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Characterization of a virulent *Lactobacillus sake* phage PWH2

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Abstract. A virulent bacteriophage PWH2 was isolated from fermented sausage. Out of 14 strains of *Lactobacillus sake* and 5 strains of *L. curvatus* tested as potential hosts, only *L. sake* Ls2 was sensitive. The plaques had a diameter of 0.5–1 mm. One-step growth kinetics of bacteriophage PWH2 revealed the following characteristics: a latent period of 1.5 h, a rise period of 1 h and a burst size of 110 (±10) phages per cell. From electron micrographs it was deduced that bacteriophage PWH2 has an icosahedral head, a long non-contractile tail and a complex base plate. The genome is linear and 35 kbp in length. The structural proteins consist of three major and two minor proteins. After an infection of *L. sake* Ls2, isolates were obtained that were resistant to PWH2. By treatment with mitomycin C, isolate R4a was found to be lysogenic. DNA/DNA hybridisation proved homology of phage PWH2 with the chromosomal DNA of strain R4a.

Introduction

The application of starter cultures for production of fermented sausages has become a well-accepted tool for controlling the fermentation process. Since sausage producers use the same starter preparations over a long period, the potential risk arises of building up a bacteriophage population in the environment of the production area. On the other hand, no direct proof has ever been shown for a detrimental role of phage attack in sausage fermentations.

Strains of *Lactobacillus sake* are common constituents of starter cultures for sausage fermentation (Hammes and Knauf 1992). These strains, in addition to strains of *L. curvatus*, are characterized as being especially competitive in the fermentation process. Their application as starter organisms ensures the dominance of the starter during the whole ripening process (Hammes et al. 1990). To create optimal starter cultures composed of lactobacilli, it is necessary to understand the phage-host interactions involved in these cultures. In addition, this knowledge might become a useful natural tool for genetic analysis and for the development of in-vivo gene transfer systems. Therefore, we studied the presence of phages of *L. sake* in fermented sausages and isolated a virulent bacteriophage PWH2 for *L. sake* Ls2 (Heidel and Hammes 1990). In this communication the bacteriophage is characterized together with a phage-resistant lysogenic mutant R4a derived from the host strain *L. sake* Ls2.

Materials and methods

Bacterial strains. The host strain *L. sake* Ls2 and all other strains of *L. sake* and *L. curvatus* tested for determination of the host range were isolates from fermented sausages.

Media. Propagation of lactobacilli was carried out at 30° C in MRS medium (de Man et al. 1960). Solid media contained 1.6% agar. The top layer for investigation of plaques was composed of 1.4 times concentrated MRS medium containing 0.8% of agar.

Bacteriophage titering. Plaque-forming units (PFU/ml) were determined as follows: a 1-ml phage-containing sample and 0.04 ml $CaCl_2$ (1 M) was added to a 0.1-ml aliquot of a growing *L. sake* Ls2 culture drawn at an optical density at 578 nm (OD_{578}) of 0.4. The suspension was incubated for 15 min at ambient temperature, added to 2.9 ml soft agar and poured onto an MRS agar plate. The plates were incubated at 30° C in an atmosphere consisting of 10% CO_2 and 90% N_2. Plaques were counted after 12 h.

Preparative phage isolation. The concentration and purification of the phage was carried out with polyethylene glycol (PEG) 6000 and 1 M NaCl according to Yamamoto et al. (1970) with two modifications: phages were concentrated by a two-step precipitation with 5% and 10% PEG 6000 and the caesium chloride stepgradient was overlayed with 20% sucrose (Maniatis et al. 1982).

Phage induction by mitomycin C (MC). Induction was carried out with minor modifications according to the method described by

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Cluzel et al. (1987). To a culture of exponentially growing R4a cells 1 μg/ml of MC was added, except to the control. The growth of the cultures was measured by determining the OD_{578}.

Phage adsorption. An overnight culture of *L. sake* was prepared and an aliquot of 4 ml was added to a mixture of 0.16 ml $CaCl_2$ solution (1 M) and 4 ml phage-containing solution (10^5 PFU/ml). This mixture was incubated at ambient temperature and after 5, 10, 15, 20, 30, 60 min aliquots of 0.2 ml were diluted in 3.8 ml ice cold MRS broth. The samples were subjected to centrifugation at 8000 *g* for 10 min and the supernatant was tested for unadsorbed phages. Adsorption was expressed as the decrease in phage titre as compared with the control (phage only).

