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Molecular Studies on Bacteriophages of the Food

Starter Culture *Streptococcus thermophilus*

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Content

1	Abbreviations.....	10
2	Introduction	12
3	Main objectives of this research.....	24
4	Materials & Methods	25
4.1	Bacterial strains, phages, plasmids and growth conditions.....	25
4.2	Media.....	27
4.3	Antibiotics and additives	29
4.4	Chromosomal DNA extraction.....	29
4.4.1	Reagents	29
4.4.2	Procedure.....	29
4.5	Agarose gel electrophoresis.....	30
4.5.1	Reagents	30
4.5.2	Procedure.....	31
4.6	Phage assay.....	31
4.6.1	Induction and isolation of bacteriophages.....	31
4.6.1.1	<i>UV-light induction.....</i>	<i>31</i>
4.6.1.2	<i>Mitomycin C induction.....</i>	<i>32</i>
4.6.2	Phage morphology.....	32
4.6.3	Phage concentration and DNA extraction.....	32
4.6.3.1	<i>Bacteriophage concentration and purification</i>	<i>32</i>
4.6.3.2	<i>Bacteriophage DNA extraction</i>	<i>35</i>
4.6.4	Plaque test.....	36
4.6.5	Spot test.....	37

4.6.6	Pulsed-field gel electrophoresis (PFGE).....	37
4.6.7	Host range screening using micro-titer plate method.....	38
4.6.8	Removal of free DNA from phage lysates	38
4.6.9	Phage adsorption test.....	39
4.6.10	Cell survival test for <i>Lactococcus lactis</i>	40
4.7	Plasmid DNA purification for Gram-positive bacteria	40
4.7.1	Procedure.....	40
4.8	“NucleoSpin [®] Plasmid QuickPure” protocol for <i>Escherichia coli</i>	41
4.9	Southern blot analysis (DNA-DNA hybridization).....	41
4.9.1	Reagents for Southern hybridization.....	42
4.9.2	DIG labeling of DNA probes	44
4.9.2.1	<i>Labeling of DNA sizes <10 kb</i>	44
4.9.2.2	<i>Labeling of DNA sizes >10 kb</i>	45
4.9.2.3	<i>Estimation of the efficiency of probe labelling</i>	45
4.9.3	Blotting.....	47
4.9.4	Hybridization protocol	48
4.9.4.1	<i>Pre-hybridization</i>	48
4.9.4.2	<i>Hybridization</i>	48
4.9.4.3	<i>Detection of hybridization signals</i>	48
4.10	Polymerase chain reaction (PCR).....	49
4.10.1	Primers.....	49
4.10.2	PCR programs	51
4.10.2.1	<i>High fidelity PCR program</i>	52
4.10.3	Colony PCR.....	52
4.10.3.1	<i>Colony PCR-buffer (Fermentas GmbH, St. Leon-Rot, Germany)</i>	52
4.10.3.2	<i>Procedure</i>	52

4.10.4	Purification of PCR product.....	52
4.11	Cloning.....	53
4.12	Transformation.....	53
4.12.1	Preparation of competent cells and transformation of <i>L. lactis</i> cells.....	53
4.12.2	Preparation of competent <i>E. coli</i> cells.....	53
4.12.3	Electroporation of <i>E. coli</i> Easy pores.....	54
4.13	Nucleotide sequencing.....	55
4.14	Restriction enzyme analysis.....	55
4.15	SDS-PAGE.....	55
4.15.1	Reagents.....	55
4.15.2	Procedure.....	57
4.16	MALDI-TOF MS.....	59
4.17	Western blot analysis (Immunoblotting).....	59
4.17.1	Reagents.....	60
4.17.2	Preparation of polyclonal antibodies specific for the lipoprotein encoded by <i>S. thermophilus</i> phage TP-J34.....	61
4.17.3	Transfer of proteins from SDS polyacrylamide gel to membrane by capillary blotting.....	61
4.17.4	Detection of protein (Ltp) on PVDF membrane.....	62
5	Characterization of <i>S. th.</i> phage TP-778.....	63
5.1	Introduction.....	63
5.2	Results and Discussion.....	67
5.2.1	<i>Streptococcus thermophilus</i> SK778.....	67
5.2.2	Induction of phage TP-778 with mitomycin C and ultraviolet (UV) light.....	68

5.2.3	Morphology of <i>S. thermophilus</i> phage TP-778.....	69
5.2.4	DNA restriction endonuclease pattern	70
5.2.5	Prophage curing experiments	71
5.2.6	Detection of host strains for phage TP-778.....	71
5.2.7	Adsorption test	73
5.2.8	Genetic relationship of phage TP-778.....	75
5.2.8.1	<i>DNA homology of phage TP-778 with other S. thermophilus phages.....</i>	75
5.2.8.2	<i>DNA homology of phage TP-778 DNA with chromosomal DNA of S. thermophilus strains.....</i>	75
5.2.9	Partial DNA sequence analysis of <i>S. thermophilus</i> phage TP-778 DNA flanking the lysogeny module.....	77
5.2.9.1	<i>PCR amplification of the lysin gene-attR region of prophage TP-778.....</i>	78
5.2.9.2	<i>DNA sequence analysis of the lysin-attR region of prophage TP-778.....</i>	78
5.2.9.3	<i>PCR amplification of the attL-cro DNA region of prophage TP-778.....</i>	81
5.2.9.4	<i>Sequence analysis of the attL-cro DNA region of prophage TP-778.....</i>	82
5.2.9.5	<i>DNA sequence analysis of the attP flanking region of the lytic phage derivative TP-778L.....</i>	82
5.2.9.6	<i>Hybridization with integrase gene probe</i>	86
5.2.10	General transduction mediated by the temperate <i>S. thermophilus</i> phage TP-778 and its lytic derivative TP-778L.....	88
5.2.10.1	<i>Screening of phage DNA for 16S rDNA by PCR.....</i>	90
5.2.10.2	<i>Southern blot analysis for screening of 16S rDNA in S. thermophilus phages and strains.</i>	91
5.3	Conclusion.....	92
6	Cloning of <i>ltp</i> gene of TP-778	93
6.1	Introduction	93
6.2	Results	94
6.2.1	Amplification of the <i>ltp</i> _{TP-778} gene from the TP-778 phage genome.....	94

6.2.2	Cloning of <i>ltp</i> _{TP-778} in the expression vector pMG36e.....	95
6.2.3	Transformation of pYAL1 in <i>E. coli</i> Easypores.....	96
6.2.4	Transformation of <i>L. lactis</i> Bu2-60 with pYAL1 DNA.....	97
6.2.5	Phage-sensitivity test for the transformants	98
6.2.6	Western blot analysis of the lipoprotein coded by the <i>ltp</i> _{TP-778} gene from <i>S. thermophilus</i> phage TP-778	101
6.2.7	Cell survival test of <i>L. lactis</i> Bu2-60 transformants carrying the <i>ltp</i> _{TP-778} gene..	102
6.2.8	Discussion and Conclusion	104
7	Multiplex PCR for <i>S. thermophilus</i> phages.....	108
7.1	Introduction	108
7.2	Results and Discussion.....	110
7.2.1	Design of PCR primers.....	110
7.2.2	Phage differentiation	112
7.2.3	Validation of the reliability of the multiplex PCR protocol to distinguish <i>cos</i> - and <i>pac</i> -type <i>S. thermophilus</i> phages.....	113
7.2.3.1	<i>Restriction endonuclease analysis of phage DNA</i>	113
7.2.3.2	<i>SDS-PAGE of the phage structural proteins</i>	115
7.2.3.3	<i>Immuno electron microscopy</i>	116
7.2.4	Determination of the detection limit of the PCR assay.....	117
7.2.5	Multiplex colony PCR for the detection of lysogenic <i>S. thermophilus</i> strains ...	119
7.3	Conclusion.....	121
8	A new <i>S. thermophilus</i> phage.....	122
8.1	Introduction	122
8.2	Results and Discussion.....	123
8.2.1	Identification of the host strain.....	123

8.2.2	Phage propagation	123
8.2.3	Electron microscopic analysis of phage P738.....	126
8.2.4	Plaque assay	126
8.2.5	Host range of phage P738	127
8.2.6	Restriction enzyme analysis of the phage P738 genome	127
8.2.7	Determination of the P738 genome by PFGE.....	128
8.2.7.1	<i>Genetic relationship of phage P738 with L. lactis and S. thermophilus phages.</i>	129
8.2.8	Partial DNA Sequencing of phage P738	131
8.2.9	Partial DNA sequence analysis of phage P738 DNA.....	133
8.2.10	Structural proteins of <i>S. thermophilus</i> phage P738	138
8.2.10.1	<i>SDS-PAGE</i>	138
8.2.10.2	<i>MALDI-TOF mass spectrometry</i>	139
8.2.11	Design of an improved and updated multiplex-PCR protocol for identification of <i>S. thermophilus</i> phages.....	141
8.3	Conclusion.....	142
9	Summary & Conclusions	143
10	Zusammenfassung	147
11	References	152
12	Appendix	163
12.1	Complete DNA sequence of the 5.2-kb lysin- $\Delta attR$ region of <i>S. thermophilus</i> prophage TP-778	163
12.2	Complete DNA sequence of the 2.7-kb region of the <i>attL-cro</i> region of <i>S. thermophilus</i> prophage TP-778	165
12.3	Complete DNA sequence of the 1.4-kb region of the <i>S. thermophilus</i> TP-778L- <i>attP</i> flanking region.....	167

12.4	Complete DNA sequence of a 6.2-kb region of <i>S. thermophilus</i> virulent phage P738 coding for the large subunit of terminase and structural proteins of the head morphogenesis.....	168
13	Acknowledgments.....	171
14	Curriculum vitae	172

1 Abbreviations

ϕ	phage
APS	ammonium persulfate
bp	base pair
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease enzyme
ds	double-stranded
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
h	hour
kb	kilo base
kDa	kilodalton
M	molar
mA	milliampere
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
o/n	overnight
<i>orf</i>	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease enzyme
rpm	revolution per minute
sec	second
SDS	sodium dodecyl sulfate
ss	single stranded
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	<i>N,N,N',N'</i> - tetramethylethylenediamine
UV	ultra violet
v	volt
v/v	volume to volume
vol	volume

w/v	weight to volume
μM	micromolar

2 Introduction

Lactic Acid Bacteria (LAB) are a heterogeneous group of Gram-positive bacteria (cocci or rods) that have been widely used for centuries in the bioprocessing of foodstuffs. Growth of LAB is accompanied with acidification due to their ability to ferment lactose into lactic acid. Consequently, this drop in pH increases the preservative quality of the fermented food via growth inhibition of many spoilage and pathogenic bacteria. Furthermore, their growth results in enzymatic processes that provide flavor and texture for the food product. LAB are found as a natural micro-flora in milk and on mucosal surfaces like oral cavity, intestine and vagina of mammals. They are usually non-pathogenic bacteria that include members of the genera *Lactococcus* (milk), *Lactobacillus* (milk, meat, vegetables, cereals), *Streptococcus* (milk), *Leuconostoc* (vegetables, milk) *Pediococcus* (vegetables, meat) and *Oenococcus* (wine) (Josephsen & Neve 2004; Klaenhammer *et al.*, 2002). Some members of this group, especially lactobacilli play important roles in the gastrointestinal tract of humans and animals: as probiotic organisms they positively effect the normal micro-flora, inhibit pathogens and stimulate/modulate mucosal immunity. Nowadays, LAB are used in the production of chemical and biological products such as biopolymers (*Leuconostoc*), ethanol, lactic acid, bacteriocin and bulk enzymes (*Lactobacillus*) (Gold *et al.*, 1992; Hofvendahl & Hahn-Hagerdal, 2000; Klaenhammer *et al.*, 2002).

Streptococcus thermophilus is one of the economically most important LAB (Fig. 2.1). It is a component of dairy starter cultures and used in large amounts for the manufacture of dairy products (annual market of around 40 billion USD) (Hols *et al.*, 2005). It is usually used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lb. bulgaricus*) or *Lb. helveticus* for the manufacture of yogurt and other fermented milk products like acid-curd cheeses. *S. thermophilus* is also used alone or in combination with lactobacilli for the production of mozzarella and cheddar cheeses

(Duplessis *et al.*, 2006; Binetti *et al.*, 2005). It is a gram-positive microaerophilic, moderately thermophilic bacterium which possesses a relatively small genome of approximately 1.8 Mb

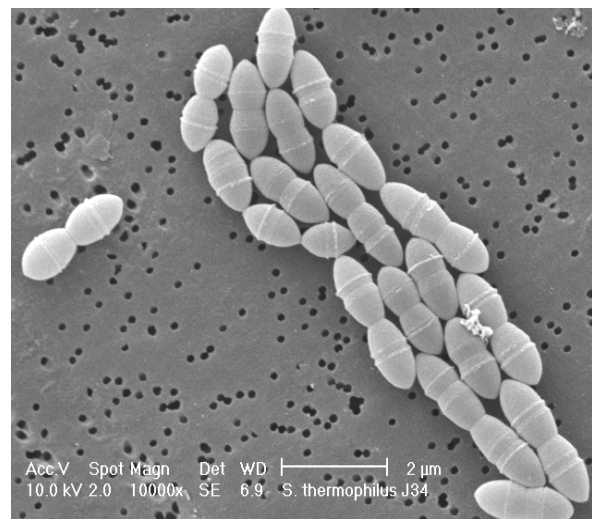


Fig. 2.1: Electron micrograph of *S. thermophilus* J34. Source: H. Neve, MRI Kiel, Germany.

with a low G+C content (Roussel *et al.*, 1997; O'Sullivan & Fitzgerald, 1998). Some insertion sequences (ISs) have been reported in its genome (Guedon *et al.*, 1995; Bourgoïn *et al.*, 1996) which may contribute to the genetic instability of some *S. thermophilus* strains (Roussel *et al.*, 1997). It has been reported that many *S. thermophilus* cultures are highly susceptible to phage infection but only 1 to 10% of them are lysogenic and contain intact temperate phages (Josephsen & Neve, 2004; Carminati & Giraffa, 1992; Neve *et al.*, 2003).

Bacteriophage (or bacteria-eater: from the Greek “phago” meaning to eat) (Fig. 2.2), are viruses, so they are unable to multiply by themselves. They grow “parasitically” inside the bacterial cell and are released after cell lysis (burst) (Neve, 1996). Bacteriophage were first described more than a century ago. In 1896, Hankin reported that river water of the Ganges and Jumna in India contained agents that killed many types of bacteria, especially *Vibrio cholerae*. They could pass through a very fine porcelain filter and were sensitive to heating at boiling temperature (Waldor *et al.*, 2005). In 1915,

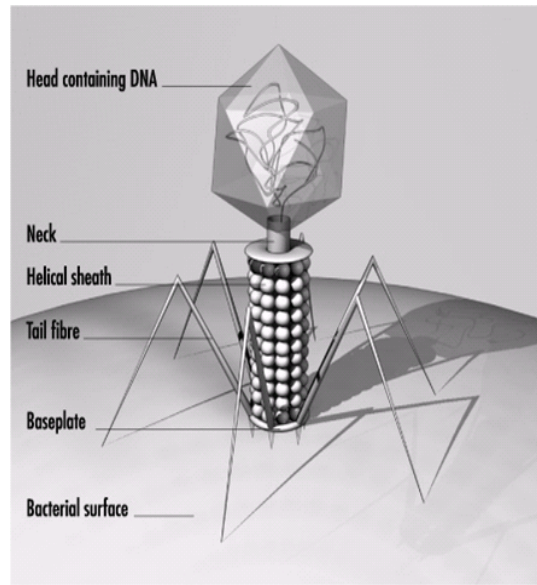


Fig. 2.2: Structure of bacteriophage. Source: Häusler, 2006.

Frederick Twort isolated filterable entities capable of destroying bacterial cultures. Independently, in 1917, Felix d'Herelle discovered a microbe that was antagonistic to bacteria causing lysis in liquid culture as well as killing of bacterial lawns on the surface of agar in discrete patches, which he termed plaques. He observed that these microbes invaded bacterial cells, multiplied inside and finally lysed them. He called them “bacteriophages” (Waldor *et al.*, 2005). Bacteriophage are probably the most abundant biological entities on earth (Breitbart & Rohwer, 2005). It is believed that bacteriophages are the most widely distributed microbial agent on the earth (more than 10^{31}) and reaching approximately 10 million phage/cm³ of any environment (i.e., soil, water and sewage), where bacteria or archaea are found (Fuhrman, 1999; Hendrix, 2003; McAuliffe *et al.*, 2007). Due to their wide distribution and abundance, they play an important role in microbial ecology, gene transfer and evolution of bacterial genomes (Chibani-Chennoufi *et al.*, 2004). Phage particles usually consist of a genetic material, which can be ssRNA, dsRNA, ssDNA or dsDNA in a circular or linear form, enclosed by an outer protein coat. According to morphology and nucleic acid content, phages are classified by the International Committee on Taxonomy of Viruses

(ICTV) into different orders. The most significant one is the order *Caudovirales* (dsDNA tailed phages) which represents 95% of all the reported phages in published literature, and includes the most important 3 families *Myoviridae* (bacteriophages possessing long contractile tails), *Podoviridae* and *Siphoviridae* phages with short and long non-contractile tails, respectively. Tailed phages of LAB are classified according to the scheme of Bradley (1967) into 3 groups: morphotype A (with contractile tails), B (with non-contractile tails) and C (with short non-contractile tails). According to their head shapes, they are further classified into 3 groups: 1 (isometric-headed), 2 and 3 (short and long prolate-headed, respectively). All phages of LAB contain linear dsDNA with cohesive ends (*cos*-type) or without (*pac*-type) (Neve, 1996).

According to their life cycles, bacteriophages can be differentiated into 2 types: virulent or temperate phage (Fig. 2.3). Usually, bacteriophages are very host-specific and attack only closely related strains. Phage infection of LAB begins with the adsorption process in which a specific interaction occurs between the receptor binding protein (RBP) located on the distal part of the phage tail and the phage receptor on the host surface (Duplessis *et al.*, 2006).

Virulent (lytic) phage inject their genomes into host cells, multiply inside and lyse the cells to release progeny phage particles. Temperate phage have the ability either to multiply in their hosts leading to lysis of the host cells or to integrate their DNAs into the chromosomes of the host bacteria. The integrated DNA replicates with cell genome replication and is distributed equally to the two daughter cells. In this cycle no virus particles are produced and the infecting viruses remain dormant in the bacterial chromosome. This process is called lysogeny and the bacteria carrying the non-infective precursor phage are called lysogens, while the integrated bacteriophage DNA is termed prophage. Lysogenic cells are immune to superinfection by the same phage (homoimmune) due to the presence of a repressor (*cI* gene product) that turns off the lytic cycle. However, it does not so with heterologous phages. Bacteriophage lambda (λ), which infects *E. coli*, is the classical example of a temperate phage. It has linear dsDNA with 48,502 bp. Phage λ is the best investigated phage, which has been used for studying gene regulation and has been applied as a cloning vector (Campbell & Botstein, 1983).

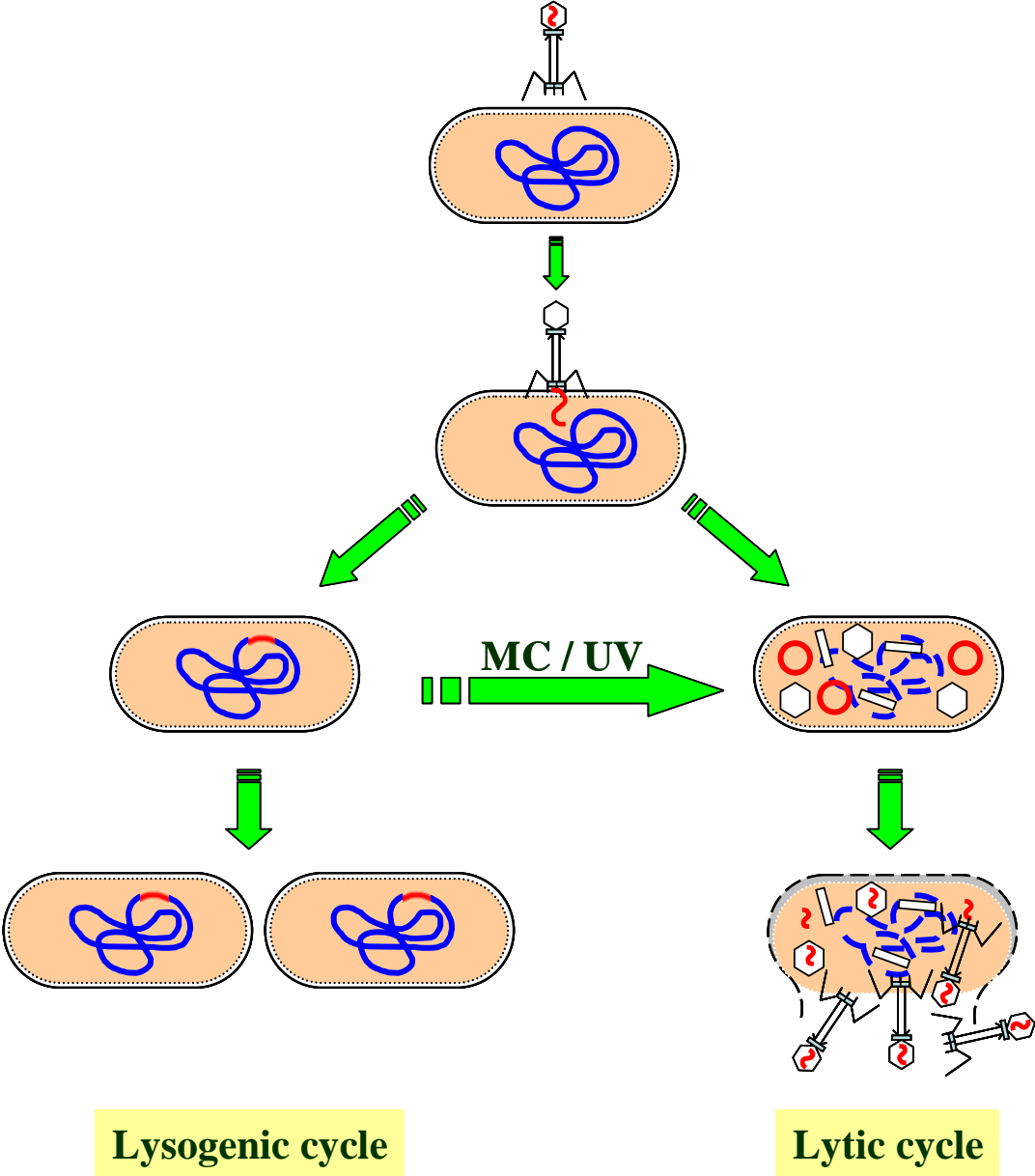


Fig. 2.3: Life cycles of bacteriophage

Lysogeny must be distinguished from pseudolysogeny, which occurs in many traditional mixed-starter cultures that are permanently infected with phages that infect phage-sensitive strains in the culture. In this case, growth of the whole culture is not significantly affected due to the presence of acid-producing cells that are non-sensitive to these phages (Neve, 1996). Temperate phages can also be a source for newly arising virulent phages, as a result of mutations in the genes required for establishing lysogeny (lysogenic module). Lysogens are not always stable, they can enter the lytic cycle either spontaneously (at a low rate) or when treated with DNA-damaging agents for example ultra violet (UV) light, mitomycin C (MC) or with other mutagenic agents (Desiere *et al.*, 1999; Birge, 2000). Occasionally, lysogenic cultures may give rise to cells that can no longer be induced, so they cannot produce infectious phage particles. This may be due to either simple mutations leading to the inactivation of prophage genes required for induction resulting in “cryptic prophages”, or to complex mutations like deletions resulting in the production of “defective prophages” (e.g., only tail genes left or other DNA remnants of a prophage) (Birge, 2000). Prophages can carry genes that control phenotype and behavior of bacterial host cells, like genes for toxins and lipoproteins which are not required for lysogenic or lytic cycle (Waldor *et al.*, 2005). Temperate phages produce “turbid plaques” in bacterial lawns of sensitive strain, which is due to the formation of immune or resistant lysogens within the majority of lysed cells (Birge, 2000).

In contrast to lactococcal phages which are morphologically completely different (Fig. 2.4), *S. thermophilus* phages (virulent or temperate) belong to the *Siphoviridae* family and they form a relatively homogenous group with the same morphology (B1 morphotype) (Ackermann, 2001). They have an isometric head (diameter, 45 to 60 nm) and a long non-contractile tail (length, 240 to 270 nm; thickness, 9 to 13 nm) (Brüssow, 2001). According to the packaging mechanism of their double-stranded DNA and their structural protein patterns on SDS-polyacrylamid gels, they are divided into two types: *pac*- (41, 25 and 13 kDa proteins) and *cos*- (32 and 26 kDa proteins) type (LeMarrec *et al.*, 1997). *S. thermophilus* phages have a linear double-stranded DNA genome ranging from 30 to 45 kb (Brüssow *et al.*, 1998).

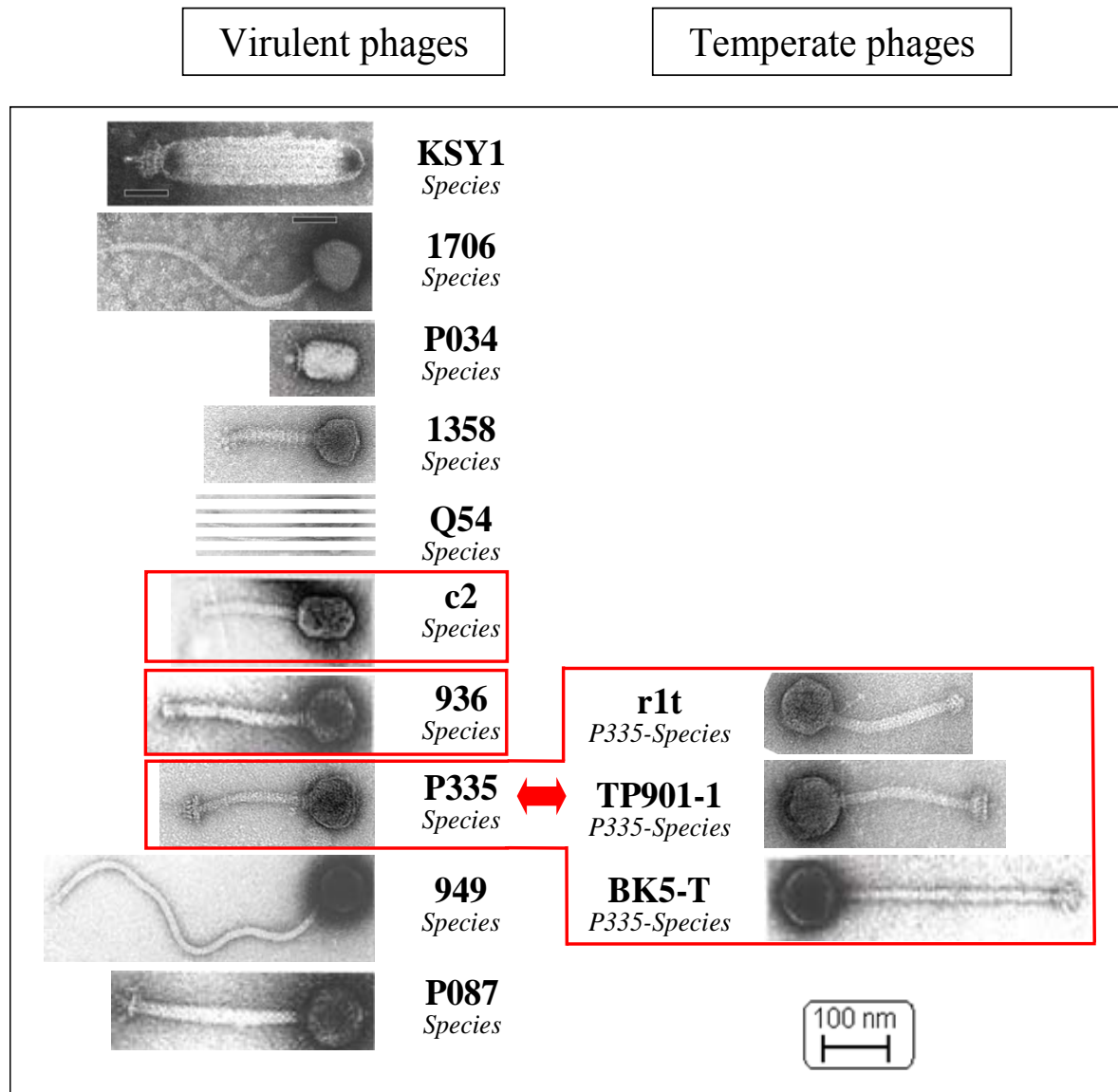


Fig. 2.4: Types of different lactococcal phages (modified from Josephsen & Neve 2004; Deveau *et al.*, 2006). Phages highlighted with red color are the most widely distributed species of lactococcal phages.

Bacteriophage attack is always a serious problem in industrial fermentation, especially in the dairy industry, causing slow fermentation or even complete starter failure with consequent loss of product. This may lead to high financial losses, because large dairy plants can process more than 10^6 l of milk per day.

Phage control, thus is very important for dairy and food fermentation. There are many phage control measures which can be applied during manufacturing processes and sanitation procedures to keep phages under control. Phages have simple and developed tools to overcome the bacterial defense mechanisms. Conversely, bacteria have several molecular weapons to defend themselves against bacteriophage infection. Therefore, the dairy industry has developed additional defense strategies depending on the use of starter cultures with reduced phage susceptibilities. These defense strategies have been summarized by Emond & Moineau (2007) and Sturino & Klaenhammer (2006):

1 Strain selection and culture rotation

This is a simple and effective strategy for prevention of bacteriophage attack by using a set of bacterial strains with distinct phage resistance mechanisms. This strategy can be used for undefined (traditional starter cultures with unknown bacterial composition) or defined starter cultures (usually composed of 2 to 5 well characterized strains via microbiological, biochemical and phage sensitivity profiles). A culture rotation program is carried out by using a series of starter cultures (usually 3 to 5) which show different phage susceptibilities.

2 Selection of bacteriophage-insensitive mutants

Starter cultures that are highly susceptible to phage infections must be removed from the starter culture rotation program. So, bacteriophage-insensitive mutants (BIMs) are obtained by exposure to one or more phages and by isolation of resistant derivatives. However, BIMs may grow slowly, may reveal low lactic acid production, may form aggregates under stress and may show high reversion rate to phage susceptibility.

3 Natural defense mechanisms

The continuous screening of starter cultures for phage insensitivity has led to the selection of bacteria which harbor one or more natural defense mechanisms. Nowadays, more than 50 such phage resistance mechanisms have been characterized in dairy *L. lactis* starter culture, and the majority of them are encoded on conjugative or mobilizable plasmids.

There are 2 types of natural defence mechanisms, host-encoded and phage-encoded phage resistance mechanisms.

3.1 Host-encoded phage resistance mechanisms

They are classified into 3 groups according to their mode of action on the phage lytic cycle (Fig. 2.5) as follows:

3.1.1 Adsorption inhibition

Phage infection begins usually with a specific interaction between the receptor binding protein (RBP) located on the distal part of the phage tail and the phage receptors on the host surface. A few plasmids mediating adsorption inhibition have been detected. There are 2 suggestions to explain the mechanism of adsorption inhibition, either through production of substances that hinder the binding process (i.e., masking of the phage receptors), or through alteration (or complete loss) of the phage receptor from the bacterial cell (Klaenhammer & Fitzgerald, 1994).

3.1.2 Restriction/Modification System (R/M)

Lactococci harbour several R/M systems. These systems include 2 enzymes, a restriction (endonuclease) enzyme which cleaves the phage (foreign) DNA at specific recognition sites, and a methylase that modifies the host DNA to be resistant against degradation. Three types of R/M (types I, II and III) systems have been detected depending on their subunit compositions, mode of DNA restriction and required cofactors (Bickle & Krüger, 1993). R/M systems are activated after injection of phage DNA. Phage which do not carry the respective DNA methylation, will be susceptible to R/M systems and their DNA will be degraded.

3.1.3 Abortive infection mechanism (Abi)

Abortive infection (Abi) describes phage-resistance mechanisms which are not due to adsorption inhibition, blockage of phage DNA ejection or R/M systems. Abi systems interfere with intracellular steps of the phage lytic cycle including DNA replication, transcription, structural proteins synthesis and lysis. No phage particles are released, however, the infected cells will die. Abi systems have been identified in many bacterial species. About 22 Abi systems have been characterized in *L. lactis* and tested against lactococcal phages of the 936-, P335- and c2-species (Chopin *et al.*, 2005). Determination of the efficiency of plating (EOP) method is usually used for evaluation of Abi defense systems through comparing of phage titers obtained from susceptible and resistant strain (Sanders & Klaenhammer, 1980). It was reported that abortive systems do not completely prevent the releasing of phages, and hosts carrying Abi system may produce plaques of reduced size but in numbers comparable to that of the susceptible host.

3.2 Phage-encoded phage resistance mechanisms (PER)

About 50% of the sequenced bacterial genomes contain prophage sequences which comprise 3-10% of the total genome of lysogens. Two genes have been reported in *S. thermophilus* prophages that are expressed during lysogeny.

3.2.1 Superinfection immunity

This effect is mediated by the *cI*-like repressor. Expression of its gene was studied in phage Sfi21 (*orf127*) and it was found that a CI-like protein prevented the multiplication of the homologous temperate phages but did not provide protection against heterologous and virulent phage (Bruttin *et al.*, 2002).

3.2.2 Blockage of phage DNA ejection (superinfection exclusion [*sie*])

In the case of superinfection exclusion, phages adsorb to the bacterial cell envelope, but DNA is not ejected into the cell. The expression of these *sie* genes protects lysogens from becoming infected with additional phages by blocking phage DNA ejection. The *orf203* of the *S. thermophilus* phage Sfi21 (Bruttin *et al.*, 1997b), the *sie₂₀₀₉* gene of the temperate lactococcal phage Tuc2009 (McGrath *et al.*, 2002) and the *ltp* gene of the *S. thermophilus* phage TP-J34 (Sun *et al.*, 2006) were shown to mediate this mechanism. The gene products are associated with the cell membrane.

4 Engineered defense mechanisms

In this case, high copy number recombinant plasmids containing genes with a specific function are used. There are many forms of engineered phage resistance mechanisms (Fig. 2.5) like:

4.1 Cloning of superinfection immunity and exclusion genes

A recombinant plasmid (pSF_{cl}) carrying the *cI*-like (*orf127*) gene of phage Sfi21 was transformed in *S. thermophilus* strain Sfi1. No protection were observed against a set of 30 virulent phages, only multiplication of Sfi21 on strain Sfi1(pSF_{cl}) was prevented (<10 plaque forming units (pfu)/ml) (i.e., homologous temperate phage infection) (Bruttin *et al.*, 2002). The superinfection exclusion gene of phage Sfi21 (*orf203*) was cloned into the pNZ124 cloning vektor and used for transformation of Sfi1 strain. The transformed Sfi1 strain showed a significant reduction of phage susceptibility (EOP $\leq 10^{-3}$) against a set of 12 virulent phages (Bruttin *et al.*, 1997b).

4.2 Anti-sense RNA

Anti-sense RNAs can be used to abort phage infection by blocking the translation of phage encoded genes that are necessary for normal replication, like genes coding for essential functions i.e., primase and helicase activities. The basis of this method is the hybridization of anti-sense RNA to the sense RNA resulting in masking ribosomal binding site (RBS) and transcriptionally inactive double-stranded RNA, which subsequently undergoes degradation (Sturino & Klaenhammer, 2002; Sturino & Klaenhammer, 2004).

4.3 Origin-derived phage-encoded resistance

After cloning of phage *ori* (origin of replication) on a high-copy-number plasmid (or cloning of multicopies of phage *ori*), the origin competes for both phage- and host-derived replication factors that catalyze phage-DNA replication resulting in reduction of the number of the replicated phage DNA (O'Sullivan *et al.*, 1993; McGrath *et al.*, 2001).

4.4 Phage-triggered suicide systems

In this system a toxic gene is expressed under the control of a phage-inducible promoter. The expression of a restriction endonuclease gene *llaIR* (from a high-copy-number recombinant plasmid) under the control of ϕ 31p, a middle-expressed inducible promoter of the virulent lactococcal phage 31 resulted in an aborted phage infection. In the same time the infected host cell was killed due to restriction of both unmethylated phage and bacterial genomes (Djordjevic *et al.*, 1997).

4.5 Subunit poisoning

This is a new and effective approach of phage-defense system in *S. thermophilus*. It depends on site-specific mutation of amino acid sequences that are essential for phage enzyme catalytic activity and/or protein-subunit oligomerization. The expression of mutant protein subunits can suppress or poison the function of native multimeric proteins. The primase gene of *S. thermophilus* phage *k3* was amplified to obtain a specific site-specific mutation within ATPase/helicase and/or oligomerization domains. Expression of mutated primase (59 kDa) in *S. thermophilus* NCK1125, after cloning into a high-copy-number plasmid, showed complete resistance to phage *k3* in 4 constructs (EOP $<10^{-9}$) (Sturino & Klaenhammer, 2007).

4.6 Host-factor elimination

The elimination of host-encoded factors that are essential for phage infection is very important for blocking of phage replication. Lucchini *et al.* (2000) have reported that knock-

out of *orf394* (encoding a putative transmembrane protein) of *S. thermophilus* provided complete resistance against a set of 15 phages.

4.7 Phage structural proteins and antibodies

Recently, a novel anti-phage strategy has been designed depending on the use of a phage neutralizing heavy-chain antibody fragment obtained from llama. De Haard *et al.* (2005) produced high titers of a neutralizing heavy-chain antibody fragment through immunization of llama with lactococcal phage p2 (936 species). These antibodies were shown to bind to the phage receptor binding protein (RBP) and prevented phage adsorption.

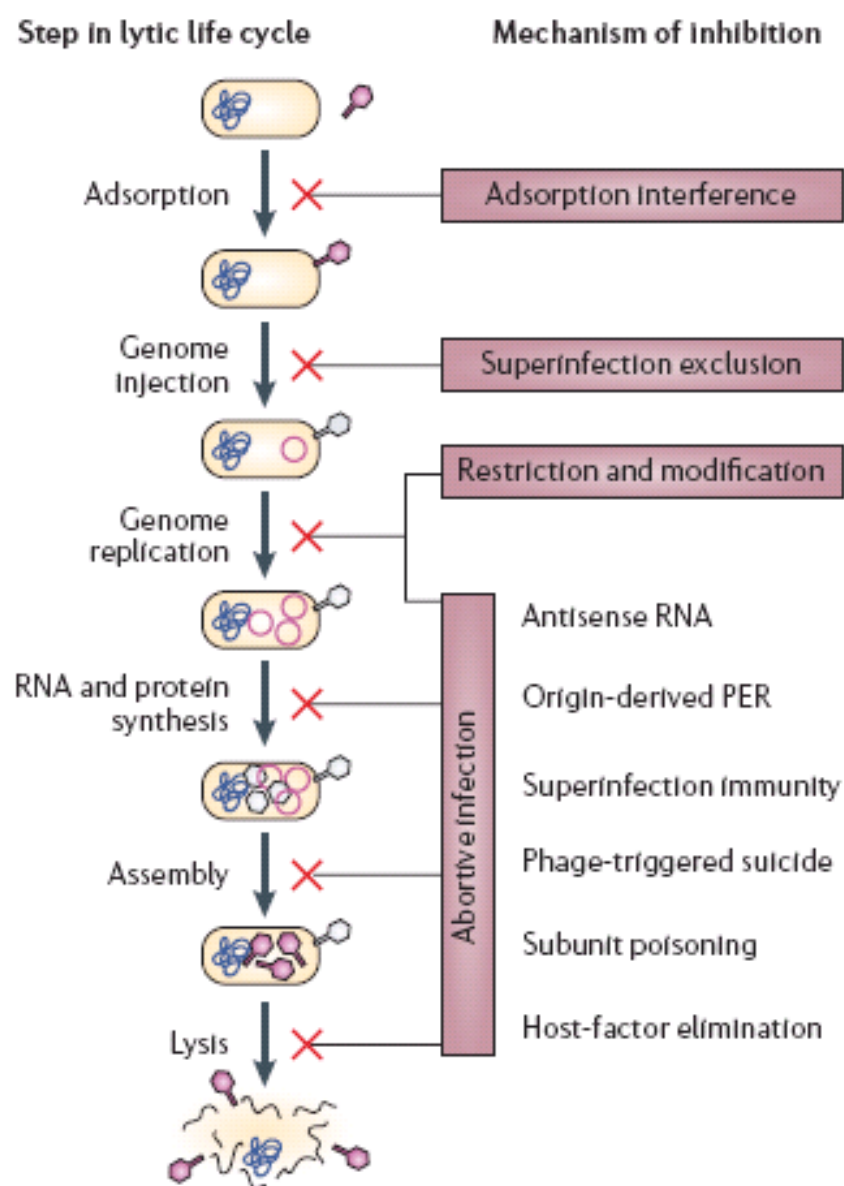


Fig. 2.5: Lytic life cycle of phage and mechanisms of phage inhibition by specific phage resistance mechanisms (Sturino & Klaenhammer, 2006).

In contrast to their harmful effect in dairy industry, bacteriophages may also have useful effect. They can be used in many applications such as in molecular genetics for studying bacterial genomes (phage transduction or as a cloning vector), in medicine and in food safety research. In the early years following the discovery of bacteriophages, there was considerable interest for using bacteriophages in treatment of bacterial diseases such as diarrheal disease and the treatment of traumatic infections during and after the second world war. During the 1920s and 1930s, therapeutic phage applications were developed to overcome bacterial infections in Western Europe and in the USA. Furthermore, patients suffering from dysentery or staphylococcal septicemia were successfully treated with phage preparations when used orally or intravenously, respectively (McAuliffe *et al.*, 2007). After the development of antibiotics for the treatment of infectious diseases in the early 1940s, the use of phage therapies for the treatment of bacterial infections was almost completely stopped except for Eastern Europe and Russia. Recently, phage therapy became interesting again for medical researchers not only due to the high increase of antibiotic-resistant bacterial strains but also due to the many advantages that it has, such as: i) a single dose of phage can be enough to treat an infection because phages will replicate themselves, ii) phage are non-toxic and highly specific for targeted bacterial populations (contrarely antibiotics kill also the normal flora), iii) antibiotic-resistant bacteria are sensitive to phage lysis. Moreover, the Food and Drug Administration approved in 2006 the use of bacteriophages that attack strains of *Listeria* as a food additive on ready-to-eat meat products.

3 Main objectives of this research

Streptococcus thermophilus starter cultures are of major importance for the food industry and are used for the manufacture of dairy products (yoghurt, mozzarella and Swiss-type cheese). Phage attacks on dairy starter cultures are always critical in dairy industry, since they may result in substantial economical losses. Therefore, it was the aim in **Chapter 1** to characterize the new temperate *S. thermophilus* phage TP-778, since this phage revealed the following unique characteristics not known from other well-described *S. thermophilus* phages: (i) This phage is packaging chromosomal host DNA during phage proliferation. (ii) In the lysogenic cells, the TP-778 prophage DNA is merged with a prophage remnant. (iii) The TP-778 prophage is using an uncommon mechanism of prophage excision which occurs by homologous recombination of the phage TP-778 integrase gene and a truncated integrase gene present on the flanking prophage remnant.

Since phage TP-778 contains a lipoprotein determinant (*orf142_{TP-778}*) in its lysogeny module, which is highly related to a corresponding gene (*orf142_{TP-J34}* designated as *ltp*) of the well-described phage TP-J34, the *orf142_{TP-778}* should be cloned and transferred to a lactococcal host strain (**Chapter 2**). The Ltp lipoprotein of the temperate *S. thermophilus* phage TP-J34 has been shown to confer a phage resistance phenotype (super infection exclusion) to lactococcal strains (Sun *et al.*, 2006). The effects of *orf142_{TP-778}* on the lactococcal phage resistance phenotype were also studied in detail in Chapter 2.

It was the aim of the work described in **Chapter 3** to develop a reliable, fast and sensitive multiplex PCR tool for identification and differentiation of all virulent and temperate *S. thermophilus* phages in dairy samples (whey samples). The tool was also shown to allow the identification of lysogenic *S. thermophilus* strains harbouring prophages.

Since the *S. thermophilus* phage P738 could not be targeted by the PCR protocol developed in Chapter 3, it was the main objective of the work written in **Chapter 4** to gain knowledge on this new phage species. By DNA sequence analysis of the structural genes specifying its head morphogenesis, it was documented that this new phage species was closely related to prophages of *S. pyogenes*. The PCR tool from Chapter 3 was redesigned by including an additional set of primers targeting the major head gene of the new phage P738.

4 Materials & Methods

4.1 Bacterial strains, phages, plasmids and growth conditions

Bacteriophages, bacterial strains, and plasmids used in this study are listed in Table 4.1, 4.2 & 4.3. *S. thermophilus* was grown in LM17 medium modified for growth optimization of *S. thermophilus* strains (i.e., LM17th) at 40°C, *L. lactis* strains were grown in GM17 medium at 30°C and *E. coli* strains were grown in Luria Bertani (LB) medium (Sambrook *et al.*, 1989) at 37°C with shaking. Transformants were grown in media supplemented with the recommended antibiotic(s).

Table 4.1: Bacterial strains used in this study

Bacterial Strain	Characteristics	Source / Reference
<i>S. thermophilus</i> SK778	Lysogenic host strain of TP-778, 2 plasmids.	Neve <i>et al.</i> , 2004
<i>S. thermophilus</i> SK778-Y	Derivative of strain SK778 containing non-inducible TP-778 prophage	This study
<i>S. thermophilus</i> J34	Lysogenic strain of TP-J34, 3.4-kb plasmid (pJ34).	Neve <i>et al.</i> , 1998
<i>S. thermophilus</i> J34-12	Derivative of <i>S. thermophilus</i> J34, contains non-inducible prophage	Neve <i>et al.</i> , 2003
<i>S. thermophilus</i> J34-6	Prophage cured derivative of strain J34.	Neve <i>et al.</i> , 2003
<i>S. thermophilus</i> strains: S15, YA4, YA5, YA6 and 124	Wild-type strains containing a prophage remnant (2.8 kb).	MRI-Kiel
<i>S. thermophilus</i> strain YA20	Wild-type strain without prophages.	MRI-Kiel
<i>S. thermophilus</i> strains: YA7, YA8, YA9, YA10, YA11, YA12, YA13, YA14, YA15, YA16, YA17, YA18	Wild-type strains	MRI-Kiel
<i>S. thermophilus</i> strain YA19	Host strain of phage P572	MRI-Kiel
<i>L. lactis</i> Bu2-60	Plasmid-free derivative of wild-type strain Bu2, lysogenic, chromosomally labelled (Str ^R Rif ^R)	Andresen <i>et al.</i> , 1984; Neve <i>et al.</i> , 1984; Kleinschmidt <i>et al.</i> , 1993
<i>E. coli</i> Easypores	Competent strain for transformation	Eurogentec GmbH, Köln, Germany

Table 4.2: Bacteriophages used in this study

Phage	Genotype / Phage type	Source
<u>S. thermophilus phages:</u>		
TP-J34	<i>pac</i> temperate	Neve <i>et al.</i> , 2003 Sun <i>et al.</i> , 2006
TP-778	<i>pac</i> temperate	Neve <i>et al.</i> , 2004
TP-778L	<i>pac</i> lytically propagated derivative of TP-778	This study
P53	<i>cos</i> virulent	Neve <i>et al.</i> , 1989
P572	<i>cos</i> virulent	MRI-Kiel
P640	<i>pac</i> virulent	MRI-Kiel
TP-YA1	<i>pac</i> temperate	This study
TP-YA2	<i>pac</i> temperate	This study
TP-YA3	<i>pac</i> temperate	This study
O1205	<i>pac</i> temperate	Stanley <i>et al.</i> , 1997
P738	<i>pac</i> virulent	This study
<u>Lactococcal phages:</u>		
P001	<i>cos</i> virulent, prolate-headed, c2 phage species	Jarvis <i>et al.</i> , 1991
P008	<i>cos</i> virulent, isometric-headed, 936 phage species	Jarvis <i>et al.</i> , 1991
P446	* isometric-headed, 1483 species	MRI-Kiel
BK5-T	<i>cos</i> temperate, isometric-headed, P335-[BK5T] phage species	Mahanivong <i>et al.</i> , 2001
r1t	<i>cos</i> isometric-headed, P335 phage species	van Sinderen <i>et al.</i> , 1996
sk1	<i>cos</i> virulent, isometric-headed, 936 phage species	Chandry <i>et al.</i> , 1997
TP901-1	<i>pac</i> temperate, isometric-headed, P335 phage species	Brondsted <i>et al.</i> , 2001
P335	P335 phage species	Jarvis <i>et al.</i> , 1991

pac: head-full mechanism of DNA packaging, no cohesive ends.

cos: cohesive ends.

* Unknown

Table 4.3: Plasmids used in this study

Plasmid	Description	Source
pJET1.2/blunt	CloneJET™ PCR cloning kit, 2,974 bp vector, Ap ^R	Fermentas GmbH, St. Leon-Rot, Germany
pJET.TP-778L-attP	pJET1.2/blunt + 1.4 kb TP-778L-attP fragment	This study
pJET.P738EV6	5.8 kb (pJET1.2/blunt + 2.8 kb P738-EcoRV fragment)	This study
pMG36e	3.6 kb lactococcal vector, Em ^R	van de Guchte <i>et al.</i> , 1989
pYAL1	4.1 kb (pMG36e + <i>ltp</i> _{TP-778} gene), Em ^R	This study
pXMS2	4.6 kb (pMG36e + <i>ltp</i> _{TP-J34} gene), Em ^R	Sun <i>et al.</i> , 2006
pS1	<i>cbh</i> (conjugated bile salt hydrolase gene) cloned into pGEM-T-easy vector	Ismail, 2007

4.2 Media

Luria Bertani (LB) (Sambrook *et al.*, 1989)

1.0% (w/v) peptone, 0.5% (w/v) yeast extract,
1.0% (w/v) NaCl
pH 7.0, sterilization: autoclave at 121°C for 20 min.

