Characterization of ϕ 393-A2, a bacteriophage that infects *Lactobacillus casei*

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Bacteriophage ϕ 393-A2, isolated from an artisanal cheese whey sample, is a temperate phage able to generate stable lysogens through integration of its DNA into the bacterial genome. One-step growth kinetics of its lytic development revealed eclipse and latent periods of 100 and 140 min, respectively, with a burst size of about 200 p.f.u. per infected cell. ϕ 393-A2 virions have an isometric head and a long, non-contractile tail terminating in a baseplate. The capsid is composed of two major and at least nine minor structural polypeptides. The phage genome consists of a double-stranded DNA molecule of 44 kbp bearing 3'-protruding cohesive ends. A physical map of the phage DNA has been constructed for six restriction enzymes. The whole ϕ 393-A2 genome has been cloned in *Escherichia coli* using plasmid- and phage-derived cloning vectors.

Keywords: *Lactobacillus casei*, bacteriophage, lysogenization, development, cloning

INTRODUCTION

Lactic acid bacteria are used as starters in the fermentation of dairy products, processed meats, fermented vegetables, and other food products. In spite of the intensive attention devoted over the last 50 years to the development of mechanisms and strategies of defence against phage attack, phage infection of starter strains continues to be an important problem, since it causes the failure of the fermentations or the production of low-quality products (Sing & Klaenhammer, 1993). Studies on bacteriophages of lactic acid bacteria have preferentially focused on those infecting the genus *Lactococcus*, because of its importance in the cheesemaking industry (Mercenier & Lemoine, 1989; Jarvis, 1989; Jarvis *et al.*, 1991). *Lactobacillus* phages have also caused failures in food fermentations, although their incidence is not as extensively documented as that of lactococcal phages (Séchaud *et al.*, 1988; Jarvis, 1989).

Recent reports (González de Llano *et al.*, 1992) have demonstrated that mesophilic lactobacilli, mainly *Lactobacillus plantarum* and *Lactobacillus casei*, are the prevailing lactic acid bacteria found in some of the artisanal fresh and blue cheeses produced in the Asturias region, Spain. Therefore, we undertook the isolation of phages from such home-made products, using these two *Lactobacillus* species as indicators, as a first step towards the evaluation of their importance in the manufacturing process. Phages specific for *L. plantarum* will be described elsewhere (J. L. Caso, C. G. de los Reyes-Gavilán,

M. Herrero, A. Montilla, A. Rodríguez & J. E. Suárez, unpublished results). The *L. casei* phage ϕ 393-A2 was chosen for a more extensive characterization, because it is temperate and its genome has cohesive ends; both properties could be useful in the future development of phage-derived cloning vectors and mechanisms of phage resistance.

METHODS

Bacterial strains and culture conditions. *Lactobacillus casei* ATCC 393 was the usual host for propagation of phage ϕ 393-A2. *L. casei* ATCC 27092 was also used in the lysogenization studies. Medium MRS (Biokar) supplemented with 10 mM CaCl₂ and 10 mM MgSO₄ (MCM) was employed throughout. Incubations were done at 30 °C unless specified otherwise. *Escherichia coli* HB101 (Bolívar *et al.*, 1977) and *E. coli* XL-1 Blue (Bullock *et al.*, 1987) were grown in 2 × TY broth (Sambrook *et al.*, 1989) and in 2 × TY + 0.2% maltose, respectively. Tetracycline (25 µg ml⁻¹) and chloramphenicol (35 µg ml⁻¹) were added when required. Solid and soft agar media were those described above supplemented with 2 or 0.5% (w/v) agar, respectively. Bacteria were stored as frozen stocks at -20 °C in the culture media supplemented with 20% (v/v) glycerol.