One-step growth. Strain Ls2 was grown in MRS broth to an OD_{578} of 0.1 (early exponential growth). To 1 ml of this culture, 0.02 ml $CaCl_2$ (1 M) and 0.1 ml phage-containing solution (6×10^8 PFU/ml) were added, mixed and incubated at 30° C for 10 min. The mixture was centrifuged (8000 *g*, 10 min), the supernatant containing unabsorbed phages was discarded and the precipitate was suspended in 10 ml prewarmed MRS. Serial dilutions were made, and samples of the 10^{-3} to 10^{-6} dilutions were titred at 15-min intervals.

Host range. The host range was determined by agar spot-tests: 0.5 ml of 12 h cultures of *L. sake* or *L. curvatus* were added to 3.5 ml MRS soft agar and poured onto an MRS agar plate. After 1 h, 0.1 ml phage solution (10^9 PFU/ml) was dropped onto the soft agar. The plates were incubated at 30° C in an atmosphere of 10% CO_2 and 90% N_2.

Isolation of phage DNA and restriction analyses. The procedure of Maniatis et al. (1982) was used for the extraction of the DNA. The DNA was digested with restriction enzymes (Boehringer, Mannheim, Germany) according to the supplier's recommendations. Fragments were separated on a 0.8% agarose gel in TBE buffer (Maniatis et al. 1982).

Electron microscopy. The phages were negatively stained with 2% uranyl acetate and examined in a Zeiss CEM 902 at an acceleration voltage of 80 kV.

Determination of phage genome size. The aqueous spreading technique was as described by Arendt et al. (1990).

DNA/DNA hybridisation. Restriction-enzyme-generated DNA fragments were blotted onto a nitrocellulose membrane (BA 85, 0.45 μm Schleicher and Schuell, Dassel, Germany) following the protocol of Southern (1975) and hybridized with digoxigenin-labelled phage DNA. The final stringent washing of the nitrocellulose membrane was performed in 0.1 SSC (NaCl 3 mM/l; Na-citrate $\times 2H_2O$ 0.3 mM/l; pH 7.0)/0.1% sodium dodecyl sulphate (SDS) at 68° C to allow hybridisation of sequences with 100% homology. The procedure was carried out according to the supplier's recommendations (Boehringer).

SDS-polyacrylamide gel electrophoresis (PAGE). The purified phage particles were dialysed against double distilled water and disintegrated by boiling for 10 min in an equal volume of sample buffer (Laemmli 1970). The samples were subjected to electrophoresis on a 12% (w/v) polyacrylamide gel at 200 V and stained with Coomassie blue (Merck, Darmstadt, Germany). Low Range Markers (Bio-Rad, München, Germany) were used as molecular mass standards.

Isolation of phage-resistant mutants. A cell culture of *L. sake* Ls2 was infected with PWH2. After cell lysis resistant cells were isolated. Their resistance was confirmed by exposure of MRS-grown cultures to phage PWH2 and by the overlay method according to bacteriophage titering. All resistant isolates were examined with the aid of the API-50 CH-test (BioMérieux, Nürtingen, Germany).

Results

Dependence of plaque formation on the growth phase

Occasionally phage PWH2 failed to form plaques on *L. sake* Ls2. However, reproducible results were obtained when cells from a defined growth phase were utilized. The highest efficiency of plating was observed by using growing cells from $OD_{578} = 0.4$, as is shown in Fig. 1.

Dependence of cell lysis on growth phase and on $CaCl_2$ concentration

As depicted in Fig. 2, cell lysis depends on the growth phase of *L. sake* Ls2 infected with PWH2 at a constant multiplicity of infection. Furthermore, lysis moderately depends on the $CaCl_2$ concentration and increases from 1×10^9 without $CaCl_2$ to 3×10^9 PFU/ml at a concentration of 20 mM/l. The best results were obtained with concentrations of 20–50 mM/l.

Morphology

An electron micrograph of phage PWH2 is shown in Fig. 3. The phage has an isometric head of 81 nm in diameter, a non-contractile tail 270 nm long and 18 nm wide and a complex base plate of 36 nm diameter.

