UNIVERSITÀ DEGLI STUDI DI NAPOLI

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DIVERSITY OF LACTIC ACID BACTERIA BACTERIOPHAGES FROM DAIRY ENVIRONMEMENT

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DIVERSITY OF LACTIC ACID BACTERIA BACTERIOPHAGES FROM DAIRY ENVIRONMENT

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CONTENTS

CHAPTER 1

Introduction

General introduction 1

Baccteriophages 2

The *Streptococcus thermophilus* bacteriophages 5

Bacteriophage /host interaction 6

Genome organization 11

Transcription 14

Integration/excision 15

Replication 17

DNA packaging 18

Head proteins 21

Tail proteins 22

Lysis cassette 22

Bacteriophage defense strategies 23

Improving sanitation and manufacturing processes 24

Phage inhibitory media 24

Strain- rotation 24

Molecular strategies 26

Outlook of thesis 27

CHAPTER 2

Investigation on the presence of LAB bacteriophages in Natural Whey Cultures used for traditional water-buffalo Mozzarella Cheese manufacture

INTRODUCTION 28

EXPERIMENTAL PROCEDURES 29

NWC samples 29

Enumeration, bacterial strain isolation and culture conditions 29

Analysis of the NWC samples for the presence of phages 30

Detection of Streptococcus thermophilus phages by PCR 30

RESULTS 31

Enumeration of lactic acid bacteria 31

Analysis for the presence of bacteriophages 33
Detection of Streptococcus thermophilus phages by PCR 33
DISCUSSION 33

CHAPTER 3

Characterization of *Streptococcus thermophilus* Lytic Bacteriophages from Mozzarella Cheese Plants

INTRODUCTION 36
EXPERIMENTAL PROCEDURES 37
Bacterial strains, bacteriophages and culture conditions 37
Phage multiplication 38
Host range 39
Phage DNA isolation 39
Restriction analysis 40
Determination of DNA packaging mechanism 40
Amplification and sequencing of the antireceptor variable region 41
RESULTS 42
Host range 42
Restriction analysis 44
Determination of DNA packaging mechanism 44
Bacteriophage clustering based on VR2 sequences 45
DISCUSSION 47

CHAPTER 4

Evidence of a temperate bacteriophage from lysogenic *Streptococcus macedonicus* AI4

INTRODUCTION 50
EXPERIMENTAL PROCEDURES 52
Induction of bacteriophages 52
Screening for indicator strains 52
Determination of DNA packaging mechanism 53
Dot blot hybridization 54
RESULTS 54
Evidence of temperate bacteriophages and screening of indicator strains 54
Determination of DNA packaging 56

Dot blot hybridization 56
DISCUSSION 57

CHAPTER 5

Genomic sequence of *S. macedonicus* temperate phage PZ1φ by genome walking method - work in progress

INTRODUCTION 59
EXPERIMENTAL PROCEDURES 60
Construction of Genome Walker™ Libraries 60
PCR amplification 61
RESULTS and DISCUSSIONS 62

REFERENCES 64

Chapter 1

INTRODUCTION

General introduction

Lactic Acid Bacteria (LAB) have always played a very important role in diary industry for their fermentative capacities. LAB comprise a wide range of genera and include lactococci, lactic streptococci and lactobacilli. When these microorganisms are present in the food products, they also have a function as biopreservatives and probiotic characteristic (Salminen *et al.*, 1996).

Beside *Lactococcus lactis, Streptococcus thermophilus* is the major starter bacterium in cheese industry (Brussow, 2001). It widely occurs in commercial starter cultures as well as in natural milk or whey cultures traditionally used in the manufacture of several protected designation of origin (PDO) and artisanal cheeses (Coppola *et al*., 1988). They are used during the manufacture of Italian-style cheese varieties such as Asiago, Mozzarella, Parmigiano and ripened cheeses including Limburger, Port du Salut and Trappist (Olson 1969). *S. thermophilus* is also used with *Lactococcus lactis* for the prduction of Cheddar cheese. This microorganism is perhaps best known for its use in combination with *Lactobacillus delbrueckii* spp. *bulgaricus*, during the manufacture of yogurt. It is well known these two microorganisms share a synergistic relationship when mixed, growing faster and producing more lactic acid. The application of lactic acid bacteria, specially *S. thermophilus*, in large-scale diary fermentation was an incentive to the unravelling of molecular mechanism and genetics underlying some of its significant industrial traits. This has resulted in the improvement of existing strains and the development of novel strains by genetic modification (McKay and Baldwin, 1990).

The recognition that the bacteriophages could be a main risk to starter culture performances led to the research aimed to the development of strategies to effectively protect starter strains.

In recent years significant progress has been made in the elucidation of genetic organization of several *S. thermophilus* phage genomes. The aim of this overview is to describe the current state-of-art about *S. thermophilus* bacteriophage research and exploitation of this knowledge in both fundamental and applied research.

Bacteriophages

Virus that infect bacteria, bacteriophages (from Greek φαγειν= to eat, bacteriophage = bacteria eater) are obligate intracellular molecular parasites belonging to *Myoviridea*, *Podoviridea* or *Siphoviridea*. On the basis of specific features of their heads and tails the bacteriophages can be differentiated in three basic morphotypes (Ackerman *et a*l., 1984, Figure 1). Phage of morphotype A is characterized by a contractile tail allowing active injection of their genome into the host cells. Morphotypes B and C have respectively, a long and small non-contractile tail.

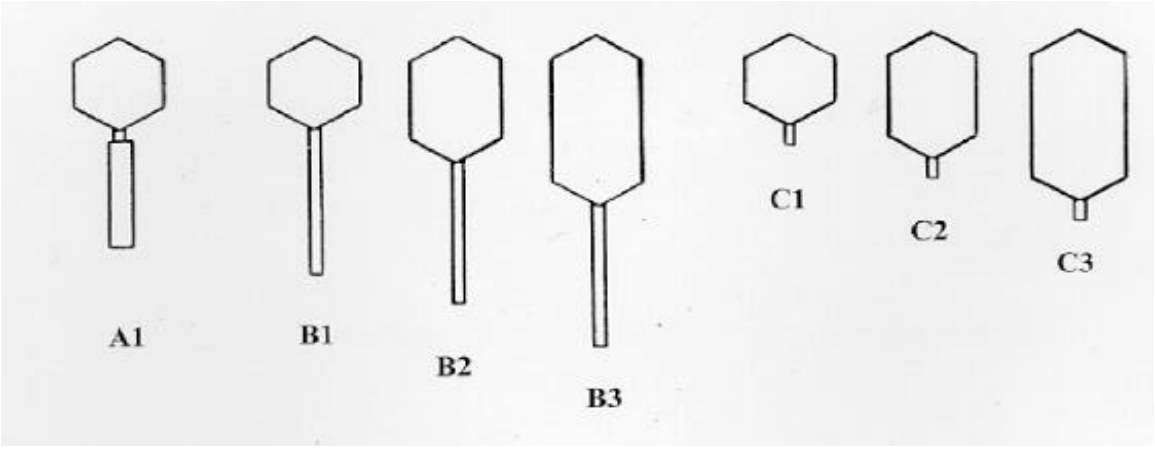

Figure 1: *Basic morphotypes of compositive viruses belonging to* Myoviridea *(A),* Siphoviridea *(B)* and Podoviridea *(C).*

The latter two groups are further subdivided on the basis of the size and shape of their capsids as either small-isometric, prolated-headed or elongated bacteriophages. Although

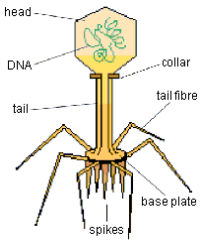

Figure 2

phages can possess specific appendages such as a collar, a baseplate, whiskers, spikes and/or fibers, taxonomically not significant structures (Figure 2).

The genome of tailed phages constitutes either a single- or double–stranded DNA or single stranded RNA molecule, encapsulated in a head (capsid) composed of proteins. Furthermore, the bacteriophages can be distinguished on the basis of their specific life cycle after infection (Figure 3). Lytic (or virulent) bacteriophages have only one strategy for the replication. At the beginning of infection a phage adsorbs to its host and injects its DNA in linear form from the phage capsid to the inside of a cell. During the latent period of a lytic cycle, phage DNA is replicated and the phage genes are transcribed in strict order: proteins of tailed phages are assembled through separate pathways into heads, tails and fibers (Ackermann and Dubow, 1987). Phage genome replication often leads to accumulation of head-to-tail arrays of phage genomes. This so called concatemeric DNA is then cut by headful cutting during the packaging process into DNA-free proheads. All cuts occur either precisely at *cos*-sites, or the first cut occurs at the packaging sequence *pac* and then the cutting process continues by imprecise headful measuring leading to terminal redundancy (Black 1989.) Phage heads and tails are connected together to finish phage maturation and phages are released due to cell bursts or lysis as a result of cell wall degradation by a phage lysozyme (Ackermann and DuBow, 1987). A virulent phage can only initiate a lytic cycle.

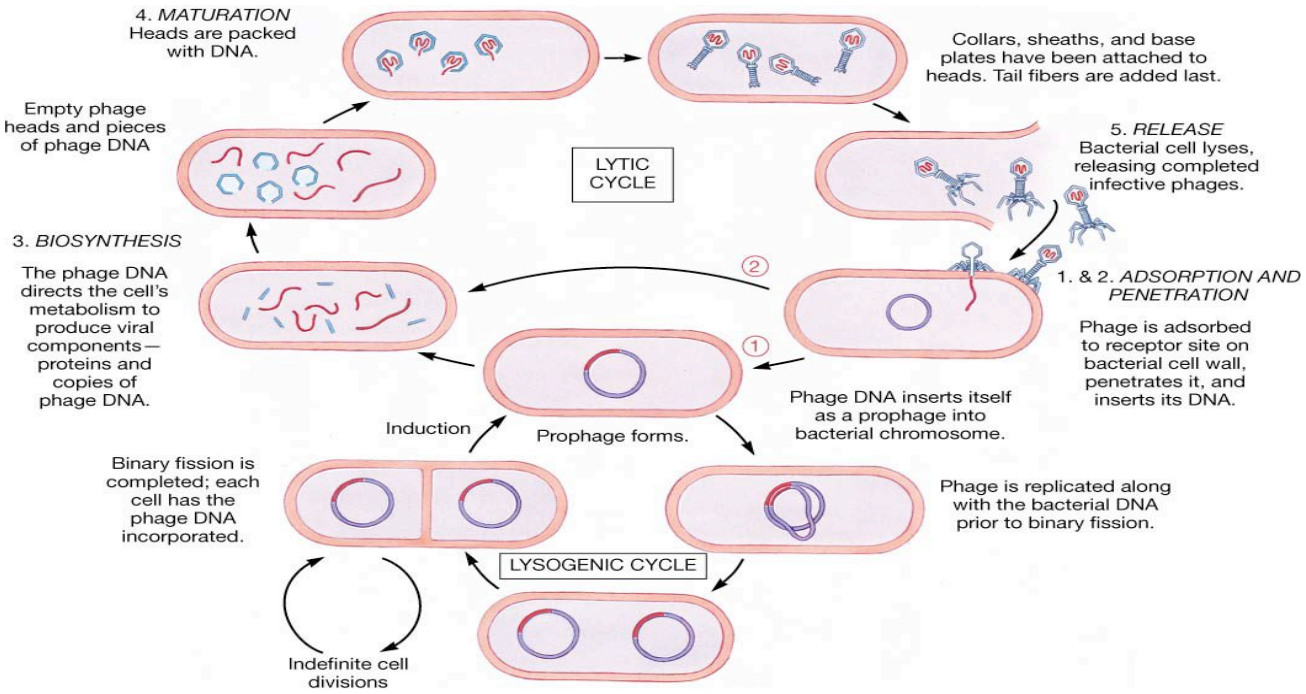

Figure 3. *The lifestyle of temperate and lytic bacteriophages.*

Temperate bacteriophages, instead, have an alternative mode of existance. Their genomes can be integrated into that of the bacterium. Thus the DNA is replicated along with the bacterial chromosome and this latent form of the bacteriophage is called "prophage". In this so called "lysogenic" state of the host cell, the genes involved in lytic growth of the phage are repressed by a phage genome-encoded repressor. Relief of repressor (induction) results in excision of the prophage from bacterial genome and directs the phage into to the lytic life stile cycle. When a temperate phage infects a host cell the decision is made between the lytic cycle and lysogeny. Prophage DNA is integrated into the host genome or it exists as a free plasmid. The circularized phage λ DNA is integrated into its host genome by reciprocal recombination at specific sites (phage and bacterial attachment sites, *attP* and *attB*) by λ integrase and the host enzyme IHF (Weisberg and Landy, 1983). For phage λ the decision

between the lytic cycle and the lysogenic state depends on relative levels of two antagonistic phage regulatory proteins, Cro and cI. After spontaneous induction or induction by DNA damaging agents like mitomycin C, prophages sometimes escape the control of the cI repressor, excise and initiate the lytic cycle (Ackermann and DuBow, 1987). Furthermore, there is the chronic bacteriophage life ciycle. A chronically infecting phage can release progeny phages into extracellular environment without killing its host (Maniloff *et al*., 1981). In this case the phage and the host coexist and bacteriophages are shed over the infection.

By the genetic organization of the conserved head cluster, it has recently been proposed a new LAB phage classification (Brussow and Desiere, 2001, Proux *et al*., 2002). According to this new proposal the sequenced LAB phages could be divided into five genera: c2-, sk1-, Sfi11-, Sfi21- and r1t-like phages (Desiere *et al*., 2002, Proux *et al*., 2002).

The Streptococcus thermophilus *bacteriophages*

S. thermophilus phages have the same basic morphology belonging to the B1 *Syphoviridae* group of *Caudovirales* order (Brussow *et al.,* 1994) with few exceptions appear to be very similar with isometric heads (generally 42 to 63 nm in diameter), tails 200-300 nm long, no obvious collars and only small base plates, often with a central fibre. Variants with larger heads, shorter tails and exceptionally long tails (polytails) (Reinbold *et al.,* 1982) are also known to occur. *S. thermophilus* phages have a linear double-stranded DNA genome ranging in size from 30 to 45 kb (Brussow *et al*., 1998). DNA-DNA hybridization studies showed homology between all *S. thermophilus* phages, including virulent and temperate phages.

Brussow and Bruttin (1995) proposed classification of *S. thermophilus* phages into four lytic groups based on host range and type-specific antisera. An alternative approach for classifying *S. thermophilus* phages is originated from the analysis of phage genomes and it is based on their evolutionary descendent. Mercenier (1990) proposed that all *S. thermophilus* phages are derived from a common ancestor. Corroborating this idea is the cloning DNA fragment from ΦS1, which hybridized to all *S. thermophilus* phage isolates. The diversity in phage genomes observed by different researchers may result from multiple rearrangements occurring within the phage population. Brussow *et al*. (1994b) proposed that the evolution of *S. thermophilus* phages takes place by means of module exchange between phage genomes, a hypothesis that was proposed previously for other bacteriophages. Le Marrec *et al*. (1997) proposed a further classification of *S. thermophilus* phages into two groups based on the number of major structural protein (MSP) and the mode of DNA packaging. The first group comprises phages with two MPS and cohesive genome extremities (*cos*-type). The second cluster includes phages with three MPS and a DNA packaging scheme that proceeds via a headful mechanism (*pac*-type).

Bacteriophage/host interaction

The obligatory use by the LAB bacteriophages of their hosts is reflected by the numerous phage/host interactions (Garvey *et al*. 1994). These interactions are often subject to change due to development of new defence mechanisms by host cells. The natural defence mechanisms in LAB against phage infection are often plasmid-coded and can be classified into four main categories:

- adsorption interference;
- injection blocking;

- restriction/ modification (R/M) systems;
- abortive-infection (Abi).

The adsorption interference is the mechanism prevailing in spontaneous phage resistant mutants of thermophilic LAB, whereas R/M systems are widely represented within *Lactococcus* genus (Moineau S., 1999). It was proven that the modification and restriction enzymes both recognize the same target, a specific nucleotide sequence. The modification enzyme is the DNA methyltransferase that methylates specific bases within the target sequence. In the absence of the specific methylation in the target sequence, DNA becomes sensitive to the restriction endonuclease. When the DNA lacking the appropriate modification imprint, it enters a restriction proficient cell. Therefore, the DNA is recognized as foreign and degraded by the endonuclease. Restriction/modification systems are classified into three types on the basis of their composition and cofactor requirements, the nature of their target sequence, and the position of the site of DNA cleavage with respect to the target sequence (Murray, 2000). In the first R/M system, designated type I, the enzymes are hetero-oligomeric. They require ATP hydrolysis for the restriction and cut at sites remote from the recognition sequences. In the R/M system type II, the endonucleases and the methyltransferases are separate enzymes and they cut the DNA within the recognition sequence. The type III is characterized by hetero-oligomeric endonucleases, which require ATP for restriction and they cut the DNA close to recognition sequence.

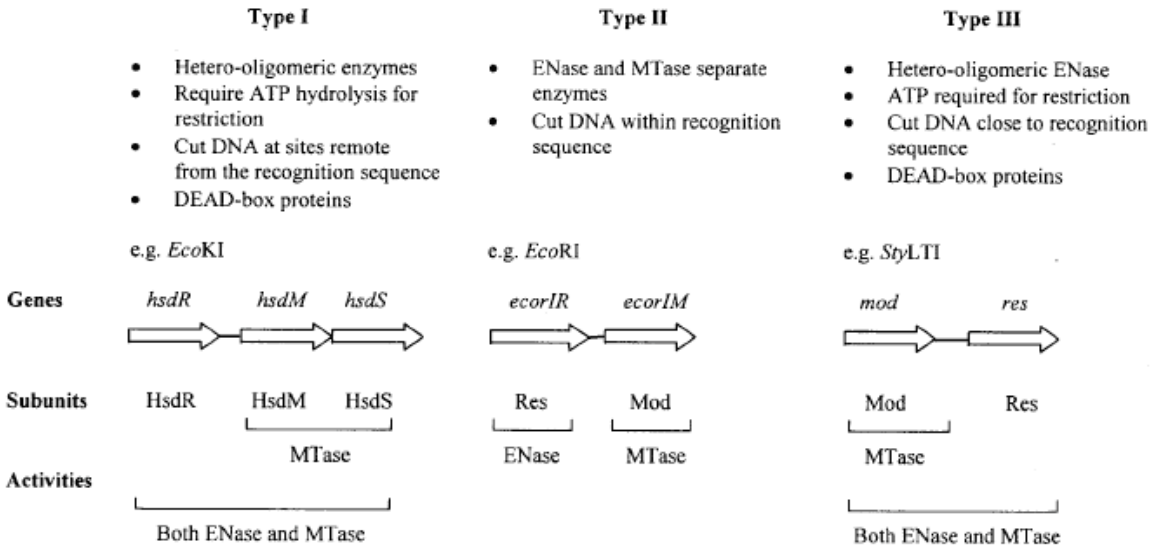

Figure 4. *Restriction/Modification system (modified from Murray 2000)*

By adsorption interference, most phage resistant strains achieve their resistance through a loss or a modification of phage receptors due to mutation in corresponding genes (Riipinen *et al*., 2007).

Furthermore, Duplessis and Moineau (2001) studied the phage genetic determinant (antireceptor) involved in the recognition of *S. thermophilus* hosts. Most of the information on the antireceptor and phage-host receptor interaction comes from studies carried out on coliphages, such as T-even (*Myoviridae* family) and lambdoid (*Siphoviridae*) phages. For T4 phage, the major host range determinant is the gene product of *orf37*, which encodes the large subunit of distal tail fibre. Specific interaction between the tip of these long fibres and receptors on the surface of *Escherichia coli* host bacterium result in rapid and efficient phage adsorption (Goldberg *et al*., 1994). The C terminal extremity of *gp37* can recognize a variety of bacterial receptor molecules, such as OmpC, OmpF, and *E. coli* B lipopolysaccharide (Tétart *et al*., 1998). In the phage λ, the gene product J constitutes the

receptor-binding protein that allows phage adsorption to the surface of *E. coli* K-12 by interaction with the outer membrane protein LamB. The J protein is the target of λ-neutralizing antibodies as well as a structural component of the tail fibre. Analysing lambda mutants it was shown that host range mutations occurred in the last distal (5-10 %) portion of gene *J* (*gpJ*) (Werts *et al*., 1994). Wang *et al.* (2000) demonstrated only 249 amino acids at carboxy-terminal part of the antireceptor protein is involved in host specificity.

Duplessis and Moineau (2001) characterized the gene of *S. thermophilus* phage DT1 that encodes the structural protein involved in host recognition. Bio-informatic analysis by Tremblay and Moineau (1999) suggested the gene product of *orf18* as the antireceptor: it has the same position within its genome as the anti-receptor of phage lambda gene *J*, the pI and molecular weight of ORF18 are similar to the those gpJ of λ and when compared with homologues, *orf18* has an organization that resembles some T-even antireceptor genes with conserved and non-conserved regions. Furthermore, they sequenced the *orf18* of *S. thermophilus* DT1 and of other six lytic, *cos*-type phages. By amino acid alignment, they could understand that these open reading frames (ORFs) were divided into three domains: the first domain of the seven ORF18s corresponded to the amino-terminal portion of the protein up to the collagen-like repeats. This portion of 491 amino acids resulted highly conserved among the seven phages, with 83-100% amino acid identity and the first domain of these phages was identical also at the nucleotide level.

The second domain could be present or absent depending on the phage and has a length of approximately 400 amino acids. This domain resulted flanked by two motifs called collagen-like repeats. This second domain was called VR1 containing. The third domain, which corresponds to the carboxy-terminal part of the protein, starts after fourth collagen-like repetition and has a length of approximately 400 amino acids. It also showed an internal

variable region VR2 of about 145 amino acids and it resulted the most divergent region within the deduced ORF18 (Figure 5). The overall comparisons of the seven *orf18*s revealed that the variations could be attributed to point short mutations, short deletions or insertions. The comparisons of host range of the seven phages with their corresponding ORF18s revealed that the ORF18 of phage DT1 has a genetic organization similar to that of *S. thermophilus* phage MD4 and both has very distinct host ranges. To provide biological evidence that *orf18* is involved in host recognition, Duplessis and Moineau (2001) used the distinctive features of these two phages for the construction of chimeric phage in which the *orf18* of phage DT1 was swapped for the *orf18* of phage MD4. By this construction, DT1 acquired the host range of MD4. Prior to this, a spontaneous deletion mutants of phage Sfi21, called D3, was isolated by routine serial propagation. In this case, the second domain between the collagen-like repeats III and I was deleted in the mutant, indicating these recombination hotspots contribute to the allelic diversity within the population (Bruttin and Brussow, 1996). The nucleotide sequences encoding the collagen-like reapeats suggested to be hotspots for recombination-mediates gene shuffling (Desière *et al*., 1998). These motifs consist of repeated amino acids triplets where glycine is the first residue in each triplet (Beck and Brodsky, 1998).

Comparisons of other VR2 regions characterized in the same study also revealed that additional phage factors are involved in the host specificity of other *S. thermophilus* phages. Although VR2 is clearly responsible for the specificity of phages MD4 and DT1 to their *S. thermophilus* hosts, this finding cannot be generalized to other *S. thermophilus*. The VR1 region may also interact with the VR2 region to confer host specificity. However, the phage receptors in *S. thermophilus* are currently unknown. A first effort consists in submitting a strain to mutagenesis with the thermolabile insertional vector pG+host9:ISS1 (Lucchini *et*

al., 2000). Vector insertion into four different sites led to a phage resistant phenotype, but all mutated strains adsorbed the phages, suggesting the elements involved in phage adsorbtion remain unidentified. Unfortunately, no biological evidence has allowed the identification of the phage antireceptor. It is also important noting that the collagen-like repeats are found in sugar-binding proteins, such as maltose-binding protein. Furthermore, it is interesting to speculate that ORF18 could bind to a carbohydrate component of the *S. thermophilus* cell wall.

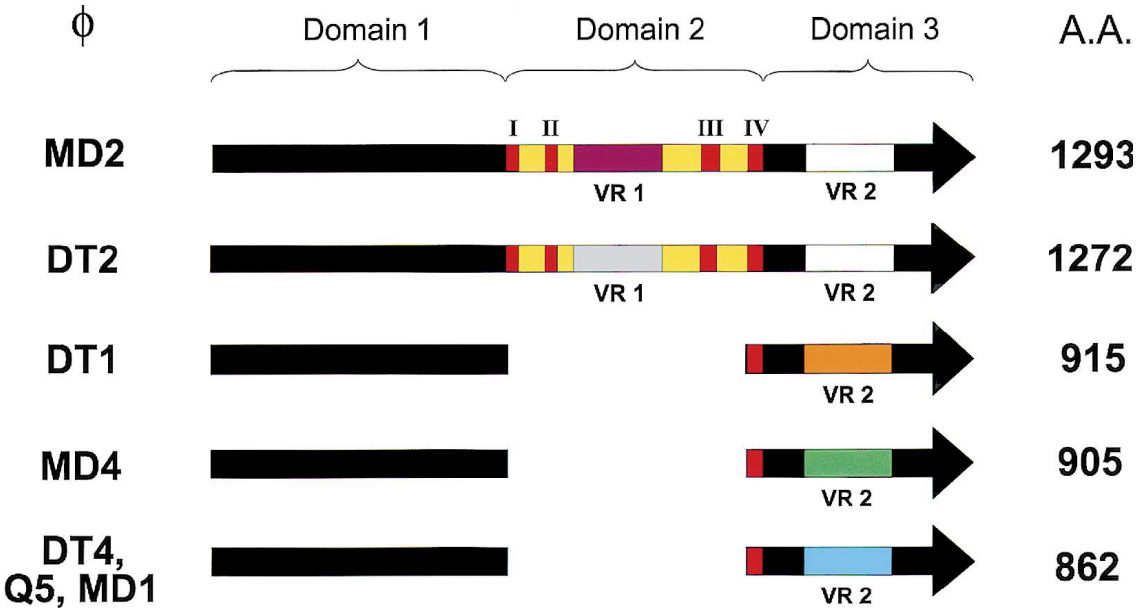

Figure 5: *Alignment and comparison of the ORF18 of seven* S. thermophilus phages. *VR1 and VR2 indicate variable region 1 and 2. Homologous sequences (>80) are indicated by similar colours. The collagen- like repeats are represented in red as motifs I, II, III, and IV (Duplessis and Moineau, 2001).*

Genome organization

Similar to most other bacteriophages, the DNA content of streptococci bacteriophages, is rather small. Genomes analyzed are double–stranded DNA linear molecule ranging in size from 30 to 45 kb (Brussow *et al*., 1998). The DNA sequence data are rapidly accumulating 4for *S. thermophilus* phages . However, the genomes sequenced so far were from isolated in

two countries (France and Germany) and in the same ecological niche (yogurt) (Tremblay and Moineau ,1999). As stated by Brussow *et al*. (1998), a greater coverage of phages from other areas is needed because evolution and population studies could be influenced by a strong geographical and ecological bias.

The first complete genome sequenced for *S. thermophilus* phage, was that of temperate phage O1205. It belonged to the 3MSP/*pac*-group and was isolated from the lysogenic strain CNRZ1205 used in the yogurt production in France. Phage O1205 has a genome size of 43075 bp organized in 57 open reading frames (ORFs) (Stanely *et al.* 1997). Later, the structural genes and the lysis module of another phage of 3MSP/*pac* group have also been determined in Sfi11 by Lucchini *et al*. (1998). The lytic phage Sfi11, also from France, differed from the phage O1205 at nucleotidic level by about 10% and the majority of the changes were point mutations and only one gene differed substantially in the two phages.

Nucleic acid sequences are also available for two other French yogurt isolates but of 2MSP/*cos* group, the temperate phage Sfi21 and the lytic phage Sfi19 (Brussow *et al*., 1994b; Bruttin and Brussow, 1996; Bruttin *et al*., 1997; Desière *et al*., 1997, 1998). The sequences of two phages differed only for 10% and these differences were due to punctual mutations as well as short deletion and insertions (Desière *et al*., 1998).

Tremblay and Moineau (1999) reported the first complete genomic characterization of a *S. thermophilus* lytic phage, DT1, isolated from a North American mozzarella cheese plant. The phage DT1 is recognized member of 2MSP/*cos* group and has a linear DNA of 34820 bp with 46 ORFs of more 40 codons. They underlighted that all these ORFs, with exclusion of ORF8, ORF27 and ORF35, were preceded by potential Shine-Delgarno (SD) sequences complementary to the 3' end of the 16S rRNA of *S. thermophilus*. These SD sequences were at an appropriate distance from one of the common initiation codons (AUG, UUG, GUG).

The 46 ORFs were compared with database and significant homologies were detected only with the phages of Gram-positive bacteria, including *Lactobacillus casei* (Garcia *et al*., 1997), *Lactobacillus delbrueckii* (Mikkonen and Alatossava, 1994), *Lactobacillus plantarum* (Kodaira *et al*.,1997) *Lactococcus lactis* (Arendt *et al*., 1994) *Leuconostoc oenos* (Sutherland *et al*., 1994), *Staphilococcus aureus*, *Streptococcus thermophilus* (Brussow *et al*., 1994b; Desière *et al*., 1997;1998; Neve *et al*., 1998; Stanley *et al*., 1997). Based on amino acid homology, putative functions were assigned to five ORFs and they included the terminase (ORF4), holin (ORF24), lysine (ORF25), helicase (ORF33) and primase (ORF36) (Tremblay and Moineau, 1999). It was also compared the genome of phage DT1 with partial sequence available for two phages of 2MSP/*cos* group, showing a great homology over 17-kb region for the lytic phage Sfi19 and a 29-kb region of temperate phage Sfi21. The percentage of nucleotide identity for 15 putative genes of phage Sfi19 ranged from 67 to 93%. For phage Sfi21 the percentage extended over 66 to 91 in 21 shared genes. All three phages showed a similar genomic organization and detailed investigation between two lytic phages Sfi19 and DT1 showed that most discrepancies were due to point mutation and they were not located at specific base position. Small insertion and deletions also occurred within some predicted genes such as *orf*15 (minor tail protein) and *orf*18 (host specificity protein). Instead, the most conserved regions of phage DT1 when compared to the putative genes of Sfi19, resulted to be the *orf*13 (major tail protein) and *orf*21 (unknown function).