Isolation, propagation and purification of phage ϕ 393-A2. Bacteriophage ϕ 393-A2 was isolated from whey of 'Gamoneu' cheese, an artisanal, long-ripening blue cheese made without addition of external starters. Sample enrichment and phage isolation were essentially as described by Séchaud *et al.* (1992), using *L. casei* ATCC 393 as the primary host strain. ϕ 393-A2 was isolated by a double cycle of resuspension, dilution and replating of individual plaques of lysis. Phage stocks were prepared from

MCM plates showing semiconfluent lysis by standard methods. Large-scale isolation of highly purified ϕ 393-A2 virions was done as follows. One litre of liquid MCM medium was inoculated with *L. casei* ATCC 393 and incubated at 30 °C without aeration until it reached an OD₆₀₀ of 0.15 (about 2×10^8 c.f.u. ml⁻¹). Then ϕ 393-A2 was added at a multiplicity of infection (m.o.i.) of 0.1, and incubation continued for a further 12 h. The lysed culture was centrifuged at 10000 *g* for 15 min, and the phage was recovered from the supernatant and purified as described for bacteriophage lambda in Sambrook *et al.* (1989), i.e. by precipitation with NaCl and PEG 8000, resuspension in SM buffer and purification through discontinuous plus continuous CsCl density gradients.

Electron microscopy. Highly purified phage particles obtained as described above were negatively stained with 2% (w/v) phosphotungstic acid, pH 7, and observed in a JEOL 2000 EX-II electron microscope at an acceleration of 80 kV. Electron micrographs were obtained in Agfa Scientia film plates.

SDS-PAGE of structural polypeptides. Samples (15 μ l) of highly purified virions of ϕ 393-A2 were boiled for 3 min in the presence of 20 mM EDTA. After cooling, they were adjusted to 10 mM Mg²⁺ and incubated with DNase I (4 μ g ml⁻¹; Boehringer Mannheim) at 37 °C for 30–60 min. Electrophoresis was conducted at 100 V in 15% (w/v) SDS-polyacrylamide gels as described by Laemmli (1970). The gels were fixed and stained in a solution of 50% (v/v) methanol, 10% (v/v) acetic acid, 0.01% Coomassie Brilliant Blue R-250 (Bio-Rad), or alternatively, they were silver-stained according to Morrissey (1981).

Host-range determination and isolation of lysogens. The host-range of ϕ 393-A2 was determined by serial dilution and plating of concentrated phage stocks (up to 10^{12} p.f.u. ml⁻¹) on a series of *Lactobacillus* strains in conventional double-layer MCM plates. The formation of plaques of lysis was tested at 30 and 37 °C.

Lysogenic derivatives of *L. casei* ATCC 393 and *L. casei* ATCC 27092 were obtained by picking several lysis plaques and streaking them on MCM plates. After 48 h incubation at the appropriate temperature, between 15 and 20 isolated colonies were resuspended in 1 ml MCM, incubated for 6 h, and centrifuged at low speed. Bacterial pellets were then resuspended in 20 mM Tris/HCl, pH 8.5, containing 100 mM sodium pyrophosphate and incubated at 37 °C for 15 min. This chelating treatment was repeated and then the bacterial cells were washed twice with the same solution and three times with MCM. Drops of the treated cultures or their supernatants were spotted on lawns of the corresponding wild-type strain and incubated at the appropriate temperature, looking for the appearance of lysis haloes. The potential lysogens were afterwards subjected to superinfection immunity tests by challenging them with high-titre phage suspensions able to produce total lysis of the wild-type ancestor strain. Their lysogenic status was verified by induction of liquid cultures with 0.5 μ g mitomycin C ml⁻¹ and titration of the released viral particles.