SOB (Hanahan, 1985)

2.0% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl
2.5 mM KCl, 10 mM MgCl₂
pH 6.8-7.0, sterilization: autoclave at 121°C for 20 min.

SOC medium

SOC medium is identical to SOB medium but also contains 20 mM glucose.

Belliker Broth

2.0% (w/v) tryptone, 1.0% (w/v) beef extract,
0.5% (w/v) yeast extract, 0.5% (w/v) dextrose,
0.5% (w/v) sucrose, 0.5% (w/v) lactose,
0.25% (w/v) gelatin, 0.05% (w/v) ascorbic acid

Sterilization: autoclave at 121°C for 20 min.

M17 medium (for *L. lactis*) (Terzaghi & Sandine, 1975)

0.5% (w/v) phytone peptone, 0.5% (w/v) poly peptone,
0.5% (w/v) beef extract, 0.25% (w/v) yeast extract,
0.05% (w/v) ascorbic acid, 0.05% (w/v) MgSO₄ x 7 H₂O
1.9% (w/v) Na-β-glycerophosphate

Sterilization: autoclave at 121°C for 20 min.

LM17th (for *S. thermophilus*) (Krusch *et al.*, 1987)

M17 medium containing 0.5% (w/v) lactose and 0.95% (w/v) Na-β-glycerophosphate

GM17

M17 medium containing 0.5 % (w/v) glucose

SGM17

GM17 medium containing 0.5 M sucrose

SGM17MC

SGM17 medium containing 20 mM MgCl₂ and 2 mM CaCl₂

SR-Agar

1.0% (w/v) tryptone, 0.5% (w/v) yeast extract,
20% (w/v) sucrose, 1.0% (w/v) glucose,
0.25 % (w/v) gelatin, 1.5% (w/v) agar,
0.037% (w/v) CaCl₂ x 2 H₂O, 0.051% (w/v) MgCl₂ x 6 H₂O
pH 6.8, sterilization: autoclave at 121°C for 20 min.

BSR-Agar

SR-Agar containing 1.9% (w/v) Na-β-glycerophosphate

4.3 Antibiotics and additives

Table 4.4: Antibiotics and other additives used for the media

Antibiotics & additives	Final concentration in medium for:			Dissolving agent
	<i>S. thermophilus</i>	<i>L. lactis</i>	<i>E. coli</i>	
Ampicillin 20 mg/ml	-	-	200 µg/ml	Deionized water
Erythromycin 10 mg/ml	1-2 µg/ml	5 µg/ml	150 µg/ml	Deionized water
Mitomycin C 20 mg/ml	0.2- 0.3 µg/ml	-	-	Deionized water

4.4 Chromosomal DNA extraction

4.4.1 Reagents

- DL-threonine (1 M)
- Solution A (20% (w/v) sucrose, 10 mM Tris-HCl [pH 8.0], 50 mM NaCl)
- Lysozyme (50 mg/ml)
- SDS (10% (w/v))
- Mutanolysin (5000 U/ml)
- Proteinase K (20 mg/ml)
- RNase A (10 mg/ml)
- Phenol
- Chloroform / isoamylalcohol (24:1)
- Na-acetate (3 M; pH 5.2)
- TE Buffer (10 mM Tris, pH 8.0, 1 mM Na-EDTA)

4.4.2 Procedure

- a) 10 ml of LM17th supplemented with 40 mM DL-threonine were inoculated with 3% of *S. thermophilus* o/n culture and incubated at 40°C until $O.D_{620\text{ nm}} = 0.7$.
- b) Cells were pelleted in 2-ml reaction tubes and washed once with autoclaved, deionized water.
- c) The pellets were resuspended in 0.5 ml of solution A containing 5 mg/ml lysozyme and 5 µl mutanolysin.
- d) The resuspended cells were frozen at -20°C for 30 min, thawed and incubated at 55°C for 10 min.

- e) 60 µl proteinase K were added, mixed by inversion (6-8 times) and incubated at 60°C for 1 h until the mixture was clear.
- f) 250 µl deionized water were added.
- g) 250 µl phenol were added and mixed by inversion (30 times).
- h) 250 µl of chloroform/isoamyl alcohol (24:1 v:v) were added and mixed by inversion (30 times).
- i) The samples were centrifuged at 14,000 rpm (Eppendorf centrifuge 5415D) for 5 min and the aqueous phase was transferred to a new tube.
- j) Steps f to i were repeated.
- k) 500 µl chloroform/isoamyl alcohol (24:1 v:v) were added and mixed by inversions (30 times).
- l) The samples were centrifuged at 14,000 rpm (Eppendorf centrifuge 5415D) for 3 min and the aqueous phase was transferred to a new tube.
- m) The DNA was precipitated by adding 1/10 volume of 3 M Na-acetate and one volume of isopropanol and mixed by inversion until chromosomal DNA was visible. The samples were stored at -20°C for 1 h.
- n) DNA pellet was obtained by centrifugation (Eppendorf centrifuge 5417R) at 14,000 rpm / 4°C / 15 min.
- o) The supernatant was discarded. One volume of 70% (v/v) ethanol was added followed by centrifugation (Eppendorf centrifuge 5417R) at 14,000 rpm / 4°C / 5 min. Ethanol was completely removed by drying at room temperature for not more than 10 min.
- p) DNA pellet was finally dissolved in 200 µl TE buffer (pH 8.0). 5 µl RNase A was added and incubated at 37°C for 30 min.
- q) 5 to 10 µl of the DNA were loaded on an 0.8% (w/v) agarose (Serva, Heidelberg, Germany) gel to estimate the concentration and purity of the chromosomal DNA.

4.5 Agarose gel electrophoresis

4.5.1 Reagents

Tris Acetate EDTA buffer (TAE) (Sambrook *et al.*, 1989)

Stock solution (50x)

Tris base (242 g/l),

Acetic acid (57.1 ml/l),

Na-EDTA (0.5 M, 100 ml),

In final volume of 1 l deionized water.

Loading buffer (6x)

25 mg bromophenol blue (0.25% (w/v)),
3.79 ml glycerol (33% (v/v)),
In final volume of 10 ml deionized water.

Staining solution

Ethidium bromide (Carl Roth GmbH, Karlsruhe, Germany) staining solution (0.5 µg/ml) was prepared by adding 1 ml of the stock solution (0.5 mg/ml) to 1 l of deionized water.

4.5.2 Procedure

Analysis of chromosomal, plasmid DNAs and PCR products were performed by using agarose gel electrophoresis unit (Bio-Rad Laboratories GmbH, München, Germany). Agarose gels [0.8% (w/v) to 2% (w/v)] were used according to the size of DNA fragment to be analyzed. Gels were prepared by cooking the required amount of agarose in 1x TAE buffer (pH 8.0) in a microwave oven for 3-5 min. Appropriate amounts were poured into the gel chamber. Before loading, DNA samples were mixed with 6x loading buffer. After running at 76 V for 40 - 60 min, the DNAs in the agarose gels were stained for 15 min in ethidium bromide and destained for another 15 min in H₂O. The gel was then photographed using the Universal Hood II-ChemiDoc XRS unit and analyzed with the help of Quantity One software (Bio Rad Laboratories, Segrate [Milan], Italy).

4.6 Phage assay**4.6.1 Induction and isolation of bacteriophages**

S. thermophilus bacteriophages were obtained either by inducing their lysogenic host strains using mitomycin C, by ultra violet light irradiation (UV), or by lytic propagation on a susceptible strain.

4.6.1.1 UV-light induction

S. thermophilus cells were grown at 40°C until the culture reached OD_{620 nm} of 0.1-0.2, then cells were harvested by centrifugation at 6,000 rpm for 10 min. The supernatant was discarded, pellets were suspended in ½ volume of 0.1 M MgSO₄, pumped through a quartz glass tube (1.3 mm diameter, 75 cm length) placed under a laboratory 254 nm UV lamp (Schütt, Göttingen, Germany), then mixed with another ½ volume of double-concentrated LM17th medium and incubated in the dark at 40°C until complete lysis (Neve *et al.*, 1998).

4.6.1.2 Mitomycin C induction

500 ml of LM17th were inoculated with 1.5% o/n *S. thermophilus* cultures and incubated at 40°C (final OD_{620 nm} of 0.2). Then another 500 ml of LM17th medium containing 0.4 µg/ml mitomycin C (final concentration 0.2 µg/ml) were added and incubation at 40°C was continued until complete lysis.

4.6.2 Phage morphology

Phage suspensions obtained from CsCl gradient centrifugation were diluted 1:30 with phosphate-buffered saline (pH 7.2), stained with 2% (w/v) uranyl acetate and analyzed under a transmission electron microscope (Tecnai 10, Fei Company, Eindhoven, The Netherlands) at an accelerating voltage of 80 KV.

4.6.3 Phage concentration and DNA extraction

Phage concentration and DNA extraction were performed according to Sambrook *et al.* (1989) with the following modifications:

4.6.3.1 Bacteriophage concentration and purification

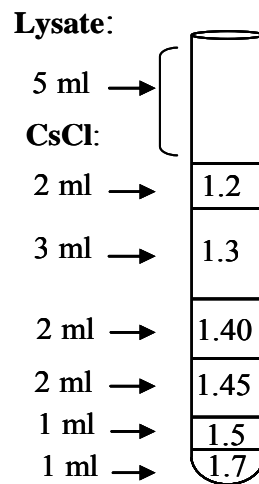
Procedure

- 1 l of completely lysed culture (obtained either from propagation of lytic phages or from induction of lysogenic strains) was cooled to room temperature, DNase I and RNase were added to a final concentration of 3 µg/ml. The batch was incubated at 37°C for 1 h.
- NaCl was added to a final concentration of 1 M and dissolved by swirling. The sample was kept cooled on ice.
- After 1 h, the cell debris were removed by centrifugation in a JA10 centrifuge rotor (Centrifuge model: J2-21, Beckman, USA) at 7,000 rpm for 10 min at 4°C and the supernatant was transferred into a clean flask. Polyethylene glycol (PEG 6000) was added to a final concentration of 10% (w/v) and was dissolved by slow stirring at room temperature and cooled on ice for at least 1 h to precipitate phage particles.
- Bacteriophages were collected by centrifugation in a JA10 centrifuge rotor (Centrifuge model: J2-21, Beckman, USA) at 8,000 rpm for 20 min at 4°C, the supernatant was discarded and the phage pellet was air-dried by leaving the centrifuge bottle in an inverted position.
- The bacteriophage pellet was gently suspended in 15 ml of SM buffer by using a wide-pore pipette or by rolling and swirling the centrifuge bottle for 10-15 min.
- CsCl stock solutions of different densities were made in SM buffer according to Table 5.

Table 4.5: Preparation of CsCl stock solutions with different densities

CsCl density (ρ)	1.2	1.3	1.40	1.45	1.5	1.7
CsCl	8.72 g	13.10 g	17.45 g	19.63 g	21.80 g	30.50 g
SM buffer	39	42	45	46.5	48	54
Final volume	30 ml	30 ml	30 ml	30 ml	30 ml	30 ml

- In a clear Beckman polypropylene centrifuge tube, a CsCl gradient was made by carefully layering CsCl solutions of decreasing density on top of each other as shown in Fig. 4.1. The borders between the different CsCl solutions were marked on the tube.

**Fig. 4.1:** Design of the CsCl step gradient. The numbers indicate the density of the CsCl solutions (g/ml).

- The SM buffer containing phage particles (5-6 ml) was loaded carefully on the surface of the CsCl gradient and centrifugation was done in a Beckman SW32.1Ti rotor (Ultracentrifuge model: Optima L-90K, Beckman Coulter, USA) at 25,000 rpm for 20 h at 10°C.
- After centrifugation, a bluish band of phage particles was visible at the border between the CsCl solutions of 1.40 and 1.45 g/ml densities. The blue band was collected by using a 21-gauge stainless-steel needle after wiping the outside of the tube with ethanol. The phage suspension was stored at 4°C and used for electron microscopic analysis or for phage DNA extraction.

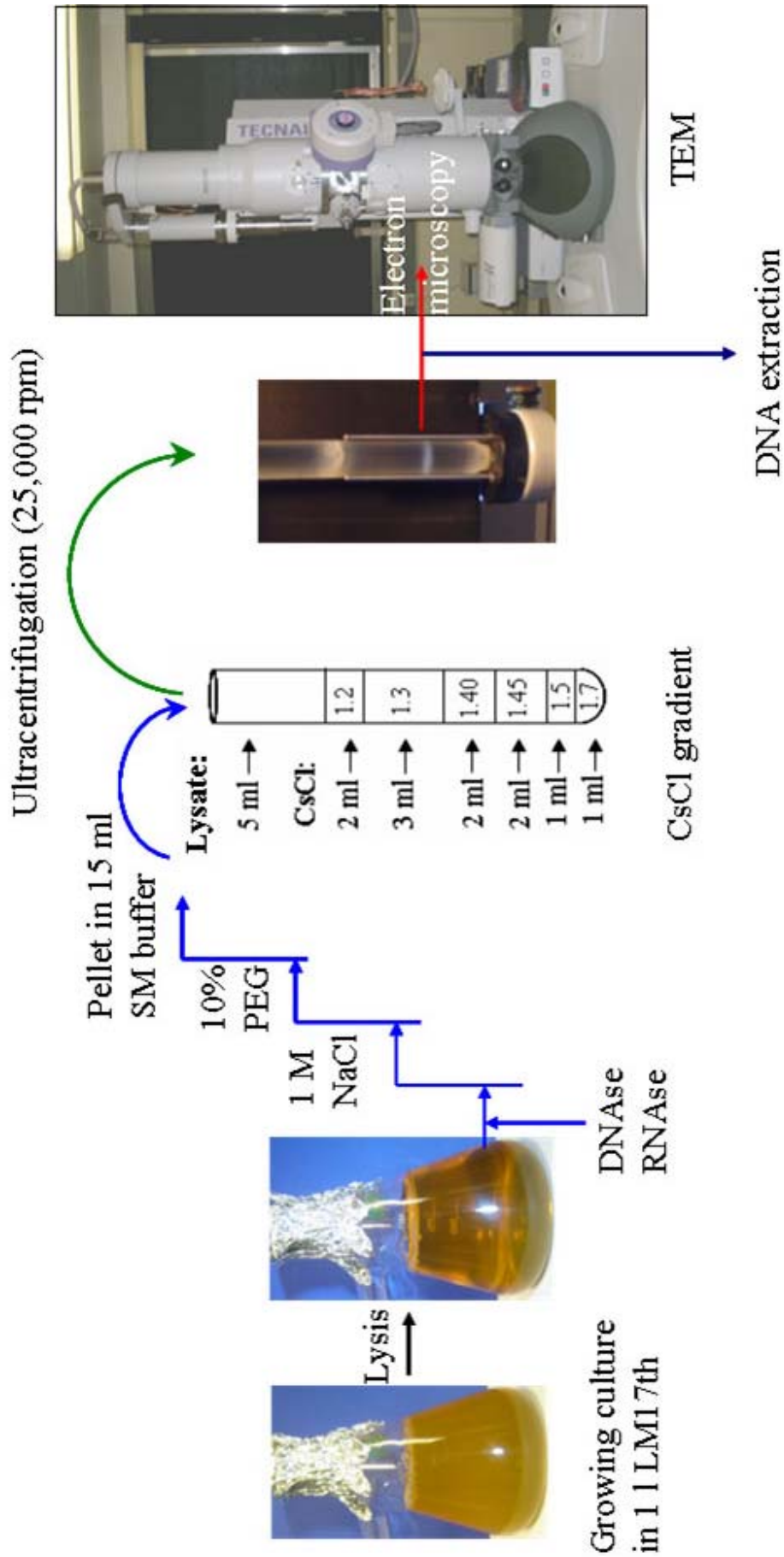


Fig. 4.2: Schematic diagram of the bacteriophage isolation protocol including CsCl step gradient centrifugation

4.6.3.2 Bacteriophage DNA extraction

Reagents

- Dialysis buffer: 10 mM NaCl
50 mM Tris-HCl (pH 8.0)
10 mM MgCl₂
- Dialysis sac (Visking® dialysis tubing 8/32, Serva, Heidelberg, Germany)
- Na-EDTA: 0.5 M
- Proteinase K: 20 mg/ml
- SDS: 10% (w/v)
- Phenol
- Chloroform/isoamylalcohol (24:1, v/v)
- TE buffer: 10 mM Tris-Cl (pH 8.0)
1 mM Na-EDTA
- Ethanol: 96% (v/v) and 70% (v/v)
- Sodium acetate (NaAc): 3 M pH 5.2

Procedure

- a) The bacteriophage suspension purified by CsCl gradient centrifugation was poured into a dialysis tube and dialyzed twice at room temperature for 1 h against a 1,000-fold volume of dialysis buffer.
- b) The bacteriophage solution was transferred into a 2-ml microcentrifuge tube in a maximum volume of one-third of the tube.
- c) Na-EDTA and proteinase K were added to a final concentration of 20 mM and 50 µg/ml, respectively.
- d) SDS (10% (w/v)) was added to a final concentration of 0.5% (w/v) and mixed several times by inversion.
- e) Then, the preparation was incubated at 56°C for 1 h and cooled to room temperature.
- f) An equal volume of equilibrated phenol was added and mixed by inversion for 30 s. The aqueous phase was transferred to a clean tube by using a wide-pore pipette after centrifugation at 13,000 rpm (Eppendorf centrifuge 5415D) for 5 min at room temperature.
- g) The aqueous phase was extracted again with 1/2 volume of equilibrated phenol and 1/2 volume chloroform, transferred to a clean tube and mixed with an equal volume of chloroform and centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 5 min at room temperature.

- h) The aqueous phase was transferred into a dialysis tube and dialyzed 3 times against a 1,000-fold volume of TE buffer (pH 8.0) o/n at 4°C.
- i) Phage DNA was precipitated with 2 volumes of 96% (v/v) ethanol and 1/10 volume Na-acetate (3 M, pH 5.2), stored at -20°C for 1 h or at -70°C for 30 min and centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) for 15 min at 4°C. DNA was washed once with 70% (v/v) ethanol and centrifuged again.
- j) The supernatant was carefully discarded, the DNA pellet was air-dried for 7 min at room temperature and dissolved in an appropriate volume of TE buffer. Samples (2-3 µl) were mixed with loading buffer and loaded on an 0.8% (w/v) agarose gel to estimate the concentration of DNA.

4.6.4 Plaque test

Plaque test was carried out by using the double layer agar technique according to Lillehaug (1997)

Reagents and Media

- LM17th bottom agar

0.5% (w/v) phytone peptone, 0.5% (w/v) poly peptone,
0.5% (w/v) beef extract, 0.25% (w/v) yeast extract,
0.05% (w/v) ascorbic acid, 0.95% (w/v) Na-β-glycerophosphate,
1.0% (w/v) agar
Sterilization: autoclave at 121°C for 20 min.

Lactose or glucose was added to a final concentration of 0.8% (w/v) using autoclaved sterile stock solutions (20% (w/v)). Then the medium was cooled to 50°C, glycine, CaCl₂ and MgSO₄ were added in a final concentration of 0.2% (w/v), 5 mM and 1 mM, respectively.

- LM17th top agar (soft agar)

The top agar (soft agar) was made similarly to the bottom agar, but only 0.45% (w/v) agar was used and lactose or glucose was omitted.

- Phage dilution buffer

1 1/4-strength Ringer solution (2 tablets/l) + 10% (v/v) LM17,
Sterilization: autoclave at 121°C for 20 min,
Before using, add 1 ml 1 M CaCl₂/100 ml.

Procedure

The phage lysate was diluted in serial dilutions from 10^{-1} to 10^{-8} in phage dilution buffer. From each dilution, 100 μ l were mixed with 300 μ l of log-phase host culture ($OD_{620\text{ nm}} = 0.5$). Then 100 μ l of 40 mM CaCl_2 and 100 μ l of reconstituted skim milk were added. The sample was incubated for 10 min at room temperature to allow adsorption of the phages to the cell surface. Subsequently, 2.5 ml of molten soft agar (47°C) were added, mixed and poured directly on LM17th bottom agar plates. After incubation for 24 h, the phage titers indicated as plaque-forming units (pfu/ml) were determined.

4.6.5 Spot test

It was performed as previously mentioned for the plaque test, but in the spot test, the soft agar was mixed with 300 μ l of host culture ($OD_{620\text{ nm}} = 0.5$) and 100 μ l of 40 mM CaCl_2 . The agar suspension then poured immediately on bottom agar. After solidification, 20 - 50 μ l of phage lysates obtained from serial dilutions were spotted on the surface and incubated at 30°C (for *L. lactis*) or at 40°C (for *S. thermophilus*) for 24 h.

4.6.6 Pulsed-field gel electrophoresis (PFGE)

- 1.1% (w/v) pulsed-field certified agarose (Bio-Rad Laboratories GmbH, München, Germany) was dissolved in 150 ml of 0.5x TBE buffer and cooled down to 50°C in a water bath.
- The gel-tray of the PFGE unit (Bio-Rad Laboratories GmbH, München, Germany) was cleaned with ethanol. After drying, 150 ml of 1.1% (w/v) agarose were poured into the tray and the comb was fixed.
- After solidification of the agarose (about 30 min), the comb was removed. 20 μ l of phage DNA mixture (40 μ l of 1.1% (w/v) molten pulsed-field certified agarose in 0.5x TE buffer at 55°C were mixed with about 150 ng phage DNA in 1.5 ml caps) were poured into the well, in addition to PFGE low range marker (New England Biolabs GmbH, Frankfurt am Main, Germany). Finally, all wells were sealed with 1.1% (w/v) agarose.
- PFGE was performed at 175 V and at 14°C (initial time: 2 sec, final time: 20 sec; running time: 20 h).
- After running time, the gel was stained in ethidium bromide (0.5 $\mu\text{g/ml}$), then destained in deionized water and photographed.

4.6.7 Host range screening using micro-titer plate method

The micro-titer plate method was used for detection of phage sensitive strains. 200 μ l of test medium (100 ml reconstituted skim milk, 20 ml Elliker broth and 1 ml of 1% (w/v) bromocresol purple) were poured into the wells of a standard microtitre plate (Fig. 4.3-A). Then, 52 wells were inoculated simultaneously with o/n cultures of *S. thermophilus* test strains and subsequently with a phage lysate using a 52-pin stainless steel inoculation device (Fig. 4.3-B). The plates were incubated for 16 h at 30°C or 40°C. Phage derived failures of lactic acid production of phage-sensitive host strains would result in the change of color of the pH indicator (i.e., bromocresol purple) from purple (pH 6.8 or above) to yellow (pH 5.2 or below).

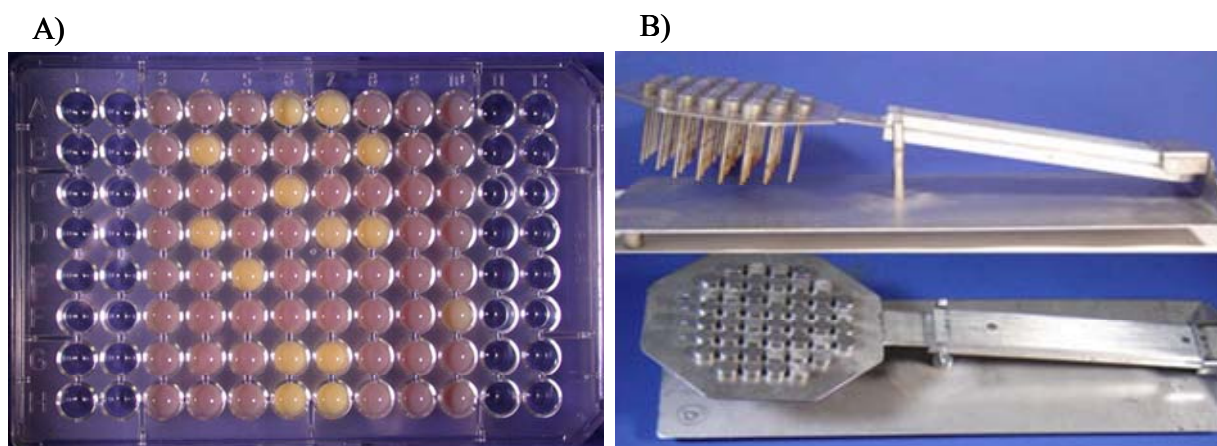


Fig. 4.3: Micro-titer plate assay for screening of 52 *S. thermophilus* strains for phage sensitivity (A). Phage-sensitive (purple) and phage-insensitive (yellow) strains were differentiated after 16 h incubation at 30°C or 40°C. (B) Photographs of the inoculation device.

4.6.8 Removal of free DNA from phage lysates

Chromosomal and free DNA were removed from phage lysates according to Sander & Schmieger (2001) with some modifications as follows:

A 500- μ l aliquot of phage suspensions harvested from CsCl step gradients were dialyzed twice against 1x DNase buffer (10x DNase buffer: 400 mM Tris, 100 mM MgSO₄, 1 mM CaCl₂) for 1 h. DNase I (30 μ l 10 mg/ml), 30 μ l RNase (10 mg/ml) and 7.5 μ l lysozyme (50 mg/ml) were added and the samples were incubated at 37°C for 1 h. An equal volume of chloroform was added, samples were mixed for 30 sec and centrifuged at 5,000 rpm (Eppendorf centrifuge 5417R) for 10 min at 4°C. The supernatant was dialysed against 1x DNase buffer for 1 h, then treated with DNase I, RNase and lysozyme as previously described. This step was repeated and the sample was incubated o/n at 4°C. Then the phage encapsulated DNA was extracted with phenol/chloroform method according to Sambrook *et al.* (1989).

4.6.9 Phage adsorption test

This test was performed according to Breum *et al.* (2007). A 100 ml-aliquot of LM17th broth was inoculated (1%) with a *S. thermophilus* test strain and incubated at 40°C until OD_{620 nm} of 0.5. Subsequently, the cultures were centrifuged at 7,000 rpm (rotor JLA-16.250; Beckman-Coulter, Krefeld) at 4°C for 10 min. The cell pellets were resuspended in 2 ml of 10 mM MgSO₄ and were subsequently kept on ice. Then 25 µl of 1 M CaCl₂ were mixed with the concentrated phage suspension and diluted with LM17th medium to a final volume of 1.8 ml. Then, 350 µl of phage suspension were mixed with 150 µl of cells giving an MOI (Multiplicity of infection = number of phage particles in relation to bacterial cells) in test tube equal to 0.01. Controls were included in which the cells were replaced by 150 µl of 10 mM of MgSO₄. Samples were incubated at 30°C (*L. lactis*) or 40°C (*S. thermophilus*) for 10 and 20 min. Subsequently, irreversible and total adsorption of phages to the host cells was performed as follows:

A- Irreversible adsorption test

↓

10 µl of test assay were rapidly diluted in 990 µl of ice-cold ¼-strength Ringer solution supplemented with 1 M NaCl.

↓

Samples were mixed by vortexing for 10 sec and stored on ice.

↓

Samples were centrifuged at 14,000 rpm for 1 min at 4°C (Eppendorf centrifuge 5417R). The supernatant was serially (10⁻¹ to 10⁻³) diluted and phage titers were determined.

B- Total adsorption test

↓

100 µl of test assay were rapidly centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) for 1 min at 4°C.

↓

10 µl from supernatant were diluted in 990 µl of ice-cold ¼-strength Ringer solution supplemented with 1 M NaCl.

↓

Samples were serially (10⁻¹ to 10⁻³) diluted and phage titers were determined.

4.6.10 Cell survival test for *Lactococcus lactis*

GM17 broth (10 ml) was inoculated either with *L. lactis* Bu2-60 or Bu2-60-transformants. Erythromycin was added (150 µg/ml) to the medium for growth of erythromycin-resistant transformants. The cultures were grown to OD_{620 nm} of 0.5 at 30°C. Cells were pelleted at 7,000 rpm in a JA20 Beckman rotor (Centrifuge model: J2-21, Beckman, USA) and resuspended in 10 ml of 10 mM MgSO₄. Aliquots of 300 µl from this cell suspension were mixed with 10 µl of 1 M CaCl₂. Phages were added to give an MOI equal to 5, then GM17 broth was added to give a final volume of 1 ml. Phage/host mixtures were incubated at 30°C for 10, 30 and 60 min. Serial dilutions were performed using ice cold ¼-strength Ringer solution and were plated on GM17 agar. Surviving colony-forming units were counted after a 48-h incubation at 30°C. The agar was fortified with 150 µg/ml erythromycin, when Bu2-60 transformants were used.

4.7 Plasmid DNA purification for Gram-positive bacteria

The “NucleoSpin[®] Plasmid Kit” protocol (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used with some modifications by S. Lick (personal communication) as follows:

Reagents

- Buffers: Nucleospin[®] plasmid kit buffer A1, A2, A3, A4, AW and AE
- Lysozyme (50 mg/ml in deionized water)
- Mutanolysin (5,000 U/ml in deionized water)
- Proteinase K (20 mg/ml in deionized water)
- Phenol (saturated with 3 M NaCl)
- Chloroform/isoamyl alcohol (24:1; v/v)
- TE buffer: 10 mM Tris, 1 mM Na-EDTA (pH 8.0)

4.7.1 Procedure

- *S. thermophilus* culture (10 ml) was grown to the logarithmic phase (OD_{620 nm} = 0.6-0.7) and cells were collected in 2-ml micro-tubes by centrifugation. Pellets were washed with sterile deionized water and were resuspended in 250 µl of buffer A1 by vigorous vortexing. 25 µl of lysozyme (50 mg/ml) and 5 µl of mutanolysin (5,000 U/ml) were added.
- Cells were incubated at 37°C for 1 h.

- Then, 250 μl of buffer A2 were added, mixed gently by inverting the tubes gently 6-8 times and incubated at room temperature for 3 min.
- Ice cold buffer A3 (300 μl) was added and mixed gently by inverting the tubes 6-8 times and placed on ice for 10 min.
- The tubes were centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) for 12 min at 4°C and the upper phase was transferred to a fresh 2-ml micro-tube.
- Then, 20 μl of proteinase K (20 mg/ml) was added, and samples were incubated at 37°C for 30 min.
- Phenol (400 μl) was added and mixed by inverting the tubes 30 times. An equal volume (400 μl) of chloroform/isoamylalcohol (24:1, v/v) was added, and the samples were mixed again by inverting 30 times.
- The tubes were centrifuged at maximum speed (13,000 rpm) for 5 min (Eppendorf centrifuge 5415D).
- The clear upper phase was transferred to a NucleoSpin[®] plasmid column and centrifuged at full speed (13,000 rpm) for 1 min (Eppendorf centrifuge 5415D).
- The filtrate in the micro-tube was discarded.
- Buffer AW (500 μl) was added to the column which was centrifuged for 1 min. The filtrate in the micro-tube was discarded.
- Then, 700 μl of buffer A4 was added and the sample was centrifuged at 13,000 rpm for 1 min. The filtrate in the micro-tube was discarded.
- The silica membrane was dried by centrifugation at 13,000 rpm for 2 min.
- Finally, the purification column was placed in a 1.5-ml Eppendorf micro-tube. Then, 50 μl of buffer AE or TE buffer were added to the column and incubated at room temperature for 1-3 min. DNA was eluted by centrifugation at 13,000 rpm and stored at 4°C or -20°C until further use.
- Aliquots (5 to 10 μl) of the plasmid DNA were mixed with loading buffer and run on an agarose gel (0.8% (w/v)) to estimate the concentration and purity of the plasmid DNA.

4.8 “NucleoSpin[®] Plasmid QuickPure” protocol for *Escherichia coli*

Plasmids from *E. coli* were purified according to the Macherey & Nagel protocol (Macherey-Nagel GmbH & Co. KG, Düren, Germany)

4.9 Southern blot analysis (DNA-DNA hybridization)

Following agarose gel electrophoresis, DNA fragmented by restriction enzyme digestion or amplified by polymerase chain reactions (PCR) was transferred to a nylon membrane (Roche

Diagnosics GmbH, Mannheim, Germany) by the capillary method (Southern, 1975; Wahl *et al.*, 1979) and hybridized with Digoxigenin-labeled probe DNA (DIG DNA Labeling kit, Roche Diagnostics GmbH, Mannheim, Germany). Probe labeling, hybridization and detection on the membrane were carried out according to the protocol provided by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany).

4.9.1 Reagents for Southern hybridization

DIG-DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany)

Hexanucleotide mix (10x concentrated)

DIG DNA labeling mix (10x concentrated)

Klenow enzyme

250 mM HCl solution

21 ml of 37% HCl were diluted with deionized water to a final volume of 1 l and sterilized by autoclaving for 20 min at 121°C.

Blotting transfer buffer (20x SSC-buffer)

20x SSC-buffer

3 M NaCl, 300 mM Na-citrate (pH 7.0)

The buffer was diluted with deionized water to a final volume of 1 l and sterilized by autoclaving for 20 min at 121°C.

20% (w/v) SDS

Sodium dodecylsulfate (20 g) was dissolved in 80 ml of deionized water at 68°C with stirring. The pH was adjusted to pH 7.2 with concentrated HCl (37%). The solution was diluted to a final volume of 100 ml and sterilized by membrane filtration using a sterile 0.2-µm filter (Whatman GmbH, Dassel, Germany).

Room temperature washing buffer

2x SSC (prepared from stock solution in deionized water)

0.1% (w/v) SDS (prepared from stock solution in deionized water)

68°C washing buffer

0.1% (w/v) SSC (prepared from stock solution in deionized water)

0.1% (w/v) SDS (prepared from stock solution in deionized water)

4 M lithium chloride

Lithium chloride (16.96 g) was dissolved in 100 ml of deionized water and autoclaved for 20 min at 121°C.

200 mM Na-EDTA

Na-EDTA (7.45 g) was dissolved in 80 ml of deionized water. After the pH adjustment to 8.0, the solution was diluted to a final volume of 100 ml and autoclaved for 20 min at 121°C.

Buffer 1 (10x)

0.10 M malic acid

0.15 M NaCl

The pH was adjusted to 7.5 with NaOH tablets, and the buffer was autoclaved for 20 min at 121°C.

Washing Buffer

A 3 ml volume of Tween 20 was filled up to 1000 ml with buffer 1 (1x).

Buffer 2 (10x)

Blocking powder (50 g) was added to 450 ml of buffer 1 (1x) and dissolved by stirring at 70°C for 1 h and filled up to 500 ml with buffer 1 (1x). The buffer was divided into 100-ml aliquots, autoclaved for 20 min at 121°C and stored at -20°C.

Buffer 3Solution (1): 1 M Tris

Tris (60.55 g) was dissolved in 400 ml of deionized water. The pH was adjusted to 9.5. The solution was diluted with deionized water to a final volume of 500 ml and autoclaved for 20 min at 121°C.

Solution (2): 1 M NaCl

NaCl (14.61 g) were dissolved in 200 ml of deionized water, filled up to a final volume of 250 ml with deionized water and autoclaved for 20 min at 121°C.

Buffer 3 for using:

A 100 ml volume of solution (2) was diluted 1: 10 with 900 ml of solution (1).

DIG Easy Hybridization Solution (Roche Diagnostics GmbH, Mannheim, Germany)

CSPD (Roche Diagnostics GmbH, Mannheim, Germany):

Di-sodium 3-(4-methoxyspiro 1,2-dioxetane-3,2'-(5'-chloro) tricyclo decan-4-yl) phenyl phosphate.

Anti-Digoxigenin-AP (Roche Diagnostics GmbH, Mannheim, Germany): Polyclonal sheep anti-digoxigenin, Fab-fragments, conjugated to alkaline phosphatase.

4.9.2 DIG labeling of DNA probes

For labeling of DNA to be used as probes in hybridization experiments, the DIG DNA labeling Mix (Roche Diagnostics GmbH, Mannheim, Germany) was used.

4.9.2.1 Labeling of DNA sizes <10 kb

One microgram of DNA was used. DNA was denatured directly before using by boiling in water for 10 min followed by chilling rapidly on an ice/salt mixture.

The following ingredients were added to the denatured DNA on ice:

Reagent	Volume	or	Volume
Hexanucleotide mix, 10x conc.	2 µl		10 µl
DIG DNA labeling mix, 10x conc.	2 µl		10 µl
Klenow enzyme	1 µl		5 µl
Deionized water to final volume of	20 µl		100 µl

- The content was mixed and spinned shortly in a microcentrifuge.
- Samples were incubated o/n at 37°C.
- The reaction was stopped by adding 2 µl (for 20 µl total volume) or 10 µl (for 100 µl total volume) of 200 mM Na-EDTA (pH 8.0).
- DNA was precipitated by adding 2 µl (for 20 µl total volume) or 10 µl (for 100 µl total volume) of LiCl followed by adding 60 µl (for 20 µl total volume) or 300 µl (for 100 µl total volume) of ice cold ethanol.
- The samples were stored at -20°C for 2 to 24 h or at -70°C for 30 min.
- The sample were centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) at 4°C for 15 min.
- The supernatant was discarded and the DNA pellet was washed with 100 µl (for 20 µl total volume) or 500 µl (for 100 µl total volume) of 70% (v/v) ethanol.
- The DNA pellet was air-dried for 10 min and dissolved in 50 µl of TE buffer and stored at -20°C until further using.

4.9.2.2 Labeling of DNA sizes >10 kb

Two micrograms of DNA were used and digested with appropriate restriction enzyme nucleases (REN) as follows:

DNA	2 µg
REN	2 U
REN buffer	2 µl
Deionized water	to a final volume of 20 µl

- Samples were incubated at temperature recommended by the supplier for 3 h.
- Sterile deionized water was added to a final volume of 100 µl.
- DNA was extracted by adding 100 µl phenol and mixed 30 times by inversion and centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 5 min at room temperature.
- The aqueous phase was removed and transferred into a new 1.5-ml microfuge tube.
- One volume of chloroform/isoamylalcohol was added and mixed 30 times by inversion and centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 5 min at room temperature.
- The aqueous phase was transferred to a clean 1.5-ml microfuge tube.
- 1/10 volume of 3 M Na-acetate (pH 5.2) and 2 volumes of ethanol were added and mixed by inversion (6-8 times). The samples were stored at -20°C for 2 to 24 h or at -70°C for 30 min.
- The samples were centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) at 4°C for 15 min.
- The supernatants were discarded and the DNA pellets were washed with 1 volume of 70% (v/v) ethanol.
- The DNA pellets were air-dried for 10 min and dissolved in 20 µl TE buffer. The DIG labeling protocol was used as described above for DNA fragments smaller than 10 kb.

4.9.2.3 Estimation of the efficiency of probe labelling

The efficiency of DIG probe labelling was estimated by comparison with a DIG labelled control DNA as follows:

- ❖ Serial dilutions were prepared from the DIG-labelled probe (Table 4.6):

Table 4.6: Serial dilutions of probe and control DNA

Ratio	Dilution	Control DNA concentration
A (1:20)	2 μ l DNA + 38 μ l TE-buffer	0.25 ng
B (1:200)	5 μ l (1:20) + 45 μ l TE	25 pg
C (1:2000)	5 μ l (1:200) + 45 μ l TE	2.5 pg
D (1:20000)	5 μ l (1:2,000) + 45 μ l TE	0.25 pg
E (1:200000)	5 μ l (1:20,000) + 45 μ l TE	0.025 pg
F (1:2000000)	5 μ l (1:200,000) + 45 μ l TE	0.0025 pg

- ❖ From each dilution (A-F), 1 μ l of control DNA was spotted on a positively charged nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany). The corresponding dilutions of the DIG labelled control DNA were spotted in parallel.
- ❖ The DNA was fixed by baking at 120°C for 30 min in a hot air oven.

Detection of labelled DNA:

The nylon membrane was submerged in buffer 1 containing 0.3% (v/v) Tween 20 for 1 min

Blocking: The membrane was shaken at room temperature in buffer 2 for 10 min.

Incubation with antibody (anti-digoxigenin): A 1 μ l volume of antibody solution (anti-digoxigenin) was added to 5 ml of buffer 2 in a Petri dish (nerpe plus GmbH, Winsen/Luhe, Germany). The membrane was transferred into this buffer and shaken gently for 10 min.

Removal of excess antibodies: The buffer was discarded and excess of antibodies was removed by washing the membrane in 50 ml of buffer 1 containing 0.3% (v/v) Tween 20 with vigorous shaking two times for 5 min each. Then the membrane was submerged in buffer 3 for 1 min.

Incubation with CSPD: CSPD (5 μ l) was added to 500 μ l of buffer 3, mixed and poured on the membrane in a transparent plastic sheet of appropriate size (Leitz®, 0.08 mm/colorless: Esselte Leitz GmbH & Co KG, Stuttgart, Germany). The membrane was incubated for 5 min at room temperature. Then it was removed from the plastic sheet and dried on filter paper. The membrane was sealed in a fresh transparent sheet and incubated for 15 min at 37°C.

Detection of hybridization signal

The membrane was placed over an X-ray film (Fuji Photo Film GmbH, Düsseldorf Germany) in a dark box for 30 min. Then the X-ray film was developed by shaking in a developing

solution (Tetenal AG & Co. KG, Norderstedt, Germany) for 3 to 5 min, washed with deionized water and fixed by shaking in a fixation solution (Adefo-Chemie GmbH, Nürnberg, Germany) for 3 to 5 min (or until bands appear). Finally, the developed X-ray film was washed with tapped water and air-dried.

4.9.3 Blotting

Blotting was performed according to the protocol provided by Roche (Roche Diagnostics GmbH, Mannheim, Germany) as follows:

1. The gel was submerged in 250 mM HCl with gentle shaking for 5 min at room temperature.
2. The gel was rinsed with deionized water for 1 min.
3. The gel was submerged in denaturation solution twice for 15 min at room temperature with gentle shaking (denaturation of double-stranded DNA into single stranded DNA).
4. The gel was rinsed with deionized water for 1 min.
5. The gel was submerged in neutralization solution twice for 15 min at room temperature.
6. Blotting of the DNA from the gel was done overnight by capillary transfer using 20x SSC (Fig. 4.4). The nylon membrane was dried on filter paper and DNA was fixed by baking at 120°C for 30 min. The membrane was stored at 4°C until further detection. Gloves and forceps were used for manipulations of the membrane.

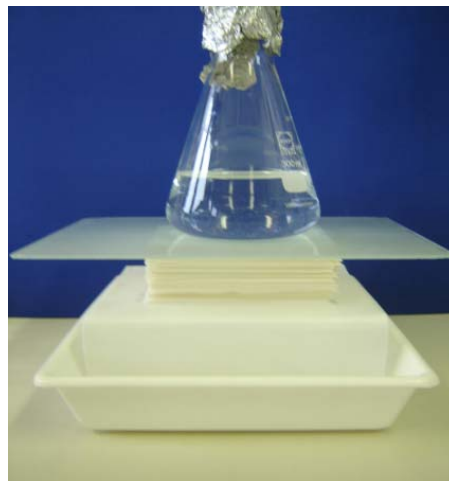


Fig. 4.4: Blotting of DNA from an agarose gel to the nylon membrane

4.9.4 Hybridization protocol

4.9.4.1 Pre-hybridization

The pre-hybridization step was performed by adding 20 ml of pre-warmed “DIG easy buffer” to the membrane for 2 h at 42°C.

4.9.4.2 Hybridization

The probe was denatured by boiling for 10 min in water. The sample was chilled immediately on ice for 3 to 5 min. The denatured probe was added to 10 ml of pre-warmed hybridization buffer and mixed (for a 10-ml volume, 200 ng probe DNA was appropriate). The nylon membrane was transferred to a hybridization glass bottle (Biometra GmbH, Göttingen, Germany), and the hybridization buffer containing probe DNA was added and incubated overnight at 42°C in a hybridization oven (Biometra OV 3: Biometra GmbH, Göttingen, Germany).

4.9.4.3 Detection of hybridization signals

The nylon membrane was removed from the hybridization bottle and washed twice at room temperature with washing buffer (2xSSC, 0.1% SDS) for 5 min.

Stringent washing

The membrane was washed twice for 15 min in washing buffer (0.1xSSC, 0.1% SDS) at 68°C by vigorous shaking. The membrane was equilibrated in buffer 1 containing 0.3% (v/v) Tween 20 for 1 min.

Blocking

The membrane was blocked in buffer 2 with gentle shaking at room temperature for 1 h.

Incubation with antibodies

A 2- μ l sample of antibody solution were added to 20 ml of buffer 2, mixed and poured on the membrane in a Petri dish (nerpe plus GmbH, Winsen/Luhe, Germany). The membrane was incubated in the antibody solution for 30 min with gentle shaking.

Removal of excess antibody

For removal of excess antibodies from the membrane, it was washed twice in 500 ml of buffer 1 containing 0.3% (v/v) Tween 20 with vigorous shaking for 15 min. Subsequently, the membrane was equilibrated for 5 min in buffer 3.

CSPD incubation

The membrane was transferred to a transparent plastic sheet of appropriate size (Leitz®, 0.08 mm/colorless: Esselte Leitz GmbH & Co KG, Stuttgart, Germany). A 2-ml aliquot of buffer 3 containing 20 µl of CSPD was added and the sample was incubated for 5 min at room temperature. Then it was dried on filter paper (complete dryness was however avoided), sealed in a new transparent plastic sheet and incubated at 37°C for 15 min.

Detection of hybridization signal

For detection of hybridization signal, the membrane was exposed for 1 h on an X-ray film (Fuji photo Film GmbH, Düsseldorf, Germany) in a dark box. Subsequently, the X-ray film was developed by shaking for 3 to 5 min in a developing solution (Tetenal AG & Co. KG, Norderstedt, Germany) until hybridization signals did appear. The film was washed with deionized water and transferred for 3 to 5 min into the fixation solution (Adefo-Chemie GmbH, Nürnberg, Germany) with gentle shaking. Finally, the developed X-ray film was washed with water and air-dried. The film was documented by scanning in a standard desk top scanner (HP ScanJet 5370C).

4.10 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed using a thermocycler (Mastercycler 5333; Eppendorf AG, Hamburg, Germany). The PCR reaction was performed in a total volume of 50 µl by using 20 µl of 2.5x PCR master mix (5 Prime GmbH, Hamburg, Germany), 3 µl from forward and reverse primer (10 pmol/µl from each), 23 µl nuclease free water and 1 µl DNA (equivalent to 100 ng). A negative control was included without template DNA. PCR products were analyzed in 1.5% (w/v) agarose gels.

4.10.1 Primers

All primers were designed with DS Gene 1.5 software (Accelrys Limited, Cambridge, England) and manufactured by Eurofins MWG Operon, Ebersberg, Germany. PCR primers used in this work are listed in Table 4.7.

Table 4.7: Primers used in this study

Primer Name	Primer sequence (5'→3')	Target DNA	Reference
ltp-XbaI	CTA CGT GCA AAA <u>TCT AGA</u> TCA CG	<i>ltp</i> -gene, phage TP-778	This study
ltp-Hind-III	CTG CAC TCA <u>AGC TTG</u> TCA AAG		
int.F	ACT GAA TTC TTG GAT AGT GAG G	TP-778 integrase gene (internal fragment: 543 bp)	This study
int.R	CGG CGA TAA GAA GCT TAA ATC T		
8F	AGA GTT TGA TCC TGG CTC AG	16S rDNA (universal primers)	Beumer & Robinson, 2005
1525R	AAG GAG GTG ATC CAG CC		
YP-F	GCT CGT CTT GAA GCT ATG C	major head protein gene of <i>pac</i> -type <i>S. th.</i> ^(a) phages	This study
YP-R	GAT AAG AGT CAA GTG ACC GTC		
YC-F	GCT ATG CTT GAC GAT TCA GT	major head protein gene of <i>cos</i> -type <i>S. th.</i> phages	This study
YC-R	AGC AGA ATC AGC AAG CAA G		
YNS-F	TTC CCT AGC TTC TGG TTC TAC	major head protein gene (<i>orf302</i>) of <i>S. th.</i> phage P738	This study
YNS-R	CAT CTG TGA AGT GAG TCA TAC C		
lys.1	CAC AAG CCT TAA AAG AGG CA	Prophage TP-778 lysin- <i>attR</i> region	This study
Primer 3	CAC AAT CTT CAT CAA GC		
Primer 4	GCA AGG TAA AGC TGC AC	Prophage TP-778 <i>cro-attL</i> region	This study
int.cro.2	TTT TTC TCC CAT GCA CTA ACC		
Yal.1	AAT TAC AAG AGT TCG GAA CGG	TP-778L- <i>attP</i> flanking region	This study
ltp.Rev	TTT GGC TTG CTA AGC CTT TC		
cbh-F	TGC TGA TGT AGA AAG CTA CCC	Indicator plasmid pS1 ^(b)	Ismail, E. 2007
cbh-R	ATA TTA ACA GCA ATG CCA GCC		

(a) *S. th.*: *Streptococcus thermophilus*(b) pS1: pGEM-T-easy vector containing *cbh* gene from *Lactobacillus gasseri*

4.10.2 PCR programs

Table 4.8: List of PCR programs used in this study

Primer		Initial denat.	Denaturation	Annealing	Elongation	Final elongation	Hold
<i>ltp-XbaI</i>	T	94°C	94°C	50°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	45 sec	7 min	∞
	C	1	30	30	30	1	-
<i>ltp-HindIII</i>	T	94°C	94°C	51°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	35 sec	7 min	∞
	C	1	30	30	30	1	-
int.F	T	94°C	94°C	51°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	35 sec	7 min	∞
	C	1	30	30	30	1	-
8F	T	94°C	94°C	51°C	72°C	72°C	4°C
	Ti	4 min	30 sec	30 sec	90 sec	7 min	∞
	C	1	30	30	30	1	-
1525R	T	94°C	94°C	51.8°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	35 sec	7 min	∞
	C	1	35	35	35	1	-
YP-F	T	94°C	94°C	51.8°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	35 sec	7 min	∞
	C	1	35	35	35	1	-
YP-R	T	94°C	94°C	51.8°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	35 sec	7 min	∞
	C	1	35	35	35	1	-
YC-F	T	94°C	94°C	51.8°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	35 sec	7 min	∞
	C	1	35	35	35	1	-
YC-R	T	94°C	94°C	51.8°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	40 sec	7 min	∞
	C	1	35	35	35	1	-
YNS-F	T	94°C	94°C	51.8°C	72°C	72°C	4°C
	Ti	4 min	30 sec	45 sec	40 sec	7 min	∞
	C	1	35	35	35	1	-
YNS-R	T	94°C	94°C	51 °C	72°C	72°C	4°C
	Ti	4 min	30 sec	30 sec	3 min	7 min	∞
	C	1	30	30	30	1	-
Y4	T	94°C	94°C	51 °C	72°C	72°C	4°C
	Ti	4 min	30 sec	30 sec	3 min	7 min	∞
	C	1	30	30	30	1	-
int.cro.2	T	94°C	94°C	51 °C	72°C	72°C	4°C
	Ti	4 min	30 sec	30 sec	90 sec	7 min	∞
	C	1	30	30	30	1	-
Yal.1	T	94°C	94°C	51 °C	72°C	72°C	4°C
	Ti	4 min	30 sec	30 sec	90 sec	7 min	∞
	C	1	30	30	30	1	-
ltp.Rev	T	94°C	94°C	54 °C	72°C	72°C	4°C
	Ti	4 min	30 sec	1 min	1 min	7 min	∞
	C	1	30	30	30	1	-
cbh-F	T	94°C	94°C	54 °C	72°C	72°C	4°C
	Ti	4 min	30 sec	1 min	1 min	7 min	∞
	C	1	30	30	30	1	-
cbh-R	T	94°C	94°C	54 °C	72°C	72°C	4°C
	Ti	4 min	30 sec	1 min	1 min	7 min	∞
	C	1	30	30	30	1	-

T: Temperature; **Ti**: Time; **C**: Cycle

4.10.2.1 High fidelity PCR program

Table 4.9: High fidelity PCR program for 5.2 kb fragment (*lys-in-attR*)

Parameter	Temperature	Time	Cycles
Initial denaturation	94°C	00:04:00	1
Denaturation	94°C	00:00:30	
Anealing	51°C	00:00:30	10
Elongation	68°C	00:04:10	
Denaturation	94°C	00:00:30	
Anealing	51°C	00:00:30	20
Elongation	68°C	00:04:30	
Final elongation	68°C	00:10:00	1
Hold	4°C	∞	-

4.10.3 Colony PCR

4.10.3.1 Colony PCR-buffer (Fermentas GmbH, St. Leon-Rot, Germany)

10x PCR-buffer:

200 mM Tris-HCl (pH 8.8 at 25°C)

160 mM (NH₄)₂SO₄

15 mM MgCl₂

The buffer was autoclaved for 20 min at 121°C and stored at -20°C in 1-ml aliquots.

