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Detection and characterization of bacteriophages attacking dairy *Streptococcus thermophilus* starter cultures

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Sixty (60) strains of *Streptococcus thermophilus* isolated from dairy traditional Egyption yoghurt samples were characterized. The yoghurt samples were collected from the local markets in Egypt. The results showed that few phages can attack *S. thermophilus* strains isolated from traditional Egyptian yoghurt (Zabady) samples, only three temperate phages were isolated. The isolated phages attacking samples were induced with mitomycin C (0.05 or 0.1 µg/ml, final concentration) and characterized by SDS-polyacrylamide gel electrophoresis, PCR and electron microscope. The characterization of phages showed that the bacteriophages belonged to the family *Siphoviridae* and they had an isometric head and long non contractile tail. SDS polyacrylamide gels and PCR amplification showed that all isolated phages belongs to *pac* phage. The PCR described in this work can detect and differentiate *S. thermophilus* phages in a short time, it is very sensitive and reveals a high limit of detection.

Key words: *Streptococcus thermophilus*, bacteriophage, SDS-polyacrylamide gel electrophoresis, PCR amplification.

INTRODUCTION

Streptococcus thermophilus is one of the most economically important lactic acid bacteria (LAB), it is a component of dairy starter cultures and used in a large amount for the manufacture of dairy products (annual market of about 40 billion USD). It is usually used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) or *Lactobacillus helveticus* for the manufacture of yoghurt and other fermented milk products like cooked cheeses (Mercanti et al., 2011; Canchaya et al., 2003; Ventura et al., 2002). *S. thermophilus* is also used alone or in combination with lactobacilli for the production of mozzarella and cheddar cheeses (Duplessis et al., 2006; Binetti et al., 2005; Hols et al., 2005). It is a Gram-positive microaerophilic, moderately thermophilic bacterium which possesses a relatively small genome of approximatley 1.8 Mb with a

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low G+C% (Roussel et al., 1997; O'Sullivan and Fitzgerald, 1998). Some insertion sequences (ISs) have been reported in its genome (Guedon et al., 1995; Bourgoin et al., 1996) and these ISs may contribute to the genetic instability in some of these strains (Roussel et al., 1997). It has been reported that many *S. thermophilus* are highly susceptible to phage infection but only 1 to 10% of them are lysogenic and contain intact temperate phages (Josephsen and Neve, 2004; Carminati and Giraffa, 1992; Neve et al., 2003). It is known that the majority of dairy phages are resistant to standard pasteurization procedures (Roussel et al., 1997). Later, other phages infecting different lactic acid species from various industerial environments were reported to exhibit extra ordinary thermal resistances (Atamer et al., 2011).

S. thermophilus phages (virulent or temperate) belong to *Siphoviridae* family and they are relatively homogenous group with the same morphology (B1 morphotype) (Ackermann, 2001). They have an isometric head (diameter, 45 to 60 nm) and a long noncontractile tail (length, 240 to 270 nm; thickness, 9 to 13 nm) (Brüssow, 2001). *S. thermophilus* phages have a linear double-stranded DNA genome ranging from 30 to 45 kb (Brüssow, 2001). According to the packaging mechanism of their double-stranded DNA and their structural proteins pattern on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), they are divided into two types, *pac*- (41, 25 and 13 kDa) and *cos*- (32 and 26 kDa) type (LeMarrec et al., 1997).

Bacteriophages attack is always a serious problem in industrial fermentation, especially in the dairy industry. It can lead to slow fermentation or even complete failure of starter with consequent loss of product leading to a high financial losses because a large dairy plant can processes more than 2 x 10^5 litres of milk per day, so phage attack could be more expensive (Sturino and Klaenhammer, 2007). However, for minimizing phage attack on LAB in dairy industry, some precautions could be followed like rotation of non-phage-related strains, using mixed starter culture, use of phage inhibiting media for culture propagation and sterile processing conditions but usually these are not enough to prevent phage infection (Binetti et al., 2005; Josphen and Neve, 2004). Using bacteriophage-resistant mutants derived from phage-sensitive strains or using genetically modified phage-resistant starter cultures harbour plasmids containing abi or R/M genes.