One-step growth curve. A mid-exponential phase culture (10 ml) of *L. casei* ATCC 393 (OD₆₀₀ 0.4–0.5) was harvested by centrifugation and resuspended in 0.25 vol. fresh MCM broth (approx. 10^8 c.f.u. ml⁻¹). Phage was added at an m.o.i. of 0.02 and allowed to adsorb for 5 min at 30 °C. The mixture was then centrifuged (6000 *g*, 2 min), the pelleted cells were resuspended in 10 ml of prewarmed MCM broth, and incubation was continued at 30 °C. Duplicate series of samples were taken at 5 min intervals, and one of them was treated with 1% (v/v) chloroform (for determination of the eclipse period) whereas the other remained untreated at 4 °C before dilution and plating for phage titration.

DNA techniques. Phage DNA was obtained from high-titre lysates as described by Suárez & Chater (1981). Plasmid DNA was extracted from *E. coli* by the boiling lysis method of Holmes & Quigley (1981) or by alkaline lysis according to Birnboim & Doly (1979). Total DNA from *L. casei* was obtained by a non-alkaline lysis protocol according to Scheirlink *et al.* (1989).

All enzymic treatments of DNA were performed as recommended by the suppliers (USB for Sequenase 2.0 and exonuclease I, and Boehringer Mannheim for all the other enzymes). DNA electrophoresis was carried out in agarose gels using standard TBE buffer (Sambrook *et al.*, 1989).

The existence of cohesive ends was checked by comparing the restriction patterns of phage DNA with and without prior treatment with T4 DNA-ligase. To determine whether the cohesive termini had 3' or 5'-protruding single strands, aliquots of phage DNA were heated at 70 °C for 10 min, to melt the cohesive ends, and then subjected to treatment with Sequenase 2.0 or exonuclease I. After heat inactivation of the enzymes (70 °C, 15 min), the treated samples, plus an untreated control, were ligated, digested with *Eco*RI, heated again at 70 °C for 10 min, and kept on ice until loading in an agarose gel.

DNA–DNA hybridizations were performed after electrophoresis and vacuum-blotting of the DNA to Hybond-N nylon membranes (Amersham). Probes were labelled with digoxigenin-dUTP (DIG DNA Labelling and Detection Kit, Boehringer Mannheim). Hybridization and immunochemiluminescent detection with Lumi-Phos 530 (Boehringer Mannheim) were performed as recommended by the supplier.

Molecular cloning of the phage genome. *Bgl*II restriction fragments of ϕ 393-A2 DNA were cloned into *Bam*HI-digested pUC18 (Yanisch-Perron *et al.*, 1985), and *Eco*RI fragments of the phage DNA were cloned in the plasmid vector pACYC184 (Chang & Cohen, 1978) or in the phagemid Lambda ZAP (Short *et al.*, 1988). The vectors were dephosphorylated before ligation. In some cases, individual phage DNA fragments were extracted from low-melting-point agarose gels (SeaKem) in TAE buffer, and purified with the GeneClean Kit (Bio 101) or with the *Pseudomonas atlantica* agarase (Boehringer Mannheim) according to the supplier's instructions. Ligation, transformation, and selection of clones were done by standard techniques (Sambrook *et al.*, 1989).

RESULTS AND DISCUSSION

Structural characteristics of the viral particles of ϕ 393-A2

Phage ϕ 393-A2 was isolated from a cheese whey on *L. casei* ATCC 393, the type strain of the species, as described in Methods. Electron microscopy of ϕ 393-A2 virions (Fig. 1) revealed particles with an isometric head, 55.2 ± 2.5 nm in diameter, and a flexuous, non-contractile tail 239.2 ± 8.1 nm long and 6.7 ± 1.4 nm wide (mean of 12 determinations), ending in a baseplate. Additional structures, such as fibres or collars, were not detected. According to its morphology, ϕ 393-A2 can be included into the Family *Styloviridae* (Matthews, 1981) or Type B of Bradley's classification (Bradley, 1967), as is the case for most *Lactobacillus* phages described up to now, including the *L. casei* phages PL-1, J-1, and the ϕ FSW group (Stetter *et al.*, 1978; Sozzi *et al.*, 1981; Watanabe *et al.*, 1982; Shimizu-Kadota *et al.*, 1983). However, ϕ 393-A2 can be differentiated from these phages because ϕ FSW has a