4.10.3.2 Procedure

One colony was picked with a sterile 1 micro LSI inoculating loop (Nunc GmbH & Co. KG Thermo Fisher Scientific, Langenselbold, Germany) from agar plates and were transferred to 1.5 ml microcentrifuge tubes containing adequate amount (50-100 µl) of 1x PCR-buffer to obtain a turbid cells suspension. Then the tubes were placed in a thermo block (Thermomixer 5436, Eppendorf) and heated at 95°C for 15 min with gentle shaking (600 rpm). Finally, 2 µl were used for PCR reaction in a 50 µl volume and the residual cells were stored at -20°C for further use.

4.10.4 Purification of PCR product

Before using the PCR products for ligation or sequencing, they were purified by “QIA quick spin” kits from QIAGEN (Qiagen GmbH, Hilden, Germany) or by “NucleoSpin® Extract II” kit (Macherey-Nagel GmbH & Co KG, Düren, Germany).

4.11 Cloning

Cloning into plasmid pJET1.2 was carried out according to the supplied protocol of “CloneJET™ PCR Cloning Kit” (Fermentas GmbH, St. Leon-Rot, Germany). Cloning into plasmid pMG36e was performed according to Sambrook *et al.* (1989). Ligation mix was dialyzed for 15 min before using for transformation assay.

4.12 Transformation

4.12.1 Preparation of competent cells and transformation of *L. lactis* cells

The protocol of Holo & Nes (1989) was used as follows:

L. lactis cells grown in GM17 ($OD_{620\text{ nm}} = 0.7$) were added (1%) to 200 ml of prewarmed SGM17 medium containing 0.2% (w/v) glycine, then incubated o/n at 30°C and grown to an $OD_{620\text{ nm}}$ 0.2-0.7. Cells were harvested at 5,000g at 4°C and washed twice in ice-cold electroporation buffer (0.5 M sucrose containing 10% (v/v) glycerol). The pellet was re-suspended in electroporation buffer (1/100 volume). The cell suspension was either used directly for transformation or was stored in 100- μ l aliquots at -80°C for further use.

For transformation, 100 μ l of competent cells were thawed on ice and mixed with 100 ng DNA (in 1 to 3 μ l). The mixture was carefully transferred to an ice-cold electroporation 0.1-cm gap cuvette (Bio-Rad Laboratories GmbH, München, Germany), pulsed once at 2.1 KV (25 μ F, 200 Ω , time constant of 3 ms) in an Electroporator 2510 apparatus (Eppendorf AG, Germany). Then, 900 μ l of ice-cold SGM17 broth were immediately added to the cuvette. The cell suspension was transferred to a microcentrifuge tube and incubated for 2 h at 30°C. The transformation solution was spread on SR-agar plates containing 3 μ g/ml erythromycin and was incubated at 30°C for 2-3 days.

4.12.2 Preparation of competent *E. coli* cells

Competent cells of *E. coli* Easypores were prepared according to Sambrook & Russel (2001) as follows:

- A single colony of *E. coli* Easypores was picked from LB agar plate and was inoculated into a flask containing 50 ml of LB medium. The culture was incubated o/n at 37°C with vigorous shaking on a rotary shaker (250 rpm).
- Two aliquots of 500 ml of pre-warmed LB medium (2-l flasks) were inoculated with 25 ml of the o/n bacterial culture and incubated at 37°C with vigorous shaking until $OD_{620\text{ nm}}$ of 0.3-0.4. The cultures were chilled on ice for 30 min.

- The cultures were transferred to ice-cold centrifuge bottles and cells were harvested by centrifugation at 4,000 rpm in a JA10 centrifuge rotor (centrifuge model: J2-21, Beckman, USA) for 15 min at 4°C. All further steps were carried out aseptically on ice. The cells were re-suspended in 500 ml ice-cold sterile deionized water.
- The cells were sedimented again by centrifugation at 4,000 rpm in a JA10 centrifuge rotor (centrifuge model: J2-21, Beckman, USA) for 20 min at 4°C and re-suspended in 250 ml of ice-cold 10% (v/v) glycerol and centrifuged again (4,000 rpm for 20 min at 4°C).
- Cells were re-suspended in 10 ml of ice-cold 10% (v/v) glycerol, harvested by centrifugation at 4,000 rpm in a JA20 centrifuge rotor (centrifuge model: J2-21, Beckman, USA) for 20 min at 4°C and re-suspended by gentle swirling in 1 ml GYT medium (10% [v/v] glycerol, 0.125% [w/v] yeast extract, 0.25% [w/v] tryptone). The optical density of this suspension was determined from a 1:100 dilution and the OD_{620 nm} value was corrected to 1.0 with ice-cold GYT medium, resulting in a final cell concentration of approximately 2.5×10^8 cells/ml.
- From this cell suspension, 40- μ l aliquots were dispensed into sterile, ice-cold 0.5- or 1.5-ml microfuge tubes, dropped immediately into a bath of liquid nitrogen and were finally stored at -70°C for further use.

4.12.3 Electroporation of *E. coli* Easypores

Electrotransformation of *E. coli* “Easypores” competent cells was done according to Sambrook & Russell (2001) as follows:

- A microfuge tube with 40 μ l of competent *E. coli* “Easypores” cells was thawed, placed on ice and mixed with 1-3 μ l ligation mix (10 pg to 25 ng DNA) and incubated on ice for 30-60 s.
- The cell-DNA mixture was transferred into an ice-cold 0.1-cm electroporation cuvette (Eurogentec GmbH, Köln, Germany) and placed in the Electroporator cuvette-holder.
- Cells were treated with one pulse using Electroporator 2510 apparatus (Eppendorf AG, Hamburg, Germany) at 1.25 kV (capacitance: 25 μ F, resistance: 200 Ω)
- Then, 950 μ l of cold SOC medium were immediately added after pulsing. Cells were transferred to a test tube and incubated at 37°C for 1 h with shaking.
- The cells were plated onto LB agar containing appropriate antibiotics (e.g., 200 μ g/ml ampicillin). The plates were incubated overnight at 37°C.

Analysis of transformants

E. coli or *L. lactis* transformants were further analyzed by plasmid extraction and restriction endonuclease analysis. Identity of the cloned inserts was also verified by DNA sequence analysis.

4.13 Nucleotide sequencing

Sequencing of cloned DNA and PCR products was done at Eurofins MWG Operon, Ebersberg, Germany. DNA sequences were analyzed using the DS Gene 1.5 software (Accelrys Limited, Cambridge, England).

4.14 Restriction enzyme analysis

Digestion with restriction enzymes was carried out according to the instructions of the suppliers (Fermentas [Fermentas GmbH, St. Leon-Rot, Germany], NEB [New England Biolabs GmbH, Frankfurt am Main, Germany] or Eurogentec [Eurogentec GmbH, Köln, Germany]). DNA restriction patterns were analyzed in 0.8% (w/v) or 1.5% (w/v) agarose gels (Serva, Heidelberg, Germany). Gels were photographed digitally using the Universal Hood II-ChemiDoc XRS unit (Bio Rad Laboratories, Segrate [Milan], Italy) and analyzed by “Quantity One” image analysis software (version 4.5.2, Bio-Rad, Laboratories, Segrate [Milan], Italy). Appropriate DNA size markers were used for calculation of sizes of DNA fragments.

4.15 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis from phage structural proteins and bacterial proteins was performed as described by Fayard *et al.* (1993) with some modifications. The “Protein Loading Buffer Pack” (Fermentas GmbH, St. Leon-Rot, Germany) was used for sample preparations. The samples were subjected to electrophoresis in 12% or 15% polyacrylamide gels at 10-20 mA (SE600 electrophoresis unit [Hoefer Pharmacia Biotech, California, USA]). The gels were stained with Coomassie Brilliant Blue R250 (Carl Roth GmbH, Karlsruhe, Germany).

4.15.1 Reagents

Reagents for analysis of bacterial proteins

TES

1 ml of 10x TE-buffer

4 ml deionized H₂O

5 ml of 1 M sucrose

Lysozyme (Serva, Heidelberg, Germany)

50 mg/ml

Mutanolysin (Sigma-Aldrich Biochemie GmbH, Hamburg, Germany)

5000 U/ml

SDS 20% (w/v)

Reagents for SDS gels:

- acrylamide mix (Serva, Heidelberg, Germany)

29% (w/v) polyacrylamide

1% (w/v) N,N'-bis-methylene acrylamide

- 1 M Tris-HCl (pH 8.8)

- 1.5 M Tris-HCl (pH 6.8)

- 10% (w/v) SDS

- ammonium persulfate (APS) 10% (w/v) (AppliChem GmbH, Darmstadt, Germany)

- TEMED (MP biomedical, Illkirch, France)

Loading buffer (sample buffer)

Protein Loading Buffer Pack

(Fermentas GmbH, St. Leon-Rot, Germany):

5x "Protein Loading Buffer"

20x reducing agent: 2 M DTT (**D**ithiothreitol). DTT was added just before buffer was used.

Tris-glycine electrophoresis buffer (running buffer)

The running buffer (1x: 25 mM Tris, 250 mM glycine [Electrophoresis grade: Merck KGaA, Darmstadt, Germany] pH 8.3; 0.1% [w/v] SDS) was prepared from a 10x stock solution (10x: 30.25 g Tris, 188 g glycine, 10 g SDS in 1 l of deionized water).

Staining solution

0.25% (w/v) Coomassie Brilliant Blue R250 (Carl Roth GmbH, Karlsruhe, Germany)

40% (v/v) methanol

10% (v/v) acetic acid (glacial)

The solution was filtered through an S & S folded 595¹/₂ filter (Schleicher & Schüll, Dassel, Germany).

De-staining solution

40% (v/v) methanol

10% (v/v) acetic acid (glacial)

4.15.2 Procedure**Preparation of phage sample**

Phage particles obtained from cesium chloride step gradients were dialyzed against dialysis buffer (10 mM NaCl, 50 mM Tris-Cl [pH 8.0], 10 mM MgCl₂) for 1 h. From this phage suspension, 30 µl were mixed with 8 µl of 5x protein loading buffer and 2 µl of 20x DTT and heated at 95°C for 5 min. Finally, 25 µl were loaded on an 12% SDS gel.

Preparation of whole-cell extracts using cell disruptor

Cell disruption was carried out according to Su *et al.* (1999) with some modifications. Ten ml of cell culture (OD_{620 nm} = 0.6-0.7) were centrifuged and the pellet were suspended in 300 µl of extraction buffer (50 mM Tris-HCl [pH 8.0], 0.1 mM Na-EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Subsequently, 0.5 g of glass beads (diameter: 0.1 mm) were added and the cells were disrupted in the FP120 Fastprep Cell Disruptor (Bio 101 Savant Instruments, Inc. USA) for 20 s, which was repeated 10 times with 2-min intervals. Finally, the glass beads and cell debris were separated by centrifugation (Eppendorf centrifuge 5417R) at 5,000 rpm for 5 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and used directly or stored at -20°C.

Preparation of SDS gels (Sambrook *et al.*, 1989)

The gel chamber equipments (i.e., multiple gel caster, glass and ceramic plates, spacers and combs) were cleaned with ethanol and assembled together (chamber size: 10 cm x 10 cm x 0.75 or 1.5 mm). The gel (12% or 15% (v/v)) ingredients (Table 4.10) were added, mixed (air bubbles were avoided) and poured quickly into the gel caster after adding TEMED. Then, 150 µl isopropanol were poured on the surface of the gel and the gel was allowed to polymerize for 45 min. After polymerization, isopropanol was drained from the surface and the surface was washed with deionized H₂O before the stacking gel was added.

Table 4.10: Solutions for preparing resolving gels for Tris-glycine SDS-PAGE

Solutions	Volume	
	12% (w/v)	15% (w/v)
Deionized H ₂ O	9.9 ml	11.5 ml
30% (w/v) acrylamide mix	12 ml	25 ml
1.5 M Tris (pH 8.8)	7.5 ml	12.5 ml
10% (w/v) SDS	0.3 ml	0.5 ml
10% (w/v) APS	0.3 ml	0.5 ml
TEMED	0.012 ml	0.02 ml
Total volume	30 ml	50 ml

The stacking gel ingredients were added according to Table 4.11, mixed and poured on top of the resolving gel. The comb was fixed quickly. The gel was allowed to polymerize for 30 min and was used immediately or stored in the refrigerator until used.

Table 4.11: Solutions for preparing 5% (w/v) stacking gels for Tris-glycine SDS-PAGE

Solutions	Volume
H ₂ O	3.4 ml
30% (w/v) acrylamide mix	0.83 ml
1 M Tris (pH 6.8)	0.63 ml
10% (w/v) SDS	0.05 ml
10% (w/v) APS	0.05 ml
TEMED	0.005 ml
Total volume	5 ml

Preparation of sample

The sample was prepared by adding 8 μ l of 5x protein loading buffer and 2 μ l of 20x DTT to 30 μ l of the cell extract, mixed and heated at 95 °C for 5 min.

Running of samples in SDS-PAGE

After polymerization, 1x running buffer was poured into the chamber and the comb was removed. The wells were washed with 1x running buffer to remove air bubbles. From every sample, 25 μ l were loaded into the wells. Electrophoresis was carried out at 10 mA until the

samples reached the resolving gel. Then, electrophoresis was continued at 20 mA and stopped when the blue lane of the sample buffer was completely removed from the gel.

Staining of SDS gel

The gel was carefully removed and immersed in at least 5 volumes of Coomassie Brilliant Blue R250 solution for overnight or at least for 4 h.

De-staining of SDS gel

The staining solution was removed and saved for further use. The gel was de-stained by repeated washing in de-staining solution (2-3x) until clear protein bands appeared (usually after 2-3 h).

4.16 MALDI-TOF MS

The P738 structural protein bands were furthermore analysed by MALDI-TOF MS in collaboration with Dr. C. Gelhaus, Zoological Institute of the University of Kiel. For this technique, the phage samples were subjected to 2 additional treatments prior to SDS-PAGE as follows: 1. incubation for 20 min at 55°C in sample buffer supplemented with 50 mM DTT, 2. addition of 150 mM iodo acetamide and incubation for 40 min at room temperature. The gel bands were excised and treated with bovine trypsin according to Gelhaus *et al.* (2005). MALDI-TOF MS was performed on a Voyager-DE STR BioSpectrometry Workstation (PerSeptive Biosystems, Framingham, USA) according to Gelhaus *et al.* (2008). The resulting spectra were processed with the Data Software version 3.5.0.0. Proteins were identified by searching the nonredundant NCBI database using the program MS-Fit (<http://prospector.ucsf.edu/ucsfhtml/msfit.htm>).

4.17 Western blot analysis (Immunoblotting)

The Western blot technique provides information about the presence, molecular weight and quantity of an antigenic protein by detection with specific antibodies. This procedure can be summarized in the following steps: (a) separation of proteins via SDS-PAGE, (b) transfer to a solid support (nitrocellulose or PVDF membrane), (c) blocking of the membrane to prevent non-specific binding of the antibodies, (d) treatment with a specific primary antibodies raised against the target antigen and finally (e) incubation with a secondary antibody. Detection of secondary antibody usually occurs indirectly by using a conjugated reporter group, e.g. enzymes such as alkaline phosphatase or horseradish peroxidase.

4.17.1 Reagents

- Transfer buffer

The transfer buffer (1x) was prepared from 10x stock solution.

10x:		1x:	
Tris-HCl (pH 8.8)	100 mM	50 ml Transfer buffer 10X	
Na-EDTA	20 mM	7.7 mg DTT	
NaCl	500 mM	450 ml deionized H ₂ O	

- **PVDF membrane (0.2 µm pore size)** (Macherey-Nagel GmbH & Co. KG, Düren, Germany)

- 100% Methanol

- TBS

10 mM Tris-HCl (pH 7)

9 g NaCl

Reagents were dissolved in 1 l deionized water.

- TBS with sodium azide

10 mM Tris-HCl (pH 7)

9 g NaCl

0.2 g NaN₃ (sodium azide)

Reagents were dissolved in 1 l deionized water.

Freshly made solutions:

- Blocking solution

The solution was prepared by dissolving 6 g albumin (fraction V & protease free - Carl Roth GmbH, Karlsruhe, Germany) in 200 ml of TBS-buffer.

- Blocking solution with sodium azide

Albumin	6 g
TBS / sodium azide	200 ml

- Primary antibody solution (1: 2,500)

Primary antibodies (Ltp _{TP-J34} -specific antibodies)	32 µl
Blocking solution / azide	80 µl

- Secondary antibody solution

Secondary antibodies (Anti-chicken IgY/AP conjugated - Promega, Madison, USA)	14 µl
Blocking solution	80 µl

- Detection buffer

Tris-HCl (pH 9.5)	100 mM
NaCl	100 mM
MgCl	5 mM

- Staining solution

Detection buffer	20 ml
NBT (Promega, Madison, USA)	132 μ l
BCIP (Promega, Madison, USA)	66 μ l

4.17.2 Preparation of polyclonal antibodies specific for the lipoprotein encoded by *S. thermophilus* phage TP-J34

Polyclonal antibodies were raised in hen's eggs (Davids Biotechnologie, Regensburg, Germany) against the Ltp lipoprotein of phage TP-J34 (Sun *et al.*, 2006).

4.17.3 Transfer of proteins from SDS polyacrylamide gel to membrane by capillary blotting

After running of protein samples in SDS polyacrylamide gel, the membrane was blotted as follows:

- The SDS-polyacrylamide gel was submerged in 1x transfer buffer for 10 min (2 times) with gentle shaking.
- A PVDF membrane (porablot PVDF, pore size 0.2 μ m: Macherey-Nagel, Düren, Germany) was cut to the desired size, laid on the surface of 100% methanol for 3-5 sec and subsequently submerged in methanol.
- Next, the membrane was completely immersed in transfer buffer (for 10-min equilibration time).
- The gel was laid in inverted position on 3 pieces of wet Whatman 3MM filter paper (Whatman GmbH, Dassel, Germany). The gel was covered with the PVDF membrane and another 3 pieces of wet Whatman 3MM filter paper with a 3-cm stack of filter papers. A 500-g weight was put on top of the filter paper stack. Protein blotting was done overnight.

4.17.4 Detection of protein (Ltp) on PVDF membrane

- After protein transfer, the PVDF membrane was incubated for 24 h at room temperature in blocking solution (containing sodium azide) with shaking.
- The blocking solution was discarded and the membrane was washed for 20 min with TBS (containing sodium azide) with shaking. This step was repeated twice.
- TBS was discarded and the membrane was incubated in primary antibody solution (anti-Ltp) with gentle shaking for 3-6 h.
- The membrane was washed with TBS for 20 min, subsequently with fresh TBS (overnight incubation with shaking).
- TBS was poured away and the membrane was incubated in secondary antibody solution (anti-chicken IgY conjugated to alkaline phosphatase - Promega, Madison, USA) for 1 h with gentle shaking.
- Then the membrane was washed thoroughly in TBS for 10 min (3 times) at room temperature with vigorous shaking to remove all non-binding secondary antibodies.
- Finally, the membrane was stained by shaking gently in a freshly prepared NBT/BCIP detection buffer in a dark place until cross-reaction signals appeared after 5-7 min.

5 Characterization of *S. th.* phage TP-778

Chapter 1: Identification and characterization of

Streptococcus thermophilus phage TP-778

5.1 Introduction

As a prerequisite for efficient phage control in dairies and other areas of the food industry, the prevailing bacteriophage populations have to be identified and characterized. Today, powerful sequencing technologies such as “shotgun sequencing” are state-of-the-art, and the DNA sequences of several hundreds of phage genomes are currently available at GenBank. The number can be expected to accumulate to thousands in short time (Canchaya *et al.*, 2007). Lactic acid bacteria are important components of starter cultures. Thermophilic *S. thermophilus* cultures are required for the production of yoghurt, mozzarella and other cheese varieties (e.g. Cheddar and Swiss-type cheeses). However they are frequently infected by lytic *S. thermophilus* phages, which have therefore been studied in detail in laboratories world-wide (Stanley *et al.*, 1997). Aside from lytic phages, temperate phages can integrate their genomes as prophages into bacterial chromosomes. These phages therefore establish a relationship with their host which is termed lysogeny. Upon prophage induction, temperate phage particles are released after lysis of the host cells. They can also propagate lytically on suitable host strains. The conditions for the lysis *versus* the lysogeny decision are not always known. It is believed that the physiological state of the cells influences this decision. Bacteriophage λ needs about 10 to 15 min after infection to make this decision. Two phage-encoded regulatory proteins, CI repressor and Cro protein, are involved. In the lysogenic cell, a high level of CI repressor and low level of Cro protein will switch off the expression of lytic phage genes and will favour the lysogenic pathway (Waldor *et al.*, 2005; Ptashne, 2004). The integration of the prophage DNA into the bacterial chromosome usually follows the Campbell model of site-specific recombination (Campbell, 1962) between two specific attachment sites, one on the bacterial chromosome (*attB*) and the other on the phage genome (*attP*). Both sites reveal the same core nucleotide sequence. As a consequence, integration of phage DNA may disrupt or modulate bacterial gene expression. The prophage DNA can also affect the fitness or virulence of the bacterial host cells. In Gram-negative bacteria, many examples of site-specific integration have been characterized, and the *E. coli* phage λ (Campbell, 1992; Landy, 1989) is the best studied prototype. Phage integration systems have also been studied for many Gram-positive bacteria. Many lactic acid

bacteria are lysogenic, e.g., *Lactococcus lactis* (Boyce *et al.*, 1995; Christiansen *et al.*, 1994; Lillehaug & Birkeland, 1993; and *Lactobacillus gasseri* (Raya *et al.*, 1992). For *Lb. delbrueckii* subsp. *bulgaricus*, a 17-bp common core sequence has been identified on the bacterial genome and the phage mv4 (Dupont *et al.*, 1995). In *S. thermophilus* host strains carrying prophages O1205 or Sfi21, a 40-bp identity region was identified in a *tRNA^{Arg}* gene which remained intact after prophage integration. The 40-bp core region was also detected in phage TP-J34 (Fig. 5.1) (Stanley *et al.*, 1997; Bruttin *et al.*, 1997c; Neve *et al.*, 2003). Although the lysogenic state is extremely stable (Little *et al.*, 1999), it can convert to the lytic development of a new temperate phage population by different mechanisms. In bacteriophage λ , the switching from the lysogenic to the lytic cycle is a result of the host regulatory SOS system, e.g. by UV light irradiation or cultivation in the presence of mitomycin C. Prophage induction and excision results in a new recombination event between the two junction sites, *attL* and *attR*, created by the integration process (Brüssow, 2001). Occasionally, induced lysogenic cultures may give rise to altered cells that are non-inducible and thus unable to produce infectious temperate phage particles. This event may be due to either simple mutations leading to “cryptic” prophages or to complex rearrangements like deletions resulting in the production of “defective” phages and prophage remnants (Birge, 2000). Prophages can carry genes that control the phenotype of the lysogenic host cells. Well-known examples are prophage-derived bacterial genes (morons) coding for toxins and for lipoproteins, which are not required for the lysogenic or lytic cycles (Waldor *et al.*, 2005). Also, it has been reported that prophages of *S. pyogenes* and *Staphylococcus aureus* contain genes which are responsible for their pathogenicity, and these genes encode toxins, superantigens, mitogenic factors and DNases (Desiere *et al.*, 2001b). The genes are located on a defined DNA region flanked by the phage lysis cassette and the attachment site of the prophage. This location may explain the acquisition of bacterial genes during abnormal prophage excision (Ventura *et al.*, 2002).

In contrast to *L. lactis* starter cultures, lysogeny is a rather rare event in *S. thermophilus*. However, a number of lytic and temperate *S. thermophilus* phages have been characterized in detail on the genomic level. The first published phage DNA sequence was a conserved 2.2-kb EcoRI fragment from the genomes of *S. thermophilus* phages Sfi18 and Sfi11, which shared homology with 60% of other phages tested. Two *orfs* were identified but no function was detected (Brüssow *et al.*, 1994b). The second phage DNA sequence published was an *XbaI* DNA fragment from *S. thermophilus* phage Sfi21. During lytic growth of phage Sfi21, a spontaneous deletion occurred within this DNA region and was possibly involved in the

control of the phage life cycle (Bruttin & Brussow, 1996). Today, many *S. thermophilus* phages have been studied and their complete genomic sequences have been determined (phage Sfi21 [Desiere *et al.*, 1998], Sfi19 [Lucchini *et al.*, 1999], DT1 [Tremblay & Moineau, 1999], 7201 [Proux *et al.*, 2002], phages 1205 [Stanley *et al.*, 1997], Sfi11 [Lucchini *et al.*, 1998], 2972 [Lévesque *et al.*, 2005] and TP-J34 [Sun *et al.*, 2006]). The genomes of all characterized virulent and temperate *S. thermophilus* phages show homologies to each other in a modular form (Mercenier, 1990).

Some *S. thermophilus* strains contain prophage remnants in their genomes, like strain Sfi16, which may have resulted from massive loss of prophage DNA regions (Ventura *et al.*, 2002). Phage genes transcribed in the lysogenic state are often avoided from this deletion process because these genes encode functions that are important to the lysogenic host (Desiere *et al.*, 2001b). The 2.8-kb Sfi16 prophage remnant sequence revealed an authentic *attL* site overlapping the prophage remnant integrase gene (*orf359*) differing from phage Sfi21 integrase gene at only 15 bp positions. The *attR* site, however, was missing (Fig. 5.2) (Ventura *et al.*, 2002). Upstream of the integrase gene, 4 *orfs* were identified, 2 transcribed in the same direction as the integrase gene (*orf168* & *orf56*) and 2 transcribed in the opposite direction (*orf117* & *orf97*). The *orf97* gene showed 90% homology to *orf2* from the virulent *S. thermophilus* phage 7201 but the other *orfs* yielded no database matches. Northern blot analysis showed transcription of *orf168*, of *orf56* and of the integrase gene in Sfi16 (Ventura *et al.*, 2002).

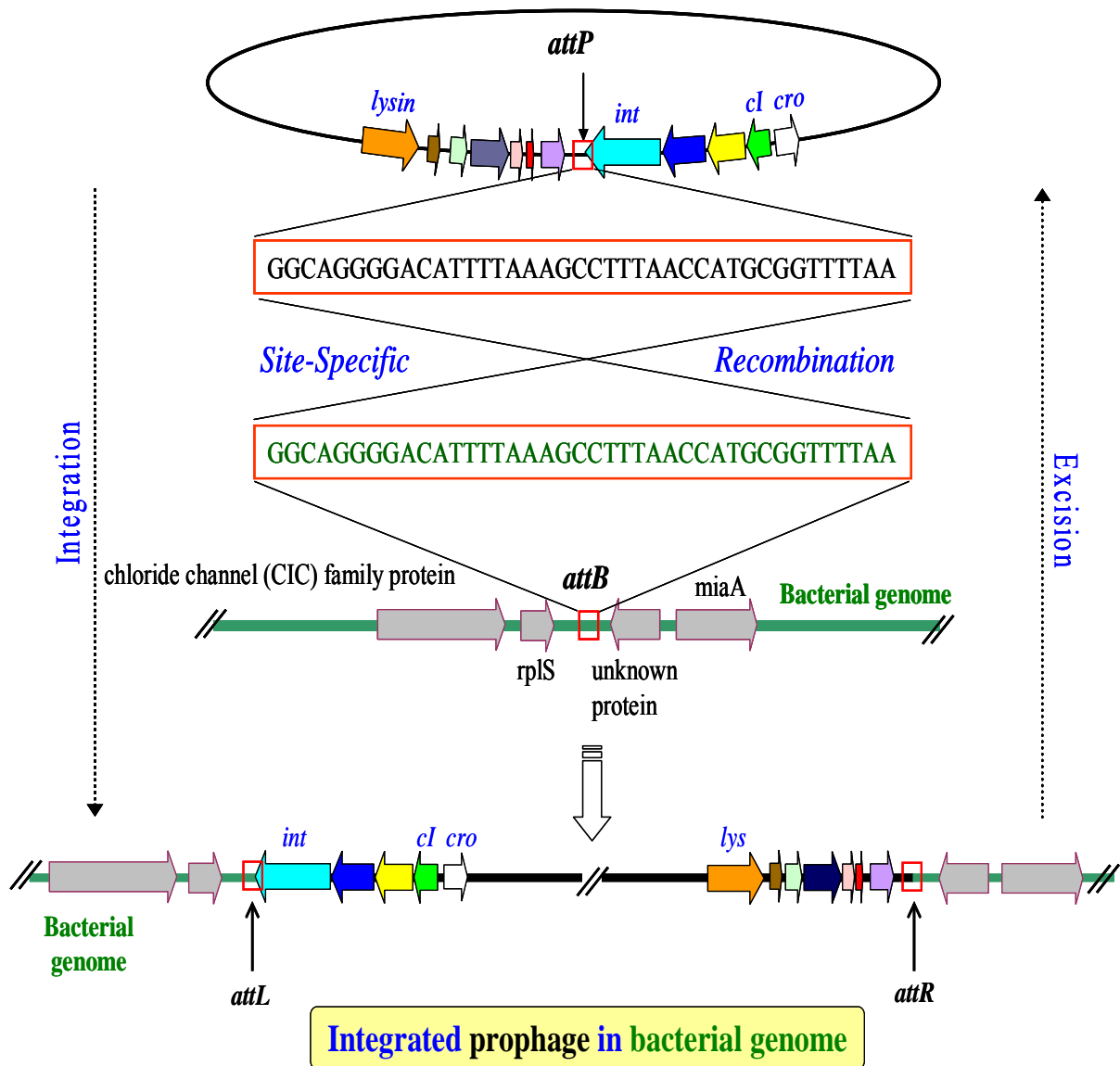


Fig. 5.1: Schematic presentation of the site-specific integration of *S. thermophilus* TP-J34 phage genome (black circle, top) into the host chromosome (green line, middle) and resulting prophage in the chromosome (bottom). Only *orfs* flanking the prophage *attP* site and the chromosomal *attB* site are shown. The 40-bp core region identical in *attP* and *attB* is shown. Abbreviations: *int* = integrase gene, *cl* = repressor gene, *cro* = cro gene and *lys* = lysin gene (Neve *et al.*, 2003; Sun *et al.*, 2006).

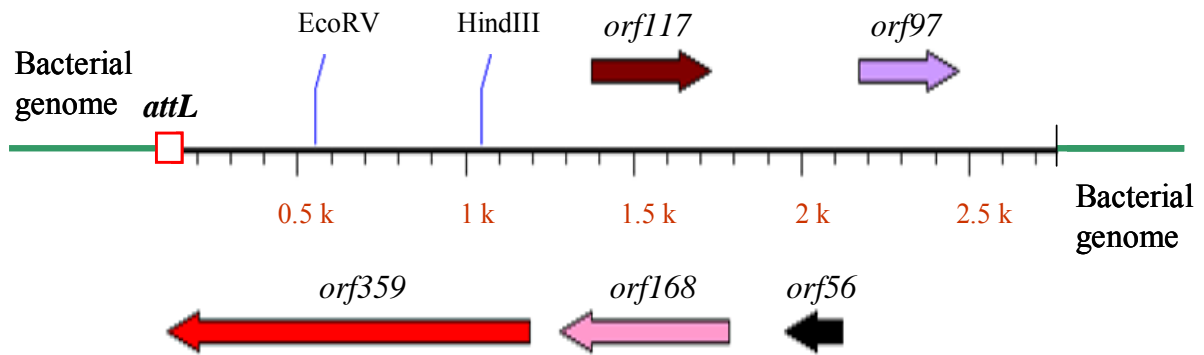


Fig. 5.2: Gene arrangement of a prophage remnant of *S. thermophilus* Sf16 (Ventura *et al.*, 2002).

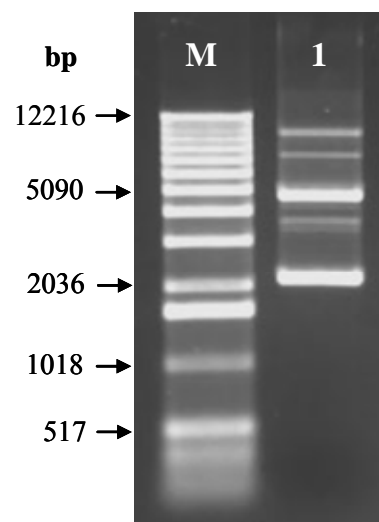
In this chapter, the detailed characterization of the *S. thermophilus* phage TP-778 and the DNA sequence analysis of its lysogeny module is shown.

5.2 Results and Discussion

5.2.1 *Streptococcus thermophilus* SK778

S. thermophilus SK778 has been shown earlier to contain a prophage (phage TP-778) (Neve *et al.*, 2004). It was the only lysogenic strain found within 150 *S. thermophilus* cultures and harbors 2 plasmids (Fig. 5.3). The morphology of SK778 cells is shown in the scanning electron micrograph in Fig. 5.4. It can be seen that some of the cells have already lysed, probably due to spontaneous prophage induction. The shape of the SK778 cells differs from the morphology of the cells from the lysogenic *S. thermophilus* strain J34 (see Fig. 5.5).

Fig. 5.3: Agarose gel electrophoresis of the plasmid DNA of *S. thermophilus* 778 (lane 1). The 2 major bands represent the native ccc-form (covalently closed circular) of the 2 plasmids of strain SK778 (sizes: approximately 2.2 and 4.8 kb). Lane M: DNA molecular weight marker X (Roche, Mannheim, Germany).



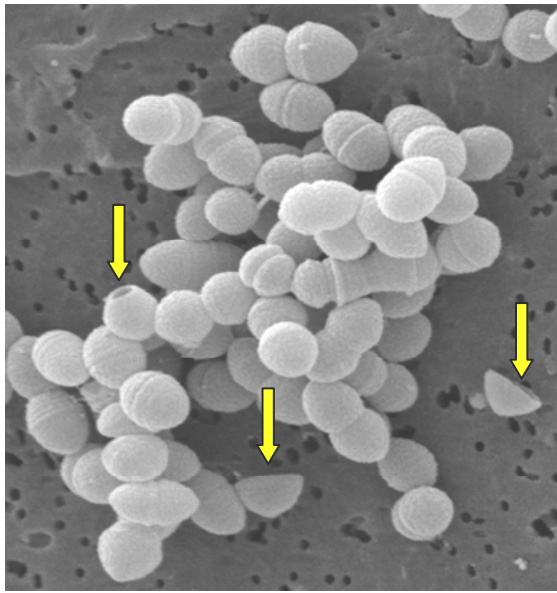


Fig. 5.4: Scanning electron micrograph of *S. thermophilus* SK778. Arrows show spontaneously lysed cells. Source: H. Neve, MRI-Kiel, Germany.

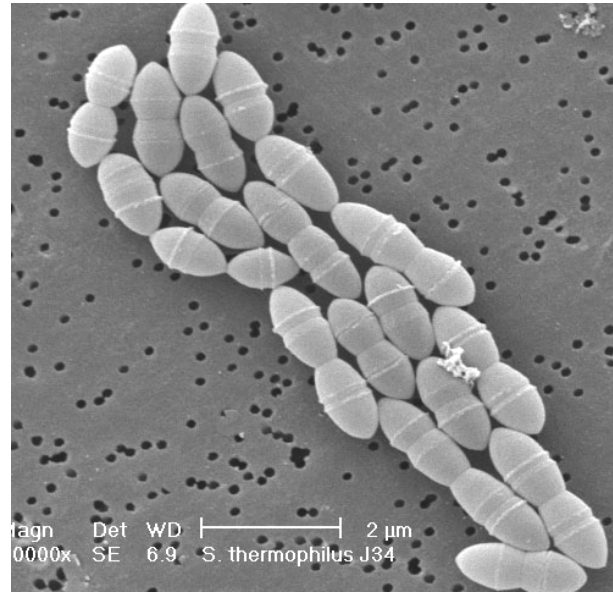


Fig. 5.5: Scanning electron micrograph of *S. thermophilus* J34. No lysed cells were observed. Source: H. Neve, MRI-Kiel, Germany.

5.2.2 Induction of phage TP-778 with mitomycin C and ultraviolet (UV) light

S. thermophilus SK778 was grown in LM17th broth at 40°C and treated either with different concentrations of mitomycin C or by UV light irradiation during the early logarithmic growth phase. Optimal induction and lysis of host cells due to the release of temperate phages was obtained either with 0.2 μg/ml mitomycin C (see Fig. 5.6 left) or by a standard laboratory UV lamp (254 nm, Schütt, Göttingen, Germany) positioned in 3-cm distance to the culture pumped through a quartz glass tube (see Fig. 5.6 right).

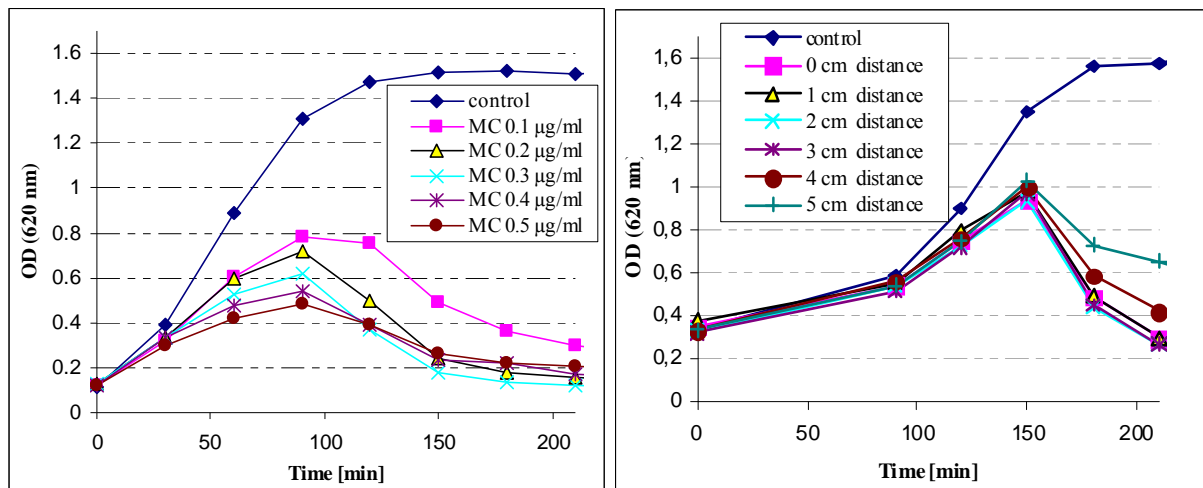
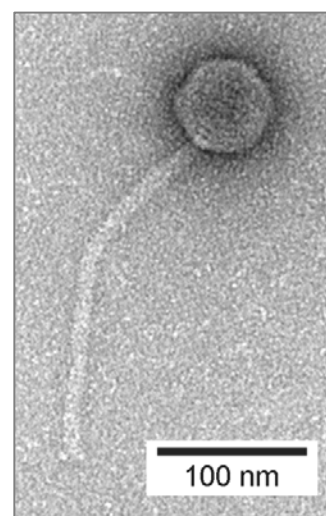


Fig. 5.6: Growth curves of *S. thermophilus* SK778 in LM17th broth upon induction of phage TP-778 with either mitomycin C (MC) (left) at various concentrations or with UV light (right) at different distances of the UV lamp to the culture.

5.2.3 Morphology of *S. thermophilus* phage TP-778

TP-778 phage particles obtained from induction were analysed in the transmission electron microscope. The majority of phage particles were intact showing isometric heads and long non-contractile tails (length ca. 240 nm) (Fig. 5.7). This corresponds with the basic morphology of other *S. thermophilus* phages (Neve *et al.*, 1989; Brüßow *et al.*, 1994a; Bruttin *et al.*, 1997a). Hence, phage TP-778 is a member of the well-described *Siphoviridae* phage family (morphotype B1) (Bradley, 1967).

Fig. 5.7: Transmission electron micrograph of *S. thermophilus* phage TP-778. Source: H. Neve, MRI-Kiel, Germany.



5.2.4 DNA restriction endonuclease pattern

In order to estimate the size of the phage genome, DNA from phage TP-778 was digested with restriction enzymes EcoRI, HaeIII and HindIII (Fig. 5.8). From the fragments obtained, a genome size of about 44 kb could be calculated. *S. thermophilus* phages are grouped into 2 classes according to their mechanism of DNA packaging (LeMarrec *et al.*, 1997). Phages of the *cos*-type have cohesive ends at their terminal DNA regions, and one copy of the phage genome is packed into the phage head during phage assembly in the lytic cycle. In contrast, DNA packaging of *pac*-type phages occurs in a “head-full” manner, and their terminal genome regions usually reveal terminal redundancy. DNA of *cos*-type phages can circulate via their cohesive ends, but *cos* ends will melt at elevated temperature. Hence it is possible to identify *cos*-type phage DNA by restriction enzyme analysis, since DNA-fragments containing the “closed” *cos*-site will disappear when heated while two new DNA-fragments (each with one of the two *cos* ends) will appear. Fig. 5.8 shows that upon moderate heating (74°C for 10 min, Quiberoni *et al.*, 2003) the restriction enzyme patterns of the TP-778 phage DNA did not change, indicating that phage TP-778 is a *pac*-type phage.

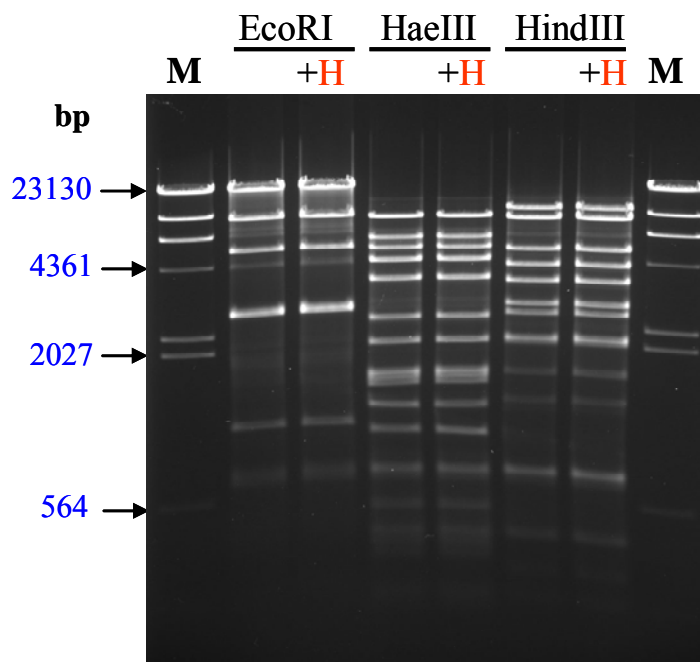


Fig. 5.8: Agarose gel (1.2%) electrophoresis of TP-778 DNA digested with EcoRI, HaeIII, HindIII. DNA was either used unheated or heated for 10 min at 74°C (+H) prior to gel electrophoresis. Lane M: phage λ DNA restricted with HindIII as size reference (Roche, Mannheim, Germany).

5.2.5 Prophage curing experiments

During the first phase of this thesis, it was not possible to find a suitable *S. thermophilus* indicator strain that would allow lytic propagation of phage TP-778. As an alternative, a prophage-cured derivative of the lysogenic SK778 culture would also allow propagation of the phage. Strain SK778 was grown in LM17th containing polyclonal antibodies raised against TP-778 phage particles (dilution of antiserum: 1:1000) at 40°C to the early log phase ($OD_{620\text{ nm}} = 0.2$) and was then induced with mitomycin C (0.2 µg/ml). Aliquots of the culture were taken during lysis and immediately diluted 1:10 and 1:100 with fresh growth broth in order to prevent re-infection of putative prophage-cured cells by phage TP-778. Aliquots (100 µl) were plated on LM17th agar and incubated at 40°C for 48 h. Residual cells of the culture after lysis were also centrifuged and the re-suspended cells were plated on agar. Surviving single-colony isolates were tested for induction with mitomycin C. It is remarkable that no prophage-cured colonies were obtained (data not shown). For other lysogenic *S. thermophilus* cultures, prophage-curing has been demonstrated (Neve *et al.*, 2003). This may indicate that phage TP-778 may have an unusual lysogeny module (see later section 5.2.9). Since re-infection was prevented by the presence of the antiserum and neutralization of the phages, efficient lysis occurring in every induced cell may be responsible for this failure.

5.2.6 Detection of host strains for phage TP-778

Since the prophage-curing experiments were unsuccessful for *S. thermophilus* SK778, a set of non-lysogenic 16 *S. thermophilus* wild-type strains were tested for their sensitivity to phage TP-778. Only *S. thermophilus* strain B106 was found to be sensitive for phage TP-778 propagation. This strain had been kindly provided by the University of Cork, Ireland, as a host strain for propagation of temperate phage 7201 (Proux *et al.*, 2002). The lytically propagated phage was named TP-778L. It was purified from a single plaque and propagated on the host strain B106 in 1 l of LM17th broth for subsequent CsCl step gradient centrifugation and phage DNA extraction. The restriction patterns of phage TP-778L (Fig. 5.9) differed from the corresponding profiles of phage TP-778 (Fig. 5.10). The absence of the largest HindIII fragment, of one specific HaeIII fragment and of an approx. 0.6-kb EcoRI fragment in the TP-778 genome illustrates that deletion events have occurred in the TP-778L phage genome during the lytic growth cycle. This is also known for other temperate *S. thermophilus* phages that had been propagated lytically on suitable indicator strains (TP-J34L, Neve *et al.*, 2003).

Fig. 5.9: Agarose gel (1.2%) electrophoresis of TP-778L DNA digested with restriction enzymes EcoRI (lane 1), EcoRV (lane 2), HaeIII (lane 3), HindIII (lane 4), HpaII (lane 5) and PvuII (lane 6). Lane M: phage DNA λ restricted with HindIII (Roche, Mannheim, Germany).

