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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 47/3 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**; **Letkiewicz *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 (**Uchiayma *et al.*, 2008a**; **Uchiayma *et al.*, 2008b**; **Uchiayma *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 **Yasmin 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* 47/3 (**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 10^7 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 46ºC 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 37ºC 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 proteinase treatment followed by ethanol precipitation (**Maniatis *et al.*, 1982**). Twenty ml of cleared lysate (of about 10^{10} plaque forming unit per ml) were treated with RNAse A (10mg per ml) and DNAse I (0.25 SU per ml) for 1 hour at 37ºC. After incubation bacteriophage particles were collected by ultracentrifugation at 100000 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 56ºC. 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 4ºC), 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 (Invitrogen).

DNA cloning and sequence analysis

Shotgun cloning was used to characterization of MSF1 bacteriophage genome. Random HincII fragments of MSF1 bacteriophage DNA were ligated into pUC118/HincII/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 $\mu g.ml^{-1}$) 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 obtained were deposited to the GenBank database under accession numbers KJ817847-KJ817854.

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 lactococci and lactobacilli (**Mc Grath *et al.*, 2007**). While there are several *E. faecalis* bacteriophages characterized (**Uchiayma *et al.*, 2008a**; **Yasmin *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 millimetre) 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 endonuclease 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.

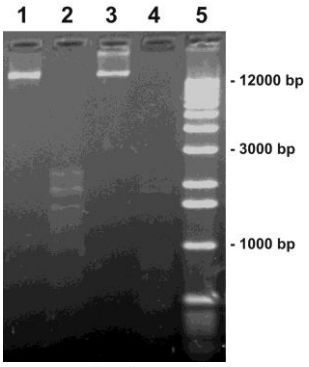

Figure 1 Restriction analysis *of E. faecalis* bacteriophage MSF1 genomic DNA. Lane 1 – uncleaved DNA, lane 2 – DNA cleaved by AluI restriction endonuclease, lane 3 – DNA cleaved by HaeIII restriction endonuclease, lane 4 – DNA cleaved by HincII restriction endonuclease. Lane 5 – standard of molecular weight, the size of selected bands is shown in base pairs.

The genome of MSF1 bacteriophage was analyzed using shotgun cloning. Random HincII fragments of MSF1 bacteriophage DNA were cloned and characterized. The gene library of MSF1 bacteriophage was prepared in *E. coli* host and selected recombinants with inserts over 500 bp were further characterized by sequence analysis. Sequence comparison (Table 1) indicated that genome of MSF1 bacteriophage is highly related to the already known bacteriophages or prophages of *E. faecalis*; even MSF1 bacteriophage came from other environment than other *E. faecalis* bacteriophages.

The sequence analysis indicated that all cloned fragments contain ORFs highly similar to the *E. faecalis* bacteriophage proteins. For all putative ORFs similarity values over 96% were observed. Fragments of genes encoding phage portal protein, phage head morphogenesis, phage tail tape measure protein, phage major capsid protein, and phage recombinase of RecT family were detected (Table 1).

Nearly complete lytic module of bacteriophage MSF1 was identified in the MS16 clone. At the late bacteriophage infection phase, bacteriolysis is caused by two proteins called holin and endolysin that are encoded by phage genomes. It is thought that holins form pores to allow endolysin access to the peptidoglycan and that endolysin degrades the peptidoglycan layer. In bacteriophages at least 11 unrelated gene families which share the functional and structural characteristics of holins have been identified (**Young *et al.*, 1995**). Bacteriophage MSF1 holin belongs to the holin family 4, which is frequently detected among holins of *E. faecalis* bacteriophages, but in genomes of streptococcal bacteriophages as well (**Ma *et al.*, 2008**). Using Blast analysis identical sequences were detected in the genomes of several *E. faecalis* strains, indicating that MSF1 related bacteriophages are found as prophages. MSF1 bacteriophage enterolysin is bacterial cell wall endolysin which cleaves the glycosidic N-acetylmuramoyl-(beta1,4)-N-acetylglucosamine bonds. Endolysins with similar enzymatic activity were detected in the genomes of *E. faecalis* bacteriophages phiEF11 (**Stevens *et al.*, 2011**) or phiFL3A (**Yasmin *et al.*, 2010**), and in the lactococcal bacteriophages as well (**Labrie *et al.*, 2008**).

M3 clone of MSF1 bacteriophage genome was found to encode either N-4 cytosine-specific or N-6 adenine-specific DNA methylase. These enzymes are usually parts of restriction and modification systems used by bacteria to protect them from bacteriophage infections, but several bacteriophages, e.g. *Bacillus subtilis* bacteriophages (**Tran-Betcke *et al.*, 1986**) were found to encode for these enzymes. Similar enzymes were detected in lactobacilli (**Durmaz *et al.*, 2008**) or in *Lactococcus lactis* phage Tuc2009 (GenBank accession number AF109874). Surprisingly, deduced MSF1 methyltransferase sequence showed high similarity (over 78 %) to methyltransferases from gram-negative bacterium *Haemophilus aegyptius* (GenPept accession number WP_006995077). HaeIII restriction endonuclease recognizing GGCC sequence was isolated from this bacterium (**Mann *et al.*, 1977**) and resistance of MSF1 genomic DNA to HaeIII endonuclease cleavage (Fig. 1, lane 3) could indicate that MSF1 encoded methyltransferase recognize the same sequence.

Table 1 BlastX analysis of cloned MSF1 genome fragments

clone	GenBank accession number	Size (bp)	BlastX best hit	
			Best hit [accession number]	Similarity
M1	KJ817847	741	*Enterococcus faecalis* SPP1 family phage portal protein [WP_010816911]	99 %
M3	KJ817848	908	*Enterococcus faecalis* TX4248 DNA (cytosine-N6_N4)-methyltransferase [WP_002363348]	100 %
M4	KJ817849	932	*Enterococcus faecalis* SPP1 gp7 family phage head morphogenesis protein [WP_010816896]	99 %
M6	KJ817850	565	*Enterococcus faecalis* phage tail tape measure protein, TP901 family, core region [WP_010826450]	100 %
M7	KJ817851	695	*Enterococcus faecalis* recombinase, phage RecT family [WP_016632349]	99 %
MS6	KJ817852	959	*Enterococcus faecalis* HK97 family phage major capsid protein [WP_010828493]	99 %
MS8	KJ817853	969	ORF1 - *Enterococcus faecalis* Cro/Cl family transcriptional regulator [WP_002395799]	98 %
			ORF2 - *Enterococcus faecalis* putative toxin-antitoxin system, toxin component [WP_016627610]	96 %
MS16	KJ817854	1081	ORF1 - *Enterococcus faecalis* phage lysis holin [WP_002378446]	99 %
			ORF2 - *Enterococcus faecalis* glycosyl hydrolase family 25, endolysin [WP_016633086]	99 %

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 MSF 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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