϕ B1.2. Electrophorèse en gel d'agarose des génomes de ϕ A1.1 et ϕ B1.2 digérés par *EcoRI*, *HindIII* et *PvuII*. b) Hybridation entre les ADN de ϕ A1.1 et ϕ B1.2. Autoradiographie préparée après hybridation avec ADN du phage ϕ B1.2 marqué au ^{32}P ; piste 1, ADN de ϕ A1.1 digéré par *PvuII*; piste 2, ADN de ϕ B1.2 digéré par *PvuII*; piste 3, ADN de ϕ A1.1 digéré par *HindIII*; piste 4, ADN de ϕ B1.2 digéré par *HindIII*; piste 5, ADN de ϕ A1.1 digéré par *EcoRI*; piste 6, ADN de ϕ B1.2 digéré par *EcoRI*; piste 7, ADN du phage lambda digéré par *HindIII*. Les hydrolysats sont chauffés pendant 10 min à 70°C et séparés par électrophorèse en gel d'agarose (1%) pendant 16 h à 1 V/cm. L'ADN du phage lambda digéré par *HindIII* est utilisé comme marqueur de taille. Le fragment *HindIII* de 1,5 kpb est indiqué par une flèche.

culated to be 35.6 ± 0.5 and 36.2 ± 0.5 kbp, respectively. Restriction maps of the 2 phage genomes were obtained by mapping the sites for 7 endonucleases and by: i), identifying the heat-sensitive end fragments; ii), comparing single and double digests. The 2 phage genomes showed only minor differences (fig 1).

Phage DNA homology

DNA from ϕ B1.2 labeled with [α - 32 P] dCTP by nick-translation was used as a probe to hybridize to the phage DNA cleaved with *Pvu*II, *Eco*RI and *Hind*III. The resulting au-

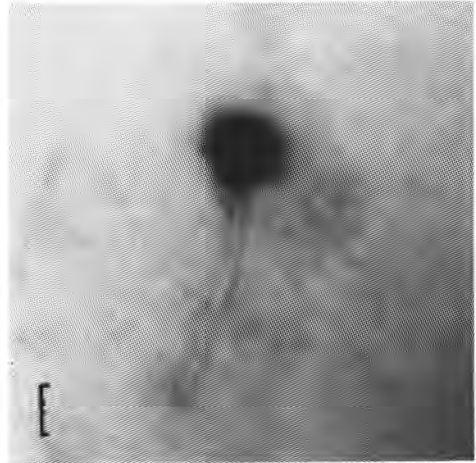
toradiogram was compared with the digestion patterns. Labeled DNA was able to hybridize strongly to all the restriction fragments except for the ϕ A1.1 DNA *Hind*III fragment of 1.5 kbp (fig 2, lane 3). It is not likely that the difference lies only within this fragment, since partial homology between the probe and the restriction fragment is sufficient to allow hybridization.

Phage morphology and biological properties of ϕ A1.1 and ϕ B1.2

The 2 bacteriophages belong to Bradley's group B (Bradley, 1967), with isometric



ϕ B1.2



ϕ A1.1

Fig 3. Electron micrographs of ϕ A1.1 and ϕ B1.2. Purified bacteriophages were negatively stained with 2% uranyl acetate; bar: 50 nm.

Observation au microscope électronique des phages ϕ A1.1 et ϕ B1.2. Les bactériophages purifiés sont colorés négativement avec 2% d'acétate d'uranyle; barre, 50 nm.

heads and long non-contractile tails (fig 3). They possess a regular 6-sided head (diameter 50 ± 1 nm), and the tail length was 246 ± 5 nm. The number of phages measured was 13 for ϕ A1.1 and 9 for ϕ B1.2. A terminal structure was observed, which appeared as a small plate.

Phage infections require the presence of the cations Ca^{2+} . In the absence of CaCl_2 the efficiency of plating considerably decreases and pinpoint plaques were observed (diameter < 0.5 mm). The optimum concentration of 25 mM was determined for the highest number of PFU (fig 4a). The growth cycle of the bacteriophages ϕ A1.1

and ϕ B1.2 was characterized by a 1-step growth on the NST3 strain under optimal conditions (42°C , 25 mM CaCl_2). Their burst size is 88 and 56 particles per cell, respectively (fig 4b). The latent period for the 2 bacteriophages is 25 min (fig 4c).