Due to the high economical losses that may result from a phage infection of starter cultures, a rapid and sensitive method is required for phage detection and identification in the dairy. In this work, a rapid and reliable multiplex-PCR method is described allowing the simultaneous detection of *S. thermophilus* phages and their differentiation into the two well known *pac*- and *cos*-type subgroups. Also, this work was designed to characterize the bacteriophages that attack *S. thermophilus* in Egyptian yoghurt (Zabady) that contaminate raw milk during milk collection in dairy farms or manufacture of yoghurt.

MATERIALS AND METHODS

Collection of yoghurt samples

Twenty yoghurt samples were collected from different sites in Egypt (Cairo, Assiut, and El Mansoura). Samples were stored at 4°C until used.

Isolation and identification of *S. thermophilus* from yoghurt samples

S. thermophilus was isolated from yoghurt samples by plating on LM17 agar plates at 40°C for 24 h (Krusch et al., 1987). The *Streptococcus* was examined and identified. Identifications of pure *S. thermophilus* isolates were made on the basis of their cultural, morphological and microscopic characteristics and by reference to Krusch et al. (1987). The bacterial identification was confirmed by API-test system (rapid ID 32 Strep, Biomerieux, Nuertingen, Germany) according to Freney et al. (1992). Five colonies from each yoghurt sample were used for bacteriophage induction and identification of lysogenic bacterial strains of *S. thermophilus*.

Induction and isolation of Bacteriophages

S. thermophilus bacteriophages were isolated by inducing their lysogenic strains using mitomycin C. Induction was performed on 0.5 L of LM17 broth which was inoculated with 1.5% overnight *S. thermophilus* culture and incubated at 40°C until OD_{620} 0.2 then another 0.5 L of LM17 broth containing 0.4 µg/ml Mitomycin C (final concentration 0.2 µg/ml) was added to the bacterial culture and incubated at 40°C until lysis occurred (Neve et al., 1998).

Bacteriophage concentration and purification

Phage concentration were performed according to Sambrook et al. (1989) with some modifications (Neve et al., 1998).

Multiplex colony PCR for detection of lysogenic *S. thermophilus* strains

A set of 60 artisanal *S. thermophilus* strains isolated from traditionally produced Egyptian yoghurt samples (by spontaneous fermentation of milk) were screened using colony-PCR (multiplex PCR) with pac- and cos-primers. To design primers for *pac*-type phages Tp-J34, O1205, Sfi11 and 2972 sequences were used and for *cis*-type phages, Sfi21, 2701, Sfi19 and DT1 were used. A set of sixty *S. thermophilus* strains, isolated from traditionally produced (using unidentified starter culture) Egyptian yoghurt (Zabady) samples was screened by colony-PCR (multiplex PCR) with primers for *cos*-phage YC-F/YC-R and primers YP-F/YP-R for *pac*-phage. All primers were designed with DS gene software and were manufactured by MWG-Biotech, Germany. The pac specific-primers were YP-F (GCT CGT CTT GAA GCT ATG C), YP-R (GAT AAG AGT CAA GTG ACC GTC) and the cos specific-primers were YC-F (GCT ATG CTT GAC GAT TCA GT) and YC-R (AGC AGA ATC AGC AAG CAA G).

Polymerase chain reaction (PCR)

Colony-PCR

Colony PCR was performed using 10X PCR-buffer (Fermentas). Three colonies were picked from agar plate into 1.5 ml centrifuge tube containing 100 µl 1X PCR-buffer to give a turbid solution using sterile toothpicks. The tubes were then placed in a thermoblock and heated at 95°C for 12 min with gentile shaking. Finally, the tubes were cooled, 2 µl were used for 50 µl PCR reaction volume and the rest was stored at -20°C for further use.

Polymerase chain reaction was performed as described previously by Mohamed et al. (2012). The amplified PCR products were analyzed using 1.5% agarose gel electrophoresis as reported by Sambrook et al. (1989). After adequate migration has occurred, DNA fragments were visualized on a ultraviolet transilluminator (Biometra, Germany) and photographed.

SDS-PAGE of the phage structural proteins

Sodium deodecyl sulfate polyacrylamide gel electrophoresis from bacteriophage structural proteins was performed as described by Fayard et al. (1993) with some modifications. The protein loading buffer Pack (Fermentas GMBH- Germany) was used for samples preparations. Bacteriophages particles obtained from Cesium Chloride step gradients were dialyzed against dialysis buffer (10 mM NaCl, 50 mM Tris-HCl PH 8, 10 mM $MgCl_2$) for 1 h. 30 µl of the extract were mixed with 8 µl of 5x protein loading buffer and 2 µl of 20x DTT, then heated at 95°C for 5 min. 25 µl of the mixture were loaded on 12% SDS gel. Electrophoresis was carried out at 10 mA until the samples reached resolving gel, then the electrophoresis was continued at 20 mA until the blue lane of the samples buffer was completely removed from the gel. The gel was stained by Coomassie Brilliant Blue R250 solution for overnight. The gel was de-stained until clear protein bands appeared.