Burst size and latent period

A typical example of a one-step growth curve is shown in Fig. 4. From this curve the following characteristics

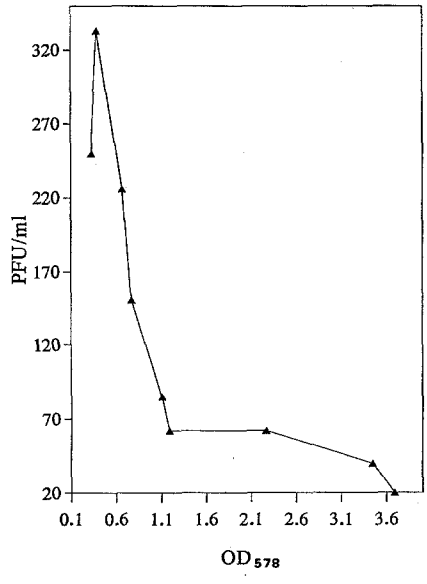

Fig. 1. The effect of growth phase of *Lactobacillus sake* Ls2 on the efficiency of plating: PFU, plaque-forming units; OD_{578}, optical density at 578 nm

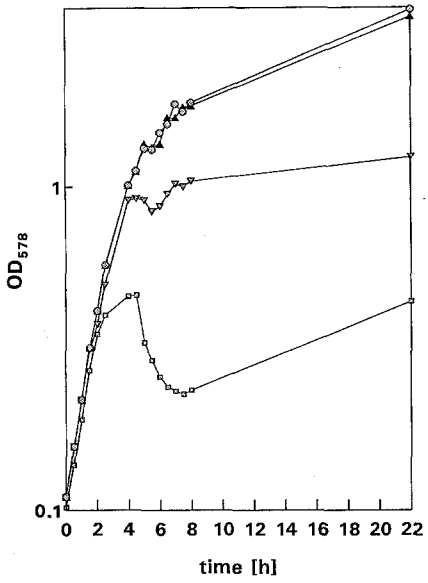

Fig. 2. Dependence of the efficiency of cell lysis on the growth phase of *L. sake* Ls2 infected with PWH2 at constant multiplicity of infection: ○, *L. sake* Ls2 without phage; □, addition of 10^8 PFU/ml at $OD_{578}=0.1$; ▽, addition of 5×10^8 PFU/ml at $OD_{578}=0.3$; ▲, addition of 1.7×10^9 PFU/ml at $OD_{578}=1$

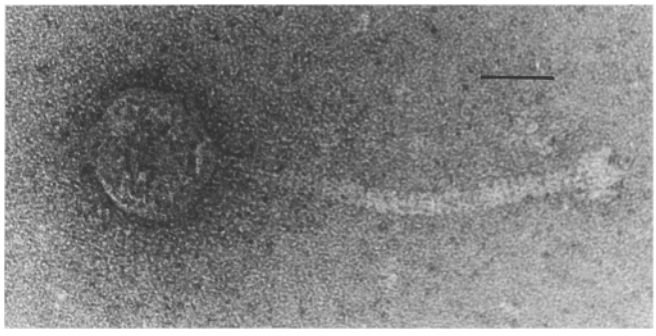

Fig. 3. Electron micrograph of bacteriophage PWH2; *bar*, 90 nm

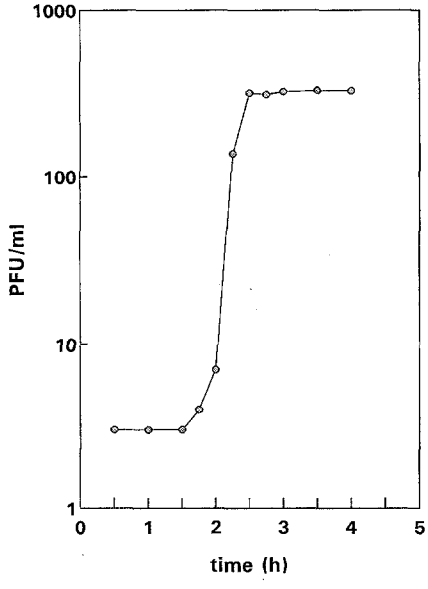

Fig. 4. One-step growth curve of bacteriophage PWH2

were derived: a latent period of 1.5 h, a rise period of 1 h and a burst size of a 110 (±10) phages per cell.

Protein composition

The structural proteins of phage PWH2 were analysed by SDS-PAGE (Fig. 5). They consist of three main proteins with molecular masses of 35, 32, 25 kDa and two minor proteins with 80 and 60 kDa.

Characteristics of phage genome

The phage genome was treated with several restriction enzymes (*Bam*HI, *Eco*RI, *Hin*dIII, *Not*I, *Sal*I, *Eco*RV, *Xba, Cla*I, *Pst*I, *Bcl*I, *Sph*I), but only *Cla*I, *Eco*RI, *Hin*dIII and *Sal*I digested the phage DNA completely (Fig. 6).