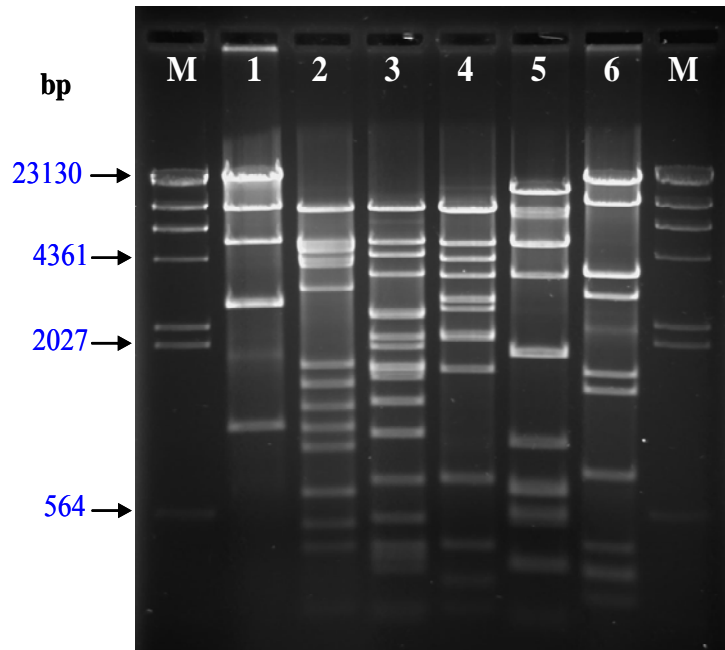
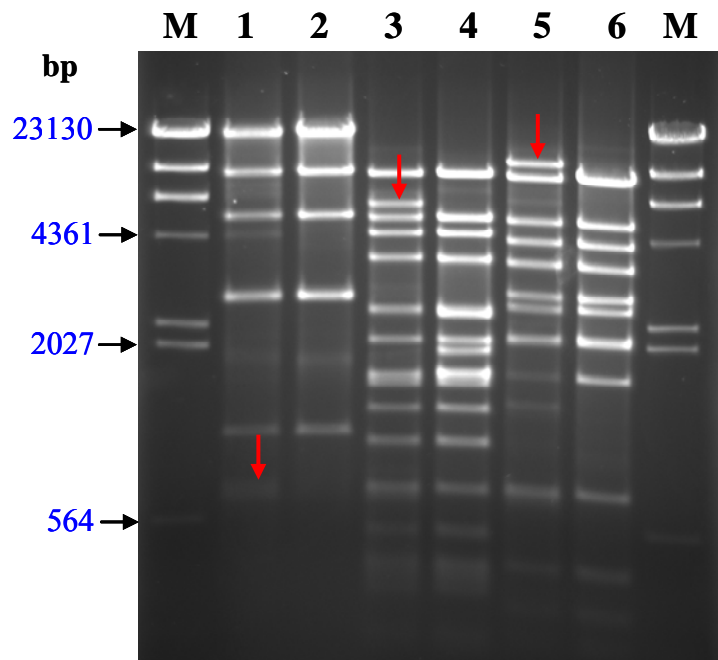


Fig. 5.10: Agarose gel (1.2%) electrophoresis of TP-778 (lane 1, 3 & 5) and TP-778L (lane 2, 4 & 6) DNA digested with restriction enzymes EcoRI (lane 1, 2), HaeIII (lane 3, 4) and HindIII (lane 5, 6). Arrows showing the DNA bands which disappeared in TP-778L. Lane M: phage DNA λ restricted with HindIII (Roche, Mannheim, Germany).



5.2.7 Adsorption test

Neve *et al.* (2003) have shown earlier that the lysogenic *S. thermophilus* strain J34 could easily be cured of its prophage TP-J34. As will be shown later (see 5.2.9.2) phages TP-J34 and TP-778 have similar lysogeny modules. Phage TP-J34 could also be propagated on the prophage-cured derivative J34-6 (Neve *et al.*, 2003). Therefore it was anticipated that this strain might also be an indicator strain for phage TP-778L. However, this phage did not infect strain J34-6. In order to test whether phage TP-778L could also adsorb to J34-6 cells, phage adsorption experiments were carried out. Since it has been shown recently that lactococcal phages bind in a 2-step manner to their host cells (first reversible, then irreversible; Breum *et al.*, 2007) both types of adsorption tests were also performed with phage TP-778L. Phage TP-778L did adsorb well (approx. 90% after 20 min) to strain J34-6, indicating that phage adsorption was not the critical step excluding phage propagation. The same adsorption behaviour was also documented for the control strain B106 which was sensitive to phage TP-778L infection. It is remarkable that no difference between reversible and irreversible adsorption was found indicating reversible adsorption to be negligible. Adsorption experiments were also performed with *S. thermophilus* strain YA4 which contains a prophage remnant identical to a phage remnant described for strain Sfi16 (Ventura *et al.*, 2002). Only poor adsorption was documented for this strain which might indicate that strain YA4 is benefitting from the prophage remnant and is protected against phage attacks. As another control, *L. lactis* strain Bu-60 was also tested for phage TP-778L adsorption. It has been shown recently that another *S. thermophilus* phage (P1109) was capable to adsorb to this lactococcal strain. It was shown for the first time that this *S. thermophilus* phage could in fact inject plasmid DNA into *L. lactis* Bu2-60 (Ammann *et al.*, 2008). As shown in Fig. 5.11, phage TP-778L did not adsorb to *L. lactis* Bu2-60.

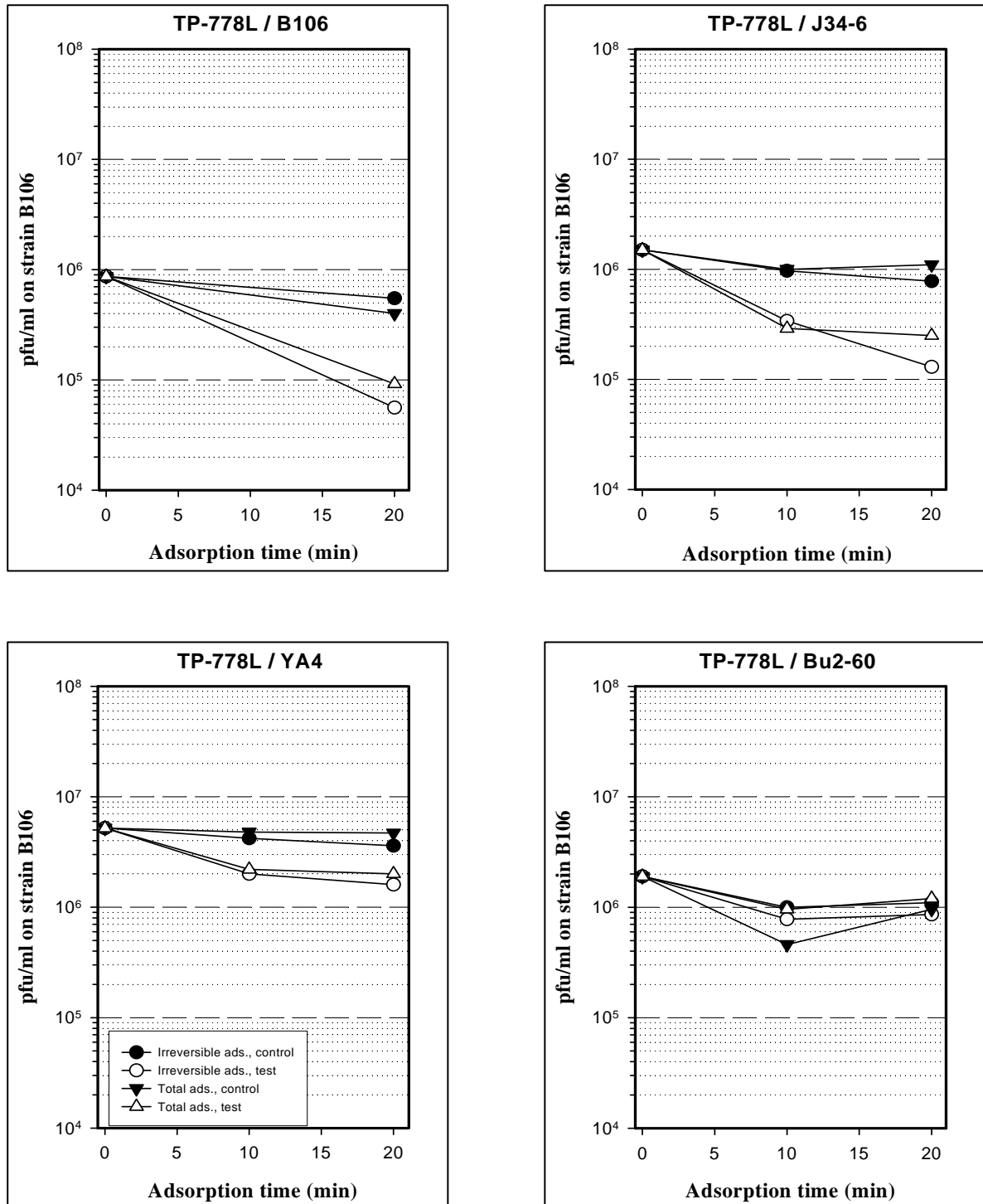


Fig. 5.11: Adsorption of phage TP-778L to *S. thermophilus* strains B106, J34-6, YA4 and to *L. lactis* Bu2-60. The graphs indicate the plaque-forming units of non-adsorbed phage particles in the supernatant after centrifugation of the cells to calculate total adsorption (—△—) and irreversible adsorption (—○—). In the controls for total (—▼—) and irreversible (—●—) adsorption, cells were replaced by corresponding volumes of buffer.

5.2.8 Genetic relationship of phage TP-778

5.2.8.1 DNA homology of phage TP-778 with other *S. thermophilus* phages

To detect DNA homologies between the genome of phage TP-778 and other *S. thermophilus* phage genomes, a Southern blot analysis was performed. Phage DNAs were isolated from the well-studied *pac*-type temperate phage TP-J34 (Neve *et al.*, 2003) and from the *cos*-type virulent phage P53 (Neve *et al.*, 1989). The TP-778 DNA was hybridized with DNA probes of these 2 phages (Fig. 5.12). Southern blot analysis showed that phage TP-778 is genetically closely related to phage TP-J34 but reveals only limited DNA homology with phage P53.

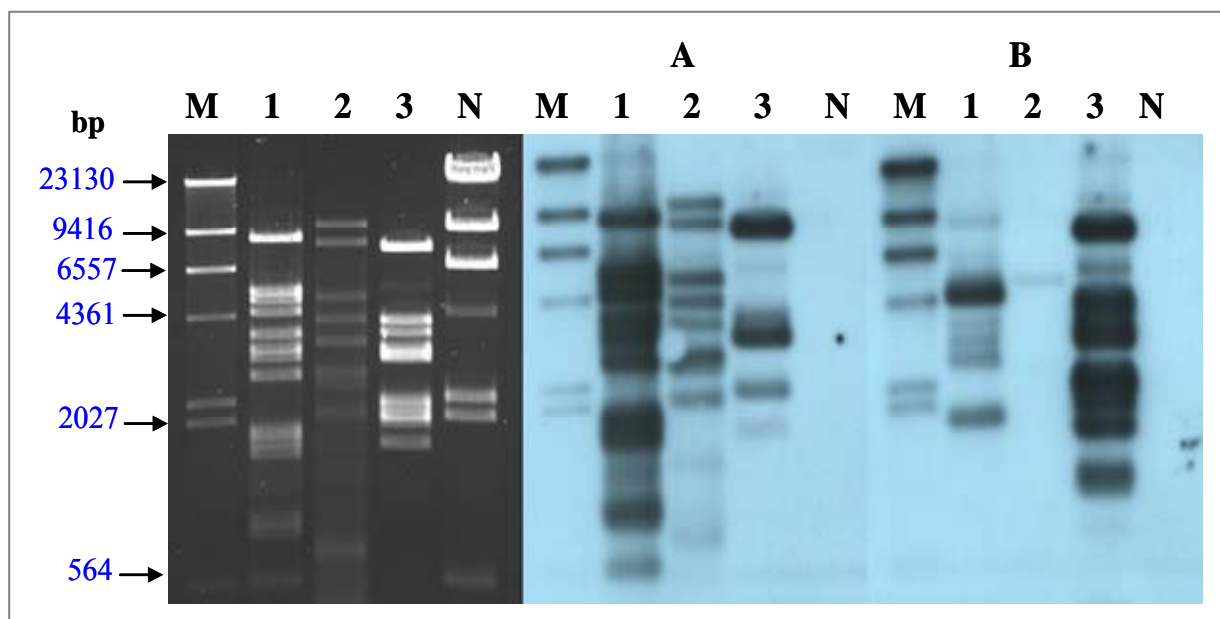


Fig. 5.12: Agarose gel (0.8%) electrophoresis (left) and Southern blot hybridization (right A & B) of HindIII-digested DNA of phages TP-J34 (lane 1), TP-778 (2) and P53 (3). The gel was blotted and probed with TP-J34 DNA (A) and with P53 DNA (B). Lane M and N: phage λ /HindIII DIG labelled (positive control) and unlabelled (negative control), respectively (Roche, Mannheim, Germany).

5.2.8.2 DNA homology of phage TP-778 DNA with chromosomal DNA of *S. thermophilus* strains

Chromosomal DNAs from 9 *S. thermophilus* strains were digested with HindIII and hybridized with TP-778 DNA (Fig. 5.13). These strains were selected in order to ensure a representative spectrum of different strains carrying (1) an inducible prophage (strain J34 [TP-J34, Neve *et al.*, 1998 and 2003] and strain SK778 [TP-778]; this thesis), (2) a non-inducible prophage (strain SK778-Y; this thesis), (3) a 2.8-kb prophage remnant (strains S15, YA4, YA5 and YA6) and (4) a control strain without any prophage DNA (i.e. strain YA20).

It was expected that the TP-778 probe should hybridize strongly to the DNA of the lysogenic strains, but only little to the DNAs with the prophage remnants and not at all to the control strain DNA without prophage DNA. However, surprisingly numerous hybridization signals were detected in all chromosomal DNAs (Fig. 5.13). The same remarkable result was obtained, when the TP-778 probe was hybridized with the DNAs of other non-lysogenic *S. thermophilus* strains (not shown). This suggests two possibilities: i) Either phage TP-778 had incorporated DNA originating from *S. thermophilus* genomic DNA or ii) this temperate phage contains chromosomal DNA packaged during its multiplication, i.e., TP-778 should be a transducing phage.

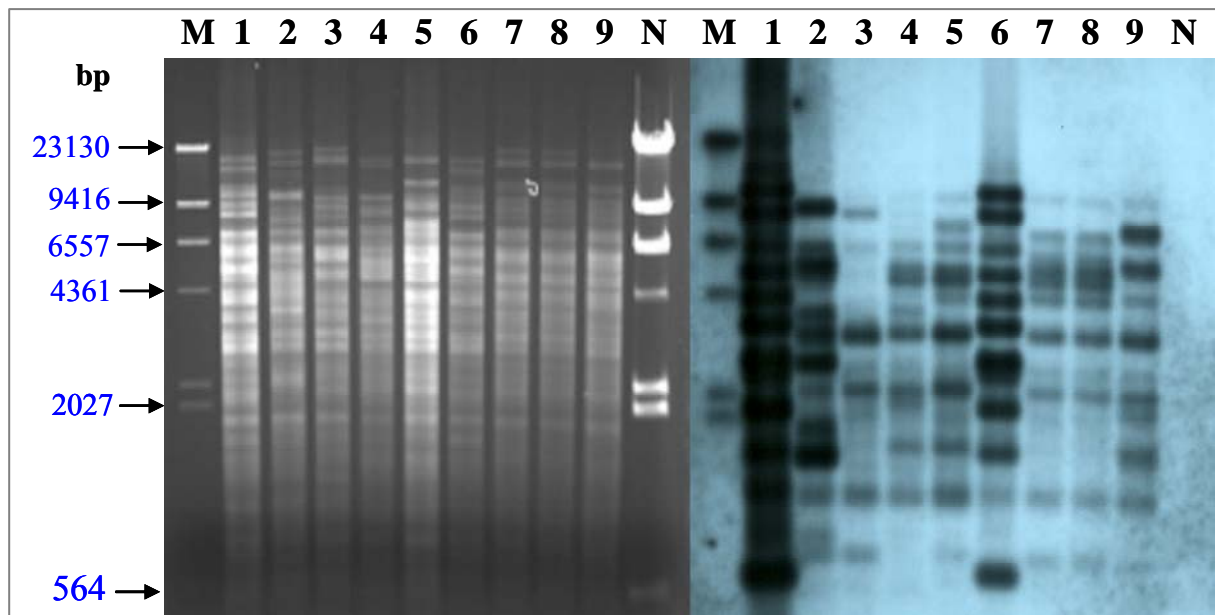


Fig. 5.13: Agarose gel (0.8%) electrophoresis (left) and Southern blot hybridization (right) of HindIII-digested chromosomal DNA of *S. thermophilus* strains SK778 (lane 1), J34 (2), 124 (3), S15 (4), YA4 (5), SK778-Y (6), YA5 (7), YA6 (8) and YA20 (9). The DNAs were probed with phage TP-778 DNA. Lane M and N: phage λ/HindIII DIG labelled (positive control) and unlabelled (negative control), respectively (Roche, Mannheim, Germany).

These characteristics were unique for the TP-778 DNA. When the DNA of the closely related temperate phage TP-J34 was used as a DNA probe, the high background of hybridization signals detected with the TP-778 DNA probe was absent and distinct hybridization signals were obtained as expected (see Fig. 5.14).

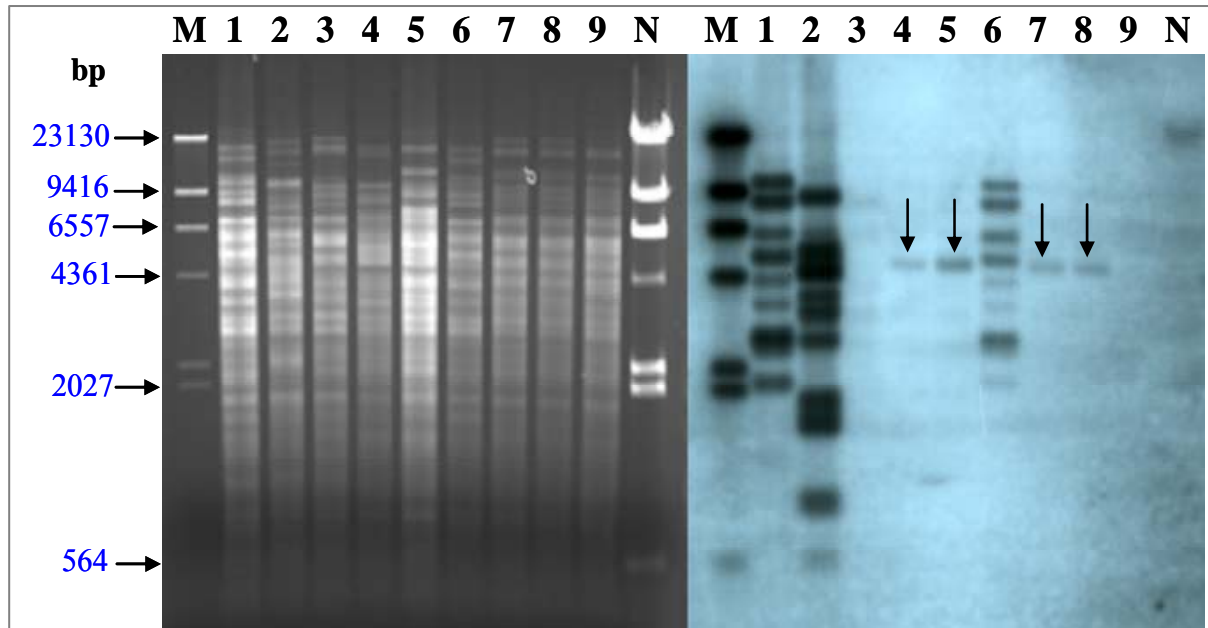


Fig. 5.14: Agarose gel (0.8%) electrophoresis (left) and Southern blot hybridization (right) of HindIII-digested chromosomal DNA of *S. thermophilus* strains SK778 (lane 1), J34 (2), 124 (3), S15 (4), YA4 (5), SK778-Y (6), YA5 (7), YA6 (8) and YA20 (9). The DNAs were probed with phage TP-J34 DNA. Lane M and N: phage λ /HindIII DIG labelled (positive control) and unlabelled (negative control), respectively (Roche, Mannheim, Germany). Arrows indicate the uniform hybridization signal (fragment size: 5 kb) of the prophage remnants present in strains S15, YA4, YA5 and YA6.

5.2.9 Partial DNA sequence analysis of *S. thermophilus* phage TP-778 DNA flanking the lysogeny module

In order to obtain knowledge on the atypical hybridisation patterns obtained with the phage TP-778 DNA probe (see Fig. 5.13), 2 core DNA fragments were amplified from the TP-778 prophage DNA for DNA sequence analysis. Specific primers were designed to amplify (1.) the TP-778 DNA located between the phage lysis cassette and the right phage attachment site *attR* and (2.) the phage DNA flanked by the left phage attachment site *attL* and the phage gene *cro* specifying the regulatory counterpart protein for the phage CI repressor. By this strategy, the DNA sequence of the complete lysogeny module and its flanking regions was obtained and analysed. As known from all other temperate *S. thermophilus* phages, the phage attachment site *attP* was also expected at the 3' end of the phage integrase gene. During prophage integration by site-specific integration, the two newly created *attL* and *attR* sites are located at the junction sites between the chromosomal and the prophage DNA.

5.2.9.1 PCR amplification of the lysin gene-*attR* region of prophage TP-778

The high-fidelity PCR technique (Fermentas, St. Leon-Rot, Germany) was used for the amplification of the *attR* containing junction region of the TP-778 genome and the SK778 host chromosome. The forward primer (lys.1) was designed from a highly conserved region within the lysin genes from different completely sequenced *S. thermophilus pac*-type phages. The reverse chromosomal binding primer Y3 was taken from the literature (Bruttin *et al.*, 1997c). A 5.2-kb PCR product was obtained (Fig. 5.15) and purified as previously described in Materials & Methods. The corresponding DNA sequence was determined using a primer walking strategy. Using this pair of primers, a significantly smaller 3.8-kb PCR fragment was obtained from the *S. thermophilus* J34 prophage DNA which was exactly matching with the TP-J34 DNA sequence (Neve *et al.*, unpublished). Hence it is notable that the TP-778 genome is carrying additional DNA in this region.

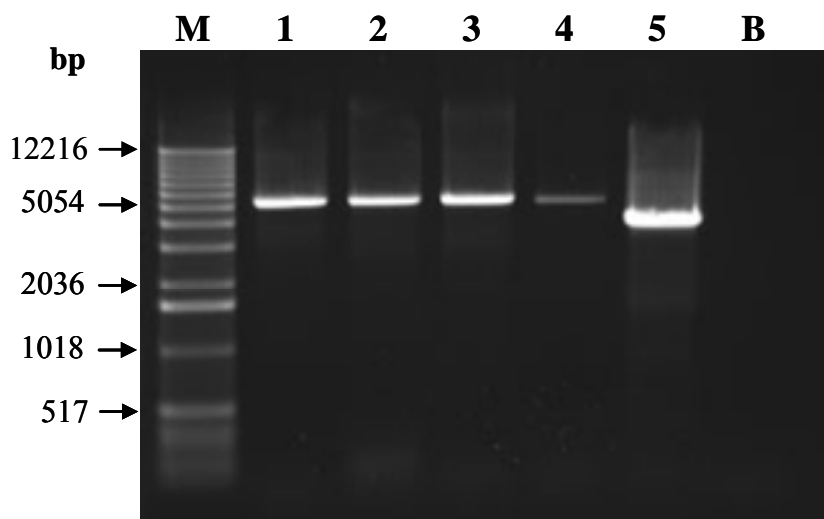


Fig. 5.15: Agarose gel (1.5%) electrophoresis of the PCR products obtained from the TP-778 prophage (lanes 1-4) and from the TP-J34 prophage (5) with primers lys.1 and Y3 specific for the *attR* containing junction regions of these prophages and the host chromosomes. Purified chromosomal DNA of *S. thermophilus* strains SK778 (lanes 1-3) and J34 DNA (5) or cells from an *S. thermophilus* SK778 single colony (4) were used. Lane B: negative „blank“ control; lane M: DNA marker X (Roche, Mannheim, Germany).

5.2.9.2 DNA sequence analysis of the lysin-*attR* region of prophage TP-778

The DNA sequence of the lysin-*attR* 5.2-kb DNA fragment amplified from prophage TP-778 was examined for open reading frames (*orfs*) larger than 50 codons, using ATG as a start codon. The *orfs* were translated into protein sequences, named according to the number of

amino acids (aa) in the predicted protein and analysed using NCBI-BLAST for protein homologies. This analysis revealed the presence of 10 *orfs* potentially coding for proteins (see Fig. 5.16 and Table 5.1) ranging from 56 to 223 amino acids. As shown in Fig. 5.16, 7 *orfs* were transcribed in one direction while 3 *orfs* were orientated in the opposite direction. Analysis of the gene product (gp) derived from the “incomplete” *orf223* (672 bp up stream to *orf73*) showed 91%, 89% and 85% identity to the phage lysin proteins of the *S. thermophilus* bacteriophages O1205 (Orf51; Stanley *et al.*, 1997), ϕ 7201 (Orf44; Proux *et al.*, 2002) and ϕ Sfi21 (Orf288, Bruttin *et al.*, 1997b), respectively. The following *orf73* gene product was 100% identical to the *orf73* gene product of phage TP-J34 (Neve *et al.*, 1998 and 2003). No matches at all, however, were found for Orf98. The putative proteins of the adjacent *orf140a* and *orf140b* genes showed high similarity to the corresponding proteins Orf140a and Orf40b of *S. thermophilus* bacteriophage Sfi21 (Bruttin *et al.*, 1997b) with 90% and 87% similarity, respectively. Surprisingly, the gene products of the next set of genes *orf132*, *orf168*, *orf117*, *orf97* and *orf56* showed 100% homology to the products of a gene cluster of the prophage remnant of *S. thermophilus* Sfi16 with the exception that Orf132 constitutes only a part (398 bp down stream to *orf168*) of Orf359 (phage integrase) of *S. thermophilus* Sfi16 prophage remnant (Bruttin *et al.*, 1997c). The non-coding 345-bp nucleotide sequence between *orf223* and *orf73* demonstrated 97% identity to the same region in TP-J34, while the unusually long 966-bp non-coding region between *orf73* and *orf98* showed 94% identity to the nucleotide sequence downstream of the lysin gene of another *S. thermophilus* phage (i.e., 7201; Proux *et al.*, 2002). It is remarkable that no *attR* sequence could be identified in the prophage DNA, indicating that phage TP-778 is lacking this DNA motif which is essential for integration and excision of the prophage DNA. Hence this lysin-*attR* 5.2-kb DNA was renamed as lysin- $\Delta attR$ 5.2-kb fragment indicating the absence of *attR*-sequence (40-bp). Apparently, integration of the TP-778 prophage has occurred adjacent to a prophage remnant which has merged with the TP-778 DNA. In the Sfi21 prophage genome, *attR* is located immediately downstream of *orf140b* which indicated the distal end of the Sfi21 phage genome (Bruttin *et al.*, 1997c). In contrast, the TP-778 prophage genome lacking *attR* has been attached to a distinct prophage remnant.

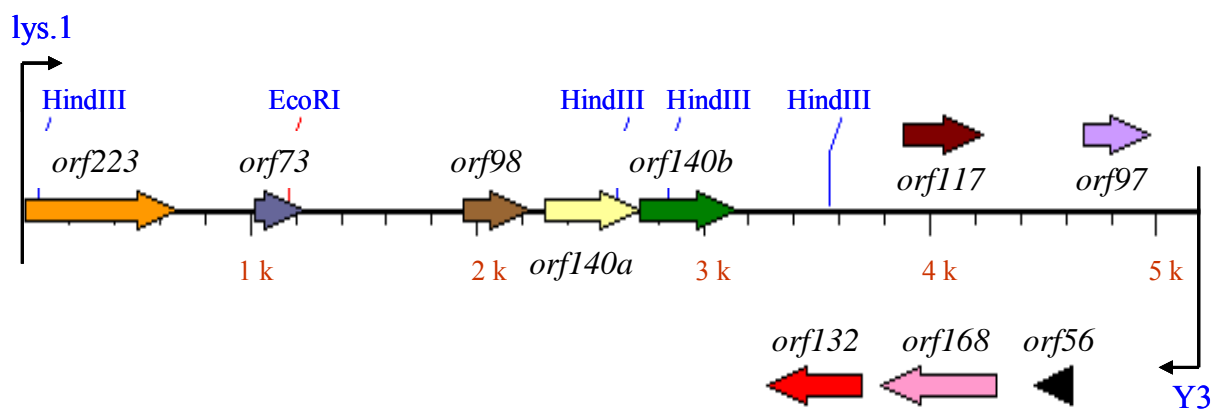


Fig. 5.16: Localization of the open reading frames (*orfs*) on the amplified lysin- $\Delta attR$ DNA fragment of prophage TP-778 obtained from PCR reaction with primers lys.1 and Y3. HindIII and EcoRI restriction endonuclease sites are also shown. Note the absence of an *attR* site.

Table 5.1: Putative proteins of the genes identified on the lysin-*attR* DNA fragment of prophage TP-778 and relationship to other *S. thermophilus* protein sequences from the GenBank database.

Gene	Size of gp (aa)	Putative function	Similarity to gp from other <i>S. thermophilus</i> phages	Identity (%) / (E-value)
<i>orf223</i>	223	lysin	O1205 (gp 281 aa)	91% (6e-115)
			7201 (gp 281 aa)	89% (1e-109)
			Sfi21 (gp 288 aa)	85% (3e-107)
<i>orf73</i>	73	unknown	TP-J34 (gp 73 aa)	100% (6e-26)
<i>orf98</i>	98	unknown	<i>No matches</i>	
<i>orf140a</i>	140	unknown	Sfi21 (gp 140 aa)	90% (1e-53)
			O1205 (gp 108 aa)	92% (4e-28)
<i>orf140b</i>	140	unknown	Sfi21 (gp 140 aa)	87% (5e-59)
			O1205 (gp 140 aa)	86% (5e-52)
			TP-J34 (gp 103 aa)	86% (5e-52)
<i>orf117</i>	117	unknown	Sfi16 prophage remnant (gp 117 aa)	100% (3e-44)
<i>orf97</i>	97	unknown	Sfi16 prophage remnant (gp 117 aa)	100% (1e-49)
			7201 (gp 175 aa)	89% (5e-27)
<i>orf132</i>	132	integrase	Sfi16 prophage remnant (gp 359 aa)	100% (5e-73)
			Sfi21 (gp 359 aa)	96% (7e-71)
			TP-J34 (gp 359 aa)	96% (7e-71)
<i>orf168</i>	168	unknown	Sfi16 prophage remnant (gp 168 aa)	100% (1e-76)
<i>orf56</i>	56	unknown	Sfi16 prophage remnant (gp 56 aa)	100% (2e-15)

5.2.9.3 PCR amplification of the *attL-cro* DNA region of prophage TP-778

In order to complete the DNA sequence analysis of the TP-778 lysogeny module, a prophage DNA fragment flanked by the phage *cro* gene and the phage *attL* site was amplified by high-fidelity PCR technology. Using the forward primer Y4 (Bruttin *et al.*, 1997c) with a chromosomal DNA binding site and the reverse phage DNA binding primer int.cro.2 with a binding site within the *cro* gene sequence of phage TP-J34, a PCR product of 2.7 kb was obtained (Fig. 5.17).

Fig. 5.17: Agarose gel (1.5%) electrophoresis of the 2.7-kb DNA fragment amplified by PCR with primers Y4 and int.cro.2 from the *attL* containing junction region of prophage TP-778 in the chromosomal DNA of *S. thermophilus* SK778. Lane M: 1-kb DNA marker (AppliChem, Darmstadt, Germany).

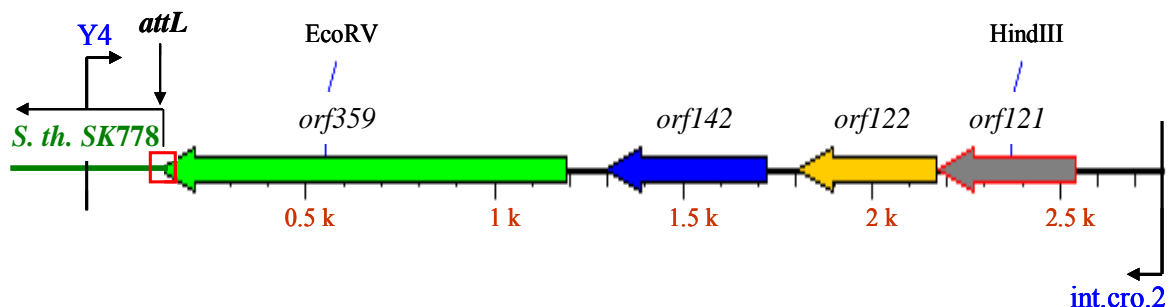
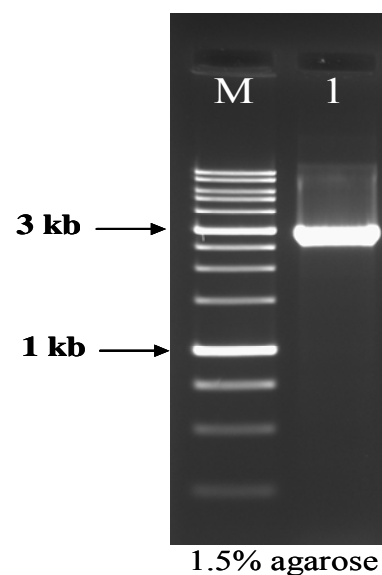


Fig. 5.18: Localization of open reading frames (*orfs*) of the amplified *attL / cro* DNA fragment of prophage TP-778 obtained from PCR reaction with primers Y4 and int.cro.2. The EcoRV and HindIII restriction endonuclease sites are also shown. The chromosomal SK778-DNA region is shown as a thin green line on the left side.

5.2.9.4 Sequence analysis of the *attL-cro* DNA region of prophage TP-778

A BLAST protein analysis at NCBI of the DNA *attL-cro* fragment of phage TP-778 indicated the presence of 4 open reading frames which were all transcribed in the same direction (Fig. 5.18). The *orf359* gene product showed 100% and 98% similarity to the phage integrases of identical sizes (i.e., 359 amino acids) of *S. thermophilus* phages Sfi21 (Bruttin *et al.*, 1997b), TP-J34 () and ϕ O1205 (Stanley *et al.*, 1997). Orf142 showed a remarkable 93% identity to the Ltp protein (Orf142) of the temperate *S. thermophilus* phage TP-J34 (Neve *et al.*, 1998 and 2003) while *orf122* gp shared 95% aa identity with the *orf122* gene product, a putative metalloproteinase, of both phages TP-J34 and Sfi21. Orf121 was 100% identical with the CI-like repressor protein of phage TP-J34 (Orf121; Neve *et al.*, 1998 and 2003).

Table 5.2: Putative genes (*orfs*) of the *attL-cro* DNA region of prophage TP-778 and relationship to other *S. thermophilus* phage genomes. Only GenBank hits with the highest scores are shown.

Gene	Size of gp (aa)	Putative function	Similarity to gp from other <i>S. thermophilus</i> phages	Identity (%) / (E-value)
<i>orf359</i>	359	Integrase	Sfi21 (gp 359 aa)	100% (0.0)
			TP-J34 (gp 359 aa)	100% (0.0)
			O1205 (gp 359 aa)	98% (0.0)
<i>Orf142</i>	142	Ltp	TP-J34 (gp 142 aa)	93% (6e-26)
<i>Orf122</i>	122	Metallo-proteinase	Sfi21 (gp 122 aa)	95% (1e-63)
			TP-J34 (gp 122 aa)	95% (1e-63)
<i>Orf121</i>	121	CI-repressor	TP-J34 (gp 121 aa)	100% (3e-63)
			Sfi21 (gp 127 aa)	50% (6e-21)

5.2.9.5 DNA sequence analysis of the *attP* flanking region of the lytic phage derivative TP-778L

The attachment sites involved in prophage integration into and excision out of the bacterial chromosome are identical in the prophage DNA (*attP*) and on the bacterial chromosome (*attB*). The *attB* core sequence is usually determined from the chromosome of a prophage-cured strain. However, since it was not possible to cure strain SK778 from its TP-778 prophage (see 5.2.5), the *attB* sequence could not be determined from the chromosomal DNA. It was attempted to obtain the DNA sequence from phage DNA isolated from freshly induced TP-778 phages. It is notable that it was not possible to obtain a distinct DNA sequence from the *attP* flanking region of the TP-778 DNA. The raw sequence, obtained as

fluorescence signals, showed normal regular fluorescence peaks till the end of *orf140b*, then many and irregular peaks were obtained (data not shown). As an alternative, DNA was isolated from the lytically propagated phage derivative TP-778L and used for amplification and sequence analysis. Using primers Yal.1 and ltp.Rev (see 4.10.1), a 1.4-kb DNA fragment was amplified from the phage genome flanked by *orf140b* and the lipoprotein determinant *orf142* (see Materials & Methods; 4.10). The PCR product was cloned in pJET1.2/blunt (“CloneJET™ PCR Cloning Kit”; Fermentas, St. Leon-Rot, Germany) and transformed in *E. coli* Easypores (Eurogentec GmbH, Köln, Germany) (Fig. 5.19). From the transformation assay, colonies were picked and screened for the presence of the recombinant plasmid designated as pJET.TP-778L-*attP*. Its identity was confirmed by DNA sequence analysis (Eurofins MWG Operon, Germany).

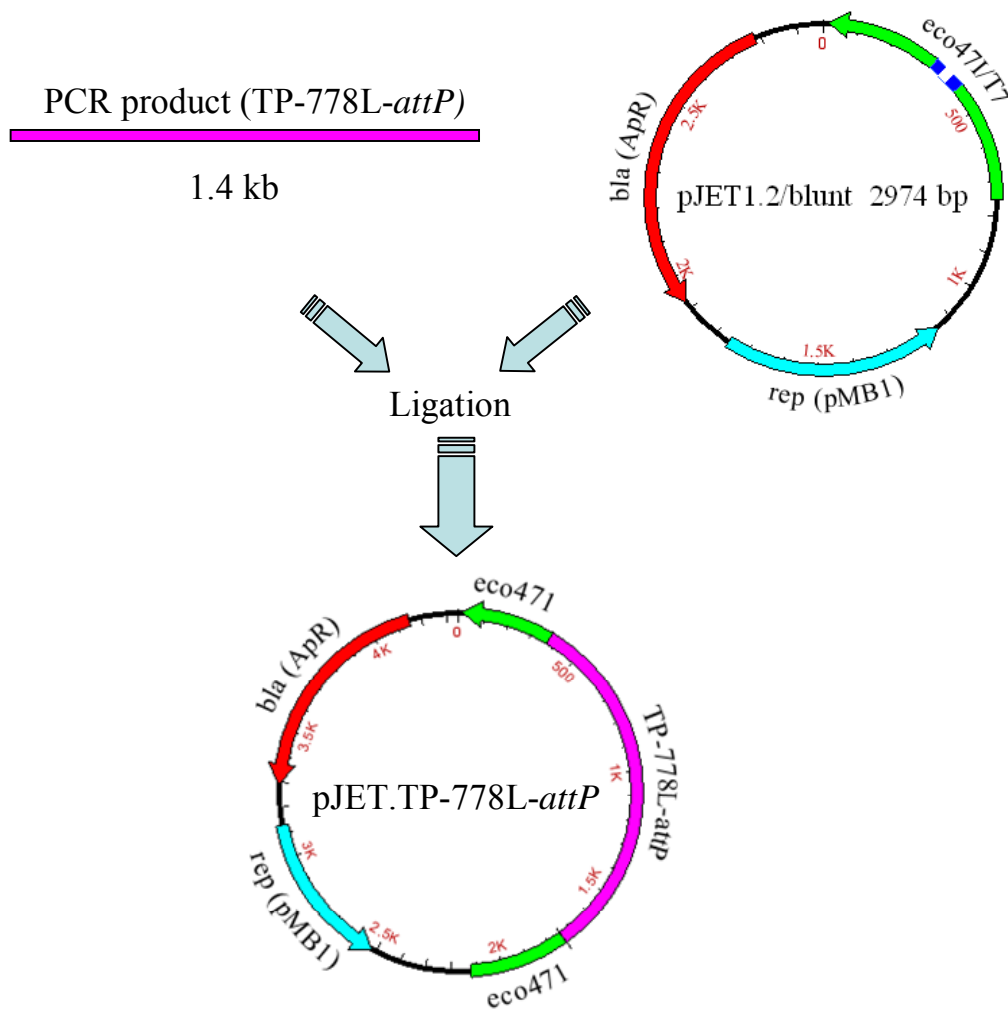


Fig. 5.19: Overview of cloning of the 1.4-kb DNA fragment from phage TP-778L containing the *attP* site in pJET1.2/blunt vector DNA (Fermentas, St. Leon-Rot, Germany). *rep (pMB1)*: replicon from pMB1 plasmid for pJET1.2 replication; *bla (Ap^R)*: β -lactamase gene conferring resistance to ampicillin; *eco471*: lethal gene for positive selection of recombinant plasmids.

Fig. 5.20: Agarose gel (1.5%) electrophoresis of intact pJET.TP-778L-*attP* DNA (lane 1) obtained by cloning of an 1.4-kb DNA region (containing the *attP* site) from phage TP-778L in pJET1.2/blunt vector. Lane 2: digestion of pJET.TP-778L-*attP* DNA with BglII. Lane M: 1-kb DNA marker (AppliChem, Darmstadt, Germany).

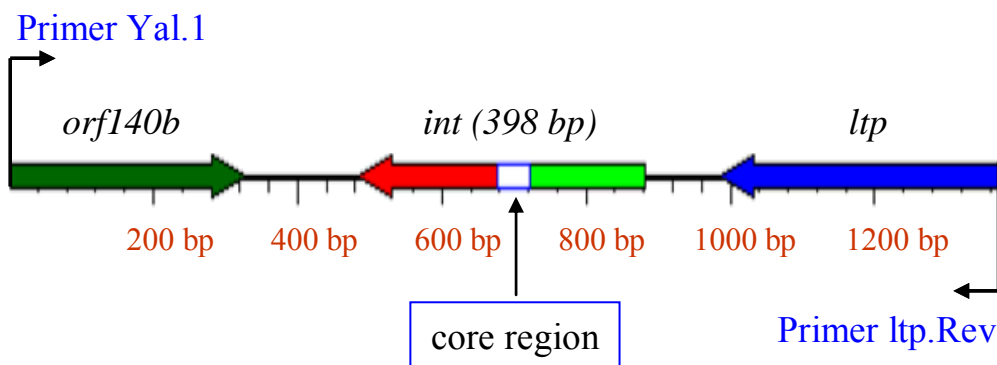
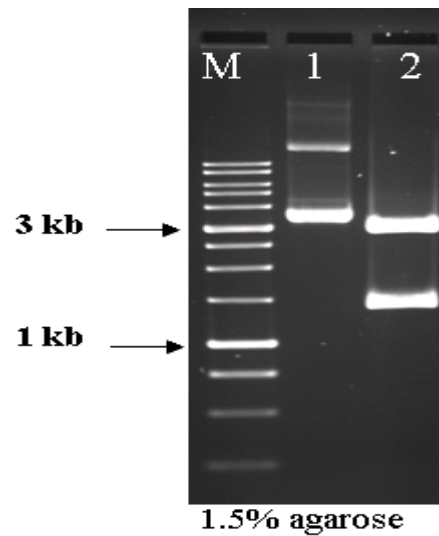


Fig. 5.21: Localization of open reading frames on the 1.4-kb PCR fragment amplified from the TP-778L-*attP* fragment obtained from the lytically propagated TP-778L phage by cloning in pJET1.2/blunt cloning vector (Fermentas, St. Leon-Rot, Germany).

The DNA sequence of the 1.4-kb *attP* containing PCR fragment was determined from the pJET.TP-778L-*attP* DNA. As expected from the primer selection, 2 open reading frames (*orf140b* and *ltp* [*orf142*]) were identified. Surprisingly, these 2 *orfs* flanked an incomplete integrase gene (i.e., Δ *int*). Two *orfs* were orientated in the same direction (*ltp* and Δ *int*) and *orf140b* was orientated divergently (see Fig. 5.21). This DNA sequence was aligned with the two formerly determined sequences of the TP-778(*lys*- Δ *attR*) and TP-778(*attL-cro*) DNA fragments. Apparently, this integrase gene fragment is originating from a homologous recombination event between the integrase gene present on the TP-778(*attL-cro*) prophage fragment and the integrase remnant *orf132* from the prophage remnant attached to the TP-778

prophage. However, a central 46-bp conserved region with sequence identity was identified that is colored in blue in Fig. 5.22.

		3470		3480
TP-778(attL-cro)	A A G T T A T	T T T T G A A	A C A G T T G	
TP-778(lysIn-attR)	A A G T T A T	T T T C G A A	A C A G T T G	
TP-778L(attP)	A A G T T A T	T T T C G A A	A C A G T T G	
Consensus	A A G T T A T	T T T c G A A	A C A G T T G	
		3490		3500
TP-778(attL-cro)	G C T T C T T	A T A A A C C	T C G C A C C	
TP-778(lysIn-attR)	G C T T C T T	A T A A A C C	T C A C A C C	
TP-778L(attP)	G C T T C T T	A T A A A C C	T C A C A C C	
Consensus	G C T T C T T	A T A A A C C	T C a C A C C	
		3510		3520
TP-778(attL-cro)	A C T G T T T	A A A A T A A	T C G T A T A	
TP-778(lysIn-attR)	A C T G T T T	A A A A T A A	T C G T A T A	
TP-778L(attP)	A C T G T T T	A A A A T A A	T C G T A T A	
Consensus	A C T G T T T	A A A A T A A	T C G T A T A	
		3530		3540
TP-778(attL-cro)	G A G T A A T	A T C T T C A	T C G A C A T	
TP-778(lysIn-attR)	G A G T A A T	A T C T T C A	T C G A C A T	
TP-778L(attP)	G A G T A A T	A T C T T C A	T C G A C A T	
Consensus	G A G T A A T	A T C T T C A	T C G A C A T	
		3550		3570
TP-778(attL-cro)	T G A C G T T	A T C T T G T	A G C T T A A	
TP-778(lysIn-attR)	T A A T G T T	A T C T T G A	A G C T T C A	
TP-778L(attP)	T G A C G T T	A T C T T G T	A G C T T A A	
Consensus	T g A c G T T	A T C T T G t	A G C T T a A	

Fig. 5.22: Alignment of the DNA sequences of prophage TP-778 integrase gene (on: *attL-cro* fragment), truncated *orf132* (on: lysin- Δ *attR* fragment) and TP-778L truncated integrase gene (on: TP-778L-*attP*-fragment). The blue color indicates the core region (46 bp) between the 3 integrase sequences, indicating the recombination site for excision of TP-778L. The yellow and green color indicate the differences within the flanking sequences.

In conclusion, normal site-specific integration and excision of the TP-778 prophage is not possible due to the absence of a functional *attR* site. However, excision of TP-778 prophage after mitomycin C induction is allowed by homologous recombination between the integrase remnant Δ *int* (*orf132*) present on the prophage remnant and the integrase gene of the TP-778 prophage. As shown in Fig. 5.21, only a new short 398-bp integrase gene remnant is created by this unusual prophage excision mechanism as identified on the genome of the lytically propagated phage TP-778L.

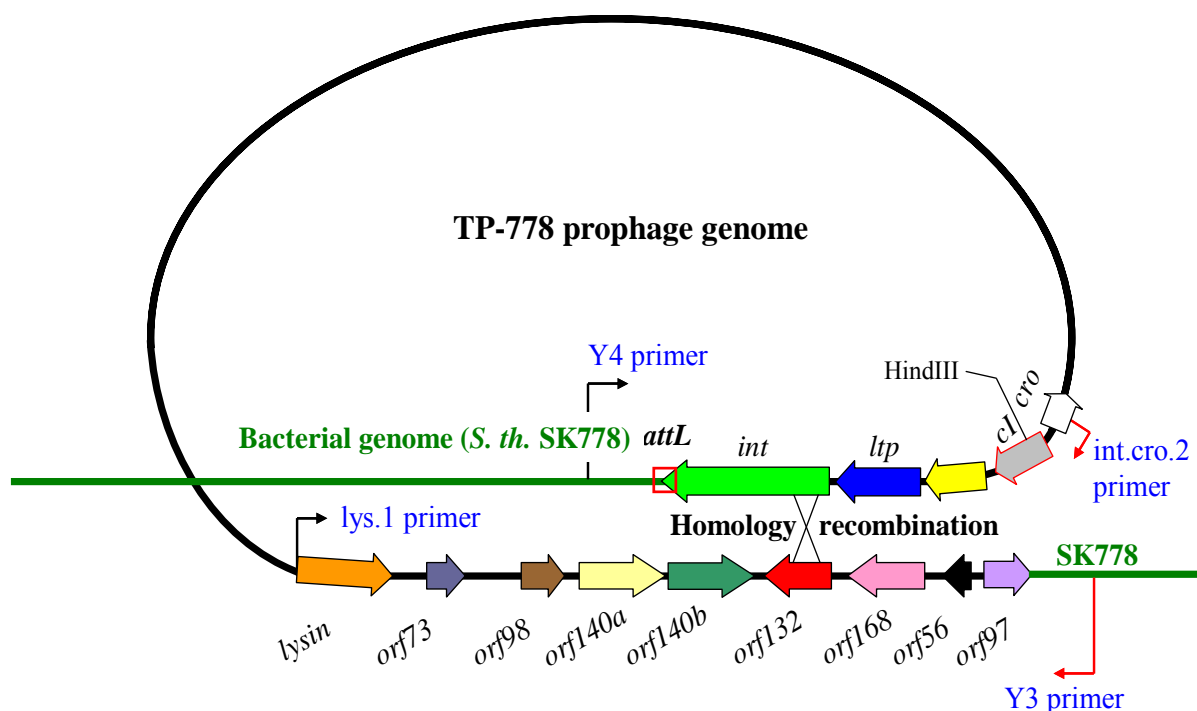


Fig. 5.23: Mechanism of TP-778L prophage excision from the *S. thermophilus* SK778 genome by homologous recombination between the distal upstream parts of the phage integrase gene (green colour) and the phage integrase gene remnant (red colour).