Electron microscopy

For transmission electron microscopy, the detected bacteriophages were purified and concentrated using CsCl gradient centrifugation as previously descriped by Sambrook and Russell (2001). Purified phages were stained negatively with 2% (w/v) uranyl acetate on freshly prepared carbon films. Grids were analyzed in a Tecani 10 transmission electron microscope (FE1 Company, Eindhofen, Netherlands) at an acceleration voltage of 80 kV.

RESULTS AND DISCUSSION

Identification of *S. thermophilus*

In this study, sixty *S. thermophilus* strains were isolated from yoghurt samples and identified based on their cultural, morphological, microscopic characteristics and by using API-test system. Three lysogenic *S. thermophilus* strains harbouring inducible *pac*-type prophages were identified. Intact temperate phages were released from two of the three strains (strains YA1 and YA2) upon mitomycin C induction (Figure 1), while defective temperate phages (phage tails) were induced from strain YA3 (Figure 4).

Induction of phages with mitomycin C

S. thermophilus strains were grown in LM17 broth at 40°C and treated with different concentrations of mitomycin C (0.00, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 µg/ml) during the early logarithmic growth phase. Optimal induction and lysis of host cells due to the release of temperate phages were obtained either with 0.1 µg/ml mitomycin C in strains 1 and 3 or with 0.05 µg/ml mitomycin C in strains 2 (Figure 1).

Multiplex colony PCR for the detection of lysogenic *S. thermophilus* strains

A rapid and reliable multiplex-PCR method is described allowing the simultaneous detection of *S. thermophilus* phages and their differentiation into the two well known *pac*- and *cos*-type subgroups. Since *pac*- and *cos*-type phages are composed of different structural proteins, two sets of primers were designed from the internal highly conserved region of the major head protein gene of eight completely sequenced *S. thermophilus*.

The two primer sets could be used simultaneously in a multiplex PCR assay to detect and distinguish *S. thermophilus* phages, because the PCR-products differed in fragment size (432 bp product for *pac*-type phages versus 514 bp product of *cos*-type phages). The PCR results identify three new lysogenic S. thermophilus strains harbouring inducible pac-type prophages (Figure 2).

SDS-PAGE of the phage structural proteins

The reliability of the multiplex PCR protocol was validated and confirmed by SDS-PAGE analysis of the structural proteins of *pac*-type phages have three major bands and *cos*-type phages have two major bands as reported previously by LeMarrec et al. (1997). The structural proteins from the *cos*-type reference phage P53 was analysed by SDS-PAGE and compared with the structural protein profiles of the new isolates YA1, YA2 and YA3. It is shown in Figure 3 that the protein profile of the *cos*-type reference phage P53 has two main bands (32 and 26 kDa), while YA1, YA2 and YA3 phages revealed three main protein bands (41, 25 and 13 kDa). These data do also confirm the classification the new phages by multiplex PCR as *pac*-type phages.

Electron microscopic analysis of phages:

Phage particles obtained from induction were examined and photographed using transmission electron microscope. The majority of phage particles were intact showing