Fig. 1. Electron micrograph of negatively stained ϕ 393-A2 virions. Bar, 50 nm.

shorter tail (156 nm; Shimizu-Kadota *et al.*, 1983), while in PL-1 and J-1 it is longer (275–290 nm; Khosaka, 1977; Stetter *et al.*, 1978; Watanabe *et al.*, 1982). Furthermore, PL-1 has a terminal tail fibre 50–60 nm long (Stetter *et al.*, 1978; Watanabe *et al.*, 1982), which was not observed in ϕ 393-A2.

SDS-PAGE of the virion proteins (Fig. 2) revealed two major polypeptides with molecular masses of about 28 and 32 kDa, which, by analogy with other known phage proteins, are postulated to be the major components of the head and tail. At least nine other polypeptide bands could also be detected in silver-stained gels (not shown), even after extensive purification of the phage particles, which suggests that they are indeed part of the viral capsid, although their role remains to be established. The protein composition of ϕ 393-A2 is different from what has been reported for the ϕ FSW group (Shimizu-Kadota *et al.*, 1983), although it resembles that of PL-1 (Stetter *et al.*, 1978).

Size and structure of the ϕ 393-A2 genome

The phage genome consists of double-stranded DNA, as deduced from its digestion with different restriction endonucleases (not shown). The mean size of the full-length phage genome was 44.0 kbp, which is higher than those from other *L. casei* phages (Khosaka, 1977; Stetter *et al.*, 1978; Watanabe *et al.*, 1980; Shimizu-Kadota *et al.*, 1983).

Comparison of *Eco*RI restriction patterns of unligated and ligated phage DNA (Fig. 3, lanes 2 and 3) showed that two DNA bands of 2.3 and 1.1 kbp present in the unligated sample disappeared after the ligase treatment, whereas a fusion fragment of 3.4 kbp was clearly visible. Other restriction enzymes gave similar results, and it was concluded therefore that ϕ 393-A2 DNA is a non-permuted, linear molecule with natural cohesive ends, as is also the case with *L. casei* phage PL-1 (Stetter *et al.*, 1978; Watanabe *et al.*, 1980), whereas phages of the ϕ FSW group have a circularly permuted DNA without cohesive ends (Shimizu-Kadota & Tsuchida, 1984).

To elucidate whether the cohesive ends were 3'- or 5'-protruding, we analysed the effect of different enzyme treatments on subsequent ligation of phage DNA under conditions of ligase limitation (enough to ligate sticky ends but not blunt ends). Treatment of the viral DNA

Fig. 2.

Fig. 3.

Fig. 2. SDS-PAGE of bacteriophage ϕ 393-A2 structural proteins, stained with Coomassie Blue. Lane 1, size standards; lane 2, phage proteins.

Fig. 3. Effect of the treatment of ϕ 393-A2 DNA with Sequenase 2.0, exonuclease *Exo*I and T4 DNA-ligase under conditions adequate to ligate sticky ends but not blunt ends (for details see the text). Lane 1, *Pst*I digest of lambda DNA; lane 2, *Eco*RI digest of untreated, linear ϕ 393-A2 DNA; lanes 3–5, *Eco*RI digests of ϕ 393-A2 DNA after treatment with ligase (lane 3), Sequenase 2.0 followed by ligase (lane 4), and nuclease *Exo*I followed by ligase (lane 5). Arrowheads mark the fragments carrying the cohesive ends, and a larger arrow marks the fusion band obtained by ligation of the former fragments. Numbers indicate the fragment sizes (in kbp). Note that the cohesive-end-carrying fragment of 1.1 kbp co-migrates with another fragment of similar length.