Hybridization between bacterial and phage DNA

In order to determine whether bacterial strains resistant to ϕ B1.2 were lysogenic, a DNA-DNA hybridization experiment was carried out using ϕ B1.2 DNA as a probe.

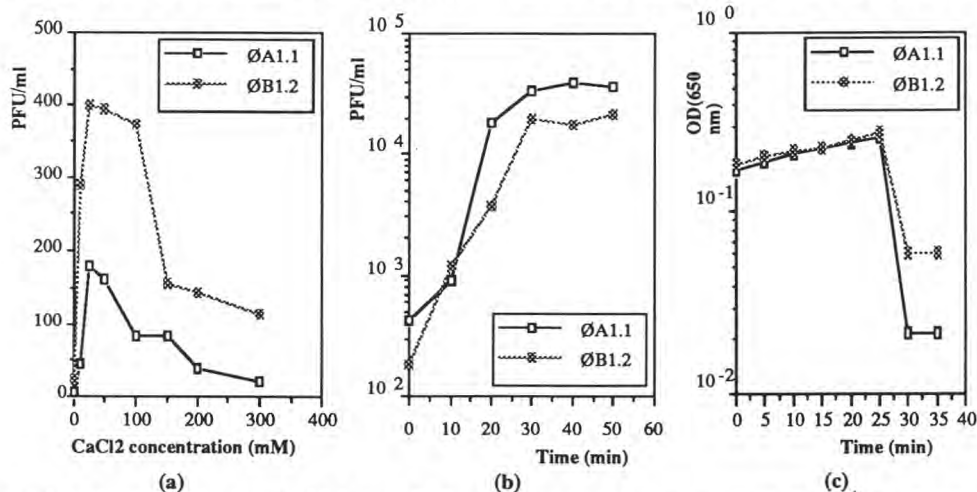


Fig 4. Biological properties of ϕ A1.1 and ϕ B1.2. a), The effect of CaCl_2 concentration on the phage infections. Infective phages were numbered on NST3 strain, in M17 broth at 42°C , in the presence of different concentrations of CaCl_2 . b), One-step growth curves of bacteriophages ϕ A1.1 and ϕ B1.2. The phages were propagated on NST3 in M17 broth at 42°C . Time 0 refers to the end of phage adsorption which was allowed for 15 min at 37°C and then diluted by 10-fold dilution in prewarmed M17 broth. c), Latent period determination. NST3 was infected by phages with a MOI of 3, at 37°C , in the presence of 25 mM of CaCl_2 . The infections were followed by reading optical density at 650 nm (OD, 650 nm).

Propriétés biologiques de ϕ A1.1 et ϕ B1.2. a) Effet de la concentration en CaCl_2 sur les infections phagiques. Les phages sont titrés sur la souche NST3, dans du milieu M17 à 42°C , en présence de différentes concentrations de CaCl_2 . b) Courbes de croissance en cycle unique des bactériophages ϕ A1.1 et ϕ B1.2. Les phages sont propagés sur NST3 dans du milieu M17 à 42°C . L'adsorption a lieu pendant 15 min à 37°C et le mélange est dilué 10 fois dans du milieu M17 préchauffé. c) Détermination de la durée du cycle de multiplication. La souche NST3 est infectée par les phages avec une multiplicité d'infection de 3 à 37°C , en présence de CaCl_2 25 mM. Les infections sont suivies par lecture de la densité optique à 650 nm (DO, 650 nm).

Under the conditions used, no bacterial DNA sequence homologous to bacteriophage ϕ B1.2 DNA was detected (data not shown). Hybridization between sensitive strains and phage DNA do not show a homologous sequence either.

DISCUSSION

In this report, 9 bacteriophages of *S salivarius* subsp *thermophilus* were differentiated according to their restriction patterns. Despite their different origins, they are closely related and are classed into 1 DNA homology group, containing 2 closely related phage strains. The phages of the same strain have the same restriction patterns. The same host range was observed for the 9 phages. Nevertheless, the determination of the efficiency of plating of the 9 bacteriophages on the sensitive bacterial strains showed that an identical restriction pattern did not imply the same host-phage interactions (unpublished results).

Krusch *et al* (1987), reported a country origin-specific host range; our results showed that the very related phages with identical host range can be detected throughout the world.