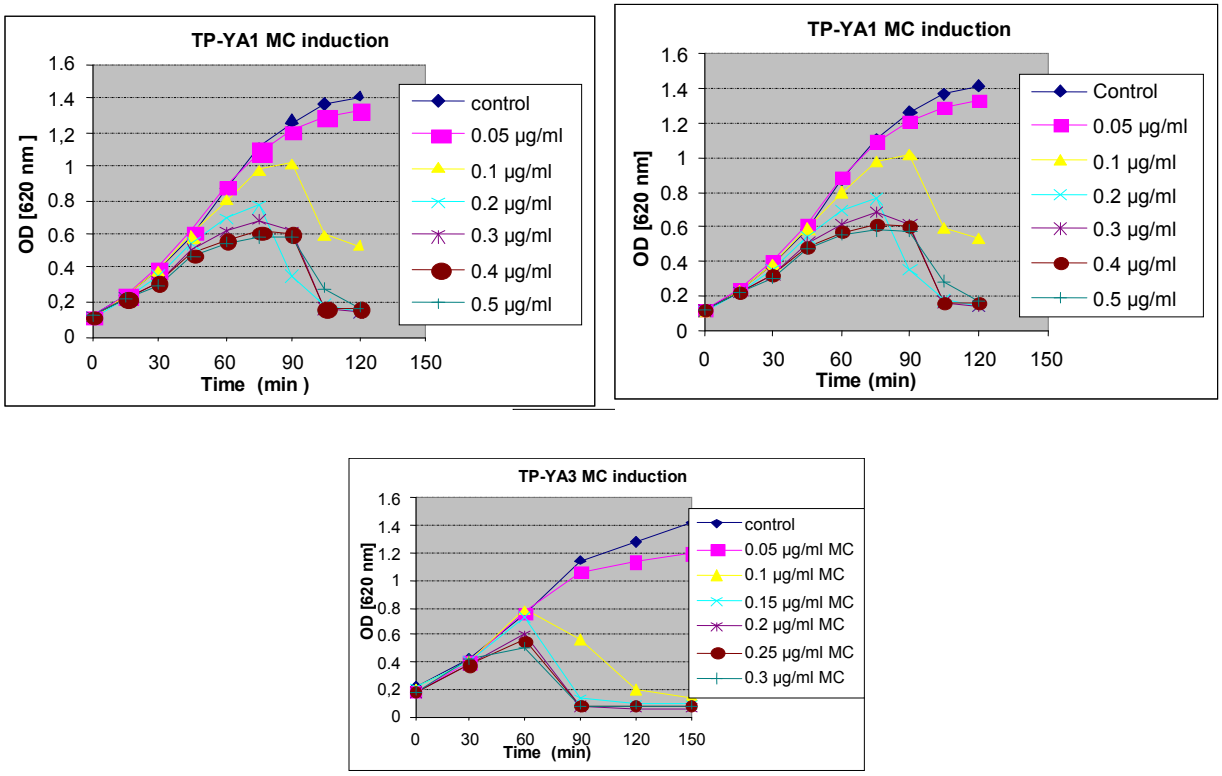

Figure 1. Mitomycin induction of TP-YA1; TP-YA2 and TP-YA3.

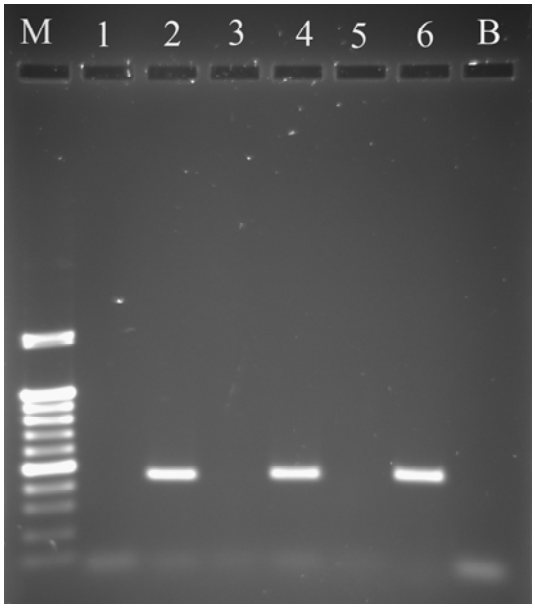

Figure 2. Agarose gel (1.5%) electrophoresis of DNA products obtained by colony multiplex-PCR for identification of three "artisanal" lysogenic *S. thermophilus* strains. Lane 2, 4 and 6 (strains: YA1, YA2, YA3) as a *pac* phage. lanes 1, 2, 3 *cos*-phage primers. Lane B: negative control. Lane M: 100 bp DNA size marker (AppliChem, Darmstadt, Germany).