with Sequenase 2.0 followed by limited ligation and digestion with *Eco*RI resulted in a restriction pattern (Fig. 3, lane 4) similar to that of ligated, circular control DNA (Fig. 3, lane 3), which is to be expected if the cohesive ends were 3'-protruding, but not if they were 5'-protruding (in this case Sequenase would fill-in the recessed strand, generating unligatable blunt ends, which should result in a restriction pattern similar to that of linear DNA). This was confirmed by treatment of ϕ 393-A2 DNA with *Exo*I, a specific 3' → 5' single-strand exonuclease, followed by limited ligation and restriction, which yielded a pattern (Fig. 3, lane 5) similar to that of linear DNA (Fig. 3, lane 2), indicating that the enzyme had indeed eliminated the 3'-protruding single-strand. Cohesive ends with 3'-protruding strands seem to be a common feature shared by phages of other Gram-positive bacteria, such as ϕ 105 of *Bacillus subtilis* (Ellis & Dean, 1985), ϕ LC3 of *Lactococcus lactis* subsp. *cremoris* (Lillehaug

Fig. 4. Restriction map of phage ϕ 393-A2 DNA. The 4.1 kbp *EcoRI* fragment which carries the *attP* site and possibly the lysin/holin genes is marked by an asterisk.

et al., 1991), and ϕ C31 of *Streptomyces* (Kobler *et al.*, 1991). In contrast, phages of Gram-negative bacteria such as lambda, HP1c1 and ϕ CTX usually bear 5'-protruding ends (Becker & Murialdo, 1990; Fitzmaurice *et al.*, 1984; Hayashi *et al.*, 1993).

Restriction map and cloning of ϕ 393-A2 genome

In order to develop a physical map of the DNA of ϕ 393-A2, the restriction sites for six enzymes (*Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Sal*I and *Xho*I) were localized in the phage genome (Fig. 4). Several complementary strategies were used to map the viral DNA: (i) identification of fragments containing the cohesive ends by comparing the restriction patterns of unligated and ligated DNA samples; (ii) elucidation of which particular fragments contained the 'left' or the 'right' cohesive ends by partial digestion with each enzyme, Southern transfer to a nylon membrane and hybridization with digoxigenin-labelled 'left-end' or 'right-end' *Bgl*II fragments (arbitrarily chosen); (iii) analysis of single and double digests; and (iv) cloning of discrete segments of phage DNA and mapping of their restriction sites.

Cloning of the ϕ 393-A2 genome in *E. coli* was preceded by ligation of the cohesive termini, and was first attempted with the multicopy vector pUC18, but only three out of the six *Bgl*II fragments could be cloned, possibly because the high gene dose of some phage products was lethal for the host cell. Hence, a second attempt was made by using the medium-copy-number plasmid pACYC184; in this way, 10 of the 11 *Eco*RI fragments were readily cloned. Still, a 4.1 kbp *Eco*RI fragment remained unclonable; this fragment was finally cloned in the phagemid vector Lambda ZAP. This fact suggests that the 4.1 kbp *Eco*RI fragment encodes a lethal product (holin?) whose expression is not fast enough to kill the cell before it is lysed by lambda ZAP, while it does so upon continuous expression from plasmid vectors.

Functional studies of ϕ 393-A2

The host-range of ϕ 393-A2 was determined by its ability to form plaques of lysis on 23 strains belonging to three *Lactobacillus* species, mainly *L. plantarum* since this is the predominant species found in the cheese from which the phage was isolated. Our results indicated that ϕ 393-A2 is species-specific, as is the case with all *Lactobacillus* phages

Fig. 5. One-step growth curve of bacteriophage ϕ 393-A2 on *L. casei* ATCC 393 at 30 °C. —■—, chloroform-treated cultures; —+—, untreated cultures.

described so far, since it could only form plaques on the two *L. casei* subsp. *casei* strains used, no plaques being produced on several strains of *L. casei* subsp. *rhamnosus*, *L. plantarum*, or *L. brevis*.