Comparison of restriction patterns of DNA strains with various endonucleases showed small but unambiguous differences between the bacterial strains studied (Colmin *et al*, 1987), notably between sensitive strains NST3 and NST5.

We determined the ability of ϕ B1.2 to adsorb to strains NST1, NST3, NST5 and NST9. All these strains, except NST1, adsorb phage with an efficiency > 90%. These indicate that NST9 resistance mechanism does not involve an alteration of the host ability to adsorb this phage. For NST1, adsorption appears to be restricted, indicating the absence of operational receptor (data not shown).

An electron microscopic examination confirmed the uniformity detected within the thermophilic streptococci described by Accolas and Spillmann (1979) and Krusch *et al* (1987). They belong to Bradley's group B.

Two phages ϕ B1.2 and ϕ A1.1 were characterized at both biological and genetic levels. Both require the divalent cation Ca^{2+} for effective infection. Many other bacteriophages have been reported to need divalent cations, usually Ca^{2+} or Mg^{2+} or both. They could be involved in different steps of the phage infection-cycle (Luria and Steiner, 1952; Watanabe and Takesue, 1972; Lawrence *et al*, 1976; Alatossova *et al*, 1987). In the case of *S salivarius* subsp *thermophilus*, the role of Ca^{2+} though essential, is still unknown. Sozzi (1972) showed that the requirement of calcium for lysis is governed by the phage-bacterium association and not only by the bacterium strain or the phage. The 2 phages have a similar latent period of 25 min. These phages with fast multiplication and high burst size may cause complete failure of industrial cultures even if they are present in only low numbers at the beginning of the fermentation process. This could be an explanation for the extent of industrial problems related to such bacteriophages.

The latent period and burst size of several group N *streptococci* phages have been reported as a function of growth in different media and at different temperatures (Keogh, 1973). Latent periods in reconstituted milk ranged from 23–56 min at 30 °C, and from 16–44 min at 37 °C. Burst sizes were from 2–113 particles at 30 °C and from 0–139 particles at 37 °C (Lawrence *et al*, 1976). The ϕ A1.1 and ϕ B1.2 latent periods and burst sizes were within these ranges. In industry, they depend on the factory and on physiological conditions of both host and phage. Studies on phage

growth characteristics in standardized laboratory conditions are of fundamental importance, since they are still unknown. In addition, they provide an approach to predicting their behaviour in the factory.

The restriction mapping of ϕ A1.1 and ϕ B1.2 genomes show strong similarities. They differ in their size: ϕ A1.1 is 0.6 kbp larger than ϕ B1.2. The difference is localized in the *Bam*HI-*Bgl*II fragment of 7.6 kbp in ϕ A1.1 genome and 7 kbp in ϕ B1.2 one. Cross-hybridization experiments show that this difference is probably contained in the 1.5 kbp *Hind*III fragment. These bacteriophages are closely related; 1 phage type could be derived from the other by a genomic rearrangement, *eg* deletion or insertion or both. The bacteriophages of lactic acid bacteria are frequently closely related; industrial fermentations might provide a dynamic situation inducing DNA exchanges. The presence of non-homologous sequence flanked by homologous sequences could promote these DNA exchanges by homologous recombination (Mata and Ritzenthaler, 1988).

A close relationship between *S salivarius* subsp *thermophilus* bacteriophages has also been described (Benbadis *et al*, 1987; Relano *et al* (cited as personal communication by Mata and Ritzenthaler, 1988, Neve *et al*, 1989). Twelve *S salivarius* subsp *thermophilus* bacteriophages were differentiated into 3 subgroups by analysis of their genomes and structural proteins (Neve *et al*, 1989). A subgroup III of this classification consists of 9 phages with a genome size of 33.8–36.7 kbp. The studied phages may fit into this subgroup. DNA–DNA hybridization studies are needed to allow a molecular comparison between the *S salivarius* subsp *thermophilus* phages so far studied. Selection of reference phages is necessary for establishing taxonomic studies (Mata, Ritzenthaler, 1988).

Hybridization experiments showed no homologous sequence between bacterial and viral DNA. Therefore, resistant bacteria were not lysogenic. Furthermore, absence of homology between bacterial and phage genome shows that the genomes of the bacterial strains we tested are not a potential source of the phage studied and related phages.

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