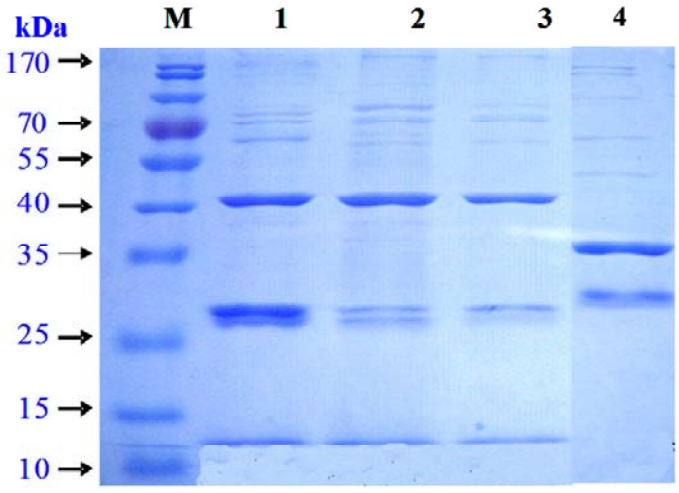

Figure 3. SDS-PAGE (12%) of the structural proteins of the isolated *pac*-type *S. thermophilus* phages YA1, YA2 and YA3 (lanes 1, 2 and 3 respectively). Lane 4 of the *cos*-type reference phage P53. Lane M: PageRuler^{TM} prestained protein marker (Fermentas, St. Leon-Rot, Germany).

isometric- headed *Siphoviridae* phages. They all revealed the same basic morphology (head diameter: 57 nm) and

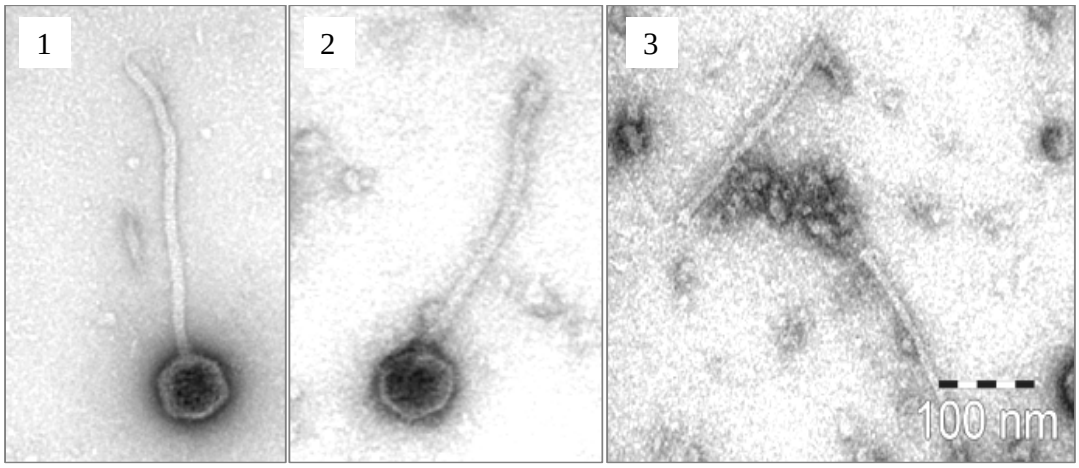

Figure 4. Electron micrographs of induced TP-YA1(1); TP-YA2 (2) and TP-YA3 (3) phage particles.

have long non contractile tails (length ca. 240 nm) (Figure 4). This corresponds with the basic morphology of other *S. thermophilus* phages (Neve et al., 1989; Brüssow, 2001; Bruttin et al., 1997). Hence, phage 1 and 2 are a member of the well described *Siphoviridae* phage family (morphotype B1) as described by Ackermann (2001) and Bradley (1967).

The data confirmed that lysogeny is a rare event in *S. thermophilus* cultures of Neve et al. (2003), who found that only 1 - 10% of the strains contain inducible prohages. However, the analysis of the traditional cultures may indicate a higher dissemination of lysogenic strains. The multiplex-colony PCR approach to detect and identify three new lysogenic *S. thermophilus* strains harbour inducible *pac*-type prophages within a set of sixty *Streptococcus thermophilus* strains isolated from traditional Egyption yoghurt (Zabady) samples.

Conclusion

From the above results, it can be concluded that the conserved DNA-regions of the structural genes for the major head proteins of *S. thermophilus* phages are reliable targets PCR for the differentiation of *pac*-type phage by multiplex PCR. The multiplex PCR described in this work can detect and differentiate *S. thermophilus* phages in a short time (2.5 h); it is very sensitive and reveals a high limit of detection. In whey samples, phages could be detected at low titers of less than 10^3 pfu per ml. Also, *S. thermophilus* starter cultures can be screened rapidly for the presence of lysogenic strains by PCR. Phage monitoring is very important in the dairy industry to avoid economical losses that may happen due to phage infection.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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