THE PARTIAL CHARACTERIZATION OF ENTEROCoccus faecalis MSF1 BACTERIOPHAGE FROM RAW MILK

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ABSTRACT
The E. faecalis MSF1 bacteriophage was isolated from the raw milk and partially characterized. The bacteriophage produces small turbid plaques on the 473 host strain of E. faecalis. The genome of bacteriophage is formed by double stranded DNA in size between 40 to 50 kbp. Despite MSF bacteriophage is the first E. faecalis bacteriophage isolated from the milk it is highly related to E. faecalis bacteriophages and prophages isolated from other environments. MSF1 bacteriophage lytic module was characterized which is composed of family 4 holin and a muramidase. Site specific DNA methyltransferase was detected in the genome of MSF1 bacteriophage indicating that site specific DNA modification is used by bacteriophage to protect it from host encoded restriction and modification systems.

Keywords: Enterococcus faecalis, phage, milk, genome characterization

INTRODUCTION
Bacteriophages (phages) are an abundant and diverse life form, universally present in the environment. Phages are viruses that infect bacteria. Their life cycle involves adsorption, production of progeny phage, and the release of progeny phage by bacteriolysis. Phages are associated with nearly all known bacterial taxa including the Enterococcus spp. Enterococci are lactic acid bacteria that are important in environmental, food and clinical microbiology. The natural habitat of enterococci is mammalian intestinal tract; however, these bacteria may play an important role in dairy industry. It is well known that enterococci are a relevant starter cultures for fermented cheeses produced from raw or pasteurized milk. Although enterococci are considered to be an important opportunistic nosocomial pathogen, there are no prescribed limits for Enterococcus spp. in foods or cheeses. However, due to their presence in human faeces, enterococci are not generally recognized as safe (Franz et al., 1999). Enterococcal bacteriophages with very effective bactericidal activity could be potentially used to control enterococcal infections in humans or animals; however in dairy industry the bacteriophage infections result in major losses in a production of fermented dairy products. Enterococcal phages have been isolated from many sources including sewage, fresh water, human faeces, human saliva and piggery effluent, milk for a variety of purposes (Bachrach et al., 2003; Uchiyama et al., 2008a, b; Letkiwicz et al., 2009; Mazaheri et al., 2010; Santiago-Rodriguez et al., 2010; Mc Lean, 2011). E. faecalis is also recognized as an opportunistic pathogen and is the most commonly clinically isolated Enterococcus sp. Several different bacteriophages have been isolated and completely characterized from clinical isolates of Enterococcus spp.; E. faecalis phage phi EF24C (Uchiyama et al., 2008a; Uchiyama et al., 2008b; Uchiyama et al., 2011), lytic bacteriophage EFAP-1 of E. faecalis (Son et al., 2010), and bacteriophage phi Ef11 (Stevens et al., 2011). The determination of morphology and complete genome sequences of eight induced bacteriophages purified from clinical isolates of E. faecalis was described by Yasmín et al. (2010).

In this study the Enterococcus faecalis MSF1 phage was isolated from the raw milk and its genome was partially characterized by molecular methods.

MATERIAL AND METHODS
Isolation of phage
Raw milk samples were obtained from Dairy Research Institute in Žilina and phages were isolated using E. faecalis 473 (Nigutova et al., 2008) as a host strain. Host strain was routinely cultivated on Todd-Hewitt agar or liquid media (Becton-Dickinson, Heidelberg, Germany). For isolation of bacteriophages double agar layer method was used (Lillehaug, 1997). One hundred microlitres of raw milk was mixed with 50 μl of actively growing host cells (of about 107 colony forming units per ml) and incubated for 15 min. at room temperature. After incubation 3 ml of molten 0.6% Todd-Hewitt agar medium at 46°C were added and the resulting suspension was poured over 1% Todd-Hewitt agar to form a thin layer which immobilizes the bacteria. The plates were cultivated at 37°C and the presence of plaques, as a visible, circular areas of clearing in the confluent bacterial growth, was scored after 24 hours incubation. A single plaque was picked from soft agar, resuspended in SM medium (Maniatis et al., 1982) and repeated rounds of double agar layer infections were used to obtain pure phage lysates.

DNA isolation and analysis
DNA of MSF bacteriophage was isolated from cleared lysates using protease treatment followed by ethanol precipitation (Maniatis et al., 1982). Twenty ml of cleared lysate (of about 109 plaque forming unit per ml) were treated with RNase A (10ng per ml) and DNase I (0.25 SU per ml) for 1 hour at 37°C. After incubation bacteriophage particles were collected by ultracentrifugation at 10000 g and phages were resuspended in SM medium (Maniatis et al., 1982). SDS was added to the phage suspension to final concentration 0.5% and the suspension was then treated with Proteinase K (50 μg/ml) for 1 hour at 56°C. The suspension was cooled to room temperature an equal volume of chloroform was added and suspension was inverted several times. After centrifugation (3000 g for 5 min at room temperature) the aqueous phase was transferred to a new tube and chloroform extraction was repeated. Purified DNA was then precipitated using ice cold ethanol (Maniatis et al., 1982). DNA was collected by centrifugation (12000 g for 10 min at 4°C), DNA pellet was resuspended in TE buffer (pH 7.6), and DNA was analyzed using agarose gel electrophoresis.

