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Efficient method for generation of bacteriophage insensitive mutants of *Streptococcus thermophilus* yoghurt and mozzarella strains

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Abstract

Bacteriophage infection of *Streptococcus thermophilus* is becoming increasingly problematic in many industry fermentations such as yoghurt and mozzarella manufacture. This study describes the development of an efficient and rapid 3-step approach for the generation of bacteriophage insensitive mutants (BIMs) of these starter strains. The method initially involves infection of a culture in solid media at a multiplicity of infection (M.O.I.) of 10 which is then incubated in milk overnight. BIMs are then isolated following successive rounds (20–25) of growth in 10% reconstituted skimmed milk (RSM) in the presence of high phage titres. The method selects for BIMs which can grow efficiently in milk. Using this approach BIMs of two industrial strains were generated, whose starter performance was comparable to the parent starters in terms of performance in milk. Genomic fingerprinting used to validate the identity of each BIM, revealed a number of restriction fragment length polymorphisms (RFLPs) in two of the resultant BIMs. This method provides a simple and reliable method for generation of BIMs of industrial starters which does not require any specialised equipment and should be widely applicable.

Keywords: *Streptococcus thermophilus*; Dairy industry; Bacteriophage insensitive mutants

1. Introduction

Streptococcus thermophilus is considered the second most important industrial starter strain after *Lactococcus lactis* (Fox, 1993; Hols et al., 2005; Rasic and Kurmann, 1978). As a member of the thermophilic lactic acid bacteria (LAB), its capacity to survive a relatively high process temperature (45 °C) has made it a vital component in the manufacture of yoghurt and so-called hard 'cooked' cheeses (Fox, 1993; Tamime and Deeth, 1980).

The extensive exploitation of *S. thermophilus* in the dairy industry has simultaneously led to an increased incidence of bacteriophage-related problems in recent years. Whey, a by-product from cheese manufacture, containing phage and lysogenic starter cultures serve as continuous industrial reservoirs for virulent phages capable of disrupting product manufacture (Bruttin et al., 1997) at the expense of both time and capital. Therefore, much effort has been invested in the study of bacteriophage resistance mechanisms for dairy starter cultures. This has certainly led to an impressive array of knowledge of *L. lactis* phages and the natural phage resistance mechanisms present in some lactococcal strains (Coffey and Ross, 2002; Mills et al., 2006). However, knowledge of similar phage resistance mechanisms in *S. thermophilus* is comparatively limited albeit some chromosomal and plasmid-borne restriction/modification systems have been identified (Burrus et al., 2001; Geis et al., 2003; Solow and Somkuti, 2001).

A number of molecular approaches have been used to introduce phage resistance mechanisms in *S. thermophilus*. Such approaches include the expression of a lactococcal restriction/modification system in various *S. thermophilus* industrial strains (Moineau et al.,

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1995), the use of *S. thermophilus* bacteriophage antisense RNA (Sturino and Klaenhammer, 2002) and insertional mutagenesis (Lucchini et al., 2000). While such methods possess huge potential for introducing protection against phage, all use recombinant DNA approaches, which undoubtedly restricts their industrial use at present. Consequently, the ability to generate spontaneous BIMs is currently the most cost effective, short term solution for many industrial strains (Coffey et al., 1998; Coffey and Ross, 2002). Indeed, this is a very important tool to maintain phage resistant derivatives of important starter cultures and has been exploited at length for both lactococcal and streptococcal starters (Coffey and Ross, 2002; Viscardi et al., 2003b,a). Acquisition of phage insensitivity in BIMs is most often as a result of non-specific point mutations in genes encoding cell receptor sites by masking of receptors through, for example, polysaccharide production (Forde and Fitzgerald, 1999). Interestingly, work by Binetti et al. (2002) indicated that streptococcal phage receptors could be carbohydrate in nature, as SDS and proteinase K treatment did not change the adsorption ability of phage particles. Interestingly, BIMs of *S. thermophilus* have been developed to date through the use of anti-phage antibodies or Hoechst 33258-labelled phages combined with flow cytometry (Viscardi et al., 2003b,a). While both methods gave rise to phage resistant derivatives within 4–5 days without requiring genetic engineering approaches, the techniques applied require specialised equipment.