The temperature of incubation affects phage propagation in lactic acid bacteria (Keogh, 1973; Sozzi *et al.*, 1978), including mesophilic lactobacilli such as *L. plantarum* (J. L. Caso, C. G. de los Reyes-Gavilán, M. Herrero, A. Montilla, A. Rodríguez & J. E. Suárez, unpublished results); this turned out to be the case with ϕ 393-A2: when incubated at 30 °C it gave rise to clear plaques (1–2 mm diameter) on the two *L. casei* subsp. *casei* strains used, whereas at 37 °C it formed turbid, smaller plaques (0.5 mm) on *L. casei* ATCC 27092 and no plaques at all on *L. casei* ATCC 393. This effect was not due to adsorption failure at 37 °C, since up to 99.5% of the particles adsorbed in 5 min at either temperature (data not shown).

The one-step growth curve of ϕ 393-A2 when propagated on *L. casei* ATCC 393 is shown in Fig. 5. Eclipse and latent periods of 100 and 140 min, respectively, were observed, followed by a relatively short rise period of 30 min; the burst size was 180–200 p.f.u. per infected cell. Similar parameters have been described for other *L. casei* phages (de Klerk & Coetzee, 1963; Stetter *et al.*, 1978). The temperate nature of ϕ 393-A2 has been established on the basis of the following criteria. (i) Stable lysogenic

Fig. 6. Southern hybridization of digoxigenin-labelled ϕ 393-A2 DNA with *Eco*RI-digested samples of: total DNA from *L. casei* ATCC 27092 (lane 1); total DNA of a lysogenic derivative of the former strain carrying ϕ 393-A2 as a prophage (lane 2); and linear DNA of phage ϕ 393-A2 (lane 3). Numbers indicate the sizes of relevant bands (in kbp). Phage DNA bands of 1.1 and 2.3 kbp carry the cohesive ends (see also Fig. 3), and hence give rise to a single fusion band of 3.4 kbp in the integrated prophage DNA. The 4.1 kbp band is missing in the prophage DNA and therefore it probably contains the *attP* site, whereas a new phage-host fusion band of about 15 kbp is visible in the lysogen DNA.

derivatives of *L. casei* ATCC 393 and of *L. casei* ATCC 27092 were obtained. The lysogens released phage particles either spontaneously or upon induction with mitomycin C and they were immune to superinfection with ϕ 393-A2 (results not shown). (ii) Southern hybridizations of total DNA of a lysogenic derivative of *L. casei* ATCC 27092 with digoxigenin-labelled ϕ 393-A2 DNA (Fig. 6) showed that the viral DNA was indeed integrated into the bacterial genome, and that the attachment site of the phage (*attP*) lies within a 4.1 kbp *Eco*RI fragment of its genome located at the middle of its physical map (Fig. 4), since this fragment was no longer detected in the DNA of the prophage. These results ruled out the possibility that the putative lysogens were simply in a phage-carrying state and confirmed that, at least in the case of *L. casei* ATCC 27092, a true integration process had occurred. It is also noticeable that the two fragments carrying the cohesive ends were substituted by their fusion product in the prophage DNA, which indicates that integration is preceded by circularization of the phage genome.

These results confirmed that ϕ 393-A2 is different from phages J-1 and PL-1, which behave as virulent on *L. casei*

ATCC 27092, although at least PL-1 is temperate on *L. casei* ATCC 334 (for a review see Séchaud *et al.*, 1988).

The fact that the 4.1 kbp *Eco*RI fragment contains the *attP* site of ϕ 393-A2, together with the already mentioned difficulty of cloning it into *E. coli* plasmid vectors, suggests that this genome region has an organization similar to that of the *Lactobacillus delbrueckii* phage mv4, in which the lysin/holin gene determinants (*lysA* and *lysB*) lie only 2 kbp apart from the *attP* site (Dupont *et al.*, 1994).

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