Restriction analysis of phage DNA was carried out for 1.5 h at 37°C in a total volume of 20 μl containing 2 U of restriction enzymes and 10 μl of bacteriophage DNA and restriction buffer. The resulting restriction fragments were separated by
gel electrophoresis at 7 V/cm for 2 h in a 1.5% agarose containing 0.1 μg of ethidium bromide per ml recorded under UV light using digital Gel Logic 212 PRO Imaging System (Carestream Health, Inc., Rochester). The sizes of DNA fragments were estimated by comparison with a 1 kb or 100 bp DNA ladder (Liogenics).

**DNA cloning and sequence analysis**

Shotgun cloning was used to characterization of MSF1 bacteriophage genome. Random HindIII fragments of MSF1 bacteriophage DNA were ligated into pUC118/HindIII/BAP vector (Takara, Japan) and used to transform *Escherichia coli* ER 2267 competent cells (NEW England Biolabs, Beverly). Transformed cells were spreaded on LB plates supplemented with ampicillin (100 μg·ml⁻¹) and cultivated at 37°C overnight. Individual transformants were analyzed for plasmid contents using alkaline lysis (Maniatis et al., 1982). Plasmid DNA isolated from transformants was cleaved with HindIII and BamHI restriction endonucleases. Recombinant plasmids containing inserts with size over 500 bp were sequenced using Sanger di-deoxy termination method with vector specific primers at GATC Biotech AG (Cologne, Germany). The sequences obtained were analyzed using BLAST algorithm (Altschul et al., 1997) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences were deposited to the GenBank database under accession numbers JK817847-JK817854.

**RESULTS AND DISCUSSION**

Bacteriophages have long been negatively associated with the dairy industry. Of the approximately 500 million tons of milk produced globally each year, an estimated one third is processed into fermented products (Brussow, 2001). The presence of dairy bacteriophages in milk leads to interruption of the fermentation of lactose by starter cultures. This can cause delays in production and alteration of product quality. Dairy bacteriophages are among the best characterized of the phages due to their potential to cause huge economic losses (Brussow, 2001). Bacteriophage-induced bacterial cell lysis leads to failed or slow fermentation, decrease in acid production and reduction of dairy product quality (Lawrence, 1978). Bacteria used in dairy industry comprise different groups of lactic acid bacteria, such as *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, and *Enterococcus* (Klaenhammer et al., 2005). Multiple bacteriophages were already identified from bacteria used in dairy industry, but most of them are bacteriophages infecting lacticocci and lactobacilli (Mc Grath et al., 2007). While there are several *E. faecalis* bacteriophages characterized (Uchilayma et al., 2008a; Yassin et al., 2010; Son et al., 2010; Stevens et al., 2011) neither of them was isolated from milk or dairy products.

Several bacteriophages infecting *Enterococcus faecalis* strain 47/3 were obtained in our laboratory from raw milk samples with frequency less than 10 pfu (plaque forming unit per millilitre of milk). The *E. faecalis* strain 47/3 strain was already shown to be a suitable indicator for isolation and enumeration of *E. faecalis* bacteriophages (Nigutova et al., 2008). Picking up the plaques and re-inoculating them into the *E. faecalis* 47/3 culture at the mid-exponential phase led to the lysis of the bacteria. The isolated bacteriophage was named MSF1 and further studied. MSF1 bacteriophage was found to produce small (less than 1 millilitre) turbid plaques on the lawn of host strains (data not shown).

Bacteriophage MSF1 genomic DNA was isolated from the cleared lysates and analyzed. MSF1 bacteriophage contains double stranded linear DNA with size between 40 and 50 kbp (Fig. 1). Surprisingly, bacteriophage MSF1 genomic DNA was resistant to the cleavage by HaeIII restriction endonuclease, while it was cleaved by other restriction endonucleases indicating the presence of site specific modification within GGCC sequences which are recognized by HaeIII restriction endonuclease. Several bacteriophages were found to possess site specific modification of GGCC sequences (Tran-Betcke et al., 1986) but for the first time this modification was detected in *E. faecalis* bacteriophage.
Sequence comparisons indicate that MSF1 bacteriophage is highly related to already known *E. faecalis* bacteriophages and prophages isolated from other environments. In dairy industry phages can originate from a variety of sources and identification of the potential sources of phage contamination is very important for protecting fermentation processes from failures. The most probable source of infecting phages is directly raw milk. Our data however indicate that very similar bacteriophages could be found in other environments as well. The better understanding of both phage and host genetics and biology is essential in order to improve the quality and effectiveness of dairy fermentations.

**CONCLUSION**

The *E. faecalis* MSF1 bacteriophage was isolated from the raw milk and partially characterized. The bacteriophage produces small turbid plaques on the 47/3 host strain of *E. faecalis*. The genome of bacteriophage is formed by double stranded DNA. For the first time site specific modification of bacteriophage DNA was observed for *E. faecalis* bacteriophage. Despite MSF1 bacteriophage is the first *E. faecalis* bacteriophage isolated from the milk it is highly related to *E. faecalis* bacteriophages isolated from other environments. MSF1 bacteriophage lytic module was characterised which is composed of holin and a muramidase. Site specific DNA methyltransferase was detected in the genome of MSF1 bacteriophage indicating that site specific DNA modification is used by bacteriophage to protect it from host encoded restriction and modification systems.

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