The size of the phage DNA was estimated to 35 kbp±2 kbp. The same size was furthermore determined by using the aqueous DNA spreading technique. Electron micrographs of the phage DNA showed that the genome of phage PWH2 is linear and has no cohesive ends (Neve, personal communication).

Phage adsorption

An adsorption experiment was carried out with *L. sake* Ls2 and phage PWH2. It could be shown that more than 90% of the phages had adsorbed to the host cells after 10 min.

Host range

The host spectrum of phage PWH2 was determined with a set of 14 strains of *L. sake* and five strains of *L. curvatus*. *L. sake* Ls2 was the only host for phage PWH2 and thus is highly specific.

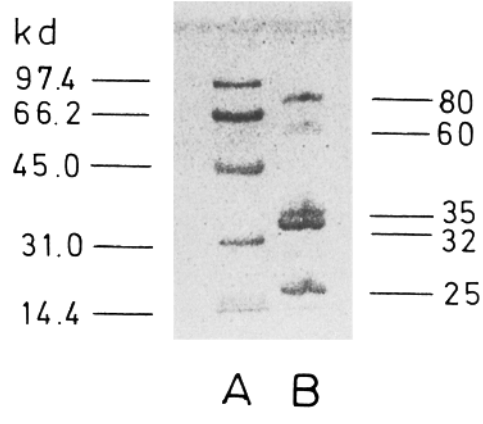

Fig. 5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of structural proteins of phage PWH2 (*lane B*) and molecular mass markers (*lane A*)

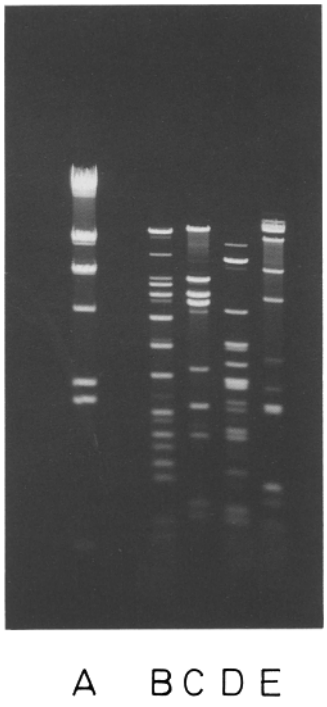

Fig. 6. Agarose gel electrophoresis of DNA fragments of phage PWH2 generated with different restriction enzymes: *lane A,* *Hin*dIII/lambda DNA marker; *lane B,* PWH2 DNA *Cla*I-generated; *lane C,* *Eco*RI-generated; *lane D,* *Hin*dIII-generated; *lane E,* *Sal*I-generated

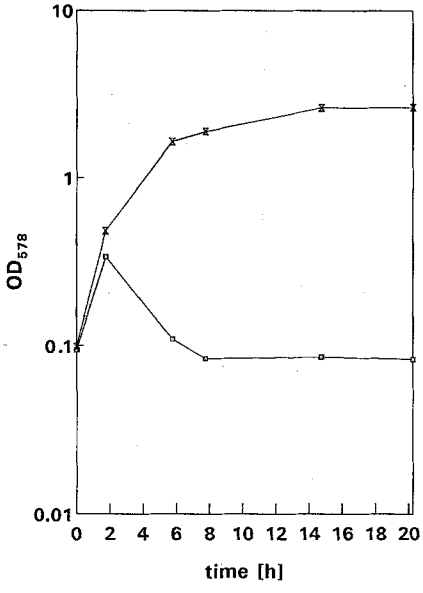

Fig. 7. Growth of *L. sake* R4a in the absence (⧖) or presence (□) of 1 μg/ml of mitomycin C

