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Geographical diversity of *Streptococcus thermophilus* phages in Chinese yoghurt plants

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ABSTRACT

The aim of this study was to investigate the diversity of phages from Chinese yoghurt plants. Restriction analysis of six isolated *Streptococcus thermophilus* (St) phages showed that Φ101, Φ102 and Φ108 with different origins but with the same host strain St1 were different while phages Φ901, Φ903 and Φ907 with host strain St9 from the same origin were homologous. DNA packaging mechanism analysis proved the isolated phages to be *pac*-type except Φ108. The sensitive strain St1 could be infected by two different packaging mechanisms. Probing and sequencing of the variable region (VR2) fragment revealed that VR2 in aggressive phages (Φ101, Φ102 and Φ108) infecting host strain St1 was deleted. Phage Φ901 has an 845 bp amplicon and the similarity coefficient of the sequence was lower than 96% compared with other reported phages, which shows phage Φ901 did not belong to any known grouping.

1. Introduction

Streptococcus thermophilus is considered to be the second most important industrial species of lactic acid bacteria (LAB) after *Lactococcus lactis* (De Vuyst & Tsakalidou, 2008). Together with *Lactobacillus* spp., it is used as a starter culture in the manufacture of important fermented dairy products, including yoghurt and cooked cheeses (Delcour, Ferain, & Hols, 2000). However, *S. thermophilus* is susceptible to bacteriophage infection during fermentation, which results in retarded fermentation and inferior quality products (Mills et al., 2011; Sturino & Klaenhammer, 2006).

Bacteriophages infecting *S. thermophilus* have been the subject of on-going interest for their negative impact on milk fermentation and positive application in strain typing. In a previous study, we reported that a commercial direct vat set (DVS) yoghurt starter was composed of two different *S. thermophilus* strains by phage typing, which indicates that *S. thermophilus* phages could be used to trace and monitor the bacterial composition of starter cultures in yoghurt fermentation (Ma et al., 2013). As the simplest biological system, phage genomes can also be exploited to develop genetic tools which are suitable for numerous biotechnological applications for industrial purposes (Mali et al., 2013; Ventura & Brüssow, 2004).

Bruttin et al. (1997) pointed out that genetic heterogeneity among different *S. thermophilus* phages with the same host range might originate from geographical difference. The study of cosmopolitan distribution of phage genetic material also showed that some phage genotypes were geographically restricted and displayed biogeographical variability (Desnues et al., 2008). Up to now, at least 345 virulent and temperate *S. thermophilus* phages have been described, mostly in North America and Europe (Quiberoni, Moineau, Rousseau, Reinheimer, & Ackermann, 2010); however, phages of *S. thermophilus* isolated from Chinese yoghurt plants have been seldom investigated.

Yoghurt is the most important fermented dairy product in China, where 9 million tons of milk was transformed into yoghurt and yoghurt drink last year. Here we investigated the diversity of *S. thermophilus* phages from different geographical origins via morphological and molecular methods.

2. Materials and methods

2.1. Strains and culture conditions

S. thermophilus St1 was isolated and identified from a commercial DVS yoghurt starter A (DVS-A, Danisco Company, Copenhagen, Denmark), and *S. thermophilus* St9 was isolated and identified from DVS-B (Chr. Hansen Company, Hørsholm,

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Denmark). They were maintained as frozen stocks (−80 °C) and grown in M17 broth (Oxoid, Basingstoke, England) supplemented with 0.5% (w/v) lactose (LM17) at 42 °C.

2.2. Phage isolation and host range determination

Yoghurt samples suspected of phage contamination from different regions (Wuhan, Qingdao and Changsha, China) produced with DVS-A starter or from different batches in the same region (Beijing, China) produced with DVS-B starter were centrifuged (5000 × *g*, 10 min) and filtered through a 0.45-μm pore size filter (Millipore, Billerica, MA, USA). Next, 0.1 mL of the corresponding decimal dilutions of the filtrates were added to 0.1 mL of corresponding indicator cell culture in exponential growth phase, and mixed with 2.5 mL LM17-Ca (10 mM $CaCl_2$) soft agar at 44–48 °C. This was poured onto the prepared bottom plate and the double-layer plates were incubated at 42 °C for 18–24 h. Typical plaques were picked up and purified by three rounds of single-plaque isolation. Host ranges of phages were investigated by spot test and turbidity test (Ma et al., 2013; Svensson & Christiansson, 1991).

2.3. Phage particles purification and electron microscopy

Phage particles were purified by using two consecutive CsCl gradient ultracentrifugation steps according to Guglielmotti et al. (2009b) with slight modifications. Phage lysate was concentrated in 10% (w/v) polyethylene glycol (PEG, molecular weight: 6000) and 0.5 M NaCl. The PEG-concentrated phages were ultracentrifuged (200,000 × *g*, 3 h at 20 °C) using an Optima MAX-XP (Beckman, Fullerton, CA, USA). A second ultracentrifugation was performed using 600,000 × *g*, 18 h at 20 °C. The purified phages were then dialysed (Spectra/Por membranes, Rancho Dominguez, CA, USA) against phage buffer (10 mM Tris–HCl, pH 7.4) and stored at 4 °C before use (Lu, Breidt, Fleminga, Altermann, & Klaenhammer, 2003).