5.2.9.6 Hybridization with integrase gene probe

The phage excision model (Fig. 5.23) as elucidated by sequence analysis was confirmed by a Southern blot analysis. An internal 543-bp DNA fragment of the integrase gene of the TP-778 prophage was used as DNA probe. Phage DNAs from the temperate phage TP-778 obtained by mitomycin C induction, from the lytically propagated derivative TP-778L, from TP-J34 (positive control) and from the lytic phage P53 (negative control) was used and cut with HindIII. As expected, the probe hybridized strongly with a 3.1-kb fragment of the control phage TP-J34. The TP-J34 integrase gene is located on this HindIII DNA fragment formerly designated as HindIII-g fragment (Neve *et al.* 2003) (Fig. 5.24). The integrase gene probe did also hybridize with a 2-kb fragment of the TP-778L DNA which is perfectly correlating with the sequence analysis data illustrated in Fig. 5.25, since the integrase remnant of phage TP-778L is located on a 2030-bp HindIII fragment of this phage genome. This hybridisation signal was also visible in the TP-778 DNA, but surprisingly – among some other bands – the major hybridization signal was detected in a gel region (approx. 6 kb), where no HindIII fragments of phage TP-778 were visible. A faint DNA smear can be seen in this gel area. When the integrase DNA probe was hybridized with the chromosomal DNA of the host strain SK778, the same strong 6-kb hybridization signal was detected. This

signal can only originate from the TP-778 prophage DNA. Its intact integrase gene is located on a HindIII fragment cut in the upstream region of the *cro* gene and a HindIII site in the adjacent chromosomal DNA at an approximately 6-kb distance. These hybridization data confirm that phage TP-778 is packaging chromosomal DNA during prophage induction with mitomycin C.

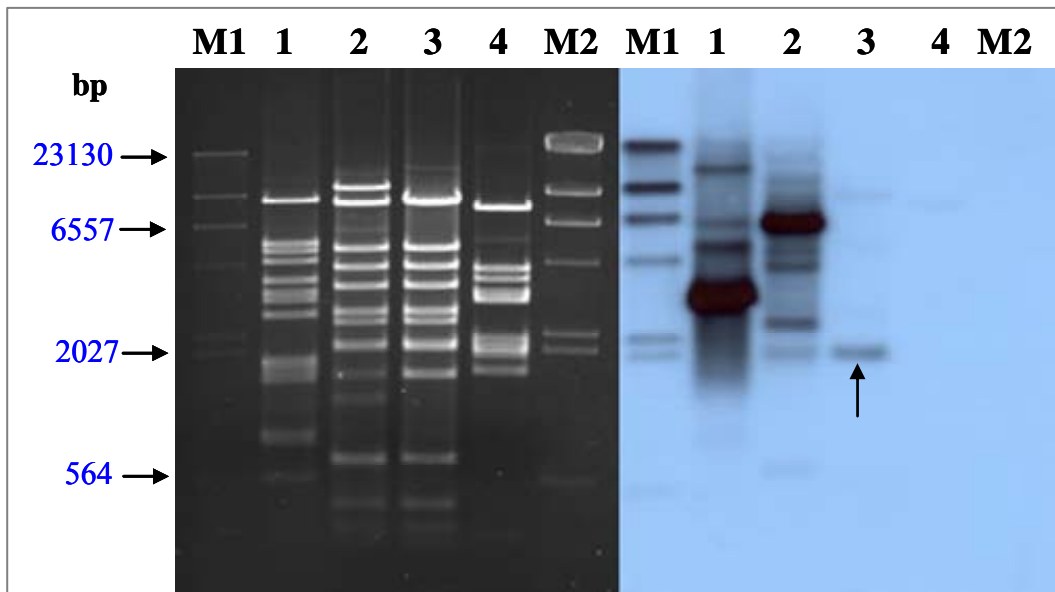


Fig. 5.24: Agarose gel (0.8%) electrophoresis (left) and Southern blot hybridization (right) of HindIII digested phage DNAs of TP-J34 (lane 1), TP-778 (2), TP-778L (3) and P53 (4). An internal 543-bp fragment of the TP-778 integrase was used as a DNA probe. Lane M1: phage λ/HindIII-DIG labelled; Lane M2: phage λ/HindIII (Roche, Mannheim, Germany).

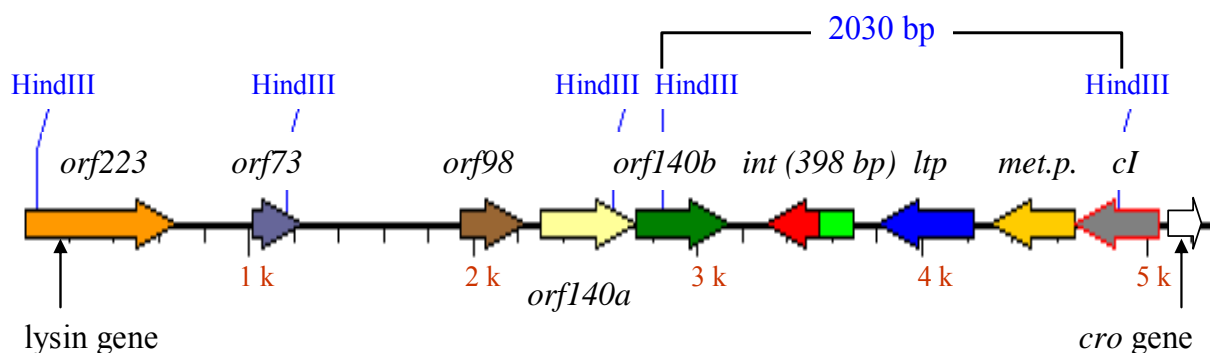


Fig. 5.25: Genetic map of lysin-*cro* fragment of the lytic phage derivative TP-778L with an integrase gene remnant (398 bp) located on a 2030 bp HindIII fragment.

5.2.10 General transduction mediated by the temperate *S. thermophilus* phage TP-778 and its lytic derivative TP-778L

Transduction is a term used to describe phage-mediated transfer of DNA between bacterial cells. Two different types of transduction are known: During specialized transduction, integrated prophages will also package chromosomal DNA flanking the *attL* or *attR* sites of the prophage during prophage induction and excision. The best example of specialized transducing phages is phage λ (Birge, 2000). In contrast, phages capable of performing generalized transduction will package chromosomal DNA instead of phage DNA into their heads during phage assembly. These phage particles are still able to attach to and inject the packaged DNA into the host bacterial cell. Transduction is a typical example of horizontal gene transfer driven to phages (Birge, 2000; Brüssow *et al.*, 2004). At least 95% of the natural *Salmonella enterica* serovar *typhimurium* strains harbour prophages and about 62% of the released phages have the potential for transduction (Sander & Schmieger, 2001). Furthermore, mobilization of antibiotic resistance genes through generalised transducing phages has been observed. Although the most transducing phages are lytic, transduction through temperate phage has been observed in staphylococci, streptococci, *Pseudomonas aeruginosa* and *Lactococcus lactis* (Mcshan & Ferretti, 2007).

As outlined before (see 5.2.9.6), the strong 6-kb hybridization signal of phage TP-778 DNA cut with HindIII and hybridized with a phage integrase probe can be explained by transduction. This hypothesis was confirmed experimentally. DNA was extracted from phage TP-778 particles purified by step gradient centrifugation. Contamination of the phage lysate with chromosomal DNA was excluded as indicated in Fig. 5.26 (Sander & Schmieger, 2001). Indicator plasmid DNA (pS1) was added to the phage before complete destruction of free DNA by DNase I treatment. The phage-encapsulated DNA was extracted and the complete removal of the spiked indicator DNA was confirmed by PCR. This DNA preparation which passed the indicator plasmid control without a positive PCR result was then tested for the presence of 16S rDNA as a suitable chromosomal marker detectable by PCR.

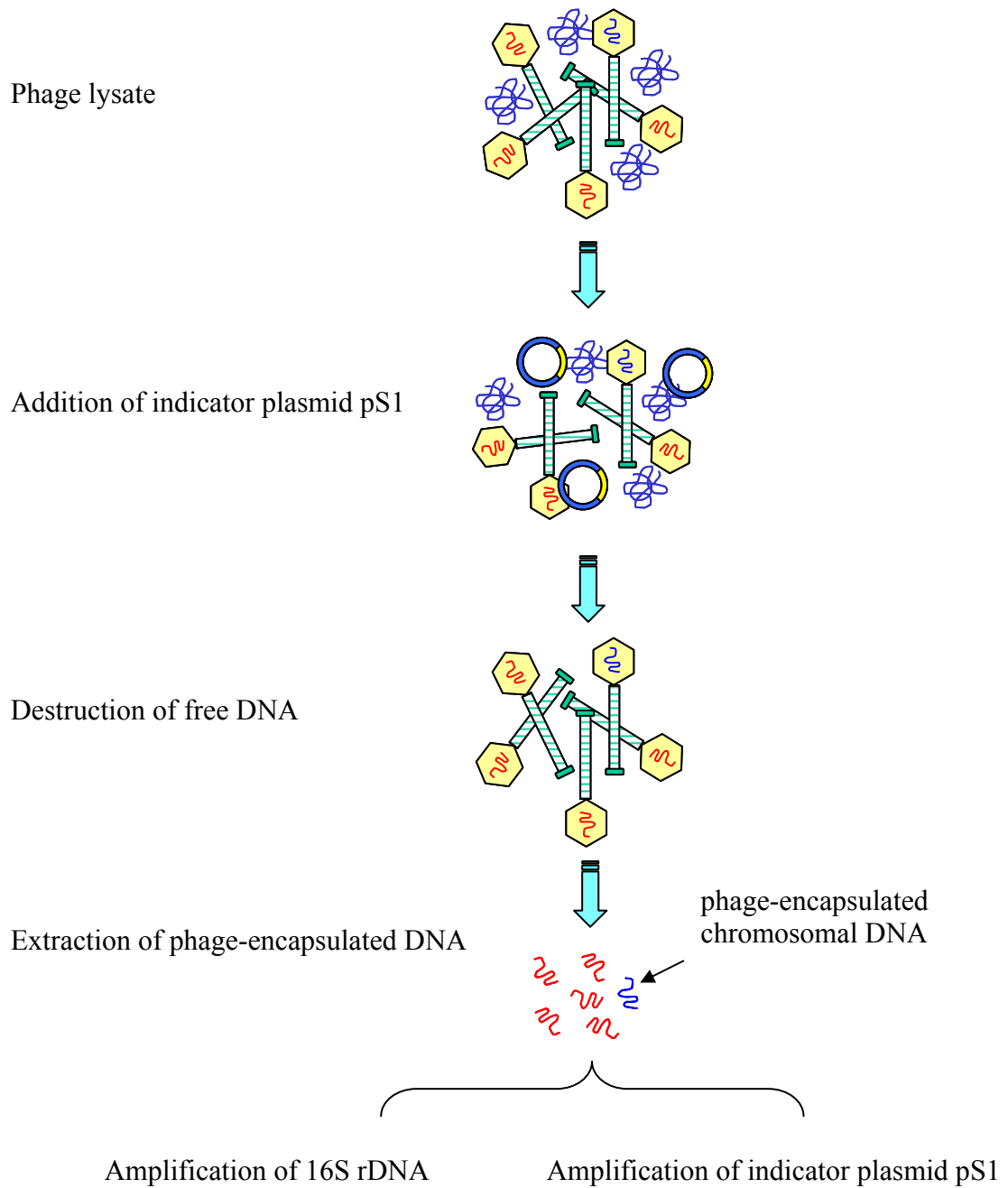
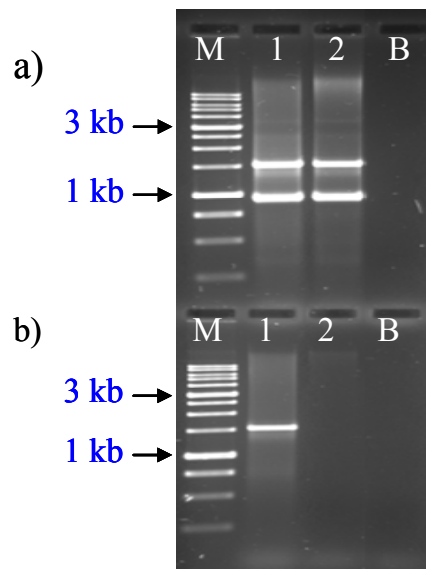


Fig. 5.26: Protocol for preparation and amplification of highly purified phage-encapsulated DNA without DNA contaminations.

5.2.10.1 Screening of phage DNA for 16S rDNA by PCR

Many bacteria carry up to 15 copies of the 16S rRNA gene which is unique to prokaryotes. So, 16S rRNA gene technology is used as a tool for phylogenetic characterisation and identification of microbes (Beumer & Robinson, 2005). 16S rDNA PCR was carried out using TP-778 and TP-J34 (control) phage lysates carefully controlled for the presence of external free DNA contaminations by the indicator plasmid pS1 protocol (see Fig. 5.26). Two universal primers (8F and 1525R) were used for 16S rDNA amplification (Beumer & Robinson, 2005). Another set of primers (cbh-F and cbh-R) was used specific for the indicator plasmid pS1 PCR control (see Materials & Methodes: 4.10). Primers were selected to ensure a differentiation of the PCR products obtained from 16S rDNA (size of 1.5 kb) and from the indicator plasmid control (size of 1 kb) for the indicator plasmid. A 16S rDNA specific 1.5-kb PCR fragment was clearly detectable in the phage TP-778 DNA before and after DNase treatment. In the corresponding control experiments performed with phage TP-J34 (Neve *et al.*, 2003), a PCR product was not obtained (Fig. 5.27). The appropriate 1-kb PCR product derived from the indicator plasmid DNA was only detectable before DNase treatment. These experiments confirmed that phage TP-778 is a transducing phage packaging DNA from the host chromosome.

Fig. 5.27: Agarose gel (1.5%) electrophoresis of PCR products [for 16S rDNA (1.5-kb PCR product) and for the spiked indicator plasmid pS1 DNA (1-kb PCR product)] obtained from highly purified TP-778L (lane 1) and TP-J34 (2) phage lysates. Gel a): PCR products were obtained before DNase I treatment. Gel b): The PCR products were obtained after DNase I treatment. Lane M: 1-kb DNA marker (Applichem), lane B: negative PCR control (“blank”).



5.2.10.2 Southern blot analysis for screening of 16S rDNA in *S. thermophilus* phages and strains.

The universal 16S rDNA primers 8F and 1525R (Beumer & Robinson, 2005) were used for amplification of an 1.5-kb fragment from the 16S rDNA of *S. thermophilus* strain SK778. This DNA was used as a DNA probe and hybridized with the DNAs of *S. thermophilus* phages TP-778, TP778L and TP-J34. As described before, the phage DNA was carefully treated with DNase I before DNA extraction. Extraction of phage and chromosomal DNA and preparation of the 16S rDNA probe were performed as described in Materials and Methods. The chromosomal DNAs of 7 *S. thermophilus* strains were also used in the Southern blot analysis (Fig. 5.28). Distinct hybridisation signals were detected in the TP-778 and TP-778L DNAs but were absent in the TP-J34 phage DNA. The probe did also hybridize with all chromosomal DNAs. This confirmed the PCR results (see 5.2.10.1) and our conclusion that the *S. thermophilus* phage TP-778 and its lytic derivative TP-778L are transducing phages performing generalised transduction. As indicated earlier in this chapter (see 5.2.10), it is not possible to use a TP-778 DNA probe to screen for the presence of prophages in the chromosomal DNAs of *S. thermophilus* phages, since this phage DNA is contaminated with chromosomal DNA.

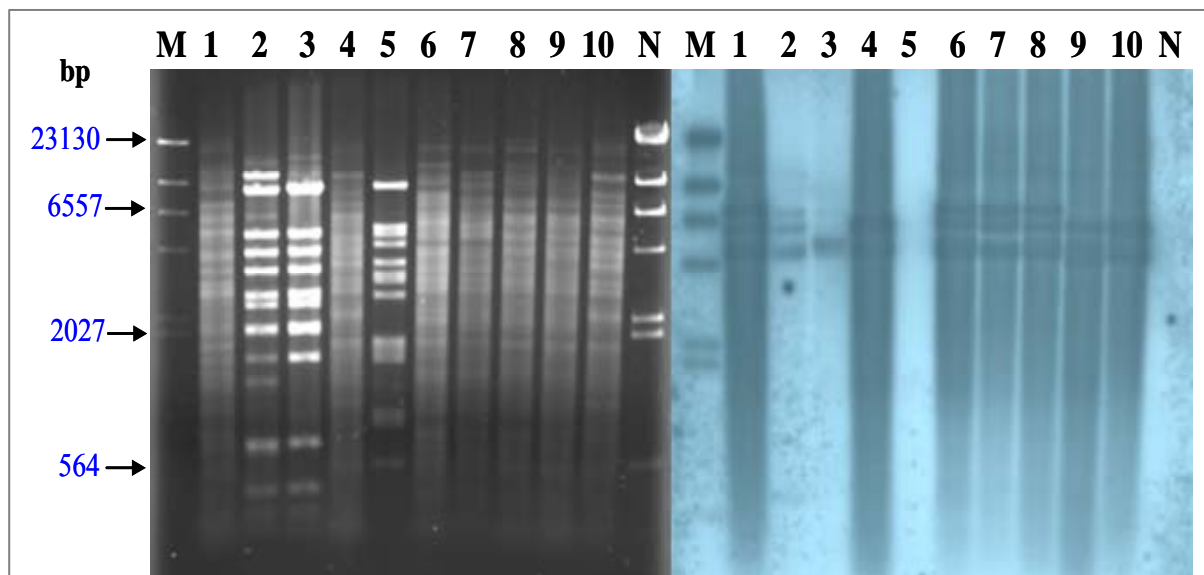


Fig. 5.28: Agarose gel (0.8%) electrophoresis of HindIII restricted phage DNA from *S. thermophilus* phages TP-778 (lane 2), its lytic derivative TP-778L (3) and TP-J34 (5) and from the chromosomal DNAs from *S. thermophilus* strains SK778 (1), J34 (4), YA4 (6), S15 (7), YA5 (8), YA20 (9) and J34-6 (10) [left panel]. DNAs were blotted and hybridized with an 1.5-kb DNA probe amplified from the 16S rDNA of *S. thermophilus* SK778 [right panel]. Lane M and N: phage λ /HindIII DIG labelled (positive control) and unlabelled (negative control), respectively (Roche, Mannheim, Germany).

5.3 Conclusion

The data represented in this chapter demonstrated that *S. thermophilus* SK778 is a lysogenic strain. In addition it contains like *S. thermophilus* Sfi16 a prophage remnant in its genome with an *attL* site but no *attR* site. It is believed that the prophage remnant is derived from the lysogeny module of an ancestor prophage. Prophages must encode genetic functions that give selective advantages to the lysogenic host. Without these function, prophages are considered as a load or burden for the host cells leading to elimination of the lysogen from the bacterial population and thereby to the loss of prophage DNA (Desiere *et al.*, 2001b). Strain Sfi16 is one of the industrial starters with a narrow phage spectrum. However, cloning of Sfi16 prophage DNA and transformation did not confer protection against phage infection (Ventura *et al.*, 2002). It is possible that the temperate *S. thermophilus* phage TP-778 had integrated its phage genome into the left attachment site (*attL*) of the prophage remnant in strain *S. thermophilus* SK778. Then the *attR* of the prophage TP-778 (= *attL* of the prophage remnant in *S. thermophilus* SK778) and part of the prophage remnant-integrase gene were deleted. Consequently, induction of prophage TP-778 with mitomycin C would have led to homologous or illegitimate but not site-specific excision due to the presence of only the *attL* site. Illegitimate recombination is a process by which 2 DNA molecules not sharing homology to each other are joined (Kegel *et al.*, 2006). It takes place at random sites between different DNAs. Illegitimate recombination may occur upon prophage induction. The majority of illegitimate recombination events were detected within open reading frames, either changing the phage genome size beyond useful limits or disrupting an essential gene (Hendrix, 2002; Brüssow *et al.*, 2004). In both cases it will render the recombinant phage non-functional. Only a few recombination events lead to viable phages, i.e. when recombination occurred in intergenic regions (Brüssow *et al.*, 2004). So, different excision sites may be used during excision from the chromosomal DNA. Therefore, after induction of phage TP-778 with mitomycin C, some prophage DNAs were excised from the bacterial genome by homologous recombination between phage- and prophage remnant-derived integrase genes as observed and analysed in the lytic phage derivative TP-778L. Thereby, phage TP-778L had become a lytic TP-778 mutant with a defective integrase gene remnant. Furthermore, it can be concluded that phage TP-778 and its lytic derivative TP-778L are generalized transducing phages as shown by the packaging of 16S rDNA. Thus, it may play an important role in phage-mediated horizontal gene transfer. Transducing phages have been observed also in many bacteria including *Salmonella*, *Streptomyces* and *Listeria* species (Brüssow *et al.*, 2004)

6 Cloning of *ltp* gene of TP-778

Chapter 2: Cloning of *ltp*_{TP-778} gene of temperate *Streptococcus thermophilus* phage TP-778 in *Escherichia coli* and *Lactococcus lactis*

6.1 Introduction

Temperate phages have the ability either to multiply in their host cells leading to lysis of the cell or to integrate their DNA into the bacterial chromosome (prophage) producing with their host a stable relationship termed lysogeny. In this state the expression of genes is prevented by the expression of the CI-like repressor protein from the phage. It is well established that prophages can provide advantages to the lysogenic cells, since they carry one or more genes that increase the fitness of the cells. The prophages influence the phenotype of their host cells, e.g. by the activity of prophage genes coding for toxins and phage resistance. Hence, they are conferring selective advantages to the hosts. These prophage-derived changes of the bacterial phenotype are generally designated as “lysogenic conversion” (Waldor *et al.*, 2005; Sturino & Klaenhammer, 2006).

Lipoprotein-coding genes are rare in phages. Two lipoproteins in phage lambda are expressed during lysogeny, Bor and Rz1, involved in serum resistance of lysogenic cells and lysis, respectively (Barondess & Beckwith, 1995, Kedzierska *et al.*, 1996). Furthermore, Cor proteins of the lambdoid, FhuA-receptor-dependent phages HK022, N15, ϕ 80, mEp167 and T1 mediate superinfection exclusion (Uc-Mass *et al.*, 2004; Roberts *et al.*, 2004) or lysogenic conversion to the lysogenic host cell (Vostrov *et al.*, 1996). In the prophage DNA of *Lactobacillus johnsonii* NCC 533 and *Lb. plantarum* strain WCFS1 two genes encoding lipoproteins have also been detected (Ventura *et al.*, 2003; Ventura *et al.*, 2004).

A lipoprotein gene has also been identified in the genome of the *S. thermophilus* temperate phage TP-J34 and was located upstream of the integrase gene (Neve *et al.*, 1998 and 2003). The *ltp* gene (lipoprotein of temperate phage TP-J34) was designated as *orf142*_{TP-J34} and is part of an operon together with 3 other genes coding for the phage integrase, a putative metalloproteinase and the phage cI repressor protein. This operon represents the core of the lysogeny module of the TP-J34 phage genome. Ltp is a lipoprotein and as such is synthesized as a precursor containing a signal sequence. Ltp processing is inhibited with globomycin showing that Ltp is indeed a lipoprotein. The Ltp protein (size: 17 kDa) is surface-exposed and is involved in a superinfection exclusion system (Sie) (Sun *et al.*, 2006).

A lipoprotein gene has also been identified in the lysogeny module of the temperate *S. thermophilus* phage TP-778 (see Chapter 1). The TP-778 lipoprotein gene is closely related to *ltp* of phage TP-J34. Both phage-derived lipoproteins are composed of 142 amino acids. Their aa sequences are identical except for a low number of 10 amino acids in the N-terminal end and in the central region of the proteins (Fig. 6.1). In this chapter, the cloning and characterization of the TP-778 lipoprotein gene is shown. For a better differentiation of the two lipoproteins, the phage designation is added as a suffix (i.e., Ltp_{TP-J34}; Ltp_{TP-778}).

Orf142-TP-778	M K K L L S F G L L	S L S I I A L A A C	S Q T K S T S S Q T
Ltp-TP-J34	M K K I L S F G L L	S L S I I A L T A C	S Q P K S T S S Q T
Consensus	M K K X L S F G L L	S L S I I A L X A C	S Q X K S T S S Q T
Orf142-TP-778	S K T S E A K T E Q	S S E S K V P K E Y	R T A V S K A K Q Y
Ltp-TP-J34	S K T S E A K T E Q	S S E S K V P K E Y	R T A V S K A K Q Y
Consensus	S K T S E A K T E Q	S S E S K V P K E Y	R T A V S K A K Q Y
Orf142-TP-778	A S T V P M S K E G	L R S Q L V S F E K	Y S Q E A S D Y A V
Ltp-TP-J34	A S T V H M S K E E	L R S Q L V S F D K	Y S Q D A S D Y A V
Consensus	A S T V X M S K E X	L R S Q L V S F X K	Y S Q X A S D Y A V
Orf142-TP-778	D N A G I D Y N K Q	A L E K A K Q Y Q D	T L A M S P D A I R
Ltp-TP-J34	E N S G I D Y N K Q	A L E K A K Q Y Q D	T L S M S P D A I R
Consensus	X N X G I D Y N K Q	A L E K A K Q Y Q D	T L X M S P D A I R
Orf142-TP-778	D Q L V S F D K F T	Q E E A D Y A V A N	L K
Ltp-TP-J34	D Q L V S F D K F T	Q E E A D Y A V A N	L K
Consensus	D Q L V S F D K F T	Q E E A D Y A V A N	L K

Fig. 6.1: Amino acid alignment of the lipoproteins of phages TP-J34 (Ltp_{TP-J34}) and TP-778 (Ltp_{TP-778}). The 10 amino acids differing in the two proteins are highlighted in yellow.

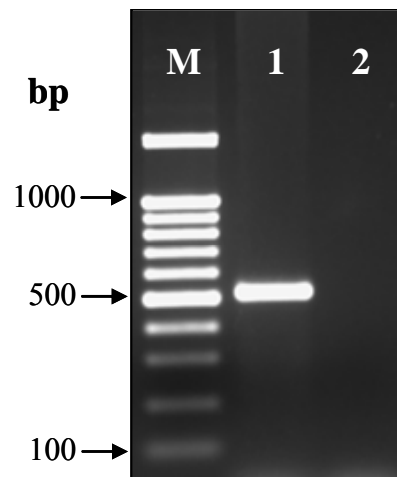
6.2 Results

6.2.1 Amplification of the *ltp*_{TP-778} gene from the TP-778 phage genome

A PCR product of 517 bp covering the intact *ltp*_{TP-778} gene of phage TP-778 DNA was amplified using the *ltp*-XbaI and *ltp*-HindIII primers (see 4.10.1) that bind upstream and downstream of *ltp*_{TP-778} within the flanking regions of the neighbouring genes *orf122* and *orf359*, respectively. The primers were designed on the basis the sequenced lysogenic module of phage TP-778 (see 5.2.9.2) to give an XbaI restriction site at the 5' end (i.e., 5' - CTA CGT GCA AAA TCT AGA TCA CG -3') and a HindIII restriction site at the 3' end (i.e., 5'-

CTG CAC TCA AGC TTG TCA AAG -3'). The 2 restriction sites are underlined in the primer sequences. The PCR reaction was performed as described previously in Materials & Methods (see 4.10). A 4- μ l aliquot of the PCR reaction were loaded on an 1.5% (w/v) agarose gel (Fig. 6.2). The PCR product was purified for cloning into a suitable shuttle cloning vector.

Fig. 6.2: Agarose gel (1.5%) of *ltp*_{TP-778} PCR fragment (lane 1) from TP-778 DNA for cloning in vector pMG36e. Lane 2: negative control. Lane M: 100 bp DNA marker (AppliChem, Darmstadt, Germany).



6.2.2 Cloning of *ltp*_{TP-778} in the expression vector pMG36e

The lactococcal expression vector pMG36e (van de Guchte *et al.*, 1989) (Fig. 6.3) has been constructed on basis of the cryptic lactococcal plasmid pWV01 and its origin of replication (*ori*). The vector contains a promoter (P32) and the corresponding ribosomal binding site from the *L. lactis* subsp. *cremoris* Wg2 chromosomal DNA. A multiple-cloning site (MCS) for the restriction enzymes *Sac*I, *Xma*I, *Sma*I, *Cla*I, *Xba*I, *Sal*I, *Acc*I, *Pst*I, *Sph*I and *Hind*III is located downstream of the P32 promoter. A terminator (T) and an erythromycin resistance gene (*EmR*) are also present on the pMG36e vector.

The pMG36e DNA and the purified 517-bp *ltp*_{TP-778} PCR fragment were digested with *Hind*III and *Xba*I for 3 h, purified with “NucleoSpin® Extract II” kit (Macherey-Nagel GmbH & Co KG, Düren, Germany) and ligated with T4 ligase enzyme yielding construct pYAL1 as illustrated in Fig. 6.3.

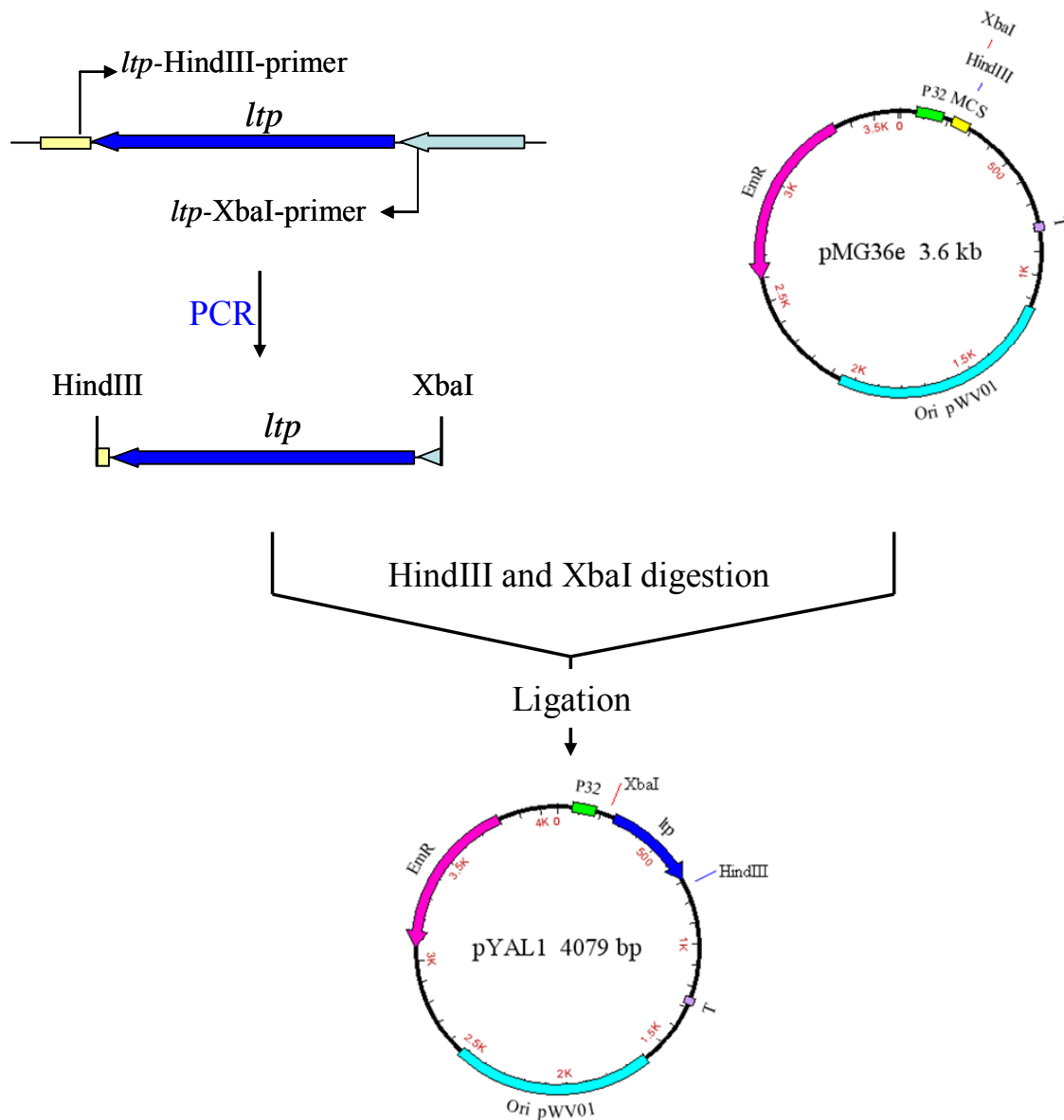


Fig. 6.3: Construction of the recombinant expression vector pYAL1. The XbaI/HindIII PCR fragment covering the *ltp*_{TP-778} gene of phage TP-778 and the cloning vector pMG36e were digested with XbaI and HindIII restriction enzymes.

6.2.3 Transformation of pYAL1 in *E. coli* Easypores

The ligation mixture was used for transformation in *E. coli* Easypores competent cells. Electroporation was done as outlined previously in the Materials & Methods section (see 4.12.3). Following transformation, erythromycin-resistant colonies were screened for the recombinant plasmid pYAL1 DNA by XbaI / HindIII restriction enzyme analysis and agarose gel electrophoresis of the plasmid DNA (Fig. 6.4 A & B).

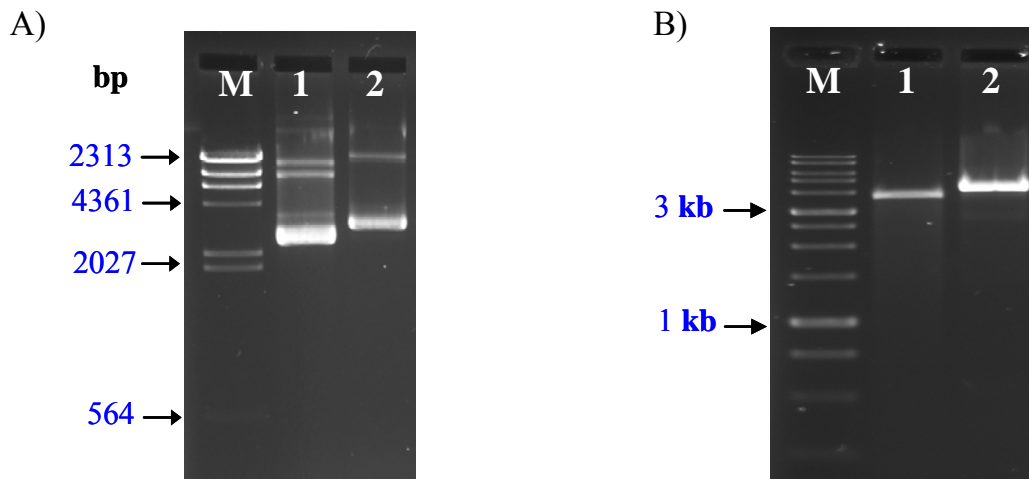


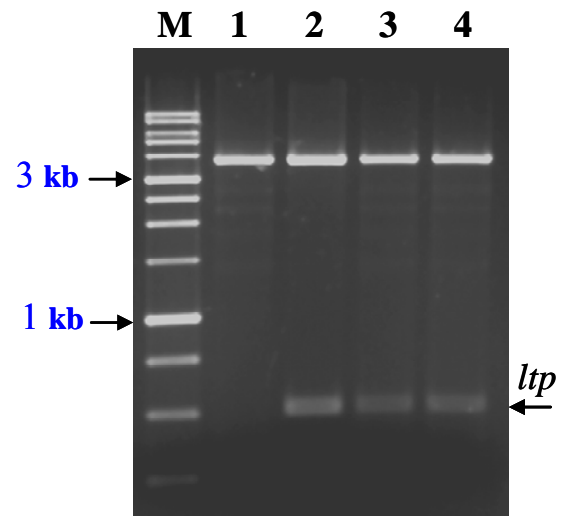
Fig. 6.4: Agarose gel (1.5%) electrophoresis of intact DNA (gel A) of cloning vector pMG36e (lane 1) and construct pYAL1 isolated from an *E. coli* Easy pores transformant (lane 2). On gel B, pMG36e DNA (lane 1) and the pYAL1 DNA (lane 2) were cut with HindIII and XbaI. The reference size DNAs used were λ -HindIII marker (gel A, lane M) (Roche, Mannheim, Germany) and 1-kb DNA marker (gel B, lane M) (AppliChem, Darmstadt, Germany).

6.2.4 Transformation of *L. lactis* Bu2-60 with pYAL1 DNA

The *ltp*_{TP-J34} gene product of *S. thermophilus* phage TP-J34 has been shown earlier to mediate a weak phage resistance in *S. thermophilus* but a surprisingly strong phage resistance against lactococcal phage P008 in *L. lactis* (Sun *et al.*, 2006). Hence it was tested whether expression of the *ltp*_{TP-778} from *S. thermophilus* phage TP-778 would also contribute to a phage resistance phenotype in a lactococcal host strain.

The plasmid-free strain *L. lactis* Bu2-60 which is sensitive to a variety of different lactococcal phages was transformed with plasmid pYAL1 as described in Materials & Methods (Holo & Nes, 1989). Three erythromycin-resistant colonies were isolated and their plasmid content was confirmed by restriction enzyme analysis using HindIII and XbaI (Fig. 6.5). From all 3 strains, the correct *ltp*_{TP-778} DNA sequence was confirmed by sequence analysis using a primer from the pMG36e DNA sequence upstream of the *ltp*_{TP-778} gene (5'-AAATGGCAATCGTTTCAGCAG-3') (data not shown).

Fig. 6.5: Agarose gel (1.5%) electrophoresis of plasmid pYAL1 extracted from 3 *L. lactis* Bu2-60 transformants Bu2-60(pYAL1)-1, -(pYAL1)-2, and -(pYAL1)-3 (lanes 2, 3, 4). The DNAs were cut with HindIII and XbaI. Plasmid pMG36e digested with HindIII and XbaI is shown on lane 1. Lane M: 1-kb DNA marker (AppliChem, Darmstadt, Germany). The arrow indicates the DNA fragment with the *ltp*_{TP-778} gene.



6.2.5 Phage-sensitivity test for the transformants

Two lactococcal phages (i.e., phage P008 and P001) from different phage species which represent the most abundant lactococcal phages were used for testing the phage sensitivity of the 3 *L. lactis* Bu2-60 derivatives -(pYAL1)-1, -(pYAL1)-2 and -(pYAL1)-3 containing the *ltp*_{TP-778} gene from phage TP-778. The *L. lactis* Bu2-60(pXMS2) strain containing the *ltp*_{TP-J34} gene from phage TP-J34 was included as a control (Sun *et al.*, 2006). As another control, Bu2-60 wild type strain and a derivative containing the cloning vector pMG36e were also used. All strains were challenged with high titer lysates of both phages (titer > 10⁹ pfu/ml). Control strains Bu2-60 and Bu2-60(pMG36e) were sensitive to both phages. As reported earlier by Sun *et al.* (2006), strain Bu2-60(pXMS2) was highly resistant to lactococcal phage P008 but sensitive to lactococcal phage P001. However, a remarkable different phage resistance pattern was shown for strains Bu2-60(pYAL1)-2 and -(pYAL1)-3 which exhibited partial resistance to phage P001 but no resistance at all to phage P008. The plating efficiency of phage P001 on these 2 strains was reduced by 4-5 log units. Although strain Bu2-60(pYAL1)-1 contained the same *ltp*_{TP-778} gene as strains -(pYAL1)-2 and -(pYAL1)-3, this transformant was sensitive to both type phages (Fig. 6.6). The reason for the different phage sensitivity of strain Bu2-60(pYAL1)-1 has not been clarified yet. It should be noted that P001-derived plaques were significantly smaller in the bacterial lawns of the Bu2-60(pYAL1)-2 and -(pYAL1)-3 transformants carrying the *ltp*_{TP-778} gene than in the lawn of the Bu2-60 control culture (see Fig. 6.6).

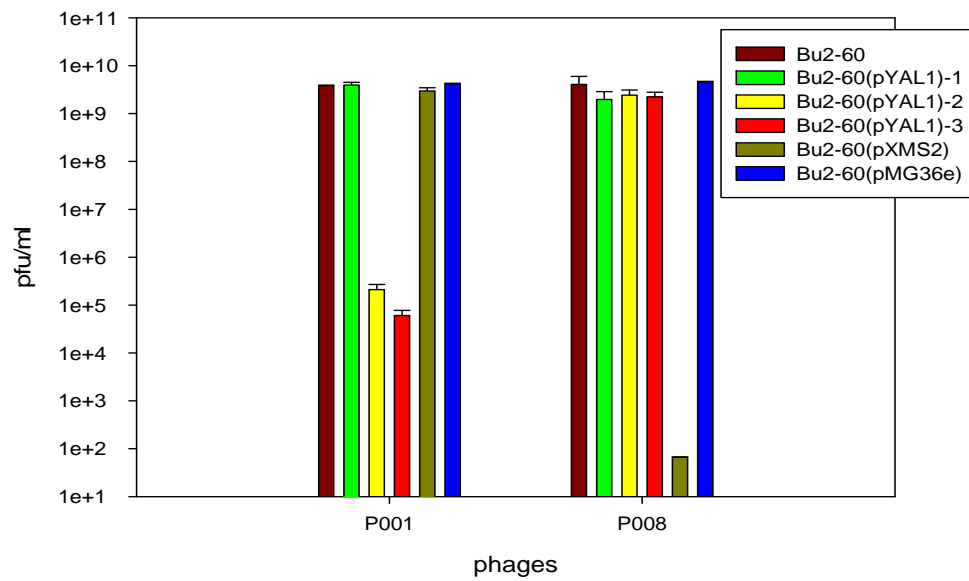


Fig. 6.6: Phage titers determined as plaque-forming units (pfu) per ml of lactococcal phages P001 and P008 on the plasmid-free strain *L. lactis* Bu2-60 and its derivatives transformed with the cloning vector pMG36e, or with the *ltp*_{TP-778} gene cloned in pMG36e (Bu2-60(pYAL1)-1, -(pYAL1)-2 and -(pYAL1)-3 or with the *ltp*_{TP-J34} gene cloned in pMG36e (Bu-60(pXMS2)).

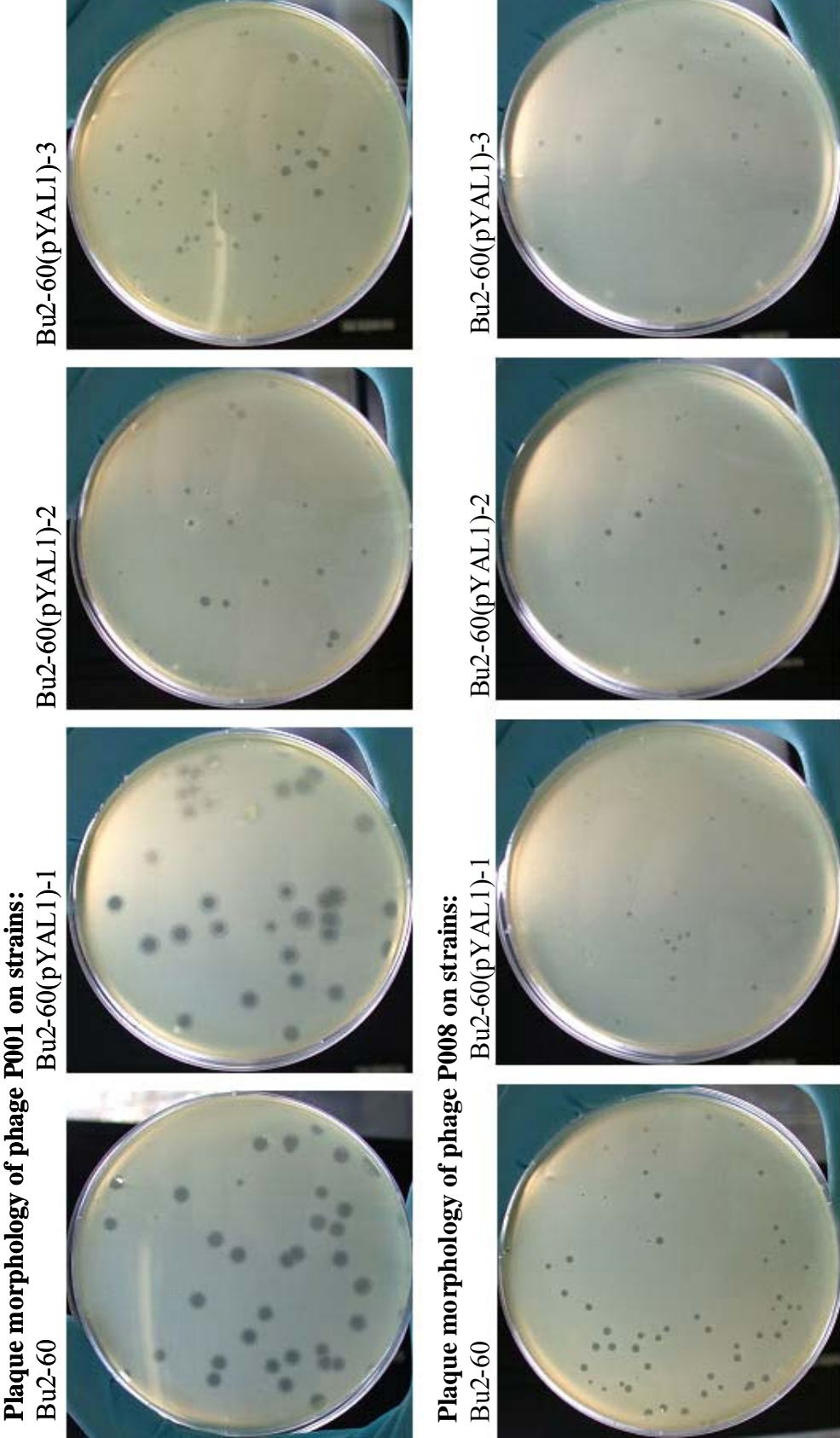


Fig. 6.7: Plaque morphology of phages P001 and P008 on *L. lactis* Bu2-60 and its derivatives.

6.2.6 Western blot analysis of the lipoprotein coded by the *ltp*_{TP-778} gene from *S. thermophilus* phage TP-778

Sun *et al.* (2006) have used a polyclonal antiserum raised against the lipoprotein coded by the *ltp*_{TP-J34} gene of phage TP-J34 to confirm expression of this phage gene in the lysogenic *S. thermophilus* strain J34 and in *E. coli* Easy pores and *L. lactis* Bu-60 transformed with the cloned *ltp*_{TP-J34} gene. Therefore it was tested whether this antiserum would also react with the *ltp*_{TP-778} gene product from *S. thermophilus* phage TP-778. Total cell extracts were prepared from *L. lactis* Bu2-60, from the *ltp*_{TP-778} containing transformants *L. lactis* Bu2-60(pYAL1)-1, -(pYAL1)-2 and -(pYAL1)-3, from *L. lactis* Bu2-60(pXMS2), from lysogenic wild-type strains *S. thermophilus* SK778 and J34 and from a J34 derivative cured from phage TP-J34 (i.e., J34-6; Neve *et al.*, 2003) as described in the Materials & Methods (see 4.15.2). It is shown in Fig. 6.8 that the antiserum specific for the *ltp*_{TP-J34} gene product did only react with the corresponding *ltp*_{TP-778} specific gene product (Ltp protein band with a molecular mass of 17 kDa). This protein band was clearly visible in the cell protein profile of strain Bu2-60(pXMS2) (see Fig. 6.8, lane 5). Notably, the antiserum did not cross-react with the corresponding lipoprotein coded by the *ltp*_{TP-778} gene of phage TP-778. However since a corresponding major 17-kDa protein band was not present in the cell extracts for the 3 Bu2-60 transformants harbouring the *ltp*_{TP-778} gene, it is not clear so far whether this gene is expressed at a far lower level in the lactococcal host than the *ltp*_{TP-J34} gene.

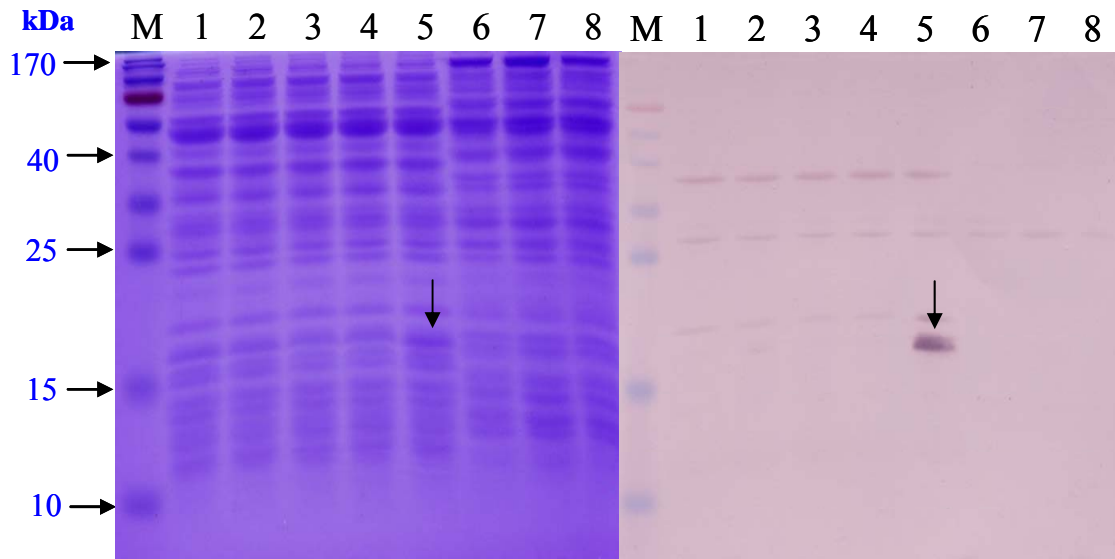


Fig. 6.8: SDS-PAGE (15%) of the total cell proteins (left) and Western blot analysis (right) of the plasmid-free wild-type strain *L. lactis* Bu2-60 (lane 1) and Bu2-60 transformants containing either the *ltp*_{TP-778} gene (Bu2-60(pYAL1)-1, -(pYAL1)-2, and -(pYAL1)-3; lanes 2-4) or the *ltp*_{TP-J34} gene (Bu2-60(pXMS2); lane 5). The lysogenic *S. thermophilus* strains SK778 (lane 6) and J34 (lane 7) were also included. The prophage-cured derivative J34-6 (lane 8) was used as another negative control. A polyclonal antiserum raised against the *ltp*_{TP-J34} gene product was used for the Western blot analysis. Lane M, PageRuler™ prestained protein marker (Fermentas, St. Leon-Rot, Germany).