The lambdoid phages genome was divided into 11 major segments of functional genes occurring in the same chromosomal order (Casjens *et al*., 1992), instead the genome of *S. thermophilus* phage could be separated into 4 large fragments (Tremblay and Moineau, 1999). The first region (heterologous) consists of DNA packaging machinery and major structural proteins. The second segment (homologous) consists of late morphogenesis genes

and lysis cassette. The third division (heterologous) is made of the lysogeny module and the fourth (homologous) corresponds to replication genes. Also for *S. thermophilus* phages the gene involved in similar functions seems to be clustered. Although the organization of many bacteriophages genomes is similar, the lytic phages have differently oriented clusters of genes required for lytic growth, whereas the temperate phages appear to have organized their genetic determinants in one large cluster.

Transcription

Transcription of lactic acid bacteria bacteriophages DNA follows a model where early, middle and late genes are sequentially transcribed as cluster.

Three temporal classes of messenger RNA transcription in lactococcal temperate phage TP901-1 were identified. Short leftward (repressor to integrase genes) and longer rightward early transcripts (DNA replication genes) were initiated in the genetic switch region. Short middle transcript of unknown attribution overlapped the end of the early region. Related to this region was initiated a long late transcript which covered two thirds of the genome that encodes structural and lysis genes (Madsen and Hammer, 1998). Detailed transcription maps were also developed for virulent *Lactococcus lactis* phage sk1, where three classes of transcript were differentiated based on their time of appearance (Chandry *et al*., 1994). Early transcripts covered the entire leftward oriented 10 kb of the sk1 genome. Middle transcripts were transcribed from a 2 kb genome region near the right *cos*-site. Instead, late transcripts were derived from the 16-kb genome region of rightward oriented gene; they started next the left of *cos*-site and terminated in the same genome region as the oppositely oriented early transcripts. Early transcription was carried out by the host RNA polymerase whereas middle and late transcription depended on a phage-encoded protein. The late promotor lacked a 35

consensus sequence and the late transcripts were processed by RNase E (Brussow, 2001). Generally, the lysogeny replacement and bacteriophage DNA replication modules are expressed as early genes (Duplessis et al. 2005). During the infection of *S. thermophilus* phage Sfi21 and Sfi19 the genes from the DNA replication module and gene encoding a Cro-like repressor are transcribed as middle genes (Ventura and Brussow, 2004). The middle region of *Lactobacillus gasseri* phage adh covers the genes from the *ori* to the end of putative terminase gene (Altermann and Henrich, 2003). The middle gene cluster of *S. thermophilus* phage 2972 and DT1 covers all the genes from the packaging genes to the end of the head morphogenesis module. The corresponding genes in the Sfi21 and Sfi19 genome are clustered as late genes (Ventura and Brussow, 2004; Duplessis *et al*., 2005)

Integration/excision

In temperate diary phages the gene functions necessary for the establishment and maintenance of the lysogeny are organized into a compact lysogeny module (Lucchini *et al.* 1999). The integration pathway of lactoccoccal phage followed the Campbell *et al.* (1983) model (Figure 6) of site-specific recombination for phage λ between two specific attachment sites (*attB*, *attP*). In the phage λ, induction of the prophage results in recombination between the two junction sites *attL* and *attR* created by the integration process and leads to the excision of the prophage DNA. Usually, the LAB phages use the tyrosine integrases for prophage integration (Brussow, 2001). In *S. thermophilus* phages Sfi21 no excisionase gene was identified upstream of the *int* gene. The upstream *orf*203 encoded a superinfection immunity function that protected the lysogen against infection by many virulent *S. thermophilus* phages (Bruttin *et al*., 1997). Prophage integration was widely studied in many *Lactobacillus*, *Lactococococcus* and *S. thermophilus* phages (Sfi21, O1205). The *att*P site is

found downstream of the *int* gene with exclusion of Sfi 21, which showed *att*P overlaps the 3'-end of the *int* gene.

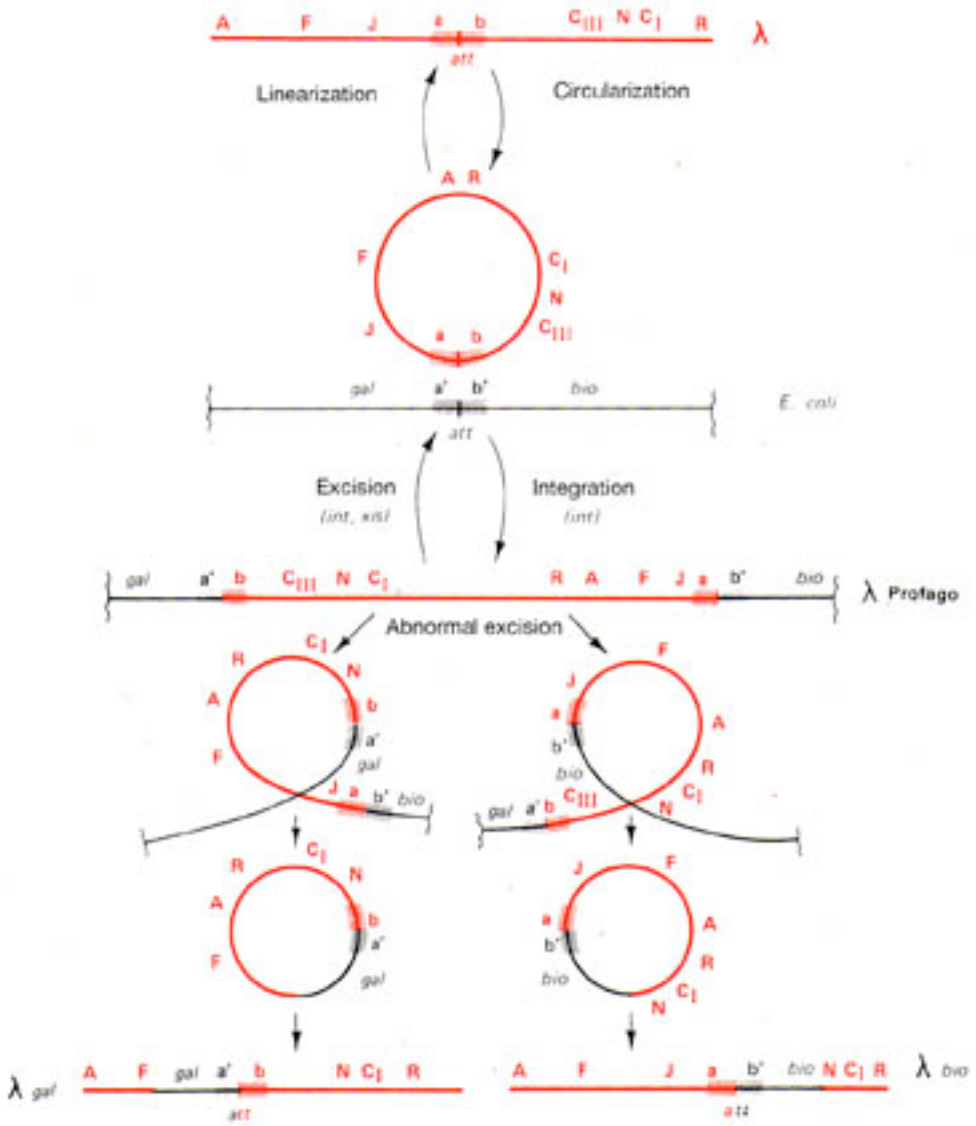

Figure 6: *Integration and excision mechanisms of DNA l phage in* E. coli.

In the *Lactobacillus* phage mv4 the 17 bp common core sequence overlaps the 3' end of $tRNA^{Ser}$. A nonreplicative vector based on *att*P and *int* from mv4 could integrate into a wide range of Gram positive bacterial hosts. The consequential isolation and sequencing of the plasmid integration site demonstrated integration into protein-coding genes and intergenic DNA in these heterologous hosts. Sequencing of the integration sites showed a flexibility of

integrase, in agreement with results obtained for other dairy bacteriophages. The phage Sfi21 integrase, in fact, mediated phage genome deletions and deletions in plasmids into which it was cloned (Brussow, 2001).

Replication

The knowledge of phage DNA replication and gene expression is still limited for *S. thermophilus* phages. Many studies about the replication deal to control phage infections. For instance, phage resistance mechanism has been engineered with phage genetic elements, such as the origin of replication (*ori*) and antisense RNA genes (Coffey and Ross, 2002). A DNA replication module containing genes encoding putative single stranded DNA binding proteins, a topoisomerase I, a methilase and a replisome organizer protein was identified in *Lactococcus* phage Tuc2009. It was also identified the origin of the replication as both region capable of supporting plasmids replication and region that interfere with phage growth by binding replisome organizers. The replisome organizer from Tuc2009 contained 160 bp repeat conferring phage growth inibition. McGrath *et al*. (1999), by mutation analysis, demonstrated the importance of the repeats for the resistance phenotype.

An alternative putative DNA replication module was identified in *S. thermophilus* phages. Three predicted proteins (Desière *et al*., 1997) showed nucleoside triphosphate binding motifs. One of them showed in also a DEAH box motif. A tree analysis classified it as a distant member of the helicase superfamily (Brussow 2001).

Phage *ori*s are mainly characterized by noncoding region containing several inverted and direct repeats (McGrath *et al*., 1999). The gene organization in the vicinity of *ori* is relatively conserved among *S. thermophilus* phages. This region is essentially composed of genes expressed early after the start of infection, such as those encoding the helicase and

primase. The *ori* of phage DT1 is located between *orf*36 and *orf*37 (Tremblay and Moineau, 1999). A non-coding region containing direct and indirect repeats, typical of phage *ori*s, was found between these two open reading frames (Lamothe *et al.,* 2005). Cloning of different restriction fragments containing this putative *ori* into *ori*-probe vector was undertaken in *E.coli* and *S. thermophilus* in order to find a DNA fragment from DT1 genome capable of sustaining plasmid replication. Despite several efforts Lamothe *et al*. (2005) could not obtain a stable plasmid containing DNA from phage DT1 that could replicate autonomously in *S. thermophilus.* They explained it by the presence of early genes in the vicinity of the *ori*.

DNA packaging

One of the most interesting issues in the field of phage morphogenesis is the mechanism by which the DNA is packaged into a preformed protein shell (prohead or procapsid). The genome packaging can be accomplished by unit-length or headful mechanism (Figure 6). The first one uses the *cos* site on the phage genome as a stop and start signal during each round of DNA packaging. This sequence, together with adjacent regions, is recognized by the terminase that cleaves off unit length chromosomes from the concatameric DNA molecule (Figure 7a). The resulting protruding self-complementary single-stranded termini (called cohesive ends) allow circularization and ligation of phage DNA (Figure 7b) and the genome is encapsitated in the procapsid (Figure 7c and Figure 7d).After its injection in the cells, the circular DNA molecule becomes the substrate for replication or integration. This mechanism has been well characterized in the phage λ, T3 and T7 (Oliveira *et al*., 2005).

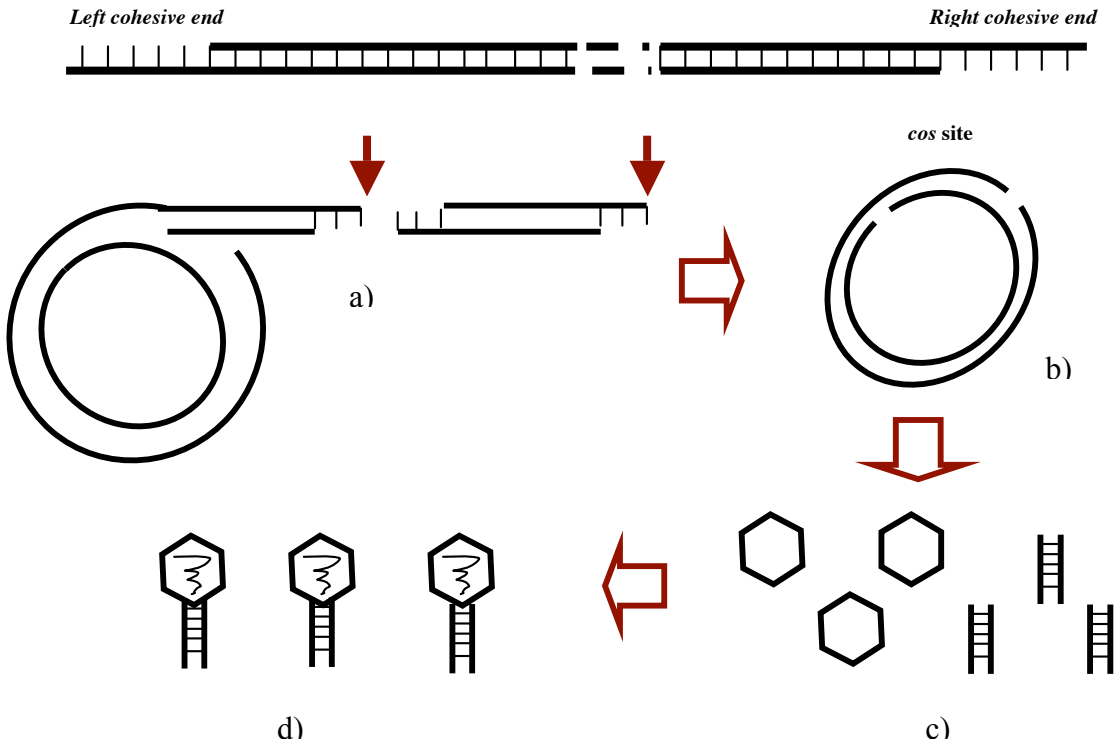

Figure 7: *Unit-lenght mechanism.*

In the headful mechanism (Figure 8), the packaging starts with the recognition of a specific sequence, called *pac*, leading to initial endonucleolytic cut (*pac* cleavege). When a threshold amount of the genome has been packaged, the terminase introduces a sequence-indipendent cut (headful cleavage). The termination cut separates the first headful from the concatamer, which serves now as a substrate to fill a second pro-head. Subsequent encapsidation begins at the end created by the previous event. This mechanism leads to the generation of terminally redundant and partially circularly permutated DNA molecules. The DNA packaging machinery thus uses two substrates for packaging: a *pac* sequence in the first packaging cycle, and a DNA end generated by headful cleavage in the following encapsidation cycles. The control to the specificity by the heedful cleavage apparatus is

essential to ensure the end of the reaction that can lead to more than 12 sequential packaging cycles along a single substrate concatemer (Oliveira *et al*., 2005).

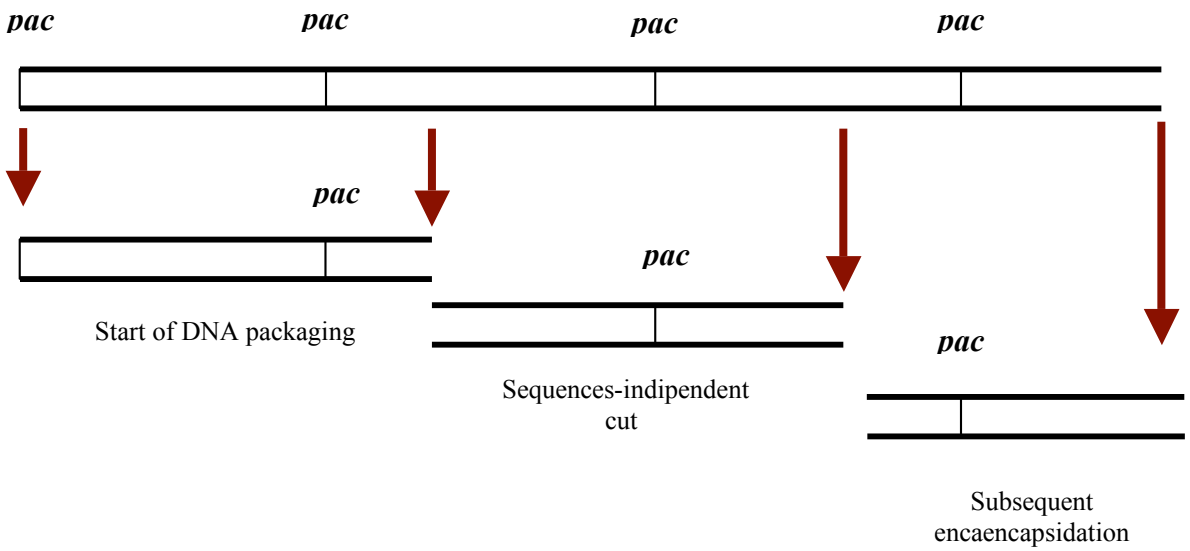

Figure 8: *Headful mechanism*

Usually, *S. thermophilus* pahges are divided in *cos*- or *pac*- type and the mechanism was determined by restriction analysis. Le Marrec *et al*. (1997) analyzed 30 *S. thermophilus* phages by restriction analysis with enzymes *Eco*RV, *Hind*III and *Pvu*II. By the use of various restriction enzymes it was found that all phage genomes contain sub-molar fragments and comparing heated and non-heated digests of linear phage DNA, it was possible to distinguish the phages within two groups. In fact, when the digests were heated for 10 minutes at 80°C the loss of one band and the appearance of two smaller bands in the restriction profile indicated that these genomes were packaged by *cos*-mechanism. On the contrary, when the treatments by heating did not give two smaller bands the genome was packaged by *pac*-mechanism. Upon examination, it was also found that the *cos*-containing and *pac*-containing phages possessed two and three major structural proteins, respectively. Further results suggested the presence of specific conserved DNA regions for each group,

which could conceivably encompass the genes specifying structural proteins and the packaging machinery. Tremblay and Moineau (1999) suggested that the availability of two packaging systems and two sets of structural proteins in *S. thermophilus* phages could represent an adaptive response to a particular dairy environment or to a specific host. The latter is of interest because lactic acid bacteria are known to possess an arsenal of anti-phage systems (Dinsmore and Klaenhammer, 1995). Because some of these phage proteins share homology to other lactic acid bacteriophages, horizontal transfer could be a possible mechanism for creating this diversity (Tremblay and Moineau, 1999).

Head proteins

The *cos*-site temperate *S. thermophilus* phage Sfi21, the lactococcal phage BK5-T and the *Lb. gasseri* phage adh have a shared gene organization for the head module indicated by the presence of the small and large terminase portal protein protease-major head protein (mhp) (Desière *et al*., 2000). It was reported that in adh (Altermann and Henrich, 2003) and Sfi21 phage (Bruttin *at al.*, 1997), the N-terminal sequence of the mature mhp in the phage particle started at aa position 104 and 105 respectively of the predicted protein. The mph from lactococcal phage c2 was identified by a combination N-terminal sequencing and immunogold electron microscopy and the comparison with the gene sequence suggested that the mhp was cleaved at aa position 205 just downstream of a protein domain with a strong coiled-coil prediction. The mhp showed to be covalently cross-linked into trimers and hexamers (Lubbers *et al*., 1995). The mhp was also identified for *pac- S. thermophilus* phage O1205 where the N-terminus of the mature protein corresponded to the predicted gene sequence.

Tail proteins

The genome organization of the putative head-to tail joining and tail genes is remarkably well conserved in temperate *cos*-site and *pac*-site dairy phages (Brussow, 2001). The gene coding the major tail protein has been identified in many dairy phages by N-terminal sequencing combined with electron microscopy. In many dairy phages the tail morphogenesis module encoded an unusually long multi domain protein which covers up to 13% of the total coding capacity of the phages. Its topological position corresponded to that of the λ gene *H* encoding the tape measure protein. The construction of an in-frame deletion or duplication of 2% in this gene from *Lactococcus lactis* phage TP901-1 shortened or lengthened the phage tail by approximately 30%, respectively (Pedersen *et al*., 2000).

The comparison of closely related *S. thermophilus* phages that differ in host range linked a single gene at the topological position of the λ *J* gene with the host range phenotype both in *cos*- and *pac*-site phages. Lucchini *et al*. (1999) by multiple alignments divided four streptococcal proteins identified in C-terminal within two highly conserved domains separated by a variable domain. The first highly conserved domain showed conspicuous collagen-like repeats (Desière *et al.,* 1998). Similarly the receptor-recognizing protein in *E. coli* T4 phage also showed a hypervariable region separated by conserved domains containing oligoglycine streches (Tètart *et al.,* 1998).

Lysis cassette

Two different classes of lytic enzymes were found in dairy bacteriophages. The *Lactobacillus* phages mv1, LL-H and phi-g1e and *Lactococcus* phages LC-3 and Tuc2009 possessed muraminidase, whereas *S. thermophilus* phage and *Lactococcus* phages BK5-T

and US3 contained amidases (Brussow, 2001). Both the classes of lytic enzymes have a two-domain structure: the N-terminal half containing the enzymatic activity and the C-terminal half that constitutes the substrate binding domain. Sheenan *et al.* (1996) demonstrated that the rearrangement of the domains by genetic engineering allowed the creation of chimeric lysins with new properties. In the *S. thermophilus* phages the lysine is actually preceded by two holins, a type I and a type II holin, defined by three and two transmembrane domains, respectively (Brussow, 2001). Sheenan *et al.* (1999) showed that the expression of the type I holin resulted in a modest decrease in cell viability, whereas expression of type II holin killed the cells. Furthermore, expression of the holin from phage phi-g1e yelded empty ghost cells with intact cell walls (Oki *et al.* 1997). In the *S. thermophilus* phage DT1 the holin (ORF24) and lysin (ORF25) of phage DT1 were closely related to lysis cassettes of other Gram-positive phages. The lysine of DT1 was considerably shorter (70 to 80 amino acids) than other analogous lysins (Tremblay and Moineau, 1999). The N- terminal DT1 phage lysine was more conserved than the C-terminal part. Loessner *et al.* (1995) reported that in the *Siphoviridae* family, the catalytic activity of the phage lysine is located at N-terminal end, whereas the target recognition is the C-terminal domain. Although the lysin genes of other *S. thermophilus* phages were preceded by two holin genes, only one gene with holin characteristics was found in phage DT1.

Bacteriophage defense strategies

To minimize the impact of phage infection, the dairy industry designed several countermeasures. In this section will be discussed briefly phage defence strategies, as specific actions that, when properly directed, reduce the number or type of phages in diary environment.

Improving sanitation and manufacturing processes

The use of higher quality milk substrate and the pasteurization and sanitation regimes are critical to control phage contamination within dairy facilities. However, most phages are not completely inactivated by standard pasteurization treatments and when survive they could infect starter cultures leading to the failure of fermentation (Binetti and Reinheimer, 2000). To inactivate the phages during the routine sanitation sodium hypochlorite (100 ppm) and peracetic acid (0.15%) are resulted very effective. Other biocides, as 75-100% ethanol and isopropanol, exhibit suboptimal biocidal activity and are generally used only in laboratory settings (Binetti and Reinheimer, 2000).

To minimize the risk of phage attack, special care should be taken in the starter room. When the bulk starter is contaminated the cultures can be lysed. Other methods, such as the use of closed cheese vats and concentrated direct vat inoculation, eliminating the need for bulk starter systems, have also used to reduce the impacts of phage contamination. Finally, the problems associated with scale up propagations can be largely limited using frozen concentrates, which eliminate the need for intermediate transfers (Klaenhammer, 1984).

Phage inhibitory media

The use of phage inihibitory media has been widely adopted. These media are supplemented with phosphates and citrates in order to chelate divalent cations, particularly calcium. When the calcium is not available, proliferation of most bacteriophages is inhibited.

Strain- rotation

The repeated use of the same defined starter culture under non-aseptic processing conditions amplifies the phage proliferation and their infection in dairy environment. Starter cultures

can be defined or undefined strain composition and can be used with or without culture or strain rotation (Cogan *et al*., 1991). The starter cultures usually contain few well-characterized phage-unrelated strains and posses defined fermentation performance.

In defined systems, rotation is a process whereby sensitive strains are replaced with one or more non-lysogenic and phage-unrelated strains with similar fermentative properties.

In recent years the use of multiple-strain starters has provided an alternative to traditional rotation programs. Multiple-strain starters are composed of three to six selected strains of lactic streptococci that are used continuously in the plant as part of the phage-monitoring program. The success of the multiple-strain starter is dependent upon selection of phage-unrelated strains that resist attack by phages present in plants targeted for use of culture.

An alternative method to traditional strain rotation is a plasmid intracellular rotation developed in lactococci, but also used in *S. thermophilus* strains (Durmaz and Klaenhamer, 1995, O' Sullivan *et al.,* 1998). By this process a number of phage-resistent derivatives of a single strain are built by introducing a variety of phage defence plasmids of different natures and specificity (e.g abortive infection and restriction and modification systems) and then they are commonly rotated. Since the most widely accepted approach for the introduction of heterologous DNA is the use of conjugation (Klaenhammer and Fitzgerald, 1994), when used properly, these rotation strategies could significantly extend the longevity of strains in the diary environment. Although the strain rotation constitutes a main tool to defence from phage infection it was documented that the concurrent use of large number of phage unrelated strains at one time would increase the size of the available gene pool and might stimulate the emergence of new virulent phages by mutation or recombination (Hull, 1985).

Molecular strategies

The specificity of phage adsorption to cell surface receptors has been well studied in *E. coli* and other Gram-negative bacteria. In Gram positive bacteria phage adsorption almost always involves the cell surface carbohydrates and specific studies have been carried out for phages of *Lactococcus* species and recently for *S. thermophilus.* Quiberoni *et al*. (2000) have characterized the *S. thermophilus* phage receptor by purifying cell walls from two *S. thermophilus* strains, YSD10 and BJ15, by treatments with sodium dodecyl sulphate and proteinase K. These treatments did not reduce the adsorption of phages CYM and 0BJ to the cell walls of YSD10 and BJ15, respectively. However, phage binding was reduced when the cell envelopes were treated with mutanolysin or 5% trichloroacetic acid, suggesting that the phage receptor component is part of the peptidoglycan or another polymer closely linked to it. In further experiments the authors tested the ability of several saccharides to inactivate both phages. These assays indicated that the phage CYM was adsorbed to a component involving glucosammine and rhamnose, while glucosamine and ribose interfered with adsorption of phage 0BJ. In *L. lactis*, the adsorption of c2-type phages involves the phage tail protein that adsorbs to a carbohydrate component (rhamnose) of the cell wall and then the phage particle becomes irreversibly anchored to a membrane associated infection protein (Pip) (Geller *et al*.,1993; Monteville *et al*., 1994).

The generation of bacteriophage insensitive mutants (BIM) by spontaneous mutation or chemical mutagenesis is widely studied (Coffey and Ross, 2002). The random introduction of pecific mutation could confer partial or total resistance to phages. Although is easy to isolate BIMs, sometimes they show a variety of negative qualities and consequently they are excluded during product manufacture. Other problems commonly associated with the use of BIMs are frequent reversion to the phage sensitive phenotype and insensitive to closely

related phages. Furthermore, it is often difficult to localize the genes that have been mutated, since they may be located anywhere in the bacterial genome. To improve the use of BIMs, Lucchini *et al*. (2000) describes the use of pG$^+$host9Iss1 based insertional mutagenesis to identify genes involved in bacteriophage sensitivity. By this mutagenesis the genes interrupted by the integrated plasmid are cloned and the vector sequences can be removed from the chromosome by recombination while leaving a single integrated copy of ISS1 in the chromosome. Using this method four encoded loci involved in bacteriophage sensitivity were identified (Lucchini *et al*., 2000). The *orf*394 that encoded a putative transmembrane protein and the gene product gp394 were identified. When mutated the gp394 conferred complete resistance to all *S. thermophilus* phages tested. Other authors (Garbutt *et al*., 1997) suggested gp394 as analogous to the lactococcal Pip, which is essential for infection of *L. lactis* c2-like bacteriophages.

Outlook of thesis

Bacteriophage infection is still a main cause of fermentation failures and consequently of economic losses for diary industry, in which lactic acid bacteria are widely used.

This study focuses on research of lytic and temperate phages from mozzarella cheese whey and their characterization in order to provide efficient tools for development and selection of phage resistant starters and to offer knowledge to apply a well organized antiphages strategy. Chapter 2 is focused on investigation LAB bacteriophages from Natural Whey Cultures used for mozzarella cheese manufacture. In Chapter 3, *S. thermophilus* bacteriophages are characterized in terms of phenotypic and genotypic features. Finally, the chapters 4 and 5 describe the evidence of a *S. macedonicus* temperate phage and a progress on its DNA sequencing by genome walking.

Chapter 2

Investigation on the presence of LAB bacteriophages in Natural Whey Cultures used for traditional water-buffalo Mozzarella Cheese manufacture

SUMMARY

Twenty-two natural whey cultures (NWCs) used for water-buffalo Mozzarella cheese manufactures were analysed for the presence of bacteriophages. One-hundred and two thermophilic and mesophilic LAB isolates from the same NWC sample were tested as indicators towards filtered NWC. No bacteriophage was found by this procedure in the samples tested.

INTRODUCTION

Most of the bacteriophage-related problems have occurred with massive industrial use of defined strain starter cultures. Consequently, the dairy industry has developed starter cultures systems to minimize the impact of phage infection (Brussow *et al*., 1994; Klaenhammer and Fitzgerald, 1994; Ravin *et al*., 2002).

Undefined cultures, both mesophilic and thermophilic, which contain unknown numbers of strains are usually less affected by the presence of virulent phages (Filosofo *et al*., 1995; Brussow, 2001). Natural (artisanal) whey cultures (NWCs), commonly used to produce water-buffalo mozzarella cheese in the South of Italy, are a typical example of undefined cultures. NWCs are commonly prepared daily in the cheese plants under non-aseptic conditions, without any protection against phages (Zago *et al*., 2006).

Water buffalo mozzarella cheese is a typical "pasta filata cheese" from Southern Italy having high moisture (55 to 62 %) and high fat in DM (>45%) and characterized by soft body and juicy appearance and by a pleasant, fresh, sour, and slightly nutty flavour (Mauriello *et al.,* 2003) and the natural whey cultures, from the manufacture of the previous day, is used as starter. For undefined starter cultures only minor effects on the ability to produce lactic acid are normally observed and the coexistence of phage and sensitive strains without evidence of acidifying activity failures has been shown in natural whey cultures for Grana Padano and Provolone cheese (Zago *et al*., 2005).

In this study, twenty-two NWCs were collected from water-buffalo mozzarella cheese plants of Caserta and Salerno provinces and investigated for the presence of phages active against mesophilic and thermophilic LAB strains.

EXPERIMENTAL PROCEDURES

NWC samples

NWC samples were collected from twenty-two different local water-buffalo mozzarella cheese plants in provinces of Caserta and Salerno. The samples collected did not show evidence of acidifying activity failures. Samples were cooled in ice immediately after collection for transportation and then kept at -18 °C until being used.

Enumeration, bacterial strains isolation and culture conditions

Standard enumeration methods were used to determine the LAB populations in the samples: serial decimal dilutions were made and microbial populations were target in duplicate as follows: M17 agar (Oxoid) incubated in aerobic condition at 30 °C for 48 h; M17 agar incubated in anaerobic condition at 43 °C for 24 h; De Man, Rogosa, Sharpe (MRS) agar (Oxoid) incubated in aerobic condition at 30 °C for 48 h, MRS agar incubated in anaerobic condition at 43 °C for 24h. Colonies of LAB strains were randomly isolated from countable M17 and MRS plates, purified, tested by KOH 3% and H_2O_2 3% and stored at –40 °C in M17 and MRS broth with 15% glycerol.

Analysis of the NWC samples for the presence of phages

The whey samples were analyzed to verify the presence of phages: the samples were centrifuged (10 min at 8000 *g*) and filtered by 0.45 μm pore size filter (Minisart®-plus, Sartorius AG, Goettingen, Germany). The filtrates were used to investigate by means the spot test and by turbidity test. Briefly, the log phase of pure cultures (isolated from the same samples) were mixed with M17 or MRS soft agar (0.5 % w/v) and plated as a thin top layer on M17-Ca/Mg or MRS-Ca/Mg (10 m*M* $CaCl_2$, 10 m*M* $MgCl_2$) agar (1% w/v) plates. Aliquots of 10 μl of filtrates suspected containing phages were spotted on the plates. After incubation at the optimal conditions of growth for bacterial strains tested, the presence or absence of lysis zones was recorded. The turbidity test was carried out inoculating M17-Ca/Mg or MRS-Ca/Mg broth with each strain at 1% and adding 1 ml of filtered NWC followed by incubation at optimal condition of growth for bacterial strains assayed. In order to enhance phages amplification, this procedure was cyclically repeated three times. The tubes were examined visually at regular time intervals.

Detection of Streptococcus thermophilus *phages by PCR*

To detect *S. thermophilus* phages directly in whey samples, it was carried out a simple protocol developed by Binetti *et al*. (2005), amplifying the variable region VR2 of antireceptor gene of tail morphogenesis module (*orf18*). The PCR reactions were performed in a total volume of 50 μL containing 125 μ*M* deoxynucleoside triphosphate, 5 μ*M* concentrations of the 2 primers, HOST1 and HOST5 (Table 1) 2.5 U of *Taq* DNA polymerase, *Taq* buffer (20 m*M* Tris-HCl pH 8.4, 1.5 m*M* magnesium chloride, 50 m*M* potassium chloride), and 1 μL of each whey sample. Whey sample inoculated with *S. thermophilus* phage DT1 at 10^5 PFU/ml was used as positive control. The PCR products were

separated on a 1% agarose gel in TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA), stained with ethidium bromide, and visualized under UV light.

Table 1: *Primers used for detection of* S. thermophilus *phages.*

Primer	Sequence, 5'- 3'	Reference
HOST1	GAATGATACTGCTGGCAGTATTTCGGTTGG	Binetti *et al*., 2005
HOST5	CAGTCATGTAGCTATCGATGAAATTCCAACG	Binetti *et al.,*2005

RESULTS

Enumeration of lactic acid bacteria

The results obtained by the enumeration of lactic acid bacteria in NWC samples analyzed are shown in table 2. The LAB load was quite heterogeneous: the mesophilic bacteria on M17 agar ranged from 1.1 10^4 (sample AB) to 1.0 10^8 (sample BM), while the mesophilic load on MRS ranged from 1.5 10^4(sample AA) to 1.8 10^7 (samples BI and BL). The thermophilic bacteria on M17 agar ranged from 1.5 10^5 (sample AM) and 2.2 10^7 (sample BF) while the thermophilic strains grown on MRS agar ranged from 1.2 10^6 (sample AB) to 3.0 10^7 (sample BL).

Table 2: *Mesophilic and thermophilic lactic acid bacteria loads in NWC samples.*

NWC Sample	M17 CFU/ml		MRS CFU/ ml	
	30 °C	**43° C**	**30 °C**	**43° C**
AA	$4.0\ 10^5$	$2.1\ 10^6$	$1.5\ 10^4$	$1.7\ 10^7$
AB	$1.1\ 10^4$	$1.5\ 10^6$	$1.1\ 10^5$	$1.2\ 10^6$
AC	$2.3\ 10^5$	$1.3\ 10^6$	$1.5\ 10^6$	$2.3\ 10^7$
AD	$1.5\ 10^6$	$1.2\ 10^7$	$1.3\ 10^5$	$1.3\ 10^7$
AE	$2.0\ 10^6$	$1.\ 3\ 10^6$	$1.3\ 10^6$	$1.4\ 10^6$
AF	$2.3\ 10^5$	$2.1\ 10^7$	$2.6\ 10^4$	$2.2\ 10^6$
AG	$1.6\ 10^6$	$1.5\ 10^7$	$1.1\ 10^5$	$1.0\ 10^7$
AH	$2.0\ 10^6$	$2.3\ 10^6$	$2.5\ 10^6$	$2.1\ 10^7$
AI	$1.0\ 10^6$	$1.8\ 10^6$	$2.8\ 10^4$	$2.1\ 10^6$
AL	$1.6\ 10^4$	$1.5\ 10^7$	$3.2\ 10^5$	$1.1\ 10^7$
AM	$1.5\ 10^5$	$1.5\ 10^5$	$1.2\ 10^5$	$1.5\ 10^7$
BA	$3.0\ 10^6$	$3.1\ 10^6$	$1.0\ 10^6$	$2.7\ 10^7$
BB	$2.1\ 10^5$	$1.8\ 10^5$	$2.1\ 10^7$	$2.0\ 10^7$
BC	$4.3\ 10^6$	$1.3\ 10^5$	$1.3\ 10^7$	$3.3\ 10^6$
BD	$1.\ 0\ 10^6$	$1.0\ 10^7$	$1.1\ 10^6$	$2.8\ 10^7$
BE	$1.0\ 10^5$	$1.0\ 10^6$	$1.0\ 10^7$	$1.3\ 10^7$
BF	$4.3\ 10^7$	$2.2\ 10^7$	$4.6\ 10^6$	$1.2\ 10^7$
BG	$1.1\ 10^5$	$1.8\ 10^7$	$2.1\ 10^6$	$1.4\ 10^7$
BH	$1.6\ 10^6$	$1.3\ 10^7$	$1.5\ 10^7$	$1.7\ 10^7$
BI	$2.0\ 10^5$	$1.0\ 10^5$	$1.8\ 10^8$	$1.3\ 10^6$
BL	$1.0\ 10^7$	$5.5\ 10^5$	$1.8\ 10^8$	$3.0\ 10^7$
BM	$1.\ 0\ 10^8$	$1.2\ 10^6$	$1.8\ 10^7$	$5.5\ 10^6$

Analysis for the presence of bacteriophages

For the detection of phages an aliquot of the natural whey starter, deprived of the bacterial cells by filtration, was used to infect 102 mesophilic and thermophilic lactic acid bacteria previously isolated from the same starters. The filtrates were used to investigate by means both spot and turbidity test. By these experiments it was not possible to make in evidence the presence of bacteriophages.

Detection of **Streptococcus thermophilus** *phages by PCR*

The method described by Binetti *et al*. (2005) was used as tool for phage detection in this study. Since by this specific PCR amplification, the detection limit for phage-contaminated milk is 10^5 PFU/ml, a whey sample inoculated with the same concentration of *S. thermophilus* phage DT1 was used as positive control. The PCR did not show the presence of an amplification product for each NWC sample assayed.

DISCUSSION

Natural whey cultures are widely used in Italy for many typical cheese manufactures and in particular for pasta filata cheeses production in South of Italy. Although they are prepared daily in cheese plants under non-aseptic conditions they show low susceptibility to the phage attack. It is probably due to their complex bacterial composition in which susceptible strains are rapidly substituted by resistant population. In these natural starters the lytic phages often coexist with bacterial strains (Daly and Fitzgerald, 1989) and both undergo to a constant selective pressure.

When the acidification is delayed or arrested during dairy fermentation due to a suspected phage infection, the first common methods to assay the whey sample for the presence of virulent phages consists to apply standard microbiological methods (Svensson and Christiansson, 1991).

In previous studies (Filosofo *et al*., 1995; Zago *et al*., 2005) it is reported that NWC samples, which did not show acidification failure, contained bacteriophages that was possible source of infection for the following manufactures. The isolation and the characterization of these bacteriophages could represent an efficient knowledge to implement strategies to prevent the loss of product.

Zago *et al*. (2005) investigated twenty-eight samples of natural whey starters used for the production of various Italian long-ripened cheeses to evaluate the presence of phage active against thermophilic lactobacilli. They reported that the phages of *Lactobacillus helveticus* and *L. delbrueckii* subsp. *lactis* were found in 16 out of 28 samples of natural whey starters which did not show delay or failure in the acidifying activity. Out of the strains tested in the same study the 15% of *L. helveticus* and early the 30% of *L. delbrueckii* subsp. *lactis* lysed when natural whey starter filtrates were added. Furthermore, only 15 of the 54 *L. helveticus* phages and 19 of the 27 *L. delbrueckii* subsp. *lactis* phages were able to lyse host strains by testing on agar medium and to form lysis plaques.

In this study twenty-two whey samples, where there was not evidence of acidification failure, collected in different cheese plants, are analyzed for the presence of bacteriophages by standard microbiological approach. These methods did not result capable to detect the phages and an alternative strategy was implemented.

Zago *et al*. (2006) optimised a PCR-based system amplifying an internal fragment of the major tail protein (MTP) gene (*g17*) for a specific detection of *Lb. delbrueckii* subsp.*lactic* phages from undefined starter cultures of Italian hard cheeses. Prior of this, Brussow (1994)

described a PCR protocol to detect *S. thermophilus* phages in whey samples. Finally, Duplessis and Moineau (2001) characterized the antireceptor gene (*orf*18) of the tail morphognesis module and designated the variable region VR2 as responsible for host specificity. This region was found in all bacteriophages and it is flanked by highly conserved region. Based on these data, Binetti *et al*. (2005) developed a PCR methods that allows *S. thermophilus* phages to be detected, providing a sensitive system useful to the dairy industry.

Since *S. thermophilus* is the most technologically important lactic acid bacteria in mozzarella cheese manufacture, to detect the *S. thermophilus* phages directly in whey samples is considered interestingly.

In this study the PCR protocol suggested by Binetti *et al*. (2005) was applied by using the natural whey starter as template to detect the *S. thermophilus* phages. The absence of amplification fragments suggested that the limit of detection of this protocol is not suitable to these samples.

Our results confirmed that the richness in the microbial composition of these natural whey starter cultures, characterized by the presence of an unknown number of LAB strains represents the first natural barrier against fatal phage infection. The quick replacement of phage sensitive strains by phage resistant mutants and the contribution of a microflora with different phage resistance patterns, probably help to renovate the starter activity.

Chapter 3

Characterization of *Streptococcus thermophilus* Lytic Bacteriophages from Mozzarella Cheese Plants

SUMMARY

In this study 26 *Streptococcus thermophilus* bacteriophages isolated from mozzarella cheese plants were characterized in terms of their host range, DNA resctriction profile, DNA packaging mechanisms and the antireceptor variable region VR2. The DNA restriction analysis was carried out by using *Eco*RV, *Pst*I and *Hind*III. The bacteriophages were classified into two main groups of *S. thermophilus* phages (*cos*- and *pac*-type) using a multiplex PCR method based on the amplification of conserved regions in the genes coding for the major structural protein. All the phages belong to the *cos*-type group, whereas only one of them gave a PCR fragment distinctive of *pac*-type group. Furthermore, the amplification of the variable region of the antireceptor gene VR2 allowed to classify the phages and verify the correlation between typing profile and host range.

INTRODUCTION

Phage attack is a main cause of fermentation failure during the manufacture of mozzarella cheese. Dairy fermentations are vulnerable to phage infection for several reasons: i) contaminating phages are dispersed in fluid milk; ii) repeated use of defined culture under non-aseptic processing conditions provides a constant host for phage proliferation (Klaenhammer and Fitzgerald, 1994; Neve *et al*., 1995); and iii) lysogenic bacteria may also be a phage source. The dairy industry has implemented many methods to reduce the consequences of phage infection such as ordinary disinfection of equipment, direct vat inoculation, propagation of starter cultures in phage inhibitory media, strain rotations and application of phage-resistant multiple strain starters (Everson, 1991). *Streptococcus thermophilus* strains are predominant in starter cultures used in the mozzarella cheese production and it is well known that they are often susceptible to phage attack resulting in slow lactic acid fermentation and loss of product quality. Since, beside *Lactococcus lactis, Streptococcus thermophilus* is considered the most technologically important lactic acid bacteria by dairy industry, the characterization of its lytic phages is an important tool for the selection of efficient starter cultures. Removing the sensitive strains and replacing them with

strains resistant to the phage infection is an efficient approach for correct management of a defined culture rotation system.

Streptococcus thermophilus phages belong to B1 Bradley's group, having a hexagonal capsid and a long noncontractile tail. They are also divided into two groups (*cos* and *pac*-types) based on the number of major structural proteins and the encapsidation mechanism of double-stranded DNA (Le Marrec *et al*., 1997). It is well known that each bacteriophage can infect different bacterial strains and the definition of the host range is an important feature of bacteriophages to assess. The phage-hosts interactions were widely studied, and Duplessis and Moineau (2001) identified the phage genetic determinant (antireceptor) likely to be involved in the recognition of *Streptococcus thermophilus* hosts. They, in fact, characterized the antireceptor gene (*orf*18) of the tail morphogenesis module, finding the variable region VR2 responsible for the host specificity. The VR2 sequence was also used to classify *Streptococcus thermophilus* phages and verify the correlation between typing profile and host range (Binetti *et al*., 2005).

EXPERIMENTAL PROCEDURES

Bacterial strains, bacteriophages and culture conditions

Bacterial strains and bacteriophages used in this study are reported in Table 1 and were from Chr. Hansen culture collection. Phages were isolated from an abnormal mozzarella cheese manufactures. All *Streptococcus thermophilus* strains out of AT1 were isolated from milk samples, instead *Streptococcus macedonicus* and *Streptococcus thermophilus* AT1 were isolated from natural whey cultures used for traditional mozzarella cheese manufacture. Bacterial strains were identified by sequence analysis of the 16S rRNA gene.

Host strains were conserved as frozen at -80 °C in M17 (Oxoid) with 2% lactose, supplemented with 15% glycerol, and routinely cultured overnight in M17 broth at 42 °C. Phage stocks were prepared by addition of phages to an actively growing M17-Ca broth (M17 supplemented with10 m*M* $CaCl_2$) culture of the appropriate host. Host cultures were incubate at 42°C until lysis was complete. Unlysed cells were removed by centrifugation at 8000 g 14 for 10 min and the surnatants were filtered by 0.45 μm pore size filters (Minisart® plus Sartorius AG, Goettingen, Germany). These phage preparations were then stored at -80°C with 15% glycerol. Phage enumeration (pfu/mL) was performed by the double-layer plaque titration method (Svensson and Christiansson, 1991), using M17-Ca agar and incubated at 42°C.

Phage multiplication

The multiplication of each bacteriophage was carried out according to the method follow described.

Overnight *S. thermophilus* host bacteria culture was inoculated (1%) into 10 mL of M17-Ca broth, infected with its virulent phage suspension at a multiplicity of infection from 0.1 to 1 and incubated at 42°C until complete lysis occurred. Then, the incubation was prolonged and 1 mL of bacterial culture was added at 1 h intervals for 4-5 times, in order to obtain a further amplification of the phage population.

Host range

The sensitivity of *Streptococcus thermophilus* and *Streptococcus macedonicus* strains to 26 phages was determined. Briefly, 0,1 mL of the log phase of each cultures was mixed with M17 soft agar (0.5 % wt/vol) and plated as a thin top layer on M17-Ca/Mg (10 m*M* aCl_2, 10

m*M* $MgCl_2$) agar (1% wt/vol) plates. Aliquots of 10 μL of lysates were spotted on the plates. After incubation at the conditions of growth, the presence or absence of lysis zones was recorded.

Phage DNA isolation

The DNA isolation was carried out by centrifugation of 1 mL of fresh phage lysate at 10000 g for 10 min. The RNase/DNase (1 mg mL^{-1}) was added and the preparation was incubated at 37 °C for 30 min. The supernatant was transferred to another tube and 100 μL of an SDS mixture (0.5 *M* Tris-HCl, 0.25 *M* EDTA, 2.5% SDS) were added. The solution was mixed for few seconds and then was incubated at 65°C for 30 min. Then, 125 μL of 8 *M* potassium acetate were added, and the preparation was mixed and placed on ice for 30 min. After centrifugation at 16100 g for 30 minutes each sample was extracted twice with phenol-chloroform (1:1). The DNA was precipitated with an equal volume of isopropanol and each pellet was resuspended in 20 μL of water. Phage DNA was quantified by electrophoresis on agarose (0.7 wt/vol) gel. The visualization by ethidium bromide staining was performed according to the standard protocols (Sambrook *et al*.1989).

Restriction analysis

Purified phage DNA was digested by using three endonucleases (*Hind*III, *Pst*I, *Eco*RV) (FastDigest™ Fermentas) used according to the manufacture's instructions. Restricted phages DNA were electrophoresed in a 0.8 agarose gel in TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA), stained with ethidium bromide and visualized under UV illumination.

Determination of DNA packaging mechanism

To rapidly classify *Streptococcus thermophilus* phages within one of 2 groups (*cos*– and *pac*-type) Quiberoni *et al.* (2006) have developed a multiplex PCR by using two pairs of primers (one per phage group Table 2) designed from the conserved regions of the gene coding for the major capsid protein in the phages for which the complete genome is available. The PCR reactions were performed in a total volume of 50 µL containing 125 µ*M* deoxynucleoside triphosphate, 5 µ*M* concentrations of the 4 primers, 2.5 U of *Taq* DNA polymerase, *Taq* buffer (20 m*M* Tris-HCl pH 8.4, 1.5 m*M* magnesium chloride, 50 m*M* potassium chloride), and 1 µL of the phage lysate. Phages DT1 (Tremblay and Moineau, 1999) was used as *cos*-type positive control. Phage DT1 was propagated on *Streptococcus thermophilus* SMQ-301. A negative control (without the template) was included for all PCR assays to eliminate the possibility of contamination.

The conditions of PCR amplifications were set as follows: 5 min at 94°C, followed by 35 cycles (45 s at 94°C, 45 s at 53°C, 1 min at 73°C), and a final step of 5 min at 73°C. The PCR products were separated on a 2% agarose gel in TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA), stained with ethidium bromide, and visualized under UV light. The fragments of 170 bp and 427 bp were distinctive of the *cos*-type and *pac* -type group, respectively.

Amplification and sequencing of the antireceptor variable region

The amplification of the variable region (VR2) involved in host recognition was performed as suggested by Binetti *et al*. (2005). The PCR reactions were performed in a total volume of 100 µL containing 125 µ*M* deoxynucleoside triphosphate, 5 µ*M* concentrations of the 2 primers, HOST1 and HOST5 (Table 2), 2.5 U of *Taq* DNA polymerase, *Taq* buffer (20 mM Tris pH 8.4, 1.5 m*M* magnesium chloride, 50 m*M* potassium chloride), and 2 µL of the phage

lysate. The PCR products were separated on a 1% agarose gel in TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA), stained with ethidium bromide, and visualized under UV light.

The PCR products, whose sizes were between 700 and 800 bp, were purified by using QIAquick PCR purification kit (QIagen, Milan, Italy) and sequencing was performed by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). Research for DNA similarity was performed with the National Centre of Biotechnology Information GenBank (Altschul *et al*. 1997). Phylogenetic analysis was performed using MEGA version 4.0 (Tamura *et al.,* 2007) after multiple alignment by the ClustalW 1.8 programme (Thompson *et al.*, 1994). Distance matrix and neighbour-joining methods (Saitou and Nei, 1987) were applied for a dendrogram construction.

Table 2. *List of primers used in this study.*

Primer	Sequence, 5'- 3'	Reference
cos FOR	GGTTCACGTGTTTATGAAAAATGG	Quiberoni *et al*. (2006)
cos REV	AGCAGAATCAGCAAGCAAGCTGTT	Quiberoni *et al*. (2006)
pac FOR	GAAGCTATGCGTATGCAAGT	Quiberoni *et al*. (2006)
pac REV	TTAGGGATAAGAGTCAAGTG	Quiberoni *et al*. (2006)
HOST1	GAATGATACTGCTGGCAGTATTTCGGTTGG	Binetti *et al*. (2005)
HOST5	CAGTCATGTAGCTATCGATGAAATTCCAACG	Binetti *et al.* (2005)

RESULTS

Host range

Host range of 26 lytic phages on 17 *Streptococcus thermophilus* strains is reported in Table 1. Phages 574, 576, 604, 616 and 620 were capable to infect only the strain *Steptococcus thermophilus* CHCC2134. Also the couple of phages 641,671 and 1027,1033 and 1034,1040 infected only one strain, CHCC3049, CHCC4323 and CHCC4327, respectively. As reported in the Table 1, the other phages showed unique host range where the number of hosts ranged

from just 1 to 4. Out of the 11 above reported, 6 other bacteriophages showed to be virulent against only 1 strain, 3 against 2 strains, 4 against 3 strains and 2 against 4 strains. On the other hand, all *Streptococcus thermophilus* strains, with exclusion of the strain *Streptococcus thermophilus* AT1, were infected from at least one bacteriophage. No phage was able to infect the three *Streptococcus macedonicus* strains (data not shown). *Streptococcus thermophilus* CHCC2134 showed to be very sensitive to phage infection, resulting host for seven different bacteriophages. Instead, the strains *Streptococcus thermophilus* CHCC4131, CHCC4325, CHCC3048 and CHCC6592, resulted susceptible to only the phages 671, 1028, 1032 and 1042, respectively. All the other strains resulted sensitive to 2, 3 or 4 bacteriophages.

Table 1. *Host range of 26 bacteriophages against* 17 Streptococcus thermophilus *strains.*

Strains	**Bacteriophages**																									
	574	575	576	577	591	596	603	604	605	607	611	616	620	641	642	654	671	1027	1028	1032	1033	1034	1036	1040	1041	1042
CHCC3063	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-
CHCC2070	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CHCC2130	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHCC2133	-	-	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHCC2134	+	-	+	-	-	+	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
CHCC2389	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
CHCC3048	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
CHCC3049	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	-	-	-	-	-	-
CHCC3050	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
CHCC4131	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHCC4323	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-
CHCC4325	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
CHCC4327	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
CHCC4460	-	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
CHCC4895	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
CHCC6592	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
St At1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Restriction analysis

The DNA restriction analysis showed only *Hind*III and *Eco*RV as capable to give a pattern with 8-12 bands, suitable for the differentiation of the 26 bacteriophages, while the enzyme *Pst*I gave patterns with less than 3 bands (Figure 1). Most phages had a unique profile, instead the phages 604, 620 and 654 showed a similar pattern with 6 or 8 bands when their DNA were digested by *Hind*III and *Eco*RV, respectively. However, while the phages 604 and 620 had the same host range (they infected only *Streptococcus thermophilus* CHCC2134), the phage 654 revealed a different host range.

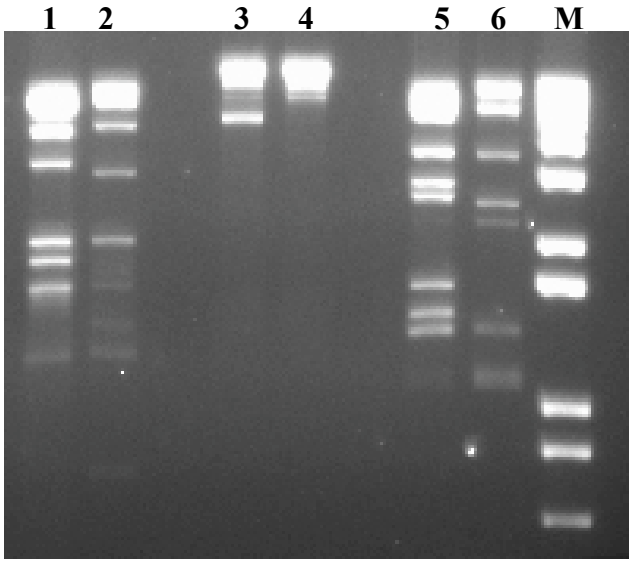

Figure 1. *Representative restriction profiles of the* Streptococcus thermophilus *bacteriophages 574 and 57* *digested by* Hind *III (lanes 1 and 2*), Pst*I (lanes 3 and 4, and* Eco*RV (lanes 5 and 6). Lane M, I Kb plus.*

Determination of DNA packaging mechanism

All the bacteriophages, with exclusion of 1042 and 575, showed an amplification fragment of 170 bp, indicating they belonged to the *cos*-type group. On the other hand only the bacteriophage 1042 yelded a PCR fragment of 427 bp, distinctive of the *pac*-type phage group. Surprisingly, the phage 575 able to infect only the strain *Streptococcus thermophilus* CHCC2070 (Table 1), showed both 170 bp and 427 bp amplification fragments (Figure 2).

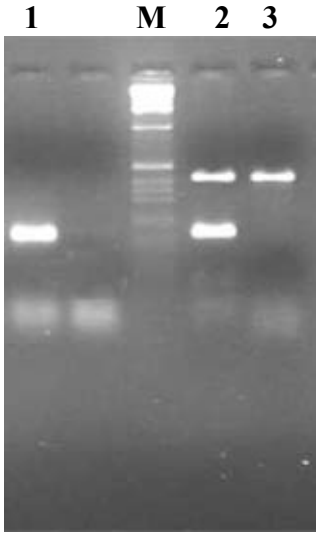

Figure 2. *Multiplex PCR for the detection of the* cos- *and* pac-*type groups of* Streptococcus thermophilus *phages.*
Lane 1, representative cos-*type phage 574; Lane 2, bacteriophage 575 showing both amplification products; Lane 3,* pac-*type phage 1042; lane M, 1 kb ladder.*

Bacteriophage clustering based on VR2 sequences

The antireceptor variable region VR2 was amplified for all bacteriophages assayed with exclusion of the phages 577, 607 642 and 1028. The PCR analysis showed an amplification fragment of 700-800 bp (Figure 3).

The cluster analysis of VR2 sequences from both bacteriophages used in this study and some *Streptococcus thermophilus* phages available in GenBank database gave a dendrogram where the bacteriophages of this study were grouped in 6 clusters at 90% similarity level (Figure 4). The cluster A grouped 7 bacteriophages at 100% similarity, the cluster B grouped 7 bacteriophages at about 92% similarity, the clusters C, D and F at 100% similarity grouped 2, 3 and 2 phages, respectively. Finally, only the phage 1041 belonged to the cluster E.

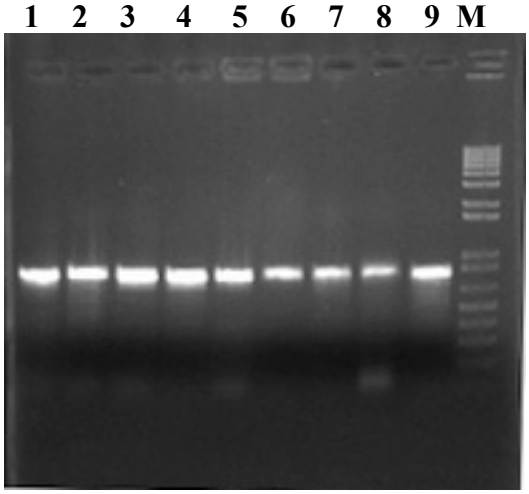

Figure 3 *Amplification of the antireceptor VR2 variable region of the bacteriophages in this study .Lanes from 1 to 9: phages 574, 575, 591, 596, 603,604,616,671, 1036, M 1kb plus ladder*

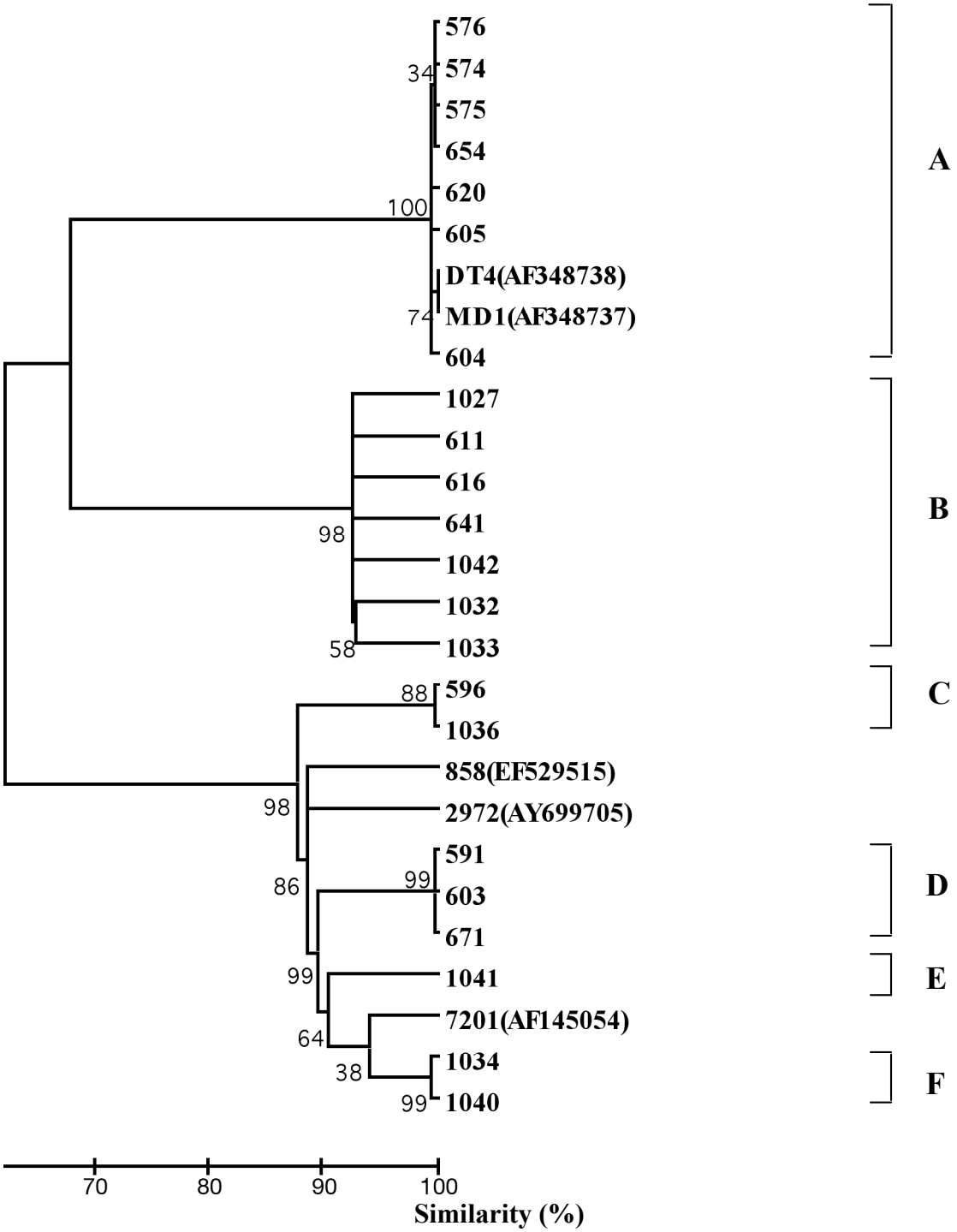

Figure 4. *Dendrogram (neighbor-joining method) showing the % of similarity of 27* Streptococcus thermophilus *bacteriophages on the basis of antireceptor VR2 sequences*

DISCUSSION

The increasing demand for dairy products in recent years has required increases in both production capacity and process efficiency. In this optic the use of starter culture properly meets these dairy industry needs. However, the repeated use of defined starter cultures under non-aseptic processing conditions amplifies the phage proliferation and their infection ability in dairy environments. The characterization of *Streptococcus thermophilus* phages is an important tool for the selection of phage-resistant dairy starter cultures. In this study 26 phages isolated from mozzarella cheese whey samples were analyzed on the basis of their host range, DNA packaging mechanism and DNA restriction profile. Furthermore, the phages were clustered considering the antireceptor variable region VR2 sequences and a correlation with host range was ascertained.

The phage population assayed showed 19 different host ranges suggesting a good variability in terms of infection capability. Interestingly, AT1 was the sole strain isolated from natural whey culture used for the production of traditional mozzarella cheese (data not shown). This environment is characterized by a very high microbial diversity, which probably represents a natural barrier against phage infection (Carminati *et al.,* 1997). This strain could be taken in account for the assessment of other industrial performances such as acidifying and proteolytic activity. On the contrary, the strain CHCC2134, as well as other multi-sensible strains, should be eliminated as a hypothetic starter culture because of the high risk of phage infection. Our results on host range were generally in agreement with Binetti *et al.* (2005) who described the host ranges of 15 bacteriophages against 14 *Streptococcus thermophilus* strains, all from dairy environment. They reported the phages had different host ranges, and the number of hosts ranged from just one to four strains. By contrast, only 16 *Streptococcus thermophilus* strains out of 36 could be found as sensitive to a number of bacteriophages ranging from 1 to 4, and

as many as 9 strains were sensitive to more of 11 among 27 bacteriophages tested (Le Marrec *et al*. 1997).

The high variability within the phage population was confirmed by restriction profile analysis. In fact, most phages showed a unique pattern, as obtained by using *Hind*III and *Eco*RV enzymes. On the contrary, as reported in results, restriction profiles obtained by using *Pst*I were not suitable for the differentiation of the 26 bacteriophages. Only the phages 604, 620 and 654 showed similar patterns when their DNA was digested by *Hind*III (6 bands) and *Eco*RV (8 bands) (data not shown). The similarity among these phages was confirmed by the results of VR2 sequence analysis, where all the three phages belonged to the cluster A (Figure 3). However, while the phages 604 and 620 showed also the same host range being able to infect the host *Streptococcus thermophilus* CHCC2134 (Table 1), the phage 654 infected a different host, i.e. *Streptococcus thermophilus* CHCC3063 strain. On the other hand, the phages 574 and 576, belonging to the cluster A obtained by VR2 sequence analysis, showed same host range (infected *Streptococcus thermophilus* CHCC2134) but different restriction profiles.

The phylogenetic tree showed 6 clusters at 90% similarity level. The bacteriophages that showed a good correlation between host range and VR2 sequence were 604, 574, 576 and 620, which belonged to the cluster A, 1027 and 1033, which belonged to the cluster B and 1034 and 1040 which belonged to the cluster F. The phages 641 and 671 had the same host range but they belonged to two very distant clusters. In agreement with Binetti *et al*. (2005) these results might indicate further phage factors are involved in host specificity.

Furthermore, the multiplex PCR allowed distinguishing the *cos*-type and the *pac*-type phages. In this study only the bacteriophage 1042 and the temperate phage from *Streptococcus thermophilus* CHCC2070 gave an amplification fragment distinctive of *pac*-type group. All the other bacteriophages belong to the *cos*-type. As reported by Quiberoni *et al*. (2006),

discriminating the *pac*-type and *cos*-type phages could be interesting to remove *Streptococcus thermophilus* strains sensitive to both phages group. In this study the unique *pac*-type phage 1042 infected only *Streptococcus thermophilus* strain CHCC6592, whereas all the other strains were sensitive to *cos*-type phages, confirming previous study demonstrating that rarely *Streptococcus thermophilus* strains were infected by members of both phage groups (Lévesque *et al*., 2005) Surprisingly, the bacteriophage 575 that infected only *Streptococcus thermophilus* strain CHCC2070 was shown to have both PCR products for *cos*-type and *pac*-type group. Inducing the *Streptococcus thermophilus* CHCC2070 by Mitomycin C the strain resulted lysogenic and the PCR performed with its lysate yielded an amplification fragment distinctive of the *pac*-type group. Definitively, the results suggested that the bacteriophage 575 lysate contained both its DNA (providing the cos-type fragment) and CHCC2070 prophage (providing the pac-type fragment).

In conclusion, the high diversity among bacteriophages highlights the serious risk of phage infection during fermented food processing. On the other hand, we registered the presence of a high percentage of low sensitive *Streptococcus thermophilus* strains. Testing a hypothetic starter culture against a phage population, characterized by a well determined high variability, is an efficient tool to exclude bacterial strains bringing on possible fermentation failure.

Chapter 4

Evidence of a temperate bacteriophage from lysogenic *Streptococcus macedonicus* AI4

SUMMARY

The bacterial strains used in this study were investigated for the presence of temperate phages by induction with mitomycin C. Only the *Streptococcus thermophilus* CHCC2070 and *Streptococcus macedonicus* AI4 showed to be lysogenic and the temperate phage PZ1φ from *Streptococcus macedonicus* AI4 was choosen for detailed investigation. PZ1φ seems to belong to Bradley's phage group B1, having a hexagonal head and a long, non-contractile tail.

INTRODUCTION

Streptococcus macedonicus, named cause of its place, Macedonia (Greece) where it was first found, was isolated during a survey of the LAB microbiota of naturally fermented Greek Kasseri cheese (Tsakalidou *et al.*, 1998). *S. macedonicus* seems to be associated with traditional dairy manufacture produced with raw milk without the use of selected starter cultures (Mora *et al*., 2003). Whereas the first strains of *S. macedonicus* have been isolated from Greek Kasseri cheese, it's reported that the other strains have been isolated from very different cheese: French cheese (Callon *et al*. 2004) and Italian cheese designated as PDO or traditional cheese, as Asiago, Montasio, Ragusano and Mozzarella cheese (Lombardi *et al.* 2004; Pacini *et al*., 2006).

The species *S. macedonicus* belongs to the thermophilic homofermentative LAB strains. It is nutritionally fastidious (multiple amino acid auxotrophies, restricted proteolityc system, *etc*.), requiring the addition of appropriate nitrogen sources. Strains of *S. macedonicus* are moderately acidifying and proteolytic probably due to weak (phospho)-β-galactosidase activity and extracellular proteolytic activity, respectively (Georgalaki *et al*. 2000). This does not allow *S. macedonicus* to completely acidify milk as the sole starter strain. However, strains of *S. macedonicus* do precipitate in fat hydrolysis, citrate consumption and casein hydrolysis in milk, underlining their usefulness as adjunct or co-culture (Georgalaki *et al.,* 2000). Furthermore, it was found that some *S. macedonicus* strains displayed antimicrobial

activity towards *Clostridium tyrobutyricum*, but no activity was detected against *Listeria innocua*. These antagonistic activities of *S. macedonicus* strains point towards their use to combat spoilage and late loss in hard and semi-hard cheeses. The strain *S. macedonicus* ACA-DC 198, for example, produces a food-grade lantibiotic, named macedonicin. Lantibiotics are a class of LAB bacteriocins that are post-traslationally modified, including the formation of didhydro amino acids and thioether amino acids; bacteriocins are defined as ribosomally synthesized antibacterial peptides that kill closely related species (De Vuyst and Vandamme, 1994). *S. macedonicus* is a food-grade streptococcus, isolated from cheese, wich does non exhibit potential pathogenicity traits. The exact role of *S. macedonicus* in cheese making has still to be elucidated. However, its natural occurrence in different European cheeses, probably originating from raw milk used, its potential contribution to cheese ripening, in particular with respect to milk fat hydrolysis and peptidolytic activity, and its potential role to combat or late loss during ripening strengthen its importance as non-starter LAB and adjunct culture in cheese manufacture (De Vuyst and Tsakalidou, 2008) has been recently investigated. To know its sensibility to bacteriophages could be interesting to evaluate a criterion to select efficient strains.

EXPERIMENTAL PROCEDURES

Induction of bacteriophages

Induction of temperate phages was performed on all bacterial strains used in this study, by addition of Mitomycin C at final concentration of 0.2 µg/mL when the cultures had an OD_{600nm}= 0.2. The presence of temperate phages was indicated by decrease of culture turbidity up to total clarification of the broth monitored by spectrophotometer measures at 600

nm. The lysate obtained was centrifuged at 8000 *g* for 10 min to remove bacterial cell debris, the supernatant was filtered through 0.45 µm membrane and stored at 4°C.

Screening for indicator strains

The possible sensitivity of 60 *S. macedonicus* strains isolated from samples collected during Provolone del Monaco making process (Aponte *et al*., 2008), was determined in two ways: i) a spot test on agar medium involved depositing drops of lysate of temperate phages or of appropriate dilutions on M17-Ca^{++} agar, with previously poured in a double layer, the second soft layer having been inoculated with the strain tested. ii) M17-Ca^{++} broth was inoculated with each strain at 1%, 0.5 ml of PZ1φ lysate was added followed by incubation at 37°C. The tubes were examined visually at regular intervals. Control and lysate containing cultures was transferred at 2% to fresh M17-Ca^{++} medium and a second or third transfer was performed.

Determination of DNA packaging mechanism

To rapidly classify *S. macedonicus* temperate phages within one of 2 groups (*cos*– and *pac*-type) it was used the Quiberoni *et al* (2006) multiplex PCR protocol by using two pairs of primers (one per phage group Table 1) designed from the conserved regions of the gene coding for the major capsid protein in the phages for which the complete genome is available. The PCR reactions were performed in a total volume of 50 µL containing 125 µ*M* deoxynucleoside triphosphate, 5 µ*M* concentrations of the 4 primers, 2.5 U of *Taq* DNA polymerase, *Taq* buffer (20 m*M* Tris-HCl pH 8.4, 1.5 m*M* magnesium chloride, 50 m*M* potassium chloride). The templates were constituted by i) 1 µL of the temperate phage lysate ii)10 ng of bacterial total DNAof *S. macedonicus* AI4 iii) 10 ng of bacterial total DNA of *S.*

gallolyticus subsp *macedonicus* 18488^{T}. Phage DT1 (Tremblay and Moineau, 1999) was used as *cos*-type positive control. Phage DT1 was propagated on *S. thermophilus* SMQ-301. Negative control (without the template) was included for all PCR assays to eliminate the possibility of contamination.

The conditions of PCR amplifications were set as follows: 5 min at 94°C, followed by 35 cycles (45 s at 94°C, 45 s at 53°C, 1 min at 73°C), and a final step of 5 min at 73°C. The PCR products were separated on a 2% agarose gel in TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA), stained with ethidium bromide, and visualized under UV light. The fragments of 170 bp and 427 bp were distinctive of the *cos*-type and *pac* -type group, respectively.

Table 1: *List of primers used for multiplex PCR*

Primer	Sequence, 5'- 3'	Reference
cos FOR	GGTTCACGTGTTTATGAAAAATGG	Quiberoni *et al*. (2006)
cos REV	AGCAGAATCAGCAAGCAAGCTGTT	Quiberoni *et al*. (2006)
pac FOR	GAAGCTATGCGTATGCAAGT	Quiberoni *et al*. (2006)
pac REV	TTAGGGATAAGAGTCAAGTG	Quiberoni *et al*. (2006)

Dot blot hybridization

Hybridazation assays and labelling of DNA to be used as probe were performed by using the enhanced chemiluminescence (ECL) system (Amersham) according to the supplier's recommendations. *S. macedonicus* AI4 total DNA and the DNA from PZ1φ was spotted on nylon membrane (Hybond-N^+ Amersham) and probed with DT1 DNA *S. thermophilus* phage.

RESULTS

Evidence of temperate bacteriophages and screening of indicator strains

Among the bacterial strains tested for the presence of temperate phages, S*treptococcus thermophilus* CHCC2070 and S*treptococcus macedonicus* AI4 exhibited cell lysis (Figure 1) suggesting temperate bacteriophages from these microorganisms were induced. All the S. *thermophilus* and S. *macedonicus* strains were tested as possible host for these temperate phages, but no bacterial strain showed to be host for the phages.

The temperate phage from *S. macedonicus* named PZ1φ was observed by transmission electron microscopy (Figure 2). PZ1φ seems to belong to Bradley´s phage group B1, having a

hexagonal head of about 64 nm in diameter and a non-contractile tail of about 187 nm in length.

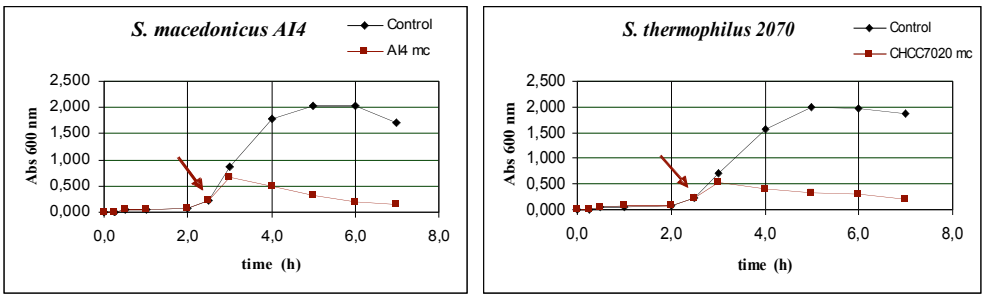

Figure 1 *Effect of mitomycin C (mc)* on S. macedonicus *AI4*(■) *and* S. thermophilus CHCC2070 (■). *Control without mc* (♦)

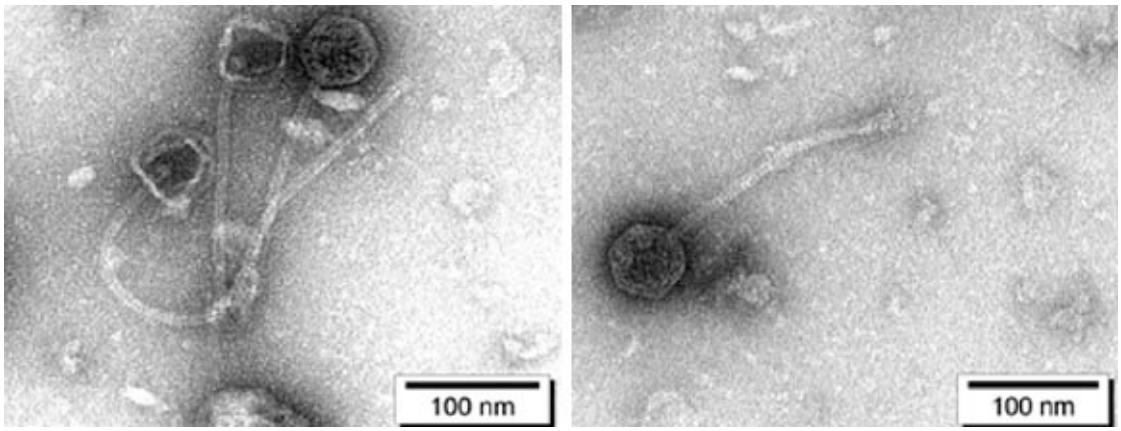

Figure 2 *Temperate bacteriophage PZ1φ from* Streptococcus macedonicus *AI4*.

Determination of DNA packaging

The multiplex PCR performed on the lysate of the PZ1φ gave an amplification product of 170 bps, indicating the temperate phage belong to the *cos*-type group. The PCR performed

on the prophage of *S. macedonicus* AI4 yielded a product of the same size. No amplification product was found for *S. gallolyticus* subsp. *macedonicus* 18488^{T} (Figure 3).

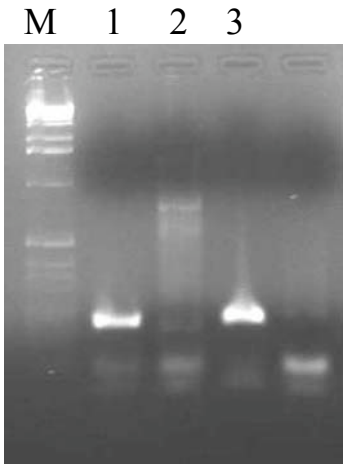

Figure 3. *Multiplex PCR for determination of DNA packaging. M 1kb ladder; 1* S. macedonicus *AI4; 2* S. gallolyticus *subsp.* macedonicus *18488.*^{T}

Dot blot hybridization

The dot blot hybridization analysis confirmed the similarity with *S. thermophilus* phages (Figure 4). However when the amount of DNA of *S. macedonicus* AI4 was diluted the signal was weak.

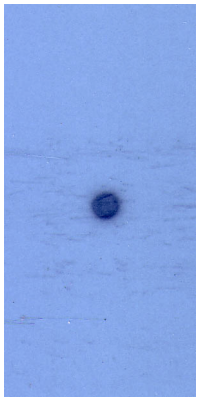

Figure 4: *Dot blot signal of* S. macedonicus *AI4 total DNA hybridizated with* S. thermophilus *DT1 probe.*

DISCUSSIONS

Lactic Acid Bacteria constitute the majority in volume and value of the commercial starter with the largest amount being applied in dairy industry (Hansen, 2002). Among the streptococci *sensu lato, S. thermophilus* strains are widely used in dairy manufactures and in the last decade several dairy *S. thermophilus*-like microogranisms have been identified, due to their unusual carbohydratate fermentation pattern and their different genotype as compared with those of *S. thermophilus* strains *sensu stricto* (Moschetti *et al*., 1998). *S. macedonicus* (Tsakalidou *et al*., 1998) and *S. waius* (Flint *et al*., 1999) have been described in detail, which has allowed some clarification of the taxonomically undefined group of *S. thermophilus*-like microrganisms. Whether these strains are discovered in natural whey starters could influence the stability of fermented dairy products other aspects need to be studied further.

Since the lysogenic starter strains are considered a major source of bacteriophages in cheese plants, the induction of all bacterial strains used in this study was carried out to evidence the presence of temperate phages. Only *S. thermophilus* CHCC2070 and *S. macedonicus* AI4 showed to be lysogenic. The importance of the role of *S. macedonicus* strains as adjunct culture (De Vuyst and Tsakalidou, 2008) in cheese manufacture has addressed the attention to its temperate phage. By electron transmission microscopy, *S. macedonicus* phage PZ1φ appears to belong to group B according to Bradley's classification having a hexagonal head and a long non-contractile tail. However, while it seems the lysogenic starter strains are main source of phages in dairy environments (Zhang *et al,* 2006), the difficulty in finding indicator strains is contradictory to this notion. Sixty *S. macedonicus* strains isolated from different sources were tested as indicator strains, but no bacterial cell was found to be a host. The multiplex PCR indicated that the temperate phage belong to the *cos*-type group and the similarity with the *S. thermophilus* phage was confirmed by dot blot hybridization.

At the best of our knowledge, this is the first evidence for a phage within the *S. macedonicus* species.

Chapter 5

Genomic sequence of *S. macedonicus* temperate phage PZ1φ by genome walking method - work in progress.

SUMMARY

The difficulties in finding indicator strains for *S. macedonicus* temperate phage PZ1φ and the impossibility to amplify the phage and to extract an adequate amount of its DNA, led to an alternative method to sequence unknown DNA regions. The PCR-based genome walking method was used starting from a known DNA *cos* fragment of PZ1φ. By taking multiple steps using new primers based on the sequence obtained in previous steps, it is possible to reveal unknown DNA sequences. The research need to be continued because of too small fragments obtained at this time.

INTRODUCTION

The genome walking method is the systematic identification of unknown regions flanking a known DNA sequence. Isolation of such unknown flanking regions can be obtained by screening a genomic library with a known DNA as probe. However, this is time consuming for preparation of libraries and their screening to obtain the desired DNA fragment (Rishi *et al.,* 2004). By using the polymerase chain reaction (PCR) is possible to isolate unknown flanking regions of known DNA sequence, efficiently and fast. Different PCR methods, such as inverse PCR (Ocham *et al*., 1988), and adaptor-specific PCR (Siebert *et al.,* 1995) have been used to isolate the unknown flanking regions. In both cases the genomic DNA is digested by restriction enzymes and ligated to self or to adaptor. The ligated product is then used as template to amplify flanking regions using PCR. The GenomeWalker ^{TM} Universal kit (Coletech, Palo Alto, CA, USA) is used by starting the known *cos*- fragment of PZ1φ. The first step is to construct pools of uncloned, adaptor-ligated genomic DNA fragments. The genome walker takes just two days after library preparation and consists of two PCR amplifications per library (Figure 1). The primary PCR uses the outer adaptor primer (AP1) provided in the kit and outer genes-specific primer (in this case that used for amplification of *cos*- fragment). The primary PCR mixture is then diluted and used as

template for a secondary, nested PCR by using a nested specific primer. This produces generally a single, major PCR product from at least one library.

The genome walking method enables to create uncloned libraries for walking by PCR in any genomic DNA. In less than a week the method provides access to the genomic DNA sequences adjacent to a known DNA sequence in any species. The method allows to walk simply by taking multiple steps using new primers based on the sequence obtained in previous steps. However, the genome walking has some limitation in amplifying (either smaller fragment amplification or no amplification) the desired size of flanking DNA, when the restriction site is close to the upstream location of the gene specific primer in the genome (Rishi *et. al*., 2004). To overcome this problem it could be possible to use different restriction enzymes, so that there is a grater chance to obtain templates wherein the location of the restriction site is distant from the gene-specific primer.

In this study the genome walking method is applied to determine the genome of PZ1φ by using the total DNA from *S. macedonicus* AI4 starting from the *cos* fragment.

EXPERIMENTAL PROCEDURES

Construction of Genome Walker™ Libraries

For each library construction the DNA of *S. macedonicus* AI4 strain was digested by restriction enzymes *Dra*I, *Eco*RV, *Pvu*II, separately, provided from kit, used according to the manufacture's instructions. Restricted phages DNA were electrophoresed in a 0.8% agarose gel in TAE buffer (40 m*M* Tris-acetate, 1 m*M* EDTA), stained with ethidium bromide and visualized under UV illumination. The digested DNA was ethanol precipitated after phenol:chloroform (1:1) extraction. The DNA pellet was in water and 0.5 μg of

completely digested DNA was used to ligate with the "Genome Walker Adaptor" according to manufacturer's protocol.

PCR amplification

The PCR reactions were carried out in a total volume of 50 µl containing the following to a final concentration: 125 µ*M* deoxynucleoside triphosphate, 5 µ*M* concentrations of the 4 primers, 2.5 U of *Taq* DNA polymerase, *Taq* buffer (20 m*M* Tris-HCl pH 8.4, 1.5 m*M* magnesium chloride, 50 m*M* potassium chloride), and 1 µl of DNA coming from each library and using the primers as described in table 2. The list of primers used for these amplifications are listed in table 3, where the *AP1*for was provided by Genome Walker^{TM} . The PCR conditions were 94°C, 25 s; 67 °C 3 min for 7 cycles; 94 °C 25 s, 67 °C 3 min for 32 cycles amd an additional one cile at 67°C for 10 minuts. The PCR products were purified by using QIAquick PCR purification kit (QIagen, Milan, Italy) and sequencing was performed by Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems).

Table 1 *Schematic plan used for first and second step during genome walking.*

DNA library	Sample No.	1 PCR primers	2 PCR primers
1 *Dra*I	**1**	*COS*rev & AP1	*WALK1rev* & AP1
2 *Eco*RV	**2**	*COSrev* &AP1	*WALK1rev* &AP1
3 *Pvu*II	**3**	*COS*rev &AP1	*COSrev* &AP1

Table 2*: List of primers used in this study.*

Primer	Sequence, 5'- 3'	Reference
COS rev	AGCAGAATCAGCAAGCAAGCTGTT	Quiberoni *et al*. (2006)
AP1 for	GTAATACGACTCACTATAGGGC	Genome Walker^{TM}
WALK1 rev	GACCTTTCTTGTCATGTGTCTTCG	This study

RESULTS and DISCUSSION

To identify the genomic DNA sequences adjacent to *cos* segment of *S. macedonicus* prophage AI4 a genome walking method was applied. By using the Genome Walker™ it was possible to identify the upstream region that result in amplification of fragments of less than 500 bp in two libraries created by using *Dra*I and *Eco*RV, but no amplification fragments was observed in the *Pvu*II library (Figure 1). Since the first PCR produced a single major product from at least one library, the following nested PCR was not performed. The amplifications of the small fragments could be due to the presence of a restriction site in the genome close to the designed primer and the negative result in the *Pvu*II library may be due to an absence of a restriction site close the gene-specific primer. Since the location of the restriction site relative to the gene-specific primer in the genome is not known, the use of different enzymes-digested libraries could result in amplifications of fragments of different sizes and relatively larger fragments in some libraries. The first step yielded a PCR product of 500 bp from *Dra*I library and the *cos* fragment was contained in this longer new fragment. The second reverse primer *WALK1* designed basing on the sequence obtained in previous step was used to walk on the genome and gave a PCR product of about 200 bp. However, the too small fragments obtained do not allow to obtain rapidly the complete sequence, considering that a phage genome ranges in size from 30 to 45 kb.

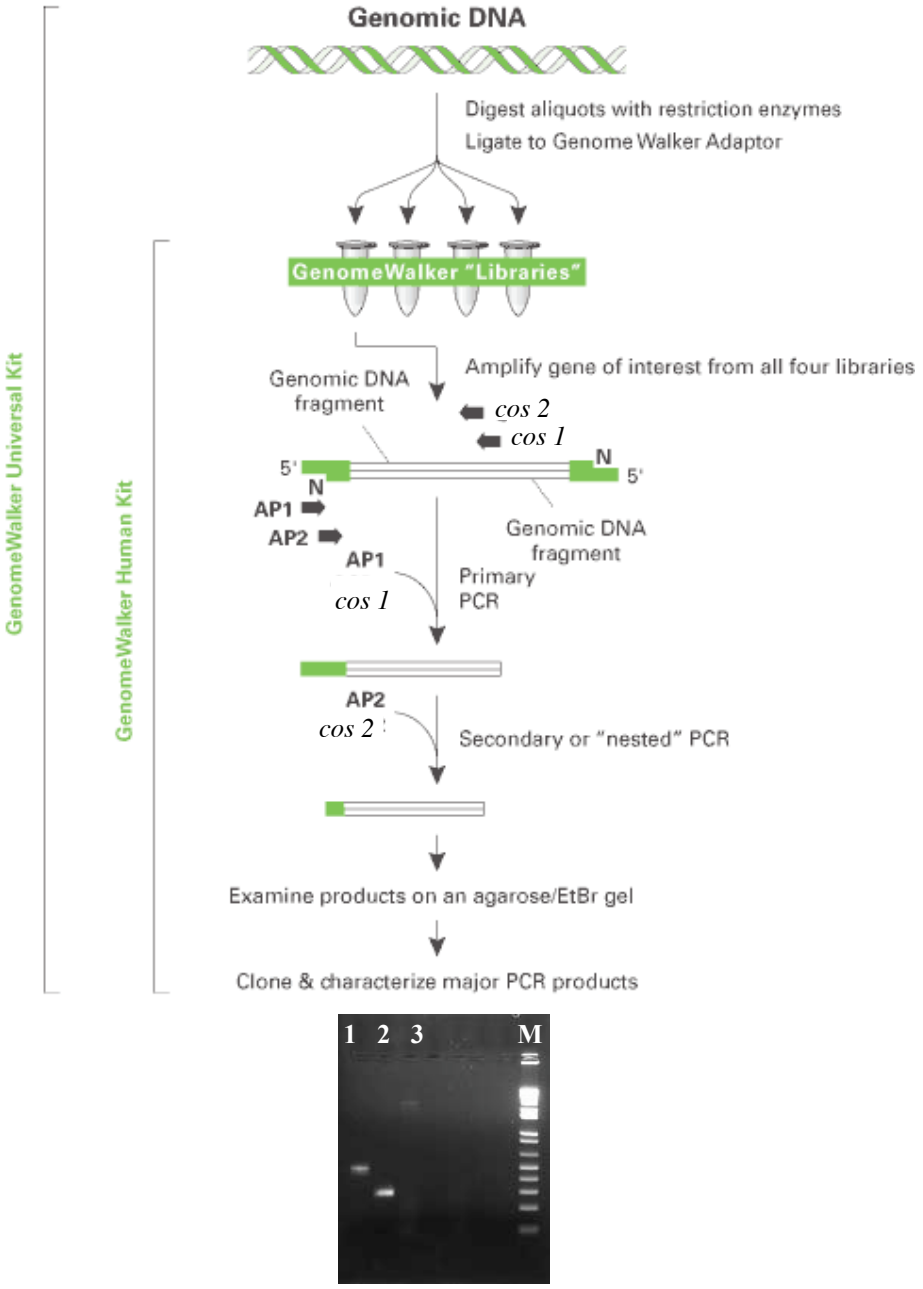

Figure 1. *The* GenomeWalker^{TM} *method, the samples loaded are listed in the table 1 and correspond to the results obtained from the first PCR. M: 1kb plus ladder.*

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