The purified phage particles in SM buffer (Sambrook & Russell, 2001) were deposited on copper grids with carbon-coated Formvar films, stained with 2% potassium phosphotungstate (pH 7.2), and examined in a H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerate voltage of 100 kV (Quiberoni, Tremblay, Ackermann, Moineau, & Reinheimer, 2006).

2.4. Phage DNA isolation and restriction analysis

DNA isolation was carried out according to Zinno, Janzen, Bennedsen, Ercolini, and Mauriello (2010). Restriction enzymes *Eco*RI, *Eco*RV, *Pst*I, *Xba*I, *Sna*BI, *Stu*I and *Dra*I (TaKaRa, Dalian, China) were used according to the manufacturer's instructions. The restriction patterns were captured and analysed using the Kodak Gel Logic 200 Imaging System (Kodak, Rochester, NY, USA). Similarity among digitised profiles was calculated using the Pearson's correlation coefficient, and dendrogram clustering was based on unweighted pair-group method with arithmetic averages (UPGMA) according to Mora et al. (2002).

2.5. DNA packaging mechanism determination

Determination of DNA packaging mechanism was carried out according to the multiplex PCR method (Quiberoni et al., 2006). PCR reactions were performed in a total volume of 20 μL with 200 μg mL^{-1} of template phage DNA, 0.5 μM primer (Table 1), 2 μL 10 × EasyTaq DNA polymerase buffer, 0.4 μL dNTPs (2.5 mM), 0.2 μL EasyTaq DNA polymerase (5 U μL^{-1}). The amplification conditions were set as follows: 5 min at 95 °C, followed by 35 cycles (45 s at 95 °C, 45 s at 53 °C, 1 min at 72 °C), and a final step of 10 min at 72 °C.

Table 1
List of primers used for packaging mechanism determination.

Primer	Sequence (5′ → 3′)	Tm (°C)	PCR product length (bp)
cos-FOR	GGTTCACGTGTTTATGAAAAATGG	58	170
cos-REV	AGCAGAATCAGCAAGCAAGCTGTT		
pac-FOR	GAAGCTATGCGTATGCAAGT	58	427
pac-REV	TTAGGGATAAGAGTCAAGTG		

2.6. Phage structural protein analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Sambrook and Russell (2001). Purified phage particles were suspended in SM buffer, and boiled for 3 min with 5 × SDS-PAGE loading buffer (250 mM Tris–HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, pH 6.8, 50% glycerol, 0.5% β-mercaptoethanol). Proteins were separated using an SDS-polyacrylamide gel (12%) with a Mini-PROTEAN II system (Bio-Rad, Hercules, CA, USA). Electrophoresis was at 100 V until samples had run through the stacking gel, then the voltage was increased to 150 V until the tracking dye reached the bottom of the gel. Proteins were stained with Coomassie brilliant blue R-250.

2.7. Amplification and sequencing of the antireceptor gene variable region

The amplification of the antireceptor gene variable region (VR2) was performed as suggested by Binetti, del Río, Martín, and Álvarez (2005). The primers, HOST1 (5′ GAATGATACTGCTGGCAGTATTTCG GTTGG 3′) and HOST5 (5′ CAGTCATGTAGCTATCGATGAAATTCCAACG 3′) were used at the concentration of 0.4 mM. Amplification conditions were set as follows: 5 min at 95 °C, followed by 35 cycles (30 s at 95 °C, 30 s at 50 °C, 1 min at 72 °C), and a final step of 10 min at 72 °C. PCR products were purified using AxyPrep DNA Gel Extraction Kit (Axygen, San Francisco, CA, USA), cloned into pMD 18-T Vector and determined at the DNA Sequencing Service of Life Technologies Corporation, Shanghai, China. Sequence data were assembled and compared with DNAMAN tools (4.03 Lynnon BioSoft, Quebec, Canada); a phylogenetic tree was constructed from the alignment using the MEGA software (http://www.megasoftware.net/) and the UPGMA method (Mora et al., 2002).

3. Results

3.1. Isolation, properties and host range of S. thermophilus phages

Six yoghurt samples suspected of phage contamination were collected from four dairy product plants, including Wuhan, Qingdao, Changsha and Beijing. According to the spot test and turbidity test, all phages were confirmed to be virulent.

Table 2
Sources and properties of *S. thermophilus* phages.

Phage	Starter supplier	Source	Diameter of phage plaque (mm)	Dimensions[a] (nm × nm)	Nature[b] (V/T)	Phage titre[c] (pfu mL^{-1})
Φ101	A	Wuhan	1.00	41.0 × 188	V	1.2×10^9
Φ102	A	Qingdao	1.10	42.2 × 228	V	1.7×10^9
Φ108	A	Changsha	1.80	42.3 × 186	V	7.2×10^8
Φ901	B	Beijing	0.50	55.1 × 211	V	2.0×10^9
Φ903	B	Beijing	0.60	53.3 × 213	V	8.2×10^8
Φ907	B	Beijing	0.50	56.3 × 213	V	9.7×10^8

[a] Capsid diameter × tail length.
[b] V-virulent phage or T-temperate phage.
[c] Values are the mean of three independent experiments.