6.2.7 Cell survival test of *L. lactis* Bu2-60 transformants carrying the *ltp*_{TP-778} gene

Since the *L. lactis* Bu2-60 transformants -(pYAL1)-2 and -(pYAL1)-3 revealed partial resistance against lactococcal phage P001, it was tested whether these strains would survive a long-term challenge of phage P001 at high multiplicity of infection (MOI) of approx. 5 within a 60-min period. As negative controls, the wild-type strain Bu2-60 and its derivative Bu2-60(pMG36e) transformed with the cloning vector pMG36e were also tested. As shown in Fig. 6.9, these 2 strains did not survive a 60-min infection of phage P001 at high MOI. In contrast, a significant number of the Bu2-60(pYAL1)-2 and -(pYAL1)-3 cells did survive a phage P001 infection for 60 min at high MOI (i.e., 73% and 35% of the infected cells).

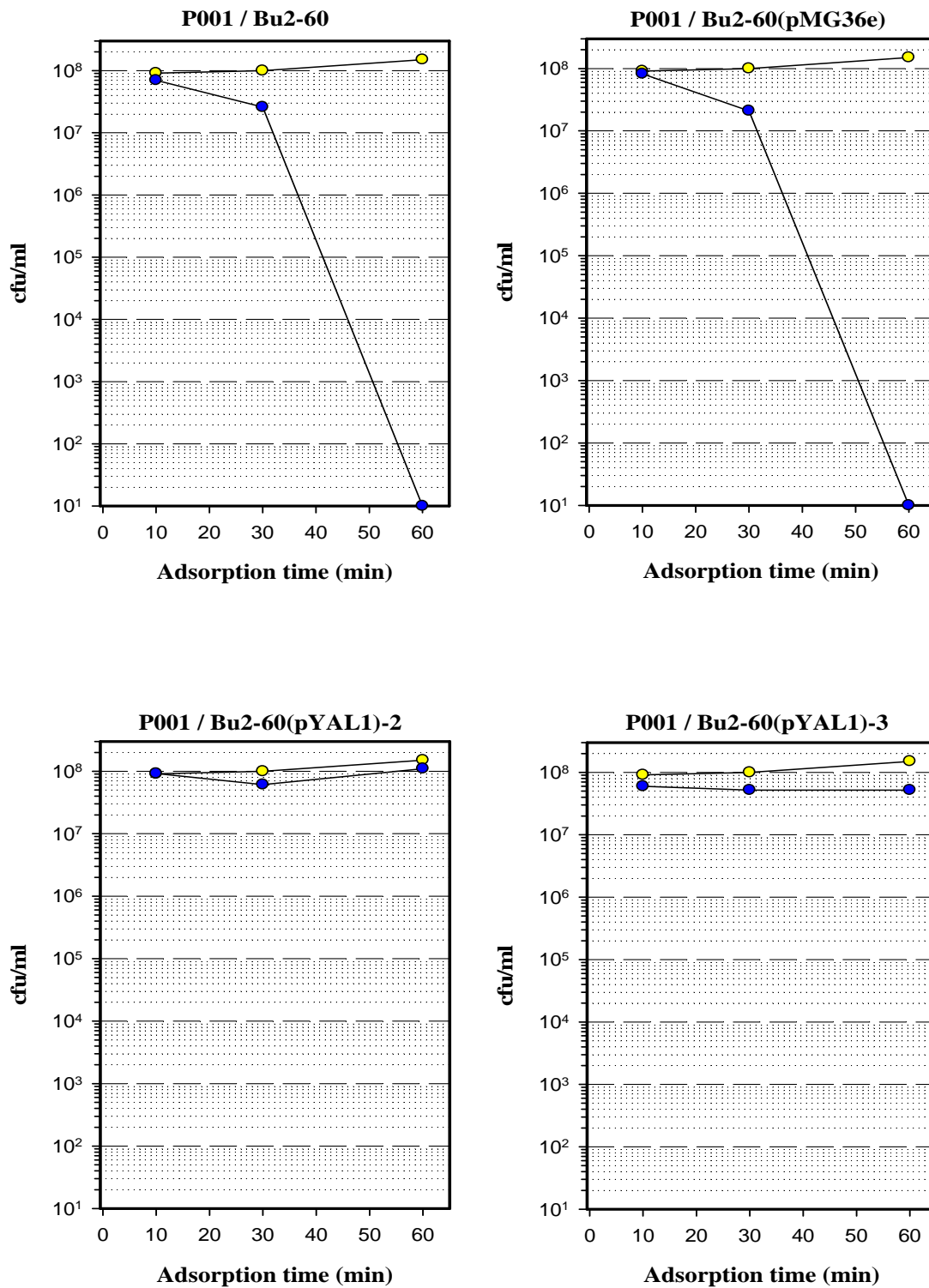


Fig. 6.9: Long-term cell survival test of *L. lactis* strains Bu2-60 and Bu2-60(pMG36e) and of strains Bu2-60(pYAL1)-2 and (pYAL1)-3 carrying the *ltp*_{TP-778} gene. The cells were challenged with lactococcal phage P001 (—●—) at high MOI for 30 min and 60 min. Bacterial cells without P001 (—●—) were used as a control.

6.2.8 Discussion and Conclusion

In this chapter it is shown that the lipoprotein coded by the *ltp*_{TP-778} gene of *S. thermophilus* phage TP-778 is highly similar to the corresponding *ltp*_{TP-J34} gene product of phage TP-J34 (Neve *et al.*, 2003, Sun *et al.*, 2006). The Ltp_{TP-778} lipoprotein of phage TP-778 has a calculated molecular weight of 15.6 kDa and reveals a significant 92% amino acids identical to the Ltp_{TP-J34} lipoprotein of the *S. thermophilus* temperate phage TP-J34. Both lipoproteins reveal the same protein secondary structures, i.e. a signal peptide (containing lipobox [LAAC in Ltp_{TP-778} and LTAC in Ltp_{TP-J34}] which is essential for lipoprotein recognition and modification [Hayashi & Wu, 1990] and 2 internal repeats. A helix form (see Fig. 6.11) (Rost *et al.*, 2004) can be derived from the majority of amino acids of both lipoproteins. The *ltp*_{TP-J34} and the *ltp*_{TP-778} genes share the same topological position within the lysogeny modules of the genomes of the *S. thermophilus* phages TP-J34 and TP-778, respectively (Fig. 6.10) and are located immediately upstream of the phage integrase genes. This location corresponds to that of genes coding for superinfection exclusion (Sie) mechanisms of *S. thermophilus* phage Sfi21 (*orf203*) and lactococcal phage Tuc2009 (*sie*₂₀₀₉) (Bruttin *et al.*, 1997b; McGrath *et al.*, 2002). Superinfection exclusion (Sie) is one of the phage-derived phage resistance mechanism which are frequently detected in temperate phages of lysogenic strains of mesophilic and thermophilic lactic acid bacteria (see Introduction). Several “Sie” systems have also been described in other Gram-positive and Gram-negative bacteria and most of them are encoded by prophages. Mahony *et al.* (2008) reported that “Sie” proteins of lactococcal phages are small proteins with hydrophobic amino terminus and have a high isoelectric point. It is notable that the genes specifying “Sie” systems show a relatively low GC content compared to the host genomic DNA. Sun *et al.* (2006) have reported that a *L. lactis* Bu2-60 transformant carrying the *ltp*_{TP-J34} gene cloned in plasmid pXMS2 revealed a remarkably high resistance to lactococcal phage P008, even when high-titer lysates of more than 10⁹ pfu/ml were applied. Furthermore, the “Sie” mechanism of the Ltp_{TP-J34} did not affect adsorption of the P008 phage to Bu2-60(pXMS2) cells but interfered with the process of DNA release from the phage and injection into the cell. This mode of action is similar to that of the Sie₂₀₀₉ protein of lactococcal phage Tuc2009 reported by McGrath *et al.* (2002).

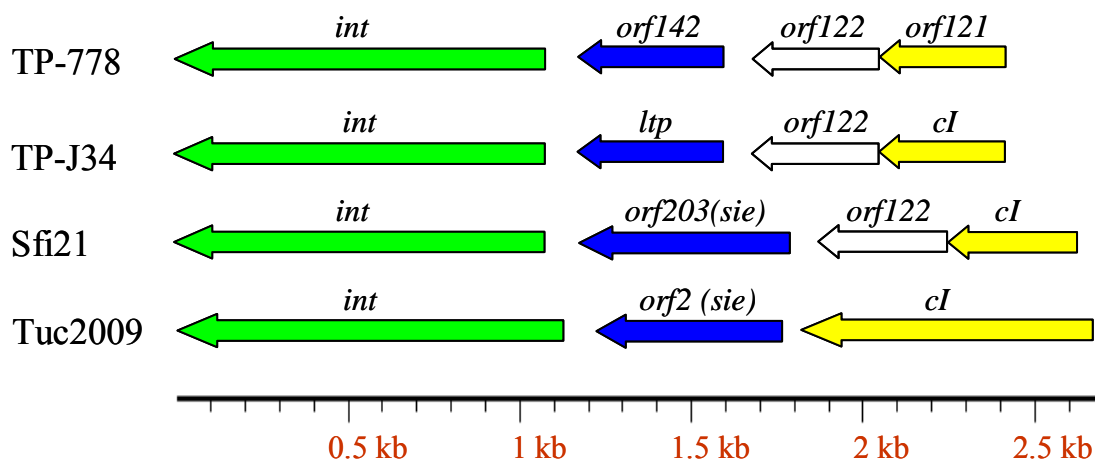


Fig. 6.10: Topological position of genes determining "Sie"-type phage resistance systems in the lysogeny modules of 3 *S. thermophilus* phage genomes and in the lactococcal Tuc2009 phage genome. The *sie* genes are coloured in blue and are located directly upstream of the phage integrase genes (green colour). The *cI*-like repressor (*cI*) genes are highlighted in yellow. An additional *orf122* gene which is highly conserved, is present in the *S. thermophilus* phage genomes only.

10.....20.....30.....40.....
Ltp _{TP-778}	MKKLLSFGLLSLSIIALAACSQTKSTSSQTSKTSEAKTEQSSES KVPKE
2 ^{ndry} structure:	EEEEEEEEEEEEEEEEEEEE HHH HH
	Signal peptide
Ltp _{TP-J34}	MKKILSFGLLSLSIIALTA CSQPKSTSSQTSKTSEAKTEQSSES KVPKE
2 ^{ndry} structure:	EEEEEEEEEEEEEEEEEEEE HH
	50.....60.....70.....80.....90.....
Ltp _{TP-778}	YRTAVSKAKQYASTV PMSKEGLRSQLV SF EKYSQEASDYAVDNAGIDYN
2 ^{ndry} structure:	HHHHHHHHHHHHHHHH HHHHHHHHH HHHHHHHHHHH HH
	51..... Internal repeat92
Ltp _{TP-J34}	YRTAVSKAKQYASTV HMSKEELRSQLV SFDKYSQDASDYAVENSGIDYN
2 ^{ndry} structure:	HHHHHHHHHHHHHHHH HHHHHHHHH HHHHHHHHHHH HH
	.100.....110.....120.....130.....140
Ltp _{TP-778}	KQALEKAKQYQDTLAMPSPDAIRDQLV SFDKFTQEEADYAVANL K
2 ^{ndry} structure:	HHHHHHHHHHHHHHHH HHHHHHHHH HHHHHHHHHHH
	99..... Internal repeat140
Ltp _{TP-J34}	KQALEKAKQYQDTLSMSPDAIRDQLV SFDKFTQEEADYAVANL K
2 ^{ndry} structure:	HHHHHHHHHHHHHHHH HHHHHHHHH HHHHHHHHHHH

Fig. 6.11: Comparative amino acids analysis of lipoproteins Ltp_{TP-J34} and Ltp_{TP-778} of *S. thermophilus* phages TP-J34 and TP-778 using PredictProtein soft ware (<http://www.predictprotein.org/>). The following secondary structures of the amino acid sequences are indicated: E=extended, H=helix and blank=other (loop). Red letters represent amino acid differences in the 2 lipoprotein sequences.

Conversely, the sensitivity of *L. lactis* transformants Bu2-60(pYAL1)-2 & Bu2-60(pYAL1)-3 carrying the *ltp*_{TP-778} gene obtained in this work, showed resistance against the virulent lactococcal prolate-headed phage P001 but not against the isometric-headed-phage P008. It is not yet clear, why transformant Bu2-60(pYAL1)-1 was sensitive to both P001 and P008 phages. Sequence analysis of the cloned *ltp*_{TP-778} gene in recombinant plasmid pYAL1 from all 3 transformants did not reveal any DNA sequence differences. Therefore, the unexpected effect of transformant Bu2-60(pYAL1)-1 may be due to an unknown mutation. The cell survival test of the 2 transformants after infection with P001 phage confirmed the P001 sensitivity result where 73% and 35% of Bu2-60(pYAL1)-2 and Bu2-60(pYAL1)-3 cells survived a 60-min phage challenge, while no surviving cells were detected in the Bu2-60 and Bu2-60(pMG36e) control cultures after such a strong phage exposure. The incomplete phage protection of the cells may be due to a low level of *ltp*_{TP-778} gene expression as discussed for other lactococcal phages (Mahony *et al.*, 2008). By SDS-PAGE of the cell protein extracts it was not possible to detect the *ltp*_{TP-778} gene product in any of the 3 transformants. In contrast, a thick Ltp_{TP-J34} protein band was clearly visible in the cell extract of *L. lactis* Bu2-60(pXMS2). However, the low cross-reactivity of the Ltp_{TP-778} lipoprotein may also simply reflect lack of binding of Ltp_{TP-J34} antibodies.

In summary, it can be concluded that cloning of the *ltp*_{TP-778} gene of *S. thermophilus* temperate phage TP-778 in the lactococcal strain Bu2-60 resulted in an enhanced phage resistance phenotype against the prolate-headed phage P001 but not against the isometric-headed phage P008 as reported by Sun *et al.* (2006) after transferring the cloned *ltp*_{TP-J34} gene of *S. thermophilus* temperate phage TP-J34 into strain Bu2-60. Apparently, this shift of phage resistance phenotype is due to one or more amino acid exchanges that were documented in the 2 different lipoproteins. This may be confirmed by mutation studies of the 2 proteins in the future.

7 Multiplex PCR for *S. thermophilus* phages

Chapter 3: A PCR tool for the simultaneous detection of lysogenic

Streptococcus thermophilus starter strains and *S. thermophilus*

bacteriophage classification

7.1 Introduction

Polymerase chain reaction (PCR) is a technique for exponentially amplifying a specific fragment of DNA, via enzymatic/thermal replication from a template DNA. It was invented by **Kary Mullis** in 1983 (Nethe-Jaenchen, 2008), and now PCR is a common technique used in biological research and medical labs for a variety of applications, such as the amplifying and sequencing of genes, diagnosis of hereditary diseases, identification of genetic fingerprints (used in forensics and paternity testing), detection and diagnosis of infectious diseases.

Bacteriophage attack, especially of lactic acid bacteria (LAB) is a serious problem in the industrial production of fermented dairy products leading to slow lactic acid production or complete failure of fermentation and consequently to significant economical losses (Josephsen & Neve, 2004). So, a rapid and sensitive method is required for phage detection and identification in dairy products during manufacturing. The susceptibility to phage infection is mainly due the use of non sterile raw material at the beginning of the fermentation process and the continuous use of a starter culture over a long period. Furthermore, most phages are not completely inactivated by standard conditions (including pasteurization). *Lactococcus lactis*, *S. thermophilus* and *Lactobacillus delbrueckii* are the most important LAB. *L. lactis* is used for the manufacture of many types of cheeses, buttermilk and sour cream. *S. thermophilus* cultures are used as starter strains for the production of yoghurt, Mozzarella and Swiss-type cheeses (del Rio *et al.*, 2007). *S. thermophilus* cultures are extensively used in the production of different cheeses, so the failure of fermentation due to phage infection has increased (Moineau, 1999). Since several years, many ecological studies have been carried out to elucidate the sources of *S. thermophilus* phages in dairy environments. A frequent recombination between different phage genomes after double infection is thought to be responsible for the variation of streptococcal phages (Fayard *et al.*, 1993; Mercenier, 1990). Bruttin *et al.* (1997a) have found that the environment, especially raw milk is the main source of phages in the cheese factory, making it impossible to eliminate phage particles completely from industrial dairy plant. Therefore, a permanent monitoring of phages is essential to make

sure that milk contains less than 10^5 pfu/ml, since higher titers will cause severe failure of milk acidification (Svensson & Christiansson, 1991).

Detection of phage is usually performed by standard microbiological methods, like plaque assay, but these techniques are time consuming and a phage-sensitive indicator strain is always required. So, the aim of this work was to develop a rapid and accurate technique in order to detect and differentiate *S. thermophilus* phages in milk and milk products by PCR (also in prophage state). PCR techniques have already been used to detect and identify viruses and bacteria in some foods (Starbuck *et al.*, 1992; Brüssow *et al.*, 1994a; Allmann *et al.*, 1995). A PCR method for detection of *S. thermophilus* phages in whey was firstly developed by Brüssow *et al.* (1994) using primers designed for a 2.2-kb EcoRI fragment which is a highly conserved DNA sequence in *S. thermophilus* phages (Brüssow *et al.*, 1994b). This EcoRI fragment was also used as a probe for detection of *S. thermophilus* phages in whey using dot blot hybridization (detection limit 10^7 pfu/ml whey). Binetti *et al.* (2005) detected and characterized *S. thermophilus* bacteriophages by a PCR protocol directed against an antireceptor gene sequence. Dupont *et al.* (2005) developed a PCR method for detection and differentiation of lactococcal 936-species bacteriophages and used primers for the gene encoding the receptor binding protein gene. Also, *L. lactis* phages can be detected using a multiplex PCR (Labrie & Moineau, 2000) which allowed the differentiation into the main lactococcal phage species 936, c2 and P335. Recently, del Rio *et al.* (2007) developed a multiplex PCR for detection of *L. lactis* and *S. thermophilus* bacteriophages in milk and dairy products. Five pairs of primers from a conserved DNA region of *L. lactis* phages species (936, c2 and P335), *Lb. delbrueckii* phages and *S. thermophilus* phages (previously designed primers Host 1 and Host 5 by Binetti *et al.*, 2005) were designed.

As previously mentioned, there are two types of *S. thermophilus* phages according to their DNA packaging mechanism: *pac*-type phages, packaging their DNAs by a “head-full” mechanism and *cos*-type phages, in which DNA is cut at a specific site (*cos*-site) and then packaged into the heads of the phage particles. All *cos*- and *pac*-type *S. thermophilus* phages share DNA homology, but they also possess distinct unrelated structural genes coding for head and tail proteins (Le Marrec *et al.*, 1997). These genes are highly conserved for both types of phage. Therefore, PCR primers were designed targeting these conserved DNA regions for *pac*- and *cos*-phages, respectively, to allow simultaneous detection of both *S. thermophilus* phage types by multiplex-PCR in milk products and in lysogenic starter cultures containing prophages.

Table 7.1: *S. thermophilus* *pac*- and *cos*-phages with complete sequenced genome

Phage	Major head protein gene	Genome type	Phage type	Reference
TP-J34	<i>orf35</i>	<i>pac</i>	temperate	Neve <i>et al.</i> , 2003; Sun <i>et al.</i> , 2006
O1205	<i>orf31</i>	<i>pac</i>	temperate	Stanley <i>et al.</i> , 1997
Sfi11	<i>orf348</i>	<i>pac</i>	virulent	Lucchini <i>et al.</i> , 1998
2972	<i>orf9</i>	<i>pac</i>	virulent	Lévesque <i>et al.</i> , 2005
Sfi21	<i>orf397</i>	<i>cos</i>	temperate	Desiere <i>et al.</i> , 1998
2701	<i>orf26</i>	<i>cos</i>	virulent	Proux <i>et al.</i> , 2002
Sfi19	<i>orf397</i>	<i>cos</i>	virulent	Lucchini <i>et al.</i> , 1999
DT1	<i>orf8</i>	<i>cos</i>	virulent	Tremblay & Moineau, 1999

7.2 Results and Discussion

7.2.1 Design of PCR primers

In order to select appropriate primers for the identification and differentiation of *cos*- and *pac*-type *S. thermophilus* phages, the complete genome sequences of 4 *S. thermophilus* *cos*-type phages (Fig. 7.1) and of 4 *S. thermophilus* *pac*-type phages (Fig. 7.2) were aligned with respect to the conserved major head protein gene (Table 7.1). 19-21 mer conserved primers were selected from the internal region of the 2 different structural genes which would give specific PCR-products of different lengths (432-bp with *pac*-phage specific primers YP-F/YP-R; 514-bp with *cos*-phage specific primers YC-F/YC-R). The primers are also indicated in Table 4.7 (see Materials & Methods: 4.10).

	380	390	400	410																															
2972-head protein	G	C	A	C	G	T	G	C	T	C	G	T	C	T	T	G	A	A	G	C	T	A	T	G	C	G	T	A	T	G	C	A	A	G	T
Sfi11-head protein	G	C	A	C	G	C	G	C	T	C	G	T	C	T	T	G	A	A	G	C	T	A	T	G	C	G	T	A	T	G	C	A	A	G	T
O1205-head protein	G	C	A	C	G	C	G	C	T	C	G	T	C	T	T	G	A	A	G	C	T	A	T	G	C	G	T	A	T	G	C	A	A	G	T
TP-J34-head protein	G	C	A	C	G	C	G	C	T	C	G	T	C	T	T	G	A	A	G	C	T	A	T	G	C	G	T	A	T	G	C	A	A	G	T
	YP-F →																																		
Consensus	G	C	A	C	G	c	G	C	T	C	G	T	C	T	T	G	A	A	G	C	T	A	T	G	C	G	T	A	T	G	C	A	A	G	T

	790	800	810																															
2972-head protein	A	A	T	T	C	T	T	C	C	C	T	G	A	C	G	G	C	C	A	C	T	T	G	A	C	T	C	T	T	A	T	C	C	C
Sfi11-head protein	A	A	T	T	C	T	T	C	C	C	A	G	A	C	G	G	T	C	A	C	T	T	G	A	C	T	C	T	T	A	T	C	C	C
O1205-head protein	A	A	T	T	C	T	A	T	C	C	T	G	A	C	G	G	T	C	A	C	T	T	G	A	C	T	C	T	T	A	T	C	C	C
TP-J34-head protein	A	A	T	T	C	T	T	C	C	C	A	G	A	C	G	G	T	C	A	C	T	T	G	A	C	T	C	T	T	A	T	C	C	C
	← YP-R																																	
Consensus	A	A	T	T	C	T	t	c	C	C	w	G	A	C	G	G	t	C	A	C	T	T	G	A	C	T	C	T	T	A	T	C	C	C

Fig. 7.1: Alignment of 2 internal regions of the structural genes coding for the major head protein (mhp) of 4 *S. thermophilus pac*-type phages. The nucleotide sequences highlighted in dark blue are highly conserved and were chosen as forward primer YP-F and reverse primer YP-R which are specific for *S. thermophilus pac*-type phages.

	80	90	100																														
7201-mpl-head protein	A	A	C	T	T	A	A	C	G	T	A	G	C	T	A	T	G	C	T	T	G	A	C	G	A	T	T	C	A	G	T	T	A
DT1-head protein-cos	A	A	C	T	T	A	A	C	G	T	A	G	C	T	A	T	G	C	T	T	G	A	C	G	A	T	T	C	A	G	T	T	A
Sfi19-head protein-cos	A	A	C	T	T	A	A	T	G	T	A	G	C	T	A	T	G	C	T	T	G	A	C	G	A	T	T	C	A	G	T	A	A
Sfi21-head protein-cos	A	A	C	T	T	A	A	T	G	T	A	G	C	T	A	T	G	C	T	T	G	A	C	G	A	T	T	C	A	G	T	A	A
	YC-F →																																
Consensus	A	A	C	T	T	A	A	y	G	T	A	G	C	T	A	T	G	C	T	T	G	A	C	G	A	T	T	C	A	G	T	w	A

	590	600	610	620																																			
7201-mpl-head protein	A	T	C	T	C	A	C	A	G	T	A	A	C	A	A	A	C	A	G	C	T	T	G	C	T	T	G	C	T	G	A	T	T	C	T	G	C	T	
DT1-head protein-cos	A	T	C	T	C	A	A	C	A	G	T	T	A	C	T	A	A	C	A	G	C	T	T	G	C	T	T	G	C	T	G	A	C	T	C	T	G	C	T
Sfi19-head protein-cos	A	T	C	T	C	A	A	C	A	G	T	T	A	C	T	A	A	C	A	G	C	T	T	G	C	T	T	G	C	T	G	A	T	T	C	T	G	C	T
Sfi21-head protein-cos	A	T	C	T	C	A	A	C	A	G	T	A	A	C	T	A	A	C	A	G	C	T	T	G	C	T	T	G	C	T	G	A	T	T	C	T	G	C	T
	← YC-R																																						
Consensus	A	T	C	T	C	a	A	C	A	G	T	w	A	C	t	A	A	C	A	G	C	T	T	G	C	T	T	G	C	T	G	A	t	T	C	T	G	C	T

Fig. 7.2: Alignment of 2 internal regions of the structural genes coding for the major head protein (mhp) of 4 *S. thermophilus cos*-type phages. The nucleotide sequences highlighted in dark blue are highly conserved and were chosen as forward primer YC-F and reverse primer YC-R which are specific for *S. thermophilus cos*-type phages.

7.2.2 Phage differentiation

The newly designed YP-F/YP-R and YC-F/YC-R primers were tested in separate PCR reactions in order to evaluate their specificity for well-characterized *pac*- and *cos*-type *S. thermophilus* phages from the institute's collection. As positive controls for *pac*-type *S. thermophilus* phages, phages TP-J34 (Neve *et al.*, 2003) and TP-778 (this study) were chosen. Phage P53 (Neve *et al.*, 1989) was used as a positive control of *cos*-type *S. thermophilus* phages. The 3 reference phages were used for the optimization of the PCR protocol (see Materials & Methods 4.10). When primers YP-F/YP-R were used, a 432-bp PCR product was obtained from the 2 *pac*-type phages, while phage P53 did not give a PCR product (Fig. 7.3, top). Contrarily, a 514-bp PCR product was exclusively derived from *cos*-type phage P53 with primers YC-F/YC-R but not from the 2 *pac*-type phages (Fig. 7.3, bottom).

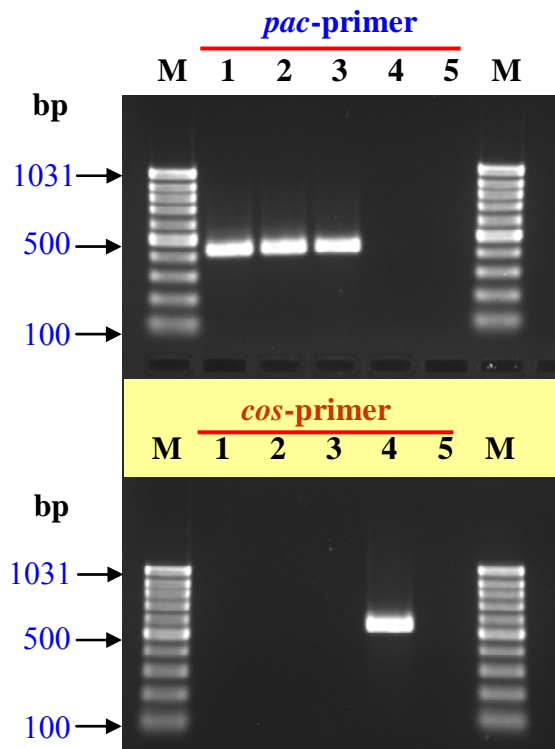


Fig. 7.3: Agarose gel electrophoresis of PCR products obtained from *S. thermophilus* *pac*-type phages TP-J34 (lane 1), TP-778 (2 & 3) and from *cos*-type phage P53 (4). Primers YP-F/YP-R were used for PCR reactions analysed in the top gel and primers YC-F/YC-R were used in PCR reactions analysed on the bottom gel. Lane 5: negative control; Lanes M: DNA size marker (100-bp ladder, AppliChem, Darmstadt, Germany).

The 2 primer pairs YC-F/YC-R and YP-F/YP-R were tested for their reliability to detect and distinguish unknown *S. thermophilus* phages from the culture collection. For this, both primer pairs were mixed and used simultaneously in a multiplex PCR experiment. PCR assays were

performed with lysates from the reference phages TP-J34, TP-778 and P53 (see also Fig. 7.3) and from the 2 phage isolates phages P640 and P572. Fig. 7.4 shows that the expected PCR product of the 2 *pac*-type reference phages TP-J34 and TP-778 and of the *cos*-type phage P53 did also appear from the multiplex PCR reactions. According to their corresponding PCR products, phage P640 was identified as a *pac*-type phage, while phage P572 was found to be a member of the *cos*-type *S. thermophilus* phage subgroup.

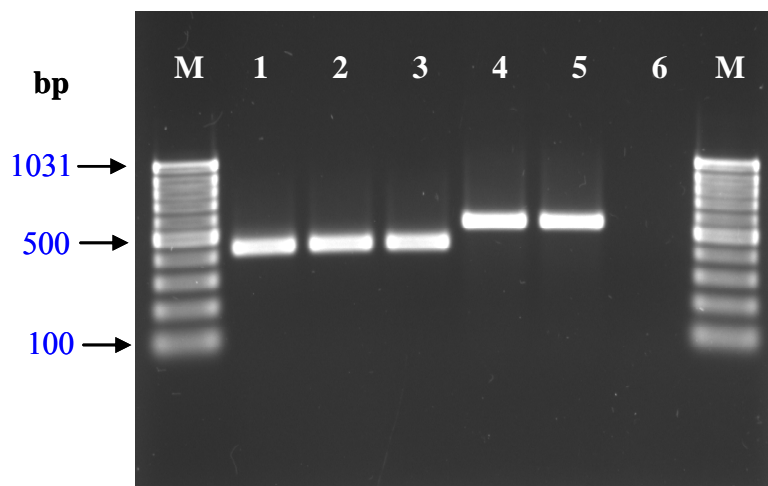


Fig. 7.4: Agarose gel (1.5%) electrophoresis of DNA products obtained by multiplex PCR with primer pairs YC-F/YC-R and YP-F/YP-R from *pac*-type reference phages *S. thermophilus* phages TP-J34 (lane 1) and TP-778 (2), of the unknown phage P640 (3), of the *cos*-type reference phage P53 (5) and of the unknown phage P572 (4). Lane 6: negative control (blank). Lane M: 100 bp DNA size marker (AppliChem, Darmstadt, Germany).

7.2.3 Validation of the reliability of the multiplex PCR protocol to distinguish *cos*- and *pac*-type *S. thermophilus* phages

7.2.3.1 Restriction endonuclease analysis of phage DNA

For the phage isolates P640 and P572, DNA restriction patterns were analysed with the restriction enzymes EcoRI, EcoRV, HaeIII, HindIII, HpaII and PvuII. The restriction digests were also incubated for 10 min at 74°C and analysed by agarose gel electrophoresis. This procedure should allow the identification of phage genomes with cohesive ends, since DNA fragments containing the *cos*-site would melt upon heating, and additional DNA fragments would become visible on agarose gels (Quiberoni *et al.*, 2003). Fig. 7.5 illustrates that – except for HaeIII digests - new DNA bands appeared after the heat treatment of restriction

digests of the P572 DNA (see arrows in Fig. 7.5). These data therefore confirmed the results of the multiplex PCR that phage P572 is a *cos*-type *S. thermophilus* phage. When the same experiment was carried out with the DNA of phage P640, no additional bands appeared after heating of the restriction digests (Fig. 7.6). Thus P640 was correctly identified as a *pac*-type *S. thermophilus* phage by multiplex PCR. Minor bands did appear in both the untreated and heat-treated EcoRV and PvuII digests of the P640 DNA (see arrows). These submolar fragments are also indicating that the P640 genome has a *pac*-site. These submolar bands are resulting from restriction enzyme cutting sites in the close vicinity of the *pac*-site (Neve *et al.*, 1993).

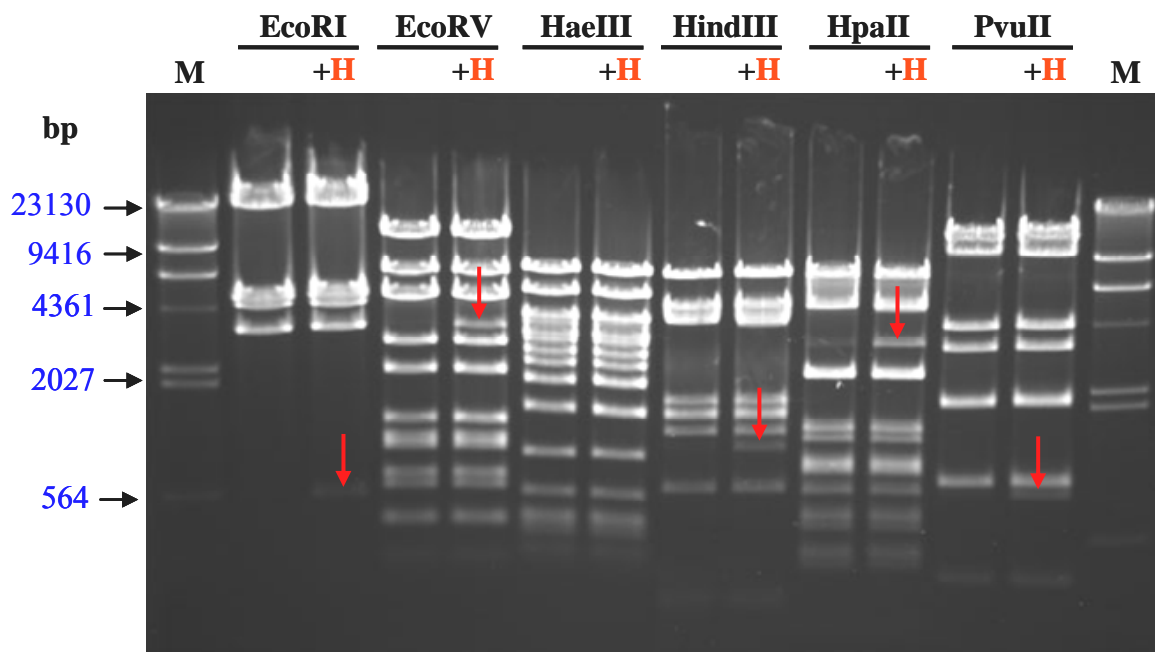


Fig. 7.5: Agarose gel (0.8%) electrophoresis of P572 DNA digested with restriction enzymes indicated on top of the gel. The restriction digests were compared with samples heated for 10 min at 74°C (+H) Lane M: λ /HindIII DNA (Roche, Mannheim, Germany). The red arrows indicate *cos*-containing DNA fragments which appear after heating of the restriction digests.

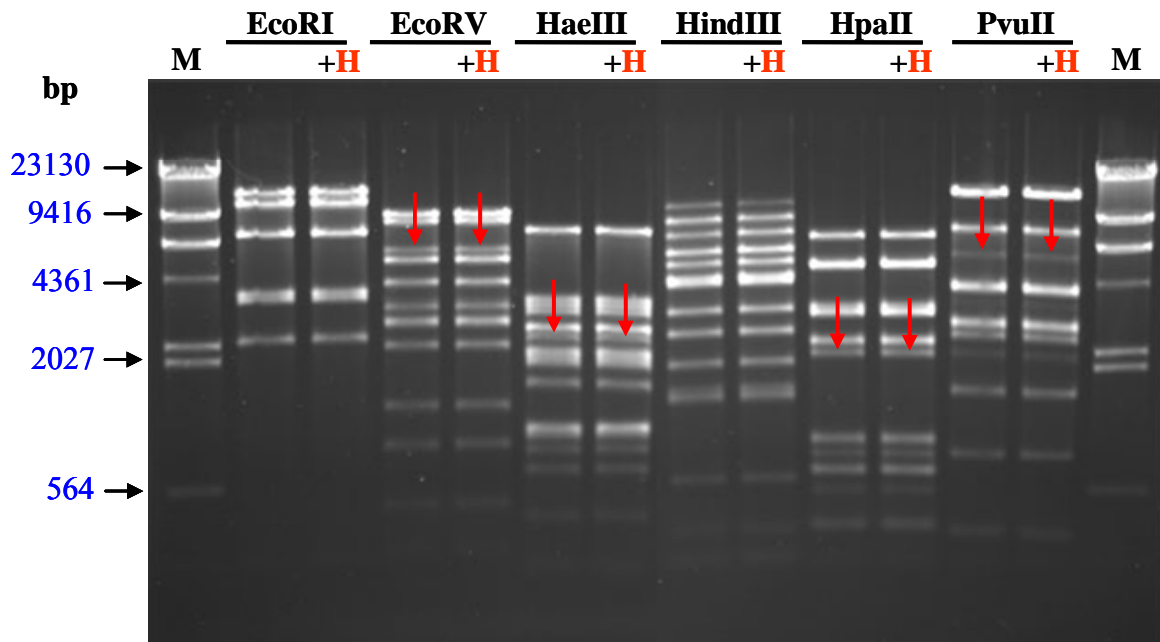


Fig. 7.6: Agarose gel (0.8%) electrophoresis of P640 DNA digested with restriction enzymes indicated on top of the gel. The restriction digests were compared with samples heated for 10 min at 74°C (+H) Lane M: λ /HindIII DNA (Roche, Mannheim, Germany). The arrows indicate submolar minor bands both in the untreated and the heated samples.

7.2.3.2 SDS-PAGE of the phage structural proteins

Cos-type and *pac*-type *S. thermophilus* phages are composed of different structural proteins. For the validation of the PCR detection and identification system, the structural proteins from the *cos*-type reference phage P53 and from the *pac*-type reference phage TP-778 were analysed by SDS-PAGE and compared with the structural protein profiles of the new isolates P572 and P640. It is shown in Fig. 7.7 that the protein profile of the *cos*-type reference phage P53 was identical to the pattern of the phage isolate P572, and both phages revealed 2 main protein bands. Thus, phage P572 is a *cos*-type phage. The protein profile of the *pac*-type reference phage TP-778 was matching the pattern of the phage P640 with 3 main protein bands. These data do also confirm the classification the new phages by multiplex PCR as *pac*-type (P640) and *cos*-type (P572).

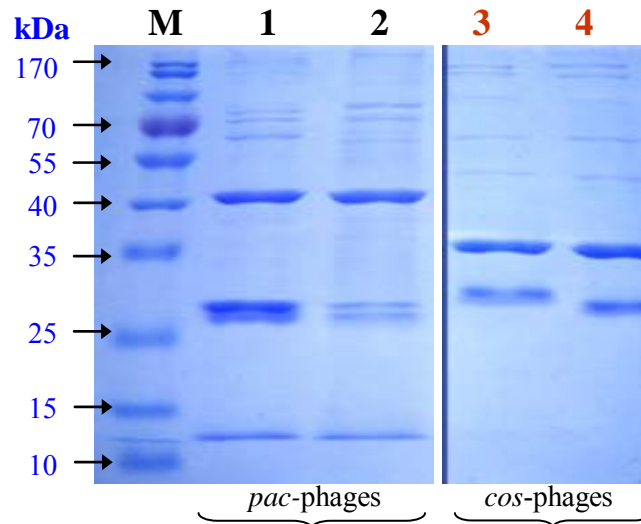


Fig. 7.7: SDS-PAGE (12%) of the structural proteins of the *pac*-type *S. thermophilus* reference phages TP-778 (lane 1), of the *cos*-type phage P53 (4) and of the new phages P640 (2) and P572 (3). Lane M: PageRulerTM prestained protein marker (Fermentas, St. Leon-Rot, Germany).

7.2.3.3 Immuno electron microscopy

Polyclonal antibodies were raised in hen's eggs (Davids Biotechnologie, Regensburg, Germany) against the structural proteins of the *pac*-type *S. thermophilus* phage TP-778. This antiserum was used in immuno electron microscopy experiments in order to differentiate *pac*- and *cos*-type *S. thermophilus* phages that are composed of different structural proteins. It is shown in Fig. 7.8 that the heads and tails of the control *pac*-type phage TP-778 were coated by a dense layer of antibodies. The antiserum did also cross-react with phage P640. Apparently this new *pac*-type phage contains non-homologous proteins at the distal part of the tail which do not react with the TP-778 antiserum. There was no binding at all of the TP-778 antiserum to the *cos*-type reference phage P53 and to the new phage P572. Therefore, the multiplex PCR differentiation of the *S. thermophilus* phages was confirmed by serological typing with immuno electron microscopy.

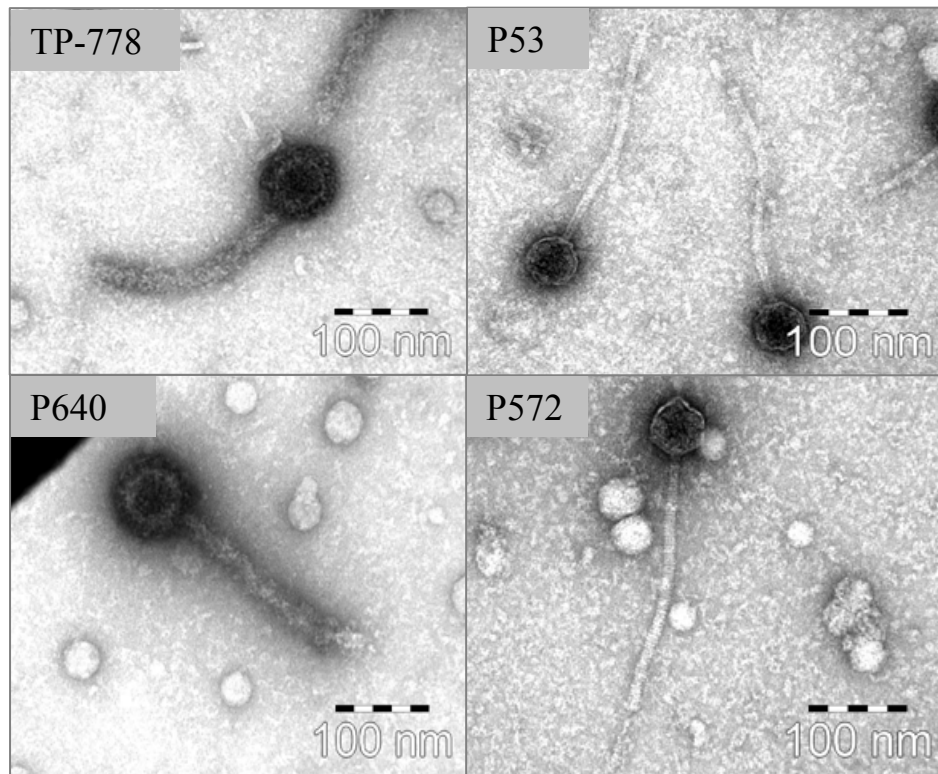


Fig. 7.8: Immuno electron micrographs of *S. thermophilus* phages treated with polyclonal antibodies raised against the *pac*-type reference phage TP-778.

7.2.4 Determination of the detection limit of the PCR assay

In order to determine the limit of detection of the multiplex PCR assay, a high-titer lysate of the *cos*-type *S. thermophilus* phage P572 was resuspended in acidic whey (final titer: 2×10^9 pfu/ml) (Fig. 7.9). Serial dilutions were made from this lysate in the same acidic whey. Whey samples spiked with different phage titers were tested in multiplex PCR assays using the *cos*- and *pac*-specific primers YP-F/YP-R and YC-F/YC-R. It is shown in Fig. 7.10 that the *cos*-type phage could be detected in acidic whey by PCR at a minimal titer of 2×10^2 pfu/ml. Previously Brüssow *et al.* (1994a) reported that the detection limit of another PCR technique developed for *S. thermophilus* phages was found by to be 10^3 pfu/ml (in whey). PCR detection of *S. thermophilus* phages has also been established in milk (Binetti *et al.*, 2005). In this medium, however, the sensitivity was rather low (i.e., 10^5 pfu/ml).

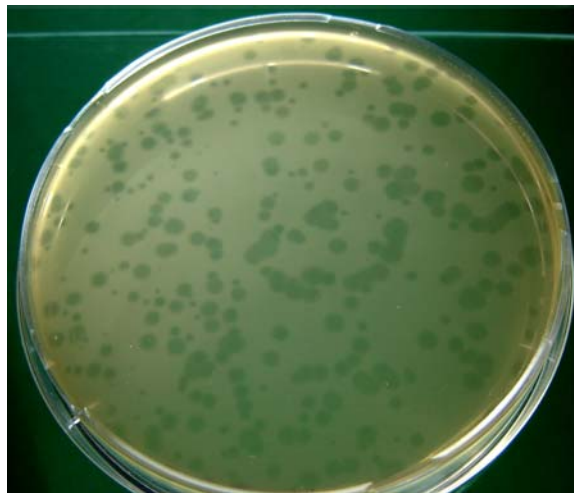


Fig. 7.9: Plaques of *S. thermophilus* *cos*-type phage P572 in the bacterial lawn of the indicator strain YA19.

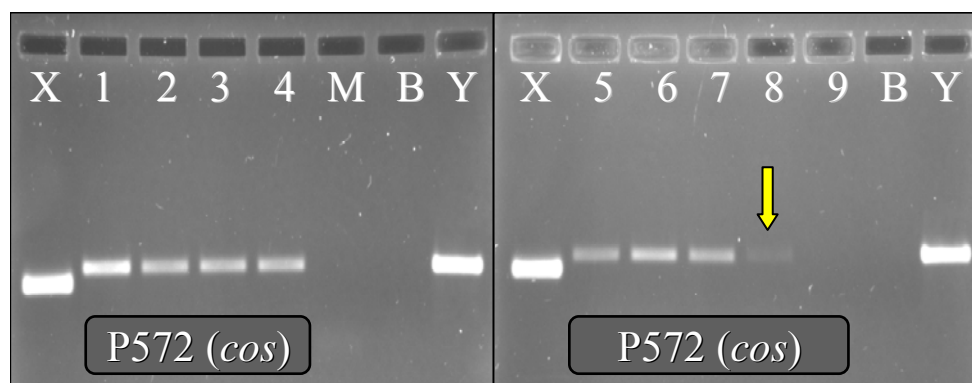


Fig. 7.10: Agarose gel (1.5%) electrophoresis of DNA products obtained by multiplex PCR using primers YC-F/YC-R and YP-F/YP-R with serial dilutions of *cos*-type *S. thermophilus* phage P572 resuspended in acidic whey. Serial dilutions were also done in acidic whey and ranged from 2×10^9 pfu/ml (lane 1) to 2×10^1 pfu/ml (9). The *pac*-type reference phage TP-J34 (lane X) and the *cos*-type reference phage P53 (Y) were used as positive controls. Lane M: whey without phages (negative control). Lane B: without whey (blank).

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

A set of 60 *S. thermophilus* strains, isolated from traditionally produced (i.e., using unidentified starter culture) Egyptian yoghurt (Zabady) samples was screened by colony-PCR (multiplex PCR) with primers YC-F/YC-R and YP-F/YP-R.

Three lysogenic *S. thermophilus* strains harbouring inducible *pac*-type prophages were identified (Fig. 7.11). Intact temperate phages were released from 2 of the 3 strains (strains YA1, YA2) upon mitomycin C induction (Fig. 7.12), while defective temperate phages (phage tails) were induced from strain YA3 (Fig. 7.13).

In order to compare the dissemination of lysogenic *S. thermophilus* strains in strains isolated from Egyptian yoghurt samples derived from industrial production, a set of 40 strains was tested with the same colony-PCR methodology. No prophages were detected in these strains (data not shown). The data confirmed that lysogeny is a rare event in *S. thermophilus* cultures (Neve *et al.*, 2003), and only 1% - 10% of the strains contain inducible prophages. However the analysis of the traditional cultures may indicate a higher dissemination of lysogenic strains.

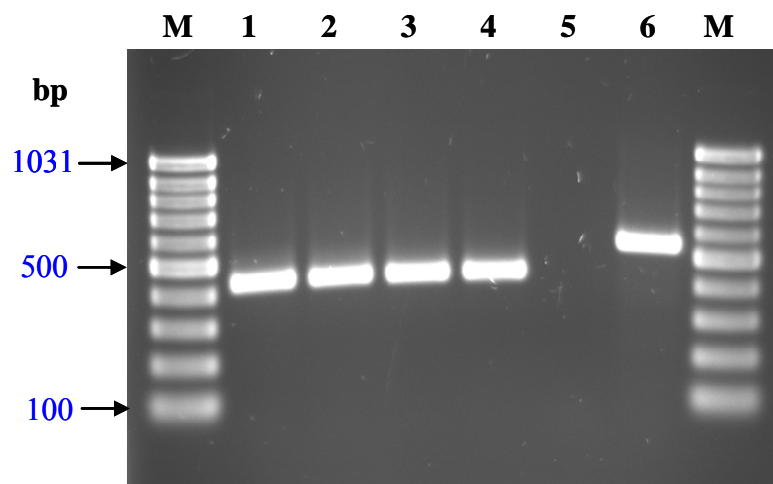


Fig. 7.11: Agarose gel (1.5%) colony multiplex PCR for identification of 3 „traditional“ lysogenic *S. thermophilus* strains YA1 (lane 2), YA2 (lane 3), YA3 (lane 4) harboring *pac*-type temperate phages. Lane 1: *pac*-phage TP-J34; Lane 6: *cos*-phage P53; Lane 5: negative control. Lane M: 100 bp DNA size marker (AppliChem, Darmstadt, Germany).