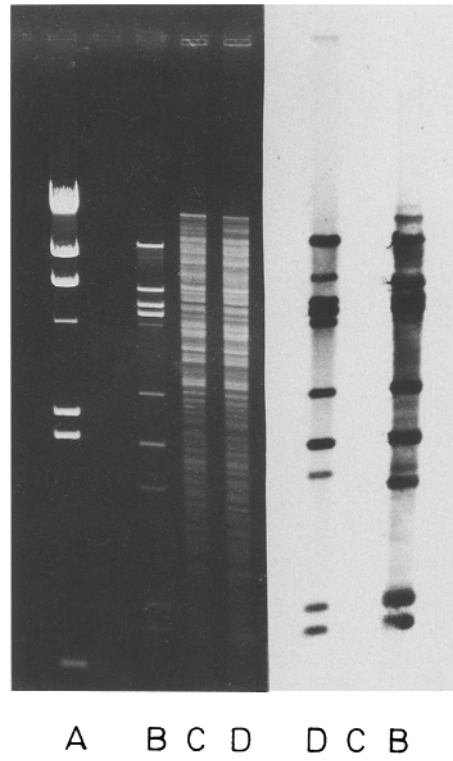

Fig. 8. Agarose gel electrophoresis of *Eco*RI-generated DNA fragments of DNA of phage PWH2 and bacterial DNA (*left*) and the corresponding Southern blot (*right*) hybridized with biotin-11-dUTP-labelled *Eco*RI-generated DNA of phage PWH2: *lanes A,* *Hin*dIII/lambda marker; *B,* phage PWH2; *C, L. sake* Ls2; *D, L. sake* R4a

Phage resistant mutant R4a

From each of three different batches of cell lysates, three resistant mutants of *L. sake* Ls2 were isolated and designated a, b, and c. In comparison to the original *L. sake* Ls2 strain, the patterns of sugar fermentation of the resistant strains show the following differences: R4a, b, and c were not able to ferment melibiose, R5a, b, and c did not ferment melibiose and gluconate and R6a, b, and c were unable to ferment gluconate. The resistant mutants R4a, R5a, and R6a were treated with 1 μg/ml of MC. Only strain R4a exhibited the characteristics of lysogeny (Fig. 7). The cell lysate was filter-sterilized and examined by electron microscopy. Complete phage particles were detected that were indistinguishable from PWH2. The wild-type strain of *L. sake* Ls2 showed cell lysis when treated with the sterile filtrate, but not when treated with mitomycin C. DNA/DNA hybridisation experiment performed with the DNA from strain R4a and a probe derived from PWH2 (Fig. 8) demonstrated that the resistant mutant R4a harbours the phage PWH2 as a prophage. No homology was present between the phage DNA and the chromosomal DNA of the wild-type strain of *L. sake* Ls2.

Discussion

Bacteriophage PWH2 is the first *L. sake* phage characterized in detail. PWH2 has an isometric head and a non-contractile tail and belongs therefore to Bradley's group B (Bradley 1967) or, according to Ackermann (1987), to the family Siphoviridae. This phage type is most common among phages of Lactobacilli (Hammes et al. 1991). An exception is phage fri of *L. plantarum*, which was isolated from a meat starter culture (Trevors et al. 1983) and belongs to Bradley's group A. It is remarkable that PWH2 has a small genome size of 35 kbp. The same size was also determined for phage ch2

(Chow et al. 1988). All other lactobacilli phages range between 40 and 73 kbp.

The efficiency of plating of PWH2 was strongly dependent on the growth phase of *L. sake* Ls2. Similar results were obtained by Chow et al. (1988). These authors also had to use host cells from the early exponential growth phase to titre phage ch2. Cell lysis of *L. sake* Ls2 infected with phage PWH2 occurs in the absence of calcium, but was three times more efficient when $CaCl_2$ was added at concentrations of 20–50 mM/l. This observation is consistent with results obtained by Sozzi (1972), who observed that lysis of lactic acid bacteria did not require calcium.

Strain R4a, a phage-resistant derivative of *L. sake* Ls2, exhibited superinfection immunity to phage PWH2 and was induceable by mitomycin C. Similar results were obtained in studies with *L. acidophilus* performed by Raya et al. (1989). In this organism exist also the classic lytic and lysogenic cycles of replication for the originally temperate phage ϕadh. A prophage-cured derivative of strain ADH, designated NCK102 was isolated. A relysogenized derivative (NCK103) of NCK102 was obtained, which exhibited mitomycin C induced lysis and superinfection immunity to phage ϕadh. Hybridisation experiments showed that the phage ϕadh genome was present in the chromosomes of strains ADH and NCK103, but absent in NCK102.

The release of phages from lysogenic starter organisms may result in severe interference with the fermentation process, as was revealed by Shimizu-Kadota et al. (1983). These authors discovered that the origin of the virulent phage ϕFSV for *L. casei* S-1 was the temperate phage ϕFSW. Our results obtained with strain R4a indicate that PWH2 can convert into a temperate phage. The resulting phage resistance due to superinfection immunity would not be desirable for practical purposes since it habours the danger of phage release, causing failure of fermentation processes performed either with mixed starter cultures or, after phage mutation, even with single strain cultures. If there actually exists a need for phage-resistant strains, resistant mutants are readily found with the potential for application in the starter preparation.

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