In this study, a highly efficient 3-step method was developed for the generation of BIMs of *S. thermophilus* starter cultures. This method initially uses a phage-infected culture in a plaque assay to generate BIMs which are subsequently isolated following multiple growth passages in milk. In total three phage-sensitive industrial *S. thermophilus* strains were investigated for phage sensitivity against nine industrial whey samples which were found to contain virulent phage against the three strains. While one of these strains was recalcitrant to BIM formation, BIMs were successfully developed and characterised for the other two strains.

Table 1
Strains used in this study

Strain	Relevant characteristics	Source
S. thermophilus		CSK, The Netherlands
CSK938	Industrial yoghurt starter culture	CSK, The Netherlands
CSK939	Industrial mozzarella starter culture	CSK, The Netherlands
CSK947	Industrial mozzarella starter culture	CSK, The Netherlands
938-BIM 5000.1a	CSK938 BIM developed with phage sample 5000	This study
938-BIM 5000.2a	CSK938 BIM developed with phage sample 5000	This study
938-BIM 5000.3a	CSK938 BIM developed with phage sample 5000	This study
938-BIM 5102.1a	CSK938 BIM developed with phage sample 5102	This study
938-BIM 5102.2a	CSK938 BIM developed with phage sample 5102	This study
938-BIM 5102.3a	CSK938 BIM developed with phage sample 5102	This study
938-BIM 5077.1a	CSK938 BIM developed with phage sample 5077	This study
938-BIM 5077.2a	CSK938 BIM developed with phage sample 5077	This study
938-BIM 5077.3a	CSK938 BIM developed with phage sample 5077	This study
938-BIM 5002.1a	CSK938 BIM developed with phage sample 5002	This study
938-BIM 5002.2a	CSK938 BIM developed with phage sample 5002	This study
938-BIM 5002.3a	CSK938 BIM developed with phage sample 5002	This study
939-BIM 5027-5093.a	CSK939 BIM developed with phage samples 5027 followed by 5093	This study
939-BIM 5027-5093.b	CSK939 BIM developed with phage samples 5027 followed by 5093	This study
939-BIM 5093-5027	CSK939 BIM developed with phage samples 5093 followed by 5027	This study
939-BIM 5093-5073	CSK939 BIM developed with phage samples 5093 followed by 5073	This study

2. Materials and methods

2.1. Bacterial strains, bacteriophages and media

All bacterial strains and phage samples used in this study are listed in Table 1. Streptococcal strains were routinely propagated at 37 °C in Elliker medium (Difco Laboratories, Detroit, MI, U.S.A.) (Elliker et al., 1956) supplemented with 1.0% (w/v) Beef extract (Oxoid, Hampshire, England) referred to as Belliker broth (O' Sullivan et al., 1999). Solid media contained 1.0% agar (Oxoid). Belliker soft agars contained 0.7% agar (Oxoid). Bacteriophages were stored in Belliker broth at 4 °C.

2.2. Propagation and enumeration of bacteriophages from industrial phage samples and isolation of pure phage preparations

Bacteriophages were propagated as follows; 10 ml of Belliker broth was inoculated with 1% of a fresh culture, 10 μl of bacteriophage-containing sample, and 0.1 M $CaCl_2$. A control culture, without bacteriophage was also prepared. The samples were incubated for up to 6 h until clearing was observed. The bacteriophage sample was then filter-sterilised prior to use (0.45 μm-pore-size filters). Bacteriophages were enumerated using the plaque assay technique described previously (O' Sullivan et al., 2000).

2.3. Generation of bacteriophage insensitive mutants

The BIMs were generated by a 3-step process as follows:

1. The sensitive host which was grown from −20 °C stock overnight, to give 10^8 colony forming units (cfu)/ml was completely lysed in a soft top agar by plaque assay following infection with industrial phage at an MOI of ≥ 1.0. However, following 24–48 h of incubation, phage resistant colonies appeared in the top agar.
2. Then the complete soft agar overlay containing the BIMs, which ranged in number from 10 to 10^3 colonies depending on the sensitive host strain, was removed with a sterile spatula and incubated overnight in 10% RSM.