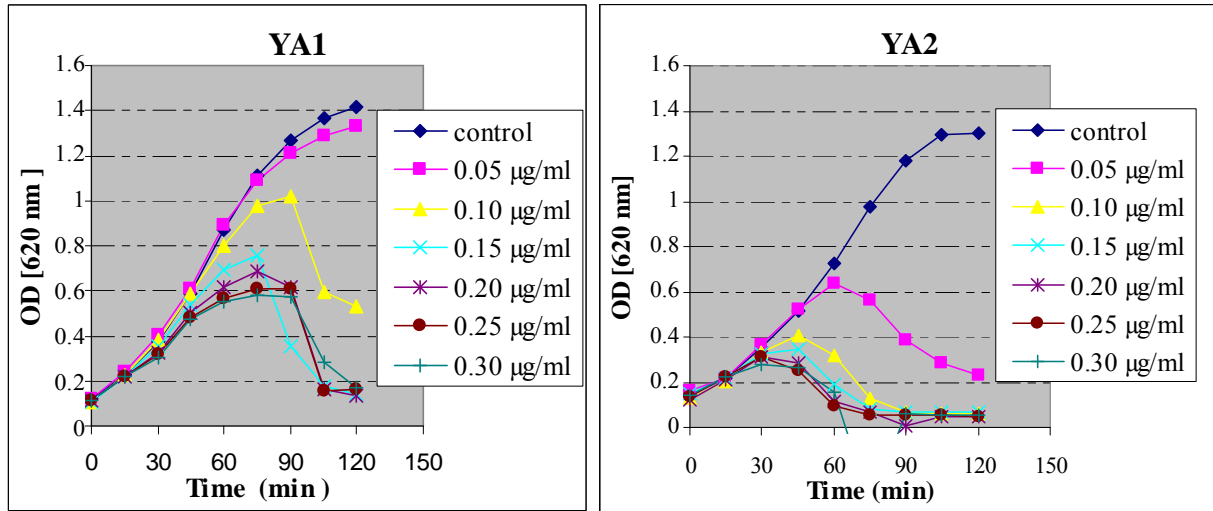


Fig. 7.12: Growth curves of lysogenic “traditional” *S. thermophilus* strains YA1, YA2 and YA3 upon induction with mitomycin C at 6 concentrations as indicated in the diagrams. No mitomycin C was added to the controls.

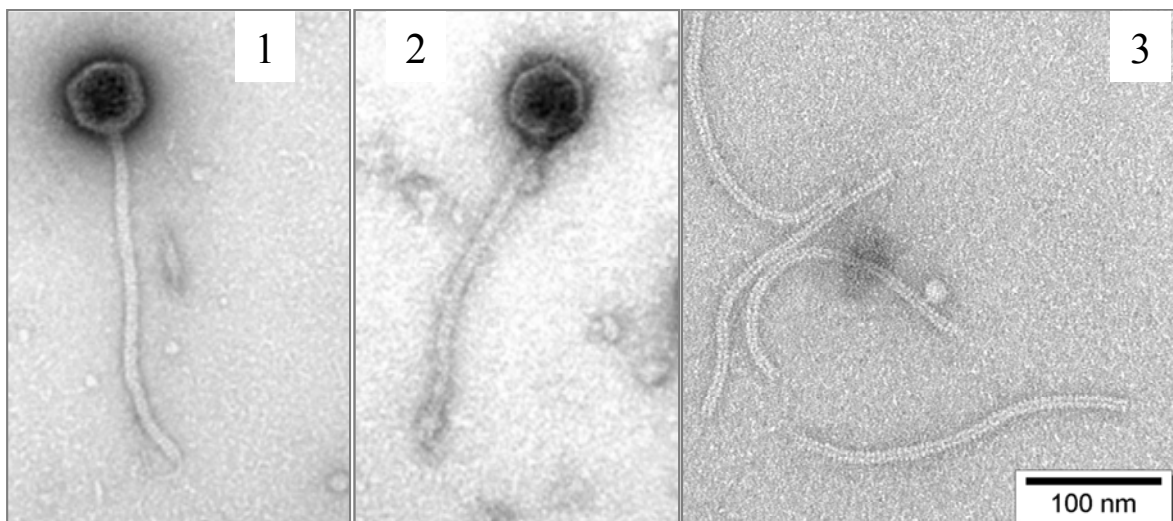
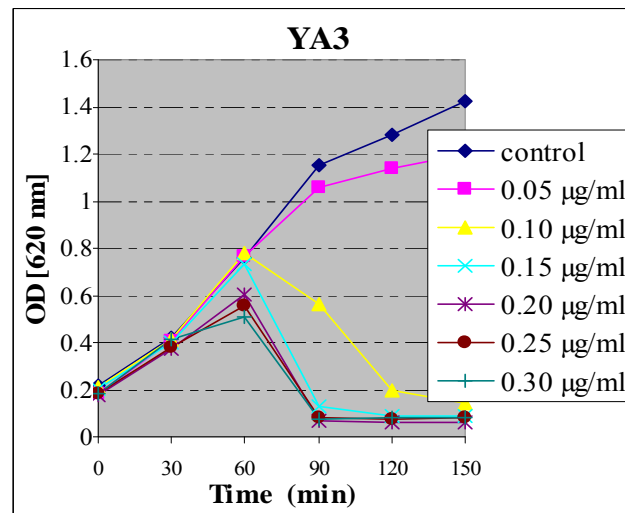


Fig. 7.13: Electron micrographs of temperate *S. thermophilus* phages TP-YA1 (1) and TP-YA2 (2) and of defective TP-YA3 phage particles consisting of tails only (3).

7.3 Conclusion

Phage monitoring is very important in the dairy industry to avoid economical losses that may happen due to phage infection. From the previously shown results, it can be concluded that the conserved DNA-regions of the structural genes for the major head proteins of *S. thermophilus* phages are reliable PCR-targets for the differentiation of *pac*- and *cos*-type phages by multiplex PCR.

The multiplex PCR described in this work can detect and differentiate *S. thermophilus* phages in a short time (2.5 h), is very sensitive and reveals a high limit of detection. In whey samples, phages could be detected at low titers of less than 10^3 pfu/ml.

Furthermore, *S. thermophilus* starter cultures can be screened rapidly for the presence of lysogenic strains by using colony PCR.

8 A new *S. thermophilus* phage

Chapter 4: Identification and characterization of the new

Streptococcus thermophilus phage P738

8.1 Introduction

The genus *Streptococcus* plays two important roles for human beings, on one side as a major bacterial pathogen (e.g., *S. pyogenes* and *S. pneumoniae*), and on the other side as a nonpathogenic and beneficial species (i.e., *S. thermophilus*) used in starter cultures for many industrial food fermentations (Canchaya *et al.*, 2003; Ventura *et al.*, 2002). Bacteriophages infecting *S. thermophilus* culture are the main cause of fermentation failures in dairy industry leading to high economical losses. Consequently, it is one of the most intensively studied phage groups. *S. thermophilus* phages belong to the *Siphoviridae* family and all *S. thermophilus* phages known so far reveal the same morphology and are genetically related. Similarities have been observed also with the genomes of some phages from other dairy starters, like the similarity between Sfi21 and the *Lactococcus lactis* phage BK5-T (60% DNA sequence identity). Also, the genome of phage Sfi21 shared 31-61% amino acid identity with the *Lactobacillus gasseri* phage adh at the protein level of the packaging, head and tail morphogenesis coding genes (Brüssow & Desiere, 2001; Desiere *et al.*, 2000). Several studies have been carried out to improve the classification of phage taxonomy. Depending on comparative genomics of the structural genes, Proux *et al.* (2002) grouped *Siphoviridae* phages of lactic acid bacteria into the 5 genera c2-, sk1-, Sfi11-, Sfi21- and r1t-like phages. As an example, the well-described lactococcal phage BK5-T (*cos*-type; Desiere *et al.*, 2001a) belongs to Sfi21-like phages, while phages Tuc2009 and TP901-1 (*pac*-type; McGrath *et al.*, 2002; Brondsted *et al.*, 2001) are members of the Sfi11-like phages. Moreover, depending on similarity of the completely sequenced phage genomes, a “phage proteomic tree” was developed by Rohwer & Edwards (2002), in which the *Siphoviridae* phages were divided into at least 5 subgroups: sk1-, λ -, TP901-1-, Sfi21- and r1t-like Siphophages. The structural gene cluster of *L. lactis* phage r1t is related to the corresponding gene cluster of the lactococcal phage phiLC3, but shows also DNA and protein similarity to the *S. pyogenes* prophages 370.3 and 8232.4 (Blatny *et al.*, 2004). From a practical point of view, all *S. thermophilus* phages described so far belong to 2 distinct subclasses, i.e., the *cos*- and the *pac*-type *S. thermophilus* phages with different phage structural proteins and with different phage DNA-packaging mechanisms (LeMarrec *et al.*, 1997, see also Chapter 3: Multiplex PCR).

In this chapter we describe a novel *S. thermophilus* phage not fitting into the current classification scheme.

8.2 Results and Discussion

8.2.1 Identification of the host strain

S. thermophilus 738 was found to be an unusual phage, since it could not be detected by the multiplex PCR method described in chapter 3. Phage 738 could be propagated on the *S. thermophilus* strain S4 from the institute's culture collection. The correct species designation was confirmed by 2 different assays:

(1) The biochemical properties of strain S4 were determined using a standard API-test system (API-test: rapid ID 32 Strep, Biomérieux, Nürtingen, Germany) (Freney *et al.*, 1992). The test confirmed with a high 99% confidence that the host strain is a member of the *S. thermophilus* species.

(2) Using universal primers 8F and 1525R (Beumer & Robinson, 2005), a 1.4-kb fragment was amplified by PCR from the 16S rDNA of strain S4 (see also Materials & Methods, 4.10). The nucleotide sequence of the fragment was determined at Eurofins MWG Operon, Ebersberg, Germany) and was confirmed to be 100% identical to the 16S rDNA specific for the *S. thermophilus* species (NCBI-BLAST).

8.2.2 Phage propagation

During the first experiments with phage P738 it was noted that this phage did only produce turbid and tiny plaques in the lawn of the host S4 in standard soft-agar overlay experiments at 40°C which was routinely used for the growth of *S. thermophilus* strains and propagation of *S. thermophilus* phages. It was not possible to propagate this phage lytically in liquid cultures (see Fig. 8.1), although the host strain did grow well at 40°C as expected. It was therefore tested whether lower incubation temperatures would increase the infectivity of this phage. Lowering the temperature from 40°C to 30°C did in fact improve the propagation of phage P738 significantly, and this temperature (usually appropriate for mesophilic cultures and their phages) was determined as the optimal temperature for propagation of phage 738 (Fig. 8.1). It should be noted that the growth rate of the host strain was significantly reduced at this temperature (see Fig. 8.1). It is shown in Fig. 8.2 that phage P738 adsorbed well to the cells of strain S4. This phage adsorption experiment was performed with a raw lysate of phage P738 in

growth broth, and it can be seen that P738 phage particles were prone to disintegrate (see tail structure in Fig. 8.2).

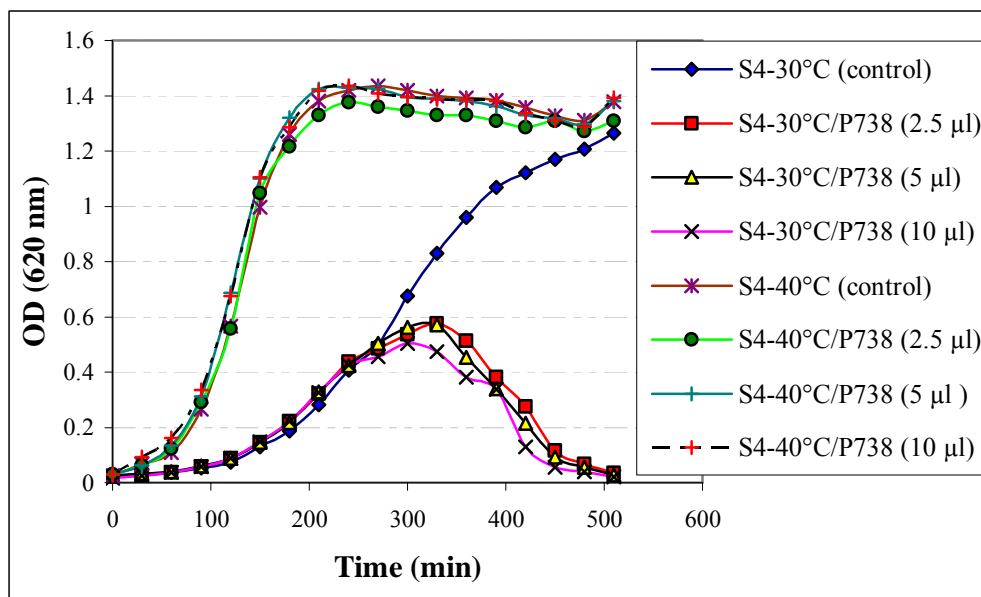
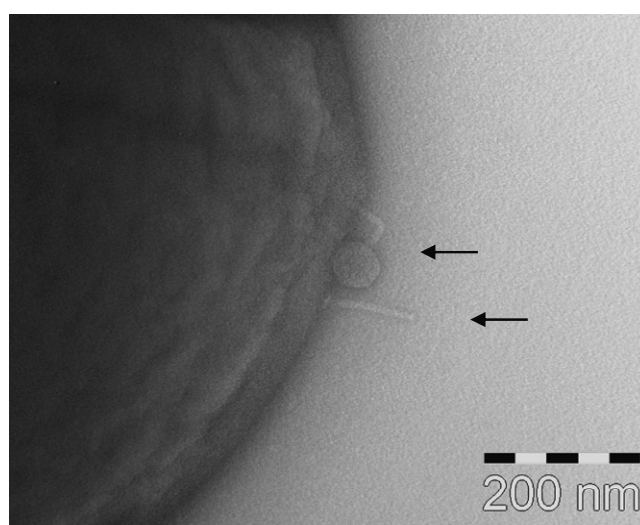


Fig. 8.1: Growth curves of *S. thermophilus* S4 in the absence and in the presence of phage P738 at different multiplicities of infection (MOI) of 0.058 – 0.235 pfu/ml. Assays were incubated at 2 different temperatures (i.e., 30°C and 40°C). The phage lysate with a titer of 3×10^8 pfu/ml was added in different volumes to the host cells at time 0. No phages were added to the control.

Fig. 8.2: Adsorption of an intact phage P738 particle (upper arrow) and of a phage tail (lower arrow) to the surface of an *S. thermophilus* S4 cell. Source: H. Neve, MRI-Kiel, Germany.



Since phage P738 revealed best cell lysis under suboptimal growth conditions for the bacterial host cells at low temperature, it was further tested if phage-mediated cell lysis was also improved at other unfavourable growth conditions. Therefore phage P738 was propagated on strain S4 at 40°C in LM17th broth containing different concentrations of carbon and energy source (i.e., 0.1%, 0.2% and 0.8% (w/v) lactose). Fig. 8.3 shows that although strain S4 grew only slowly in broth containing 0.1% or 0.2% (w/v) lactose to low optical densities, phage-derived cell lysis occurred. In medium containing the standard amount of lactose (0.8% w/v), the culture grew rapidly but failed to lyse in the presence of phage. When growth broth with 0.1% (w/v) lactose was used for phage propagation on S4 cells at 30°C and at 40°C, similar P738 phage titers were obtained (7.4×10^6 pfu/ml versus 4.6×10^6 pfu/ml). However, when the same multiplicity of infection (MOI) was used in a phage infection experiment done at 30°C in standard growth broth containing 0.8% (w/v) lactose, the phage titer obtained was much higher (approximately 10^8 pfu/ml). This may be due either to the low number of intact phages or to low burst sizes in infected cells (number of phages generated from infected bacterial cell). Müller-Merbach *et al.* (2007) reported that latency period (time from infection until release of new phages) and burst size depended on the type of phage, the host, the physiological state of the host cell as well as composition and temperature of the growth medium. Generally, the latency period is closely related to the generation time of the host, so it is shorter for higher temperature due to the shortened generation time.

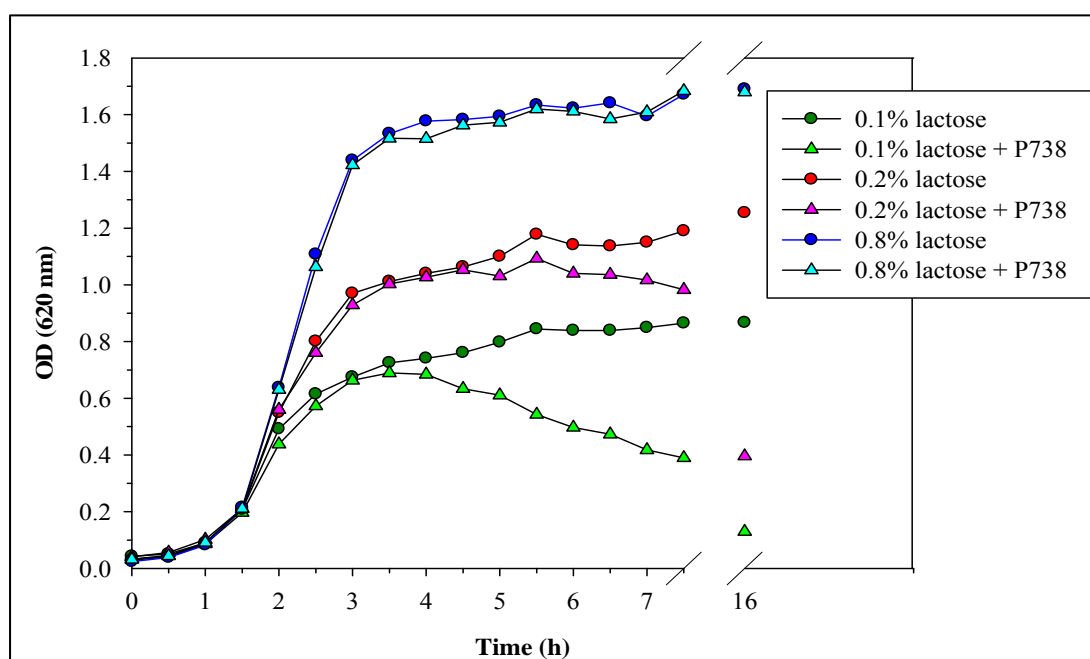


Fig. 8.3: Growth of *S. thermophilus* S4 at 40°C LM17th broth containing different lactose concentrations in the absence and presence of phage P738.

8.2.3 Electron microscopic analysis of phage P738

The morphology of phage P738 was analyzed by transmission electron microscopy. The phage particles (Fig. 8.4) had isometric heads (diameter: 57 nm) and remarkably short, non-contractile tails (length: 124 nm, diameter: 10 nm). A distinct baseplate (height: 9.5 nm, diameter: 17.9 nm) was present at the distal tail end, and a long protruding tail fiber (length: 54 nm) was attached to the baseplate. Although phage P738 belongs to the *Siphoviridae* family of phages, its morphology is unique and differs from the shape of *S. thermophilus* phages described so far (see Fig. 8.5). This phage clearly represents a new phage type capable of infecting *S. thermophilus* cultures.

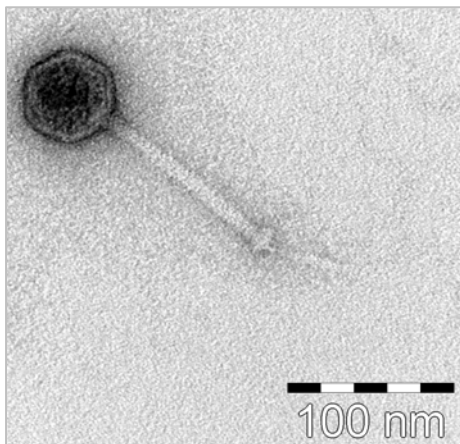


Fig. 8.4: Transmission electron micrograph of *S. thermophilus* phage P738, a new morphotype of *S. thermophilus* phages. Source: H. Neve, MRI-Kiel, Germany.



Fig. 8.5: Transmission electron micrograph of *S. thermophilus* phage TP-J34, a member of the well-established *pac*-type subgroup of *S. thermophilus* phages. Bar: 100 nm. Source: H. Neve, MRI-Kiel, Germany.

8.2.4 Plaque assay

Phage titers of P738 lysates and its plaque morphology on host strain S4 were determined using a standard plaque assay as described in Materials and Methods (see 4.6.4). Plates were incubated both at 30°C and 40°C overnight. It is notable that phage P738 plaques were observed after incubation both at 30°C as well as 40°C although no lysis of the S4 cells was

detected at 40°C in standard LM17th broth. However, the efficiency of plating (EOP) was slightly reduced by 1 order of magnitude at 40°C, and plaque sizes were also reduced in bacterial lawns incubated at 40°C compared to plates incubated at 30°C (Fig. 8.6). The small plaques which were observed at 40°C may be due to low numbers of intact phages released after burst of host cells.

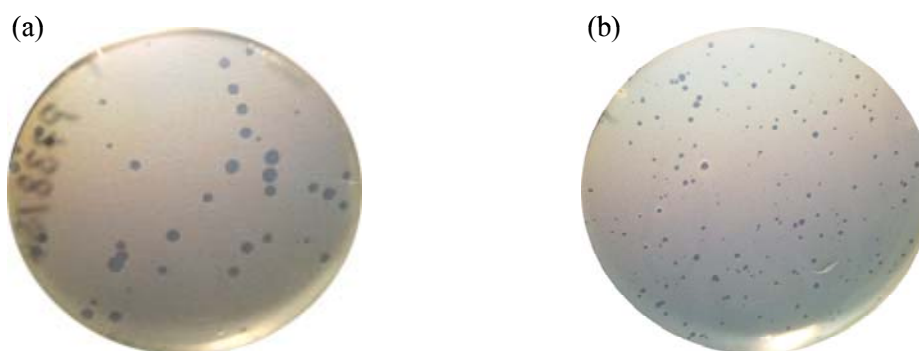


Fig. 8.6: Phage P738 derived plaque sizes and plaque morphologies in the bacterial lawns of strain S4 incubated overnight at 30°C (a) and at 40°C (b).

8.2.5 Host range of phage P738

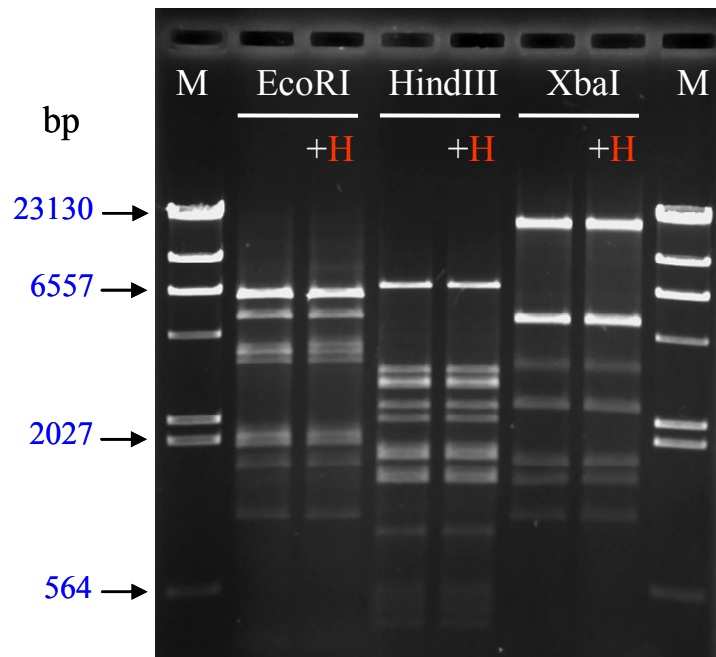
Phage sensitivity of 400 *S. thermophilus* strains from the MRI culture collection was screened against phage P738 using a micro-titer plate method (Neve *et al.*, 1994). Plates were incubated in parallel at 30°C and 40°C. A set of 14 *S. thermophilus* strains was sensitive to phage P738, i.e., strains YA5, YA6, YA7, YA8, YA9, YA10, YA11, YA12, YA13, YA14, YA15, YA16, YA17 and YA18). The phage sensitivity of these strains which exhibited delayed growth activity in the presence of P738 at 30°C, were also confirmed by spot tests with a phage P738 lysate at 30°C and 40°C. Spots were seen on agar plates incubated at 30°C and 40°C. Apparently, phage P738 is a bacteriophage with a broad host range.

8.2.6 Restriction enzyme analysis of the phage P738 genome

Phage P738 was concentrated and purified by CsCl step gradient centrifugation (see Materials & Methods, 4.6.3). Phage DNA was digested with restriction enzymes EcoRI, HindIII and XbaI (Fig. 8.7). Aliquots of digested phage DNAs were also heated at 74°C for 10 min (Quiberoni *et al.*, 2003) to differentiate between *pac*- or *cos*-type phage DNA as outlined before in Chapter 1 (see 5.2.4) and Chapter 3 (see 7.2.3.1). No extra DNA bands were found

in the heated digests, indicating that phage P738 is a *pac*-type phage. The sizes of the DNA fragments obtained from the digests which were visible in the agarose gel, were summed up using the “Quantity One” software (version 4.5.2, Bio-Rad, Laboratories, Segrate [Milan], Italy). The mean value of the P738 genome size was determined as 35 kb.

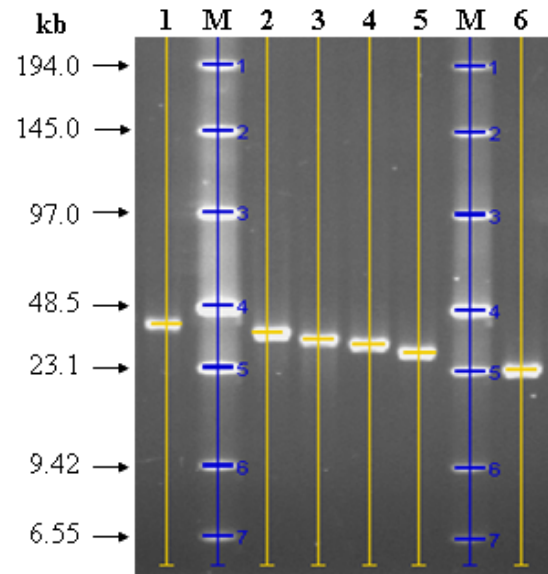
Fig. 8.7: Agarose gel (0.8%) electrophoresis of phage P738 DNA digested with 3 restriction enzymes. Samples were used without heating or after a 10-min heating at 74°C (+H). Lane M: λ / HindIII reference DNA ladder (Roche, Mannheim, Germany).



8.2.7 Determination of the P738 genome by PFGE

In order to confirm the genome size, intact DNA from phage P738 DNA and DNA from other reference phages with known genome sizes were separated by PFGE (Fig. 8.8). The sizes of the reference phage DNAs differed from 28.5 kb (lactococcal phage P008; Jarvis *et al.*, 1991) to 40 kb (lactococcal phage BK5-T, Mahanivong *et al.*, 2001; Desiere *et al.*, 2001a). The size of the phage DNA was estimated using “Quantity One” software. A “PFGE low range marker” (New England Biolabs GmbH, Frankfurt am Main, Germany) was used as an additional reference. The genome size of phage P738 was calculated to be 35 kb (Fig. 8.8).

Fig. 8.8: PFGE (1.1% agarose gel) of intact P738 DNA (lane 3) and of intact DNA of reference phages BK5-T (1; 40 kb), P53 (2; 37 kb), P446 (4; 32.5 kb), P008 (5; 28.5 kb) and P001 (6; 20 kb). Lane M: low range PFGE DNA marker (New England Biolabs GmbH, Frankfurt am Main, Germany). The horizontal and vertical lines were created by the “Quantity One” software for determination of the DNA sizes.



8.2.7.1 Genetic relationship of phage P738 with *L. lactis* and *S. thermophilus* phages

The DNA of phage P738 was used as a DNA probe and a Southern blot analysis was performed with the DNAs of well-known *S. thermophilus* and *L. lactis* phages deposited in the MRI phage collection. No hybridization signals were obtained from these hybridization experiments - neither from the lactococcal nor from the *S. thermophilus* phage DNAs. (Fig. 8.9 & Fig. 8.10). All so far described *pac*- and *cos*-type *S. thermophilus* phages are known to share DNA homology within the early transcribed gene clusters, whereas their structural proteins are totally unrelated (Luccini *et al.*, 1999). Nucleotide sequence similarity was even detected between *S. thermophilus* phage Sfi21 and the lactococcal phage BK5-T (60%) over the DNA packaging and head coding genes (Brüssow & Desiere, 2001). Thus, phage P738 does not fit into the scheme of currently characterized *S. thermophilus* phages and should become the type phage of a new *S. thermophilus* phage species.

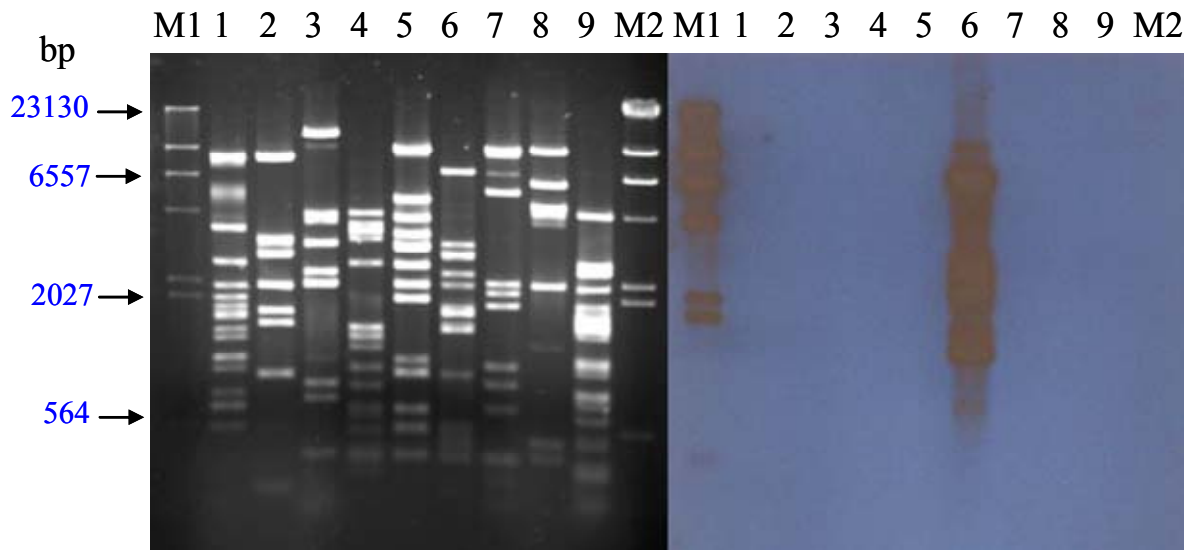


Fig. 8.9: Agarose gel (0.8%) electrophoresis (left) and Southern blot hybridization (right) of phage P738 DNA (lane 6) and of lactococcal phage DNAs digested with HindIII (1: BK5-T, 4: P335, 7: r1t, 9: TP901-1), with StyI (2: P001), with EcoRV (3: P008, 8: sk1) or with EcoRI (5: P446). Lane M1: phage λ -HindIII (DIG labelled). Lane M2: phage λ -HindIII (unlabelled). The agarose gel was blotted and hybridized with a phage P738 DNA probe digested with HindIII.

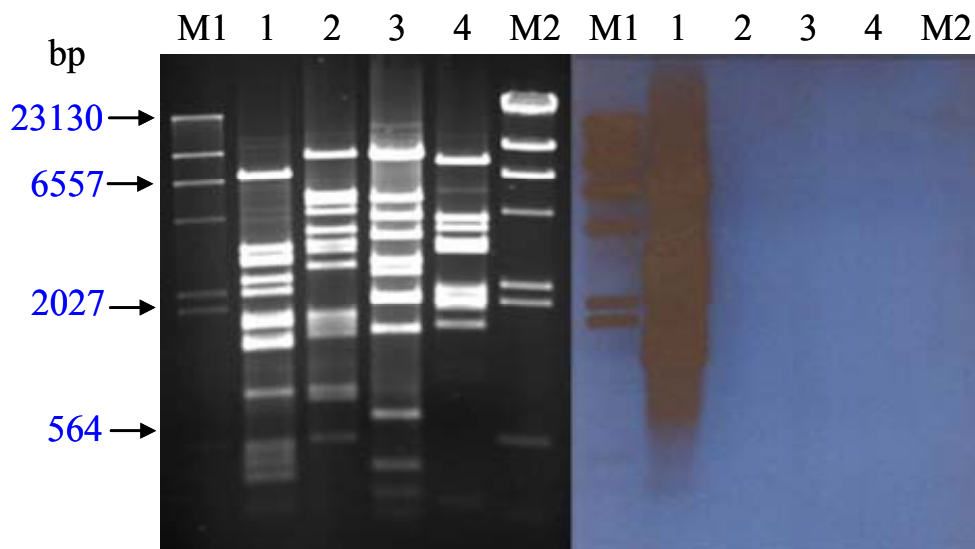


Fig. 8.10: Agarose gel (0.8%) electrophoresis (left) and Southern blot hybridization (right) of phage P738 DNA (lane 1) and of *S. thermophilus* *pac*-type phages TP-J34 (2) and TP-778L (3) and *cos*-type phage P53 (4). All DNAs were cut with HindIII. Lane M1: phage λ -HindIII (DIG labelled). Lane M2: phage λ -HindIII (unlabelled). The agarose gel was blotted and hybridized with a phage P738 DNA probe digested with HindIII.

8.2.8 Partial DNA Sequencing of phage P738

The *S. thermophilus* phage P738 did not reveal DNA homology to other *S. thermophilus* and *L. lactis* phages, as judged by the Southern blot analysis. To obtain more knowledge on the origin of phage P738, part of the DNA was sequenced. An EcoRV fragment of phage P738 was cloned into plasmid vector pJET1.2/blunt using the “CloneJET™ PCR Cloning Kit” (Fermentas, St. Leon-Rot, Germany) and transformed into *E. coli* “Easypores” cells as described previously in Materials & Methods (Fig. 8.11a). One transformant contained a recombinant plasmid (pJET.P738EV6) in which a 2.8-kb EcoRV fragment had been cloned from the P738 phage DNA. From this insert, the DNA sequence was determined at Eurofins MWG Operon, Ebersberg, Germany (Fig. 8.11b). The sequence information obtained was used for a primer walking strategy to obtain the nucleotide sequence of a 3.4-kb genomic region. Intact P738 phage DNA was used as the PCR template.

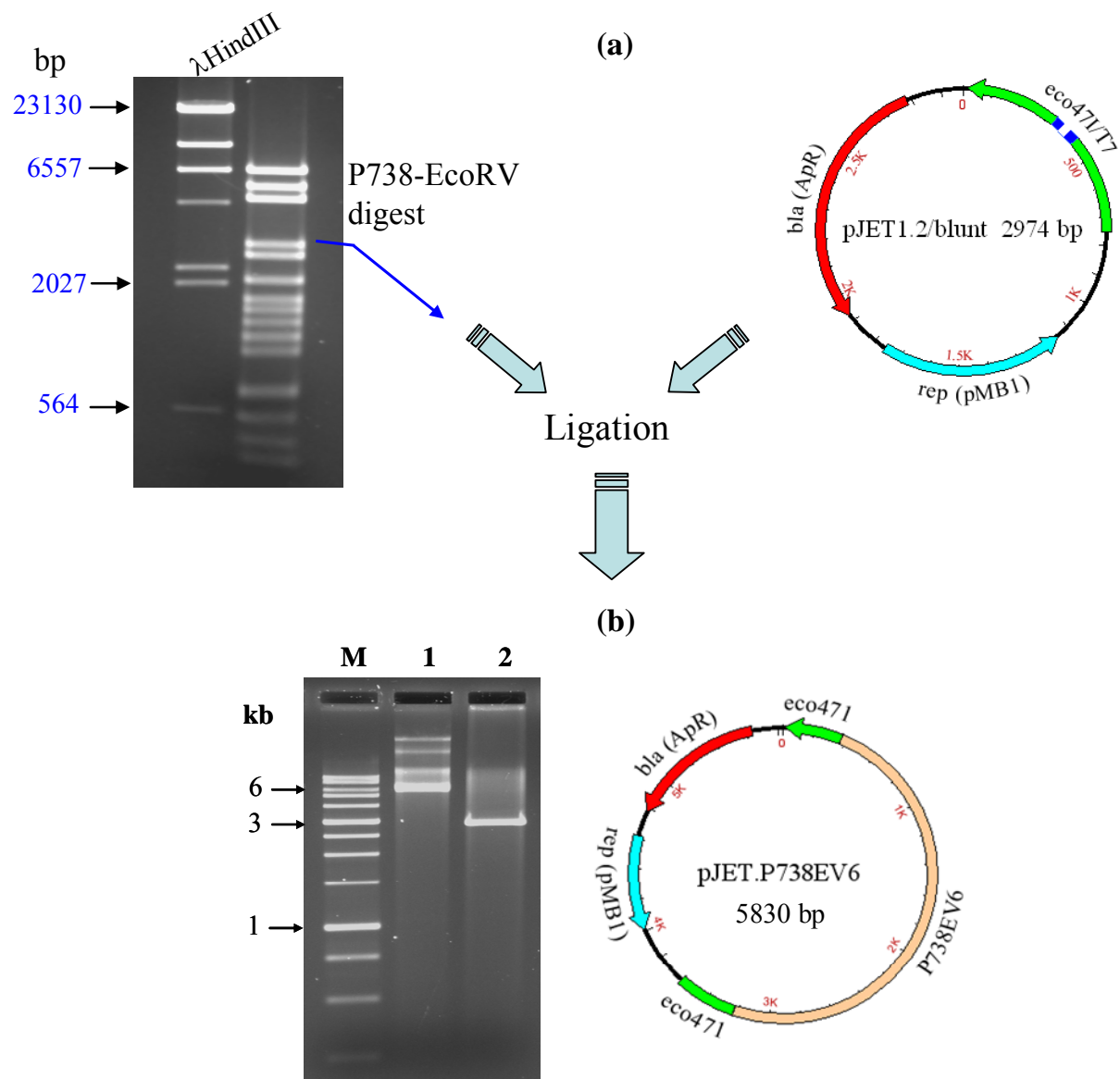


Fig. 8.11: Cloning of a 2.8-kb EcoRV fragment of phage P738 into pJET1.2 cloning vector. (a) Ligation of pJET1.2 (see physical map at the top) and 2.8 EcoRV fragment of phage P738 (indicated on the upper agarose gel). (b) Illustration of the resulting recombinant plasmid pJETP738EV6 (see physical map at the bottom) which is also shown in the agarose gel at the bottom of the diagram as intact DNA (lane 1) and upon digestion with BglII (lane 2). Lane M: 1-kb DNA marker (AppliChem, Darmstadt, Germany).

8.2.9 Partial DNA sequence analysis of phage P738 DNA

DNA sequence analysis and alignment of the amino acid sequences of the putative gene products of the P738 DNA region were performed as described previously using DS gene software and NCBI GenBank. The partial P738 DNA sequence was analyzed for open reading frames (*orfs*) longer than 60 codons using ATG as a start codon. The *orfs* (Fig. 8.12) were translated into protein sequences, named according to the number of amino acids (aa) in the predicted protein and analyzed for similar proteins using NCBI-BLAST (Table 8.1). Sequence analysis revealed the presence of 7 *orfs* potentially coding for proteins (Fig. 8.12).

Analysis of **Orf425**, the putative gene product (gp) of orf425 showed 73% and 71% similarity with the **large subunit of terminases** of *S. pyogenes* M49 591 and MGAS5005 prophage DNA and *S. pyogenes* phages 10270.2 and 10750.2. Also, it showed 70% identity to phage large terminase subunit of *Staphylococcus* phages 88 and 92 and 69% to the *Staphylococcus* phage ROSA.

Orf430 showed 59% similarity with **portal proteins** of *S. pyogenes* MGAS5005 and *S. pyogenes* phages 10270.2 and 10750.2, furthermore 59% identity to a hypothetical phage protein of *S. pyogenes* phage M49 591, *S. pyogenes* phage SSI-1 and *S. pyogenes* phage 315.5. Notably, it also showed 42% and 40% identity to minor structural proteins and to Orf32 (portal protein) of lactococcal phages Tuc2009 (McGrath *et al.*, 2002) and TP901-1 (Brondsted *et al.*, 2001), respectively. Low similarity (24%) was even detected to the portal protein of *pac*-type *S. thermophilus* phages Sfi11, O1205 and 2972 (Lucchini *et al.*, 1998; Stanley *et al.*, 1997; Lévesque *et al.*, 2005, respectively).

Orf271 demonstrated 27% identity to a phage protein of *S. pyogenes* strains MGAS5005, M49 591 and SSI-1 and *S. pyogenes* phages 315.5, 10270.2 and 10750.2. It exhibited also 17% identity to a putative **head morphogenesis protein** of *Staphylococcus* phages phiMR25 and phiNM. It also shared 37 aa and 33 aa with Orf32 and Orf33 of the lactococcal phages Tuc2009 and TP901-1, respectively. A low degree of 10% similarity with *S. thermophilus* phages Sfi11, O1205 and 2972 was also detected.

Orf195 shared 68 from a total of 199 aa (34%) with the phage **scaffold protein** of *S. pyogenes* bacteriophages 10270.2 and 10750.2 and *S. pyogenes* MGAS5005. Furthermore, 29% identity was detected to a phage scaffold protein of *Streptococcus pneumoniae* strains SP19-BS75 and SP23-BS75. It showed also 20% identity to the scaffold protein of *Staphylococcus* phages 53 and 80alpha.

Orf302 showed significant similarity (64% identity) to an anonymous phage protein of *S. pyogenes* phages 315.5, 10750.2 and 10720.2. It showed also 35% homology to the putative **major head protein** of *S. pyogenes* SSI-1.

Orf108 showed 42% identity to a phage protein with unknown function of *S. pyogenes* phages 10270.2 and 10750.2. It showed also 36% and 33% similarity with Orf38 (putative **DNA packaging**) of the lactococcal phage TP901-1 and Orf41 (with unknown function) of the lactococcal phage Tuc2009, respectively.

Orf103 shared 32 aa from a total of 103 aa (31%) of a hypothetical phage protein from *S. pyogenes* MGAS5005, SSI-1 and M49 591 (coding for glucose/sorbose dehydrogenases), and *S. pyogenes* phage 315.5. It showed also 30% identity (31/103 aa) to Orf103 of *S. pyogenes* phage 10270.2.

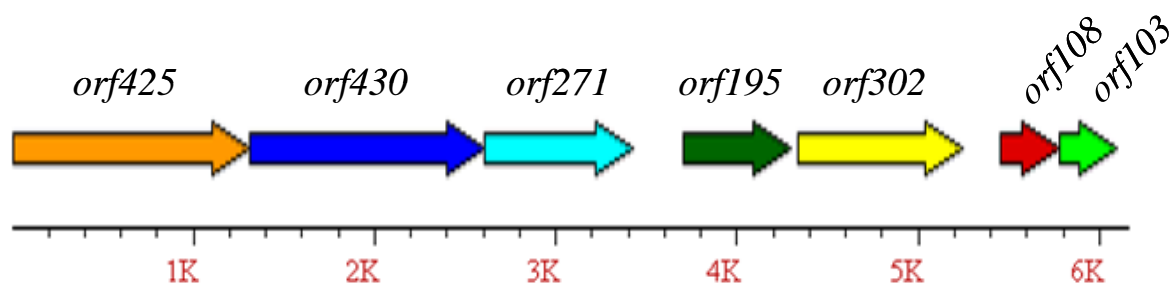


Fig. 8.12: Genetic map of a 6.2-kb genomic region of *S. thermophilus* phage P738 putatively coding for morphogenesis of head proteins and for DNA packaging. The *orfs* were translated into amino acid sequences and named according to the number of amino acids (aa) in the predicted proteins.

Table 8.1: Putative *orfs* of the partially sequenced P738 genome and comparison with other protein sequences from GenBank (red: *S. pyogenes* proteins, blue: LAB phage proteins).

<i>orf</i>	Size of gp (aa)	Putative function	Similarity to gp from other phages		Identity (%) / (E-value)
<i>orf425</i>	425	Terminase (large subunit)	<i>S. pyogenes</i> M49 591	(gp 424 aa)	73% (1e-180)
			<i>S. pyogenes</i> MGAS5005	(gp 424 aa)	73% (1e-180)
			<i>S. pyogenes</i> ϕ 10270.2	(gp 432 aa)	71% (3e-180)
			<i>S. pyogenes</i> ϕ 10750.2	(gp 432 aa)	71% (3e-180)
			<i>Staphylococcus</i> ϕ 88 & 92	(gp 421 aa)	70% (2e-171)
			<i>Staphylococcus</i> ϕ ROSA	(gp 421 aa)	69% (2e-169)
			<i>Bacillus</i> ϕ SPP1	(gp 422 aa)	54% (1e-127)
			Prophage LambdaCh01	(gp 420 aa)	42% (2e-85)
<i>Cl. botulinum</i> A-ATCC 3502	(gp 419 aa)	39% (1e-73)			
<i>orf430</i>	430	Portal protein	<i>S. pyogenes</i> phMGAS5005	(gp 441 aa)	59% (8e-150)
			<i>S. pyogenes</i> M49 591	(gp 441 aa)	59% (9e-149)
			<i>S. pyogenes</i> ϕ 10270.2	(gp 441 aa)	59% (6e-148)
			<i>S. pyogenes</i> ϕ 10750.2	(gp 441 aa)	59% (6e-148)
			<i>S. pyogenes</i> ϕ 315.5	(gp 429 aa)	59% (4e-147)
			<i>S. pyogenes</i> SSI-1	(gp 429 aa)	59% (5e-146)
			<i>L. lactis</i> ϕ Tuc2009	(gp 453 aa)	42% (1e-100)
			<i>L. lactis</i> ϕ TP901-1	(<i>orf32</i> / 452 aa)	40% (6e-100)
			<i>Lb. johnsonii</i> ϕ Lj928	(gp 470 aa)	33% (1e-67)
			<i>S. thermophilus</i> ϕ Sfi11	(gp 502 aa)	24% (1e-39)
			<i>S. thermophilus</i> ϕ O1205	(<i>orf27</i> / 501 aa)	24% (1e-37)
<i>S. thermophilus</i> ϕ 2972	(gp 501 aa)	24% (2e-37)			
<i>orf271</i>	271	Head structural protein	<i>S. pyogenes</i> MGAS5005	(gp 302 aa)	27% (2e-26)
			<i>S. pyogenes</i> ϕ 315.5	(gp 302 aa)	27% (3e-26)
			<i>S. pyogenes</i> SSI-1	(gp 302 aa)	27% (3e-26)
			<i>S. pyogenes</i> M49 591	(gp 302 aa)	27% (3e-26)
			<i>S. pyogenes</i> ϕ 10270.2	(gp 302 aa)	27% (5e-26)
			<i>S. pyogenes</i> phage 10750.2	(gp 302 aa)	27% (5e-26)
			<i>Staphylococcus</i> phiMR25	(gp 331 aa)	17% (1e-13)
			<i>Staphylococcus</i> phiNM	(gp 331 aa)	17% (2e-13)
			<i>L. lactis</i> ϕ Tuc2009	(gp 346 aa)	10% (5e-04)
			<i>L. lactis</i> ϕ TP901-1	(<i>orf33</i> / 564 aa)	6% (9e-04)

			<i>S. thermophilus</i> ϕ Sfi11	(gp 284 aa)	12% (0.0056)
			<i>S. thermophilus</i> ϕ O1205	(orf28 / 297 aa)	10% (0.028)
			<i>S. thermophilus</i> ϕ 2972	(gp 297 aa)	10% (0.028)
<i>orf195</i>	195	Scaffold protein	<i>S. pyogenes</i> ϕ 10270.2	(gp 199 aa)	34% (4e-28)
			<i>S. pyogenes</i> ϕ 10750.2	(gp 199 aa)	34% (4e-28)
			<i>S. pyogenes</i> MGAS5005	(gp 189 aa)	35% (2e-27)
			<i>S. pyogenes</i> ϕ 315.5	(gp 190 aa)	35% (2e-26)
			<i>Staphylococcus</i> ϕ 53 & 80alpha	(gp 206 aa)	20% (3e-15)
			<i>Staphylococcus</i> phiMR25	(gp 206 aa)	20% (7e-15)
			<i>S. pneumoniae</i> SP19-BS75	(gp 194 aa)	29% (2e-10)
			<i>S. pneumoniae</i> SP23-BS75	(gp 194 aa)	29% (2e-10)
<i>orf302</i>	302	Head protein	<i>S. pyogenes</i> MGAS5005	(gp 296 aa)	64% (1e-99)
			<i>S. pyogenes</i> M49 591	(gp 296 aa)	64% (1e-99)
			<i>S. pyogenes</i> ϕ 315.5	(gp 296 aa)	64% (1e-99)
			<i>S. pyogenes</i> ϕ 10270.2	(gp 296 aa)	64% (1e-99)
			<i>S. pyogenes</i> ϕ 10750.2	(gp 296 aa)	64% (2e-99)
			<i>S. pyogenes</i> SSI-1	(gp 295 aa)	35% (1e-37)
<i>orf108</i>	108	Unknown protein	<i>S. pyogenes</i> ϕ 10270.2	(gp 114 aa)	42% (8e-16)
			<i>S. pyogenes</i> ϕ 10750.2	(gp 114 aa)	42% (8e-16)
			<i>S. pyogenes</i> MGAS5005	(gp 114 aa)	42% (1e-15)
			<i>S. pyogenes</i> M49 591	(gp 114 aa)	42% (1e-15)
			<i>S. pyogenes</i> ϕ 315.5	(gp 114 aa)	42% (1e-15)
			<i>L. lactis</i> ϕ TP901-1	(orf38/110 aa)	35% (2e-9)
			<i>L. lactis</i> ϕ Tuc2009	(orf41/110 aa)	32% (9e-7)
<i>orf103</i>	103	Unknown protein	<i>S. pyogenes</i> MGAS5005	(gp 103 aa)	31% (2e-7)
			<i>S. pyogenes</i> ϕ 315.5	(gp 103 aa)	31% (3e-7)
			<i>S. pyogenes</i> M49 591	(gp 103 aa)	31% (3e-7)
			<i>S. pyogenes</i> SSI-1	(gp 103 aa)	31% (3e-7)
			<i>S. pyogenes</i> ϕ 10270.2	(gp 103 aa)	30% (2e-6)
			<i>S. pyogenes</i> ϕ 10750.2	(gp 103 aa)	30% (2e-6)

According to the DNA sequence analysis data obtained so far, *S. thermophilus* phage P738 exhibits the same gene arrangement in its DNA packaging and head morphogenesis module as other *pac*-type phages of *L. lactis* and *S. thermophilus*. (see Fig. 8.13 below). This result is also another confirmation for the presence of a *pac*-site in the genome of this new phage as concluded already before from the restriction enzyme analysis (8.2.6).

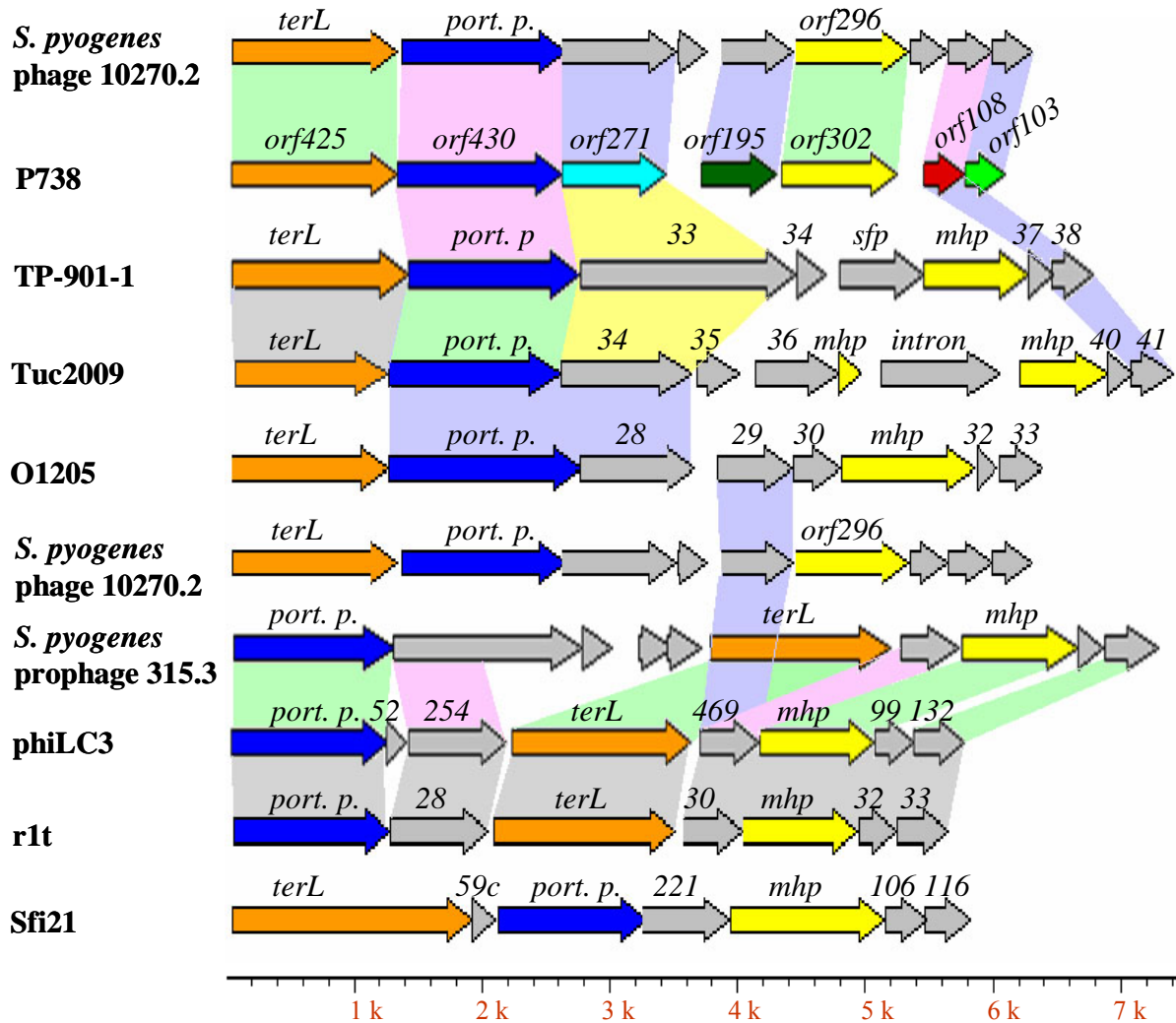


Fig. 8.13: Organization and alignment of the DNA packaging and head assembly genes of the new *S. thermophilus* phage P738 with the corresponding DNA regions of *S. pyogenes* phages 10270.2 and 315.3, of lactococcal phages TP901-1, Tuc2009, phiLC3 and r1t, and of *S. thermophilus* phages O1205 and Sfi21. Similarities between putative proteins are linked by shading colors: grey: 80-100% similarity; green: 60-80%; pink: 40-60%; blue: 20-40%; yellow: <20% similarity. *terL* = terminase large subunit; *Port. p.*= portal protein; *mhp* = major head protein.

8.2.10 Structural proteins of *S. thermophilus* phage P738

8.2.10.1 SDS-PAGE

For analysis of the phage P738 structural protein profile, phage particles purified and concentrated by CsCl gradient centrifugation were subjected to SDS-PAGE (Fig. 8.14). Phage P738 revealed a unique structural protein pattern with one major protein band (ca. 33 kDa) and 5 distinct minor protein bands with sizes of 50 kDa, 60 kDa, 72 kDa, 91 kDa and 150 kDa, respectively. Although phage P738 is also a *pac*-type *S. thermophilus* phage (see restriction genome analysis and sequence analysis), its structural proteins are clearly different from the proteins of other *S. thermophilus pac*-type phages revealing 3 major structural protein bands in SDS-PAGE (see Fig. 8.15). This is also a clear evidence for establishing a new *S. thermophilus* P738 phage species.

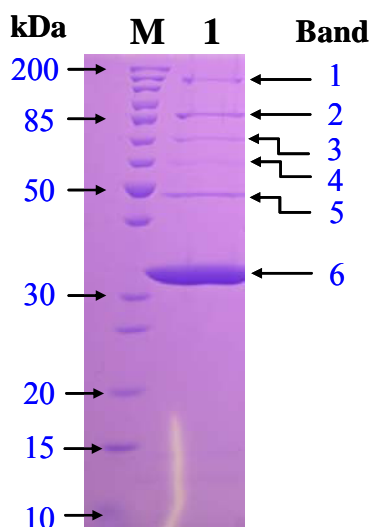


Fig. 8.14: SDS-PAGE (12%) of the phage P738 structural proteins. Lane M: PageRuler™ unstained protein marker (Fermentas, St. Leon-Rot, Germany).

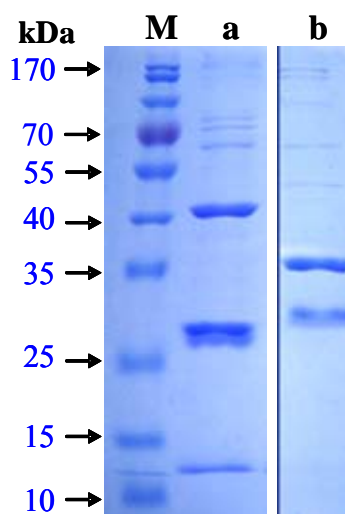


Fig. 8.15: SDS-PAGE (12%) of structural proteins of *pac*-type *S. thermophilus* phage TP-778 (lane a) and of *cos*-type *S. thermophilus* phage P53 (b). Lane M: PageRuler™ prestained protein marker (Fermentas, St. Leon-Rot, Germany).

8.2.10.2 MALDI-TOF mass spectrometry

The P738 structural protein bands were furthermore analysed by MALDI-TOF MS in collaboration with Dr. C. Gelhaus, Zoological Institute of the University of Kiel. Phage samples were subjected to 2 additional treatments prior to SDS-PAGE (see Materials & Methods: 6.14). The 6 SDS-PAGE protein bands of phage P738 were excised and analyzed using the MALDI-TOF MS method according to Gelhaus *et al.* (2005). The resulting spectra (see Fig. 8.17 & Fig. 8.18) were processed with the Data Software version 3.5.0.0. Proteins were identified by searching the nonredundant NCBI database using the program MS-Fit (<http://prospector.ucsf.edu/ucshtml/msfit.htm>). Only 2 protein bands were identified: band 5 was attributed to *orf430*, and band 6 to *orf302* gp of the new *S. thermophilus* phage P738.

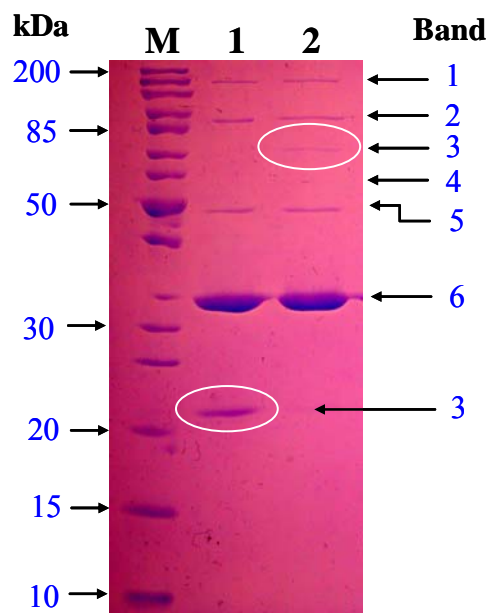


Fig. 8.16: SDS-PAGE (12%) of the phage P738 structural proteins. Lane 1: P738 sample treated with 150 mM iodo acetamide for MALDI-TOF MS analysis. Lane 2: P738 sample without treatment. Lane M: PageRuler™ unstained protein marker (Fermentas, St. Leon-Rot, Germany). The white circles indicate the band 3, which showed a lower molecular weight (21 kDa: lane 1) after treatment with 150 mM iodo acetamide.

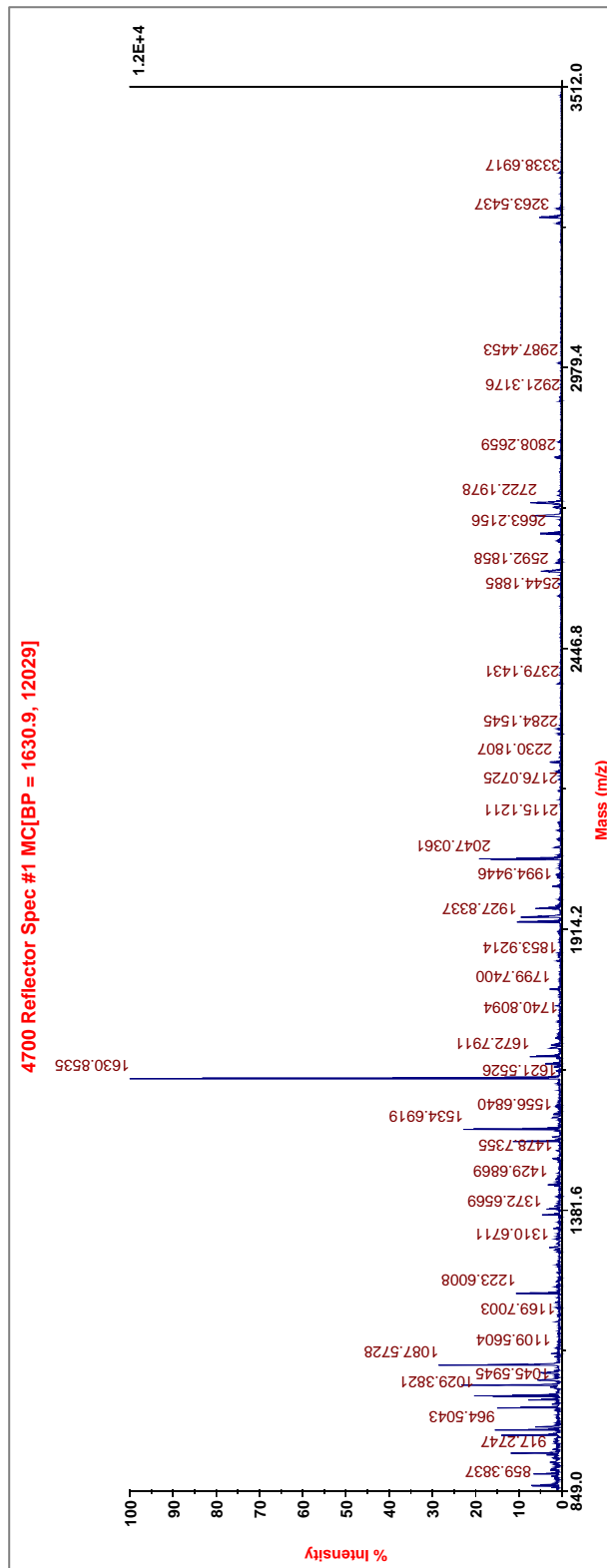


Fig. 8.17: Spectra obtained from MALDI-TOF MS of protein band 5 (50 kDa) of the phage P738 excised from an SDS polyacrylamide gel after electrophoresis.

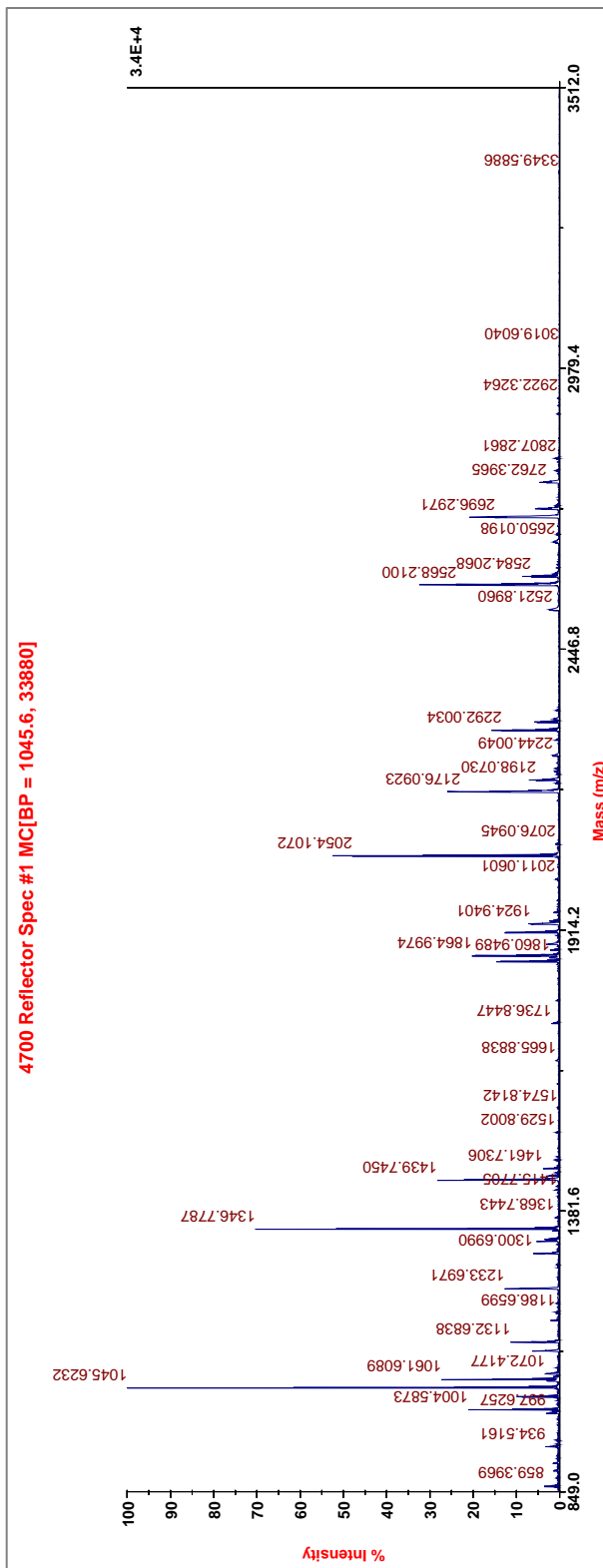
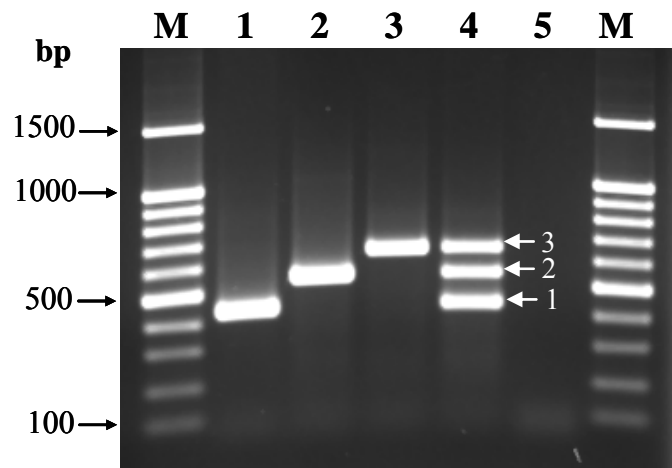


Fig. 8.18: Spectra obtained from MALDI-TOF MS of protein band 6 (33 kDa) of the phage P738 excised from an SDS polyacrylamide gel after electrophoresis.

8.2.11 Design of an improved and updated multiplex-PCR protocol for identification of *S. thermophilus* phages

Based on the nucleotide sequence of the putative major head structural gene *orf302* of phage P738, 2 primers were designed (YNS-F & YNS-R). These primers were expected to amplify a 650-bp fragment from the P738 *orf302* gene. The 2 primers were mixed with the 4 previously designed *pac*- and *cos*-primers YP-F, YP-R, YC-F & YC-R (Chapter 3) for rapid detection of all 3 types of *S. thermophilus* phages in a multiplex PCR (Fig. 8.19). Multiplex PCR was performed according to the PCR conditions that were previously mentioned in Materials & Methods (with one exception: the elongation time at 72°C was increased from 35 sec to 40 sec). DNAs isolated from the *pac*-type *S. thermophilus* phage TP-778L (see 5.2.6), from *cos*-type reference P53 (Neve *et al.*, 1989) and from the new *S. thermophilus* phage P738 were used as PCR templates. PCR products of 432 bp, 514 bp and 650 bp were obtained from reference phages TP-778L and P53 and *S. thermophilus* phage P738, respectively. Therefore, the multiplex system is suitable for detection of all 3 types of *S. thermophilus* phages which can be rapidly detected and differentiated in one single PCR reaction.

Fig. 8.19: Agarose gel (1.5%) electrophoresis of PCR products obtained in multiplex PCR reactions from *pac*-type reference phage TP-778L (lane 1), from *cos*-type reference phage P53 (2), from phage P738 (3) and from a mixture of all 3 *S. thermophilus* phages (4) with primer pairs YP-F/YP-R, YC-F/YC-R, YNS-F/YNS-R. Lane 5: negative control without phage DNA, blank; Lane M: 100-bp DNA marker (AppliChem, Darmstadt, Germany).



8.3 Conclusion

The newly detected *S. thermophilus* phage P738 (genome size: 35 kb) differed from all other known *S. thermophilus* phages, which possess the same basic morphology with isometric heads and long non-contractile tails (B1 *Siphoviridae*). P738 has a shorter tail and tail fiber than other *S. thermophilus* phages (about 50% shorter). Also, lytic propagation of phage P738 occurred at 30°C, unlike all other *S. thermophilus* phages, which are propagated at 40°C.

Using phage P738 DNA in DNA-DNA hybridization, similarity was neither detected to the known *S. thermophilus* phages nor to lactococcal phages, although P738 is morphologically similar to the lactococcal phage r1t. Also, the protein profile of phage P738 showed only one main protein band while the known *S. thermophilus* phages have either 3 (*cos*-type phages) or 2 main protein bands (*pac*-type phages).

Moreover, protein alignment of the sequenced region of the phage P738 terminase and head gene cluster showed the highest similarity (28% to 73%) to *S. pyogenes* MGAS10270 (Accession: CP000260) prophages 10270.2 and 10570.2.

Therefore, phage P738 is a member of a new *S. thermophilus* phage group.

9 Summary & Conclusions

Lactic acid bacteria play divergent and important roles for human beings: On the one hand they include major bacterial pathogens like *Streptococcus pyogenes* and *S. pneumoniae* and on the other hand many species are used as starter cultures for the manufacture of fermented food (i.e., dairy products, meat, vegetables, wine, sour dough, cocoa) and silage. *S. thermophilus* is one of the most important lactic acid bacteria required for the manufacture of yogurt, mozzarella and other cheese varieties (e.g. Cheddar and Swiss-type cheeses). Bacteriophage attacks are always a serious problem in industrial fermentations leading to significant financial losses. Characterization of bacteriophage populations is therefore the first step of efficient phage control in dairies and other food fermentation industries.

The aim of the work described in **Chapter 1** was to identify and characterize a new temperate *S. thermophilus* phage (i.e., phage TP-778) that was induced from its lysogenic host strain *S. thermophilus* SK778. Phage TP-778 revealed the typical morphology of all known *S. thermophilus* phages with isometric heads and long non-contractile tails. It was classified as a *pac*-type phage with a genome size of ca. 44 kb. No prophage-cured strain could be obtained, indicating that correct excision of prophage DNA from the host chromosomal DNA was prevented. Phage TP-778 could be propagated lytically on the non-lysogenic *S. thermophilus* strain B106, and a lytically propagated derivative (i.e., TP-778L) was isolated and also characterised as described in this chapter. When phage TP-778 DNA was used as a probe for Southern blot analysis of the chromosomal DNAs of a set of non-lysogenic *S. thermophilus* strains, an unexpected high background of unspecific hybridization signals appeared in the DNAs of all strains. This indicated that the phage TP-778 genome could (1) either carry DNA homologous to the chromosomal DNA of *S. thermophilus* starter strains or (2) that the phage would be capable to package (and consequently transduce) chromosomal DNA during phage proliferation.

In order to address these questions, a detailed DNA sequence analysis was performed for the lysogeny module of the phage genome and its flanking regions from the phage lysin gene to the phage *cro* regulatory protein gene. This genomic region was selected since it should reveal all genetic determinants for prophage integration/excision (i.e. integrase gene), for establishing the lysogenic/lytic phage life cycle (i.e., regulatory genes of the genetic switch) and furthermore the junction sites between prophage and bacterial DNA (i.e., *attL* and *attR*). The 2 neighbouring prophage regions from the left prophage/chromosome junction

site (*attL*-site) to *cro* gene (fragment *attL-cro*) and from the lysin gene to the expected *attR*-site (fragment lysin-*attR*) located on the opposite flanking region of the prophage DNA were amplified by PCR and sequenced. Sequence analysis of the lysin-*attR* prophage DNA revealed the presence of about 1.9-kb prophage remnant DNA. A truncated 398-bp integrase gene was present on the phage remnant. A functional *attR*-site was also deleted from this prophage fragment, indicating that integration or excision of the prophage could not occur by the site-specific recombination via homologous *attP* and *attB* sites. Hence, this fragment was renamed as lysin- Δ *attR* fragment. While only a non-functional (truncated) integrase was identified on the lysin- Δ *attR* prophage fragment, a complete 1080-bp integrase gene was identified on the *attL-cro* DNA fragment. This core region of the TP-778 lysogeny module was highly homologous to the corresponding region of the well-characterized *S. thermophilus* temperate phage TP-J34 (Neve *et al.*, 2003) and did also code for a unique lipoprotein (Orf142_{TP-778}). Hence it was concluded that prophage TP-778 is directly associated with a prophage remnant in the lysogenic host strain.

In order to confirm that TP-778 can package extra DNA, a 16S rDNA region was amplified from purified phage particles treated with DNase I to destroy any non-packaged chromosomal DNA contaminations. The positive PCR reaction was confirmed by Southern blot analysis using the 16S rDNA probe. It was concluded that phage TP-778 and its lytic derivative (TP-778L) were both capable of packaging chromosomal host DNA.

The core region of the lysogeny module of lytically propagated phage derivative TP-778L was also analysed by DNA sequencing. Phage TP-778L contained a 398-bp truncated recombinant integrase gene remnant. By alignment with the intact 1080-bp integrase gene of phage TP-778 and with the 398-bp integrase gene fragment present on the prophage remnant, it was shown that the TP-778L integrase fragment exhibited a recombinant structure originating from a homologous recombination event between the two integrase determinants present in the lysogenic SK778 cells.

In **Chapter 2** comparisons of genes *ltp*_{TP-778} (*orf142*_{TP-778}) of phage TP-778 and *ltp*_{TP-J34} (*orf142*_{TP-778}) of phage TP-J34 and their corresponding gene products are presented. The lipoprotein gene *ltp*_{TP-778} of phage TP-778 is closely related to the corresponding gene *ltp*_{TP-J34} of phage TP-J34. Previously, *ltp*_{TP-J34} has been shown to code for a unique superinfection exclusion system. This phage resistance mechanism was also active in a lactococcal host background against the isometric-headed phage P008 but not against the prolate-headed phage P001 (Sun *et al.*, 2006). The amino acid sequences of both deduced lipoproteins

differed only by 10 amino acids. The *ltp*_{TP-778} gene was cloned into the expression vector pMG36e. The resulting recombinant plasmid pYAL1 was also transformed into the *Lactococcus lactis* strain Bu2-60 in order to assess its effect of *ltp*_{TP-778} on the phage resistance phenotype of the cells. Gene expression of Ltp_{TP-778} in strain Bu2-60 was confirmed by SDS-PAGE analysis, but the protein did not react with a polyclonal antiserum raised against Ltp_{TP-J34} coded by phage TP-J34. When 3 Bu2-60(pYAL1) transformants were challenged with phages P008 and P001, 2 of them were partially resistant to phage P001 but not to phage P008. The 3rd transformant was still sensitive to both phages. The deviation of phage resistance phenotypes observed in Bu2-60 transformants harbouring the *ltp*_{TP-778} or the *ltp*_{TP-J34} gene was suggested to be due to the variability of one or more of the 10 amino acids that differ in the lipoproteins coded by *ltp*_{TP-778} and *ltp*_{TP-J34}.

Due to the high economical losses that may result from a phage infection of starter cultures, a rapid and sensitive method is required for phage detection and identification in the dairy. In **Chapter 3**, a rapid and reliable multiplex-PCR method is described allowing the simultaneous detection of *S. thermophilus* phages and their differentiation into the 2 well-known *pac*- and *cos*-type subgroups. Since *pac*- and *cos*-type phages are composed of different structural proteins, 2 sets of primers were designed from the internal highly conserved region of the major head protein gene of 8 completely sequenced *S. thermophilus* *pac*-type phages (TP-J34, O1205, Sfi11, 2972) and *cos*-type phages (Sfi21, 2701, Sfi19, DT1). The 2 primer sets could be used simultaneously in a multiplex PCR assay to detect and distinguish *S. thermophilus* phages, because the PCR-products differed in fragment size (432-bp product for *pac*-type phages versus 514-bp product of *cos*-type phages). The reliability of the multiplex PCR protocol was validated and confirmed by the following 3 controls: (I) restriction enzyme analysis of the DNA of *cos*- and *pac*-type DNA phages with and without a heating step at 74°C required for the melting of fragments harbouring *cos*-sites, (II) SDS-PAGE analysis of the structural proteins of *pac*-type phages (3 major bands) and *cos*-type phages (2 major bands), and furthermore by (III) immuno electron microscopy of the phages treated with a polyclonal antiserum raised against the *pac*-type phage TP-778. The multiplex PCR yielded results in a short time of approximately 2.5 h. The method is highly sensitive with a limit of detection as low as ca. 10³ phages per ml of acidic whey. The protocol was also used in a colony PCR approach to detect and identify 3 new lysogenic *S. thermophilus* strains harbouring inducible *pac*-type prophages within a set of 60 *S. thermophilus* strains isolated from traditional Egyptian yoghurt (Zabady) samples.

When a number of newly isolated *S. thermophilus* phages was screened by the multiplex-PCR developed in Chapter 3, phage P738 failed to deliver a PCR product. In order to confirm that this phage represents a new phage species for *S. thermophilus* phages, a detailed characterization was performed for this phage as described in **Chapter 4**. Phage P738 was originally propagated on the well-described *S. thermophilus* strain S4 but could also infect a set of 15 other *S. thermophilus* strains illustrating its broad host range. Notably, it was not possible to propagate phage P738 lytically in liquid cultures at the standard incubation temperature of 40°C used for these thermophilic cultures. Efficient cell lysis and phage proliferation was only possible at a lower temperature of 30°C. Phage P738 had a unique morphotype with an isometric head (57 nm Ø), a remarkably short, non-contractile tail (124 nm length x 10 nm Ø) and a distinct tail fiber (54 nm length). By SDS-PAGE analysis it was documented that phage P738 revealed a distinct structural protein profile with one major protein band (33 kDa) and 5 minor protein bands (sizes: 50 kDa, 60 kDa, 72 kDa, 91 kDa, 150 kDa). By pulsed-field gel electrophoresis and restriction enzyme analysis, phage P738 was identified as a *pac*-type phage with a genome size of ca. 35 kb. The P738 DNA did neither hybridize with the DNA from other *S. thermophilus* reference phages (phages TP-J34, TP-778L, P53) nor with the DNA of different *L. lactis* reference phages (phages BK5-T, P335, r1t, TP901-1, P001, P008, sk1, P446).

A 2.8-kb EcoRV fragment of the P738 genome was cloned into the plasmid vector pJET1.2/blunt. Using a primer walking strategy, the DNA sequence was determined from a 6.2 kb genomic region. Seven open reading frames (*orfs*) were identified which were transcribed in the same direction. By *in silico* DNA sequence analysis it was shown that the genes putatively code for the large subunit of terminase (TerL) and furthermore for the structural proteins required for head morphogenesis. These putative proteins revealed high similarity with proteins from *S. pyogenes* prophages. Only very little similarity was found to the corresponding gene products of *S. thermophilus* phages. By MALDI-TOF mass spectrometry of the phage structural proteins, 2 protein bands were identified that could be correlated with *orf430* (50-kDa product; portal protein) and with *orf302* (33-kDa product; major head protein). A P738 specific set of PCR primers was selected for targeting the structural gene of the major head protein of this new *S. thermophilus* phage species and was included in the multiplex PCR tool developed in Chapter 3.

10 Zusammenfassung

Milchsäurebakterien nehmen wichtige aber gegensätzliche Funktionen für den Menschen wahr. Einerseits findet man unter ihnen bedeutsame bakterielle Krankheitserreger (z.B. *Streptococcus pyogenes* und *S. pneumoniae*), andererseits werden sie als Starterkulturen für die Herstellung fermentierter Lebensmittel (Milchprodukte, Fleisch, Gemüse, Sauerteig, Wein und Kakao) und Silage verwendet. *S. thermophilus* ist eines der wichtigsten Milchsäurebakterien und wird für die Herstellung von Joghurt, Mozzarella und verschiedenen Käsesorten (z.B. Cheddar und Schweizer Hartkäse) verwendet. Infektion der Kulturen durch Bakteriophagen stellt stets ein großes Problem bei der industriellen Fermentation dar, aus der erhebliche finanzielle Verluste resultieren. Die Charakterisierung der Phagenpopulationen ist deshalb ein wichtiger erster Schritt zur effizienten Phagenkontrolle in Molkereien und in anderen Industriezweigen, in denen Lebensmittelfermentationen stattfinden.

Ziel der im **Kapitel 1** beschriebenen Arbeit war die Identifizierung und Charakterisierung eines neuen temperenten *S. thermophilus* Phagen (Phage TP-778), der aus dem lysogenen Wirtstamm *S. thermophilus* SK778 durch Induktion freigesetzt wurde. Der Phage TP-778 zeigte die typische Morphologie aller *S. thermophilus* Phagen mit isodiametrischen Köpfen und langen, nicht-kontraktilen Schwänzen. Dieser Phage wurde als *pac*-Typ Phage mit einer Genomgröße von ca. 44 kb klassifiziert. Es ließen sich keine prophagenkurierte Stämme isolieren – ein Hinweis darauf, dass keine normale Excision der Prophagen-DNA aus der chromosomalen DNA erfolgt. Der Phage TP-778 konnte auf dem nicht-lysogenen Wirtstamm *S. thermophilus* B106 lytisch vermehrt werden. Ein auf diesem Wirt angezüchtetes Einzelplaque-Isolat (TP-778L) wurde später genauer charakterisiert. Die TP-778 DNA wurde in einer „Southern-Blot“ Analyse mit den chromosomalen DNAs aus nicht-lysogenen *S. thermophilus* Stämmen hybridisiert, dabei traten viele unspezifische Hybridisierungssignale bei allen Stämmen auf. Dies war ein Hinweis, dass das TP-778 Phagen genom entweder (1) DNA-Regionen enthält, die Ähnlichkeiten zur chromosomalen Wirts-DNA zeigen, oder dass (2) der Phage in der Lage ist, während der Phagenvermehrung in den Wirtszellen deren chromosomale DNA mit zu verpacken (und auch zu transduzieren).

Zur Klärung wurde eine DNA-Sequenzanalyse des Lysogeniemoduls und der flankierenden Phagen genom-Bereiche (vom Phagenlysin-Gen bis zum regulatorischen *cro* Phagen gen) durchgeführt. Dieser Genomabschnitt wurde ausgewählt, da dort alle genetischen Determinanten für die Integration / Excision der Prophagen-DNA (z.B. das Integrase gen), zur

Festlegung des lysogenen / lytischen Phagenvermehrungszyklus (z.B. regulatorische Gene des genetischen Schalters) und weiterhin die Kontaktstellen zwischen Prophagen-DNA und bakterieller DNA (*attL* und *attR*) lokalisiert sind. Die beiden im Prophagen gegenüberliegenden DNA Regionen („*attL-cro*“ DNA-Fragment von der linken Prophagen/Chromosom-Kontaktstelle (*attL*-Site“) bis zum *cro* Gen; „*lysin-attR*“ DNA-Fragment vom Lysin-Gen bis zur mutmaßlichen *attR*-site auf der gegenüberliegenden Kontaktstelle) wurden mittels PCR amplifiziert und sequenziert. Durch die Sequenzanalyse der *lysin-attR* Prophagen DNA wurde ein 1,9-kb Prophagen-Überrest („Remnant“) mit einem auf 398-bp verkürzten Integrasegen identifiziert. Dieser Region fehlte eine funktionelle *attR*-„Site“, sie wurde daher in *lysin-ΔattR* Fragment umbenannt. Das bedeutet, dass der TP-778 Prophage keine normale Integration / Excision durch ortsspezifische Rekombination zwischen den homologen *attP*- und *attB*-„Sites“ ausführen kann. Während ein nicht-funktionelles, deletiertes Integrasegen auf dem *lysin-ΔattR* Prophagen Fragment gefunden wurde, wies das *attL-cro* Fragment ein komplettes Integrasegen (1080 bp) auf. Außerdem zeigt diese Kernregion des TP-778 Lysogenie-Moduls sehr hohe Homologie zu dem temperenten Phagen TP-J34 (Neve *et al.*, 2003) und kodiert – wie auch die TP-J34 DNA – für ein Lipoprotein (*Orf142_{TP778}*). Es wurde geschlussfolgert, dass der TP-778 Prophage direkt mit dem 1,9-kb Prophagen-„Remnant“ im lysogenen Wirtstamm verknüpft ist.

Zur Bestätigung, dass der Phage TP-778 zusätzliche DNA verpacken kann, wurden Phagen mit DNase I behandelt, um Kontamination mit chromosomaler Wirts-DNA auszuschließen. Von der extrahierten Phagen-DNA konnte dann ein Fragment der 16S rDNA amplifiziert werden. Die positive PCR-Reaktion wurde in einer „Southern Blot“ Analyse mit einer 16S rDNA Sonde bestätigt. Somit ist der temperente Phage TP-778 (und auch das lytische Derivat TP-778L) befähigt, chromosomale DNA der Wirtszellen zu verpacken.

Die Kernregion des Lysogeniemoduls des lytisch vermehrten Phagenderivats TP-778L wurde ebenfalls durch DNA-Sequenzierung analysiert. Dort wies der Phage TP-778L ein auf 398 bp deletiertes Integrase-Gen auf. Dessen Sequenzvergleich mit dem intakten Integrasegen des Phagen TP-778 und dem Integrase-Genfragment des 1,9-kb Prophagen-„Remnants“ dokumentierte eine rekombinante „Hybrid“-Struktur für das TP-778L Integrase-Genfragment gezeigt - resultierend aus einer homologen Rekombination der beiden Integrase-Determinanten in lysogenen SK778 Zellen.

Kapitel 2 beschreibt den Vergleich der Gene *ltp_{TP-778}* (*orf142_{TP-778}*) des Phagen TP-778 und *ltp_{TP-J34}* (*orf142_{TP-J34}*) des Phagen TP-J34 und deren abgeleitete Genprodukte. Das

Lipoproteinen *ltp_{TP-778}* des Phagen TP-778 ist eng verwandt mit dem entsprechenden *ltp_{TP-J34}* Gen des Phagen TP-J34. Kürzlich wurde gezeigt, dass *orf142_{TP-J34}* (*ltp*) für ein spezifisches „Superinfection Exclusion System“ kodiert. Dieser Phagenresistenzmechanismus war ebenfalls in Laktokokken-Wirtszellen gegen den isodiametrischen Phagen P008 wirksam, allerdings nicht gegen den prolaten Phagen P001 (Sun *et al.*, 2006). Die Aminosäuresequenz der beiden abgeleiteten Lipoproteine unterschied sich nur in 10 Aminosäuren. Das *ltp_{TP-778}* Gen wurde in den Expressionsvektor pMG36e kloniert. Der rekombinante Vektor pYAL1 wurde ebenfalls in *Lactococcus lactis* Bu2-60 transformiert, um den *ltp_{TP-778}* Effekt auf den Phagenresistenz-Phänotyp der Zellen zu bestimmen. Die *ltp_{TP-778}* Genexpression in Bu2-60-Zellen wurde durch SDS-PAGE Analyse bestätigt, jedoch reagierte das Protein nicht mit einem polyklonalen Antiserum gegen das *Ltp_{TP-J34}* Lipoprotein des Phagen TP-J34. Von 3 Bu2-60(pYAL1) Transformanten waren 2 Stämme weitestgehend resistent gegen den Phagen P001, aber nicht gegen den Phagen P008. Die dritte Transformante war sensitiv für beide Phagen. Diese abweichenden Phagenresistenz-Phänotypen der Bu2-60 Transformanten resultiert vermutlich aus den wenigen variablen Aminosäuren, in denen sich die *Ltp_{TP-778}* und *Ltp_{TP-J34}* Lipoproteine unterscheiden.

Bedingt durch die hohen finanziellen Schäden, die durch eine Bakteriophageninfektion der Starterkulturen entstehen können, besteht Bedarf für schnelle und sensitive Methoden zur Phagendetektion und -identifikation in Molkereien. In **Kapitel 3** wird eine schnelle und zuverlässige Multiplex-PCR Methode beschrieben, die die gleichzeitige Detektion von *S. thermophilus* Phagen und deren Differenzierung in die 2 bekannten *pac*- und *cos*-Typ Untergruppen erlaubt. Da *pac*- und *cos*-Typ Phagen unterschiedliche Strukturproteine aufweisen, wurden 2 Primerpaare aus der jeweils hochkonservierten Region der Hauptkopfprotein-Gene von 8 vollständig sequenzierten *S. thermophilus* *pac*-Typ Phagen (TP-J34, O1205, Sfi11, 2972) und *cos*-Typ Phagen (Sfi21, 2701, Sfi19, DT1) ausgewählt. Die 2 Primerpaare konnten simultan in einem Multiplex-PCR Ansatz verwendet werden, da sich die PCR-Produkte in ihrer Fragmentgröße unterschieden (432-bp Produkt für *pac*-Typ Phagen bzw. 514-bp Produkt für *cos*-Typ Phagen). Die Zuverlässigkeit des Multiplex-PCR Protokolls wurde durch die folgenden drei Kontrollen validiert und bestätigt: (I) Restriktionsenzym-Analyse der DNA aus *cos*- und *pac*-Typ Phagen mit und ohne Erhitzungsschritt bei 74 °C, der nötig ist zum „Schmelzen“ der DNA-Fragmente, die *cos*-Enden aufweisen, (II) SDS-PAGE Analyse der Strukturproteine von *pac*-Typ (3 Hauptbanden) und *cos*-Typ Phagen (2 Hauptbanden), und zusätzlich durch (III)

Immunoelektronenmikroskopie der Phagen, die mit einem polyklonalen Antiserum gegen den *pac*-Typ Phagen TP-778 behandelt wurden. Die Multiplex-PCR lieferte bereits nach ca. 2,5 h Resultate. Die Methode ist sehr sensitiv mit einer Nachweisgrenze von ca. 10^3 Phagen pro ml Sauermolke. Das Protokoll wurde auch in einem Kolonie-PCR Ansatz an 60 *S. thermophilus* Stämmen, die aus traditionell fermentierten, ägyptischen Joghurtproben (Zabady) isoliert worden waren, verwendet. Dabei wurden 3 lysogene *S. thermophilus* Stämme mit induzierbaren *pac*-Typ Phagen detektiert und identifiziert.

Bei dem Screening von neu isolierten *S. thermophilus* Phagen mit der Multiplex-PCR Methode, die in Kapitel 3 entwickelt wurde, wurde bei dem Phagen P738 kein PCR Produkt nachgewiesen. Um zu überprüfen, ob dieser Phage eine neue Phagenspecies der *S. thermophilus* Phagen repräsentiert, wurde, wie in **Kapitel 4** beschrieben, eine detaillierte Charakterisierung dieses Phagen durchgeführt. Der Phage P738 wurde zunächst auf dem gut beschriebenen *S. thermophilus* Stamm S4 angezüchtet, jedoch konnte der Phage auch 15 weitere *S. thermophilus* Stämme infizieren, womit ein breites Wirtsspektrum dokumentiert wurde. Es war außergewöhnlich, dass die lytische Anzucht des Phagen in Flüssigkultur bei der Standard-Inkubationstemperatur von 40°C nicht möglich war. Eine effiziente Zell-Lyse und Phagenvermehrung war nur bei einer niedrigeren Temperatur von 30°C möglich. Der Phage P738 wies eine einzigartige Morphologie auf, mit einem isodiametrischen Kopf (\varnothing 57 nm), einem bemerkenswert kurzen, nicht-kontraktilen Schwanz (Länge 124 nm, \varnothing 10 nm) und einer ausgeprägten Schwanzfaser (Länge 54 nm). Durch SDS-PAGE Analyse wurde für den Phagen P738 ein eigenständiges Muster an Strukturproteinen mit einer Hauptproteinbande (33 kDa) und 5 Minorproteinbanden (50 kDa, 60 kDa, 72 kDa, 91 kDa, 150 kDa) gezeigt. Durch Pulsfeld-Gelelektrophorese und Restriktionsenzym-Analyse wurde der Phage P738 als *pac*-Typ Phage identifiziert mit einer Genomgröße von ca. 35 kb. Die DNA des Phagen P738 hybridisierte weder mit der DNA anderer *S. thermophilus* Referenzphagen (TP-J34, TP-778L, P53), noch mit der DNA unterschiedlicher *Lactococcus lactis* Referenzphagen (BK5-T, P335, r1t, TP901-1, P001, P008, sk1, P446).

Ein 2,8-kb EcoRV Fragment des P738 Phagengenoms wurde in den Plasmidvektor PJET1.2/blunt kloniert. Damit konnte mittels „Primer-Walking“ die DNA-Sequenz eines 6,2-kb langen Genomabschnitts bestimmt werden. Sieben offene Leseraster (*orfs*) wurden erfasst, die alle in der gleichen Richtung transkribiert wurden. Durch *in silico* DNA-Analyse und Sequenzvergleich wurde gezeigt, dass die Gene mutmaßlich für die große Untereinheit der Terminase (TerL) und außerdem für die Strukturproteine der Kopf-Morphogenese

kodieren. Diese putativen Proteine wiesen eine hohe Ähnlichkeit zu Proteinen von *S. pyogenes* Prophagen auf. Zu entsprechenden Genprodukten der *S. thermophilus* Phagen wurde nur sehr geringe Ähnlichkeiten gefunden. Durch MALDI TOF Massenspektrometrie der Phagen-Strukturproteine wurden 2 Proteinbanden identifiziert, die *orf430* (50-kDa Produkt; „portal protein“) und *orf302* (33-kDa Produkt; „major head protein“) zugeordnet werden konnten. Ein P738-spezifisches PCR-Primerpaar wurde zur Amplifikation eines *orf302*-Genomabschnitts (Hauptkopffprotein-Gen) dieser neuen *S. thermophilus* Phagen-species ausgewählt. Somit wurde das in Kapitel 3 beschriebene Multiplex-PCR System zu einem universellen „Tool“ zur Erfassung aller soweit bekannten *S. thermophilus* Phagen vervollständigt.

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12 Appendix

12.1 Complete DNA sequence of the 5.2-kb lysin- $\Delta attR$ region of *S. thermophilus* prophage TP-778

orf223 →

CTTGGGCAACAAC TTGCTAAAAATGGCTTCTATCGTGTAAGTATCAATCAAGACTGGGAAGC
 TTTGACAGGAGATATCGTAATGATGTCGTGGGGTGCTGACATGTCACAACTCTGGTGGAGCAG
 GTGGACACGTTGGTGTTCATGATGGATGCTACATACTTTATTAGTTGCGACTACTCAACTCAA
 GGAGCAGTAGGTCAAGCTATCAATACATATCCGTGGAATGACTACTATGCAGCGAACCAAGCC
 TACATATATAGAGGTTTGGCGCTATGCTGACTCTGCACCACAGACTAATAACAAAGCTAAATA
 CAGCAGTAGTAACACAAGAGAAAGCATACTATGAAGCCAATGAAGTCAAAATACGTTAATGGA
 ATTTGGCAGATTAAATGTGATTACTTAAACACCAATTGGCTTCGATTGGCTTCAGAACGGGAT
 CCCAGTTTCAATGGTTAACTGGGTTGACGCTAACGGTAACGACATTCAGATGGAGCTGACC
 AAGATTTCAAAGCTGGTATGTTCTTTAGTTTCTCTGGTGATGAAACTAACATCGTAGACACC
 GGAAACGGTGGATACTATGGTGGATA TTATTGGCGTCAATTTGAA TTTGGACAATTCGGACC
 AGTATGGCTCTCTTGTGGAA TAAAGATGATTTGGTAAACTACTACAAA TAG ACCACGAAAA
 CAATAAAGGAAAAGGAGTATATCACCTCCCCTCACACTGCAATAGGGATATCATGGCAGCAG
 TGGTCGAAGCCTCAGCATT TTTGCTGGGGCTTTTTTTGTGTTATAATAGAA TAGAA TAAAAA
 ACAAATAGATGAGGTAAGAAAAACGGCTATTGACCTGTATCGTGAAC TGGATATCCAATCGT
 TGGAGCAAAGATTGGAAAAGAACGAAGAAAA TACTCAACGCTTTCTCCAACAAACAGCCAAA
 AGTTTAAACCAAGACAAGACTGAAC TATCTCTCCGCACTGACCAGTTAGGGCGTAGTGTGTA

← *orf73*

AAAGATTGAAAACAAACTAGACGAC ATG TACGCTAAGAACGAACTAGATTTGAAATTCAGA
 TGATGGATCAAAAGATTGACGCTAAA TTTGATACCTTTGGTCAACGCATGGAAAACATGTTT
 TTAGCACAAACCAATAGGCAACTTTGAGGAACAAGCCAAGAA TAGAAAAGAA TTCACATATTG
 GTTTATTTGCATTCCTTGTAGCTATCGCTGTTATTGCTATTCCTGTTTGGTTCGGCAA TAAAT
 ATCACCTTGTAACCTATTTTAGTGTTAAAA TATACATGAAACGACAAACCCCTGCCTGACG
 CAGGGCTTTTTTATTTGTTATTGCTGTAAGAA TTAATTTTGATATAAAAA TCAGAA TTTGA
 TAAAA TGTAATTGGATAACCTAGATACA TTTTATCTAGTGCAAAAGATAAAA GACTGATTAG
 GTTCAGCGCCCTTGAAAAAGGGAACATTTTGTGGGGTTAAGACCCCTTTTGT TTTTTTTT
 TGTGTTATAATATATATGAAACGACAAACCCCTGCATCCACATGGACAGATACGCTCTGAC
 GCAGGGTTTTTTATTTGCTTTATTTTGATATAAATGCTACTATATTAATGGATACAGTTAAA
 TGTTATAGTATTCGATAAACTCTCTCTCACCC TGACTTGAA TTAGTCAGGGTTTTCTTTTCA
 ATTGTAGGCTCATTAGCATTATGTTTCTTTGGATTATTTCAAAA TAGAAAAT TGAGGATTA
 GAGAAATGATTGGATTTGTTATTTGGATTTTATCTATATTA TTTGTTGATATTAATAGAA GGA
 CTTATATTTTAGGAAAAGAGGGCAATCGCTCTCTTTTTTTATTTTACAAAAAAATCTAAAT
 TTCTTTATCAAAAGTGTGACATATGTCAACGTGTGCGATATAATGTATACATAAGATAAAG

orf98 →

AGAGAGGGTAAAAAGAAATGCAAAAAATGGTAAACAAAATTTTAGGTTATCGATACACTGATGAGA
TTAAAAACGATTTCTGCAACAGAGAATAAAATGTCTAATCTTTATAACAAAATGGACAAAGAT
AGTTTACGAGAGATCCATAGTGCATTATACGGTCTATTAACAGCTGGATATGATATCAGCAA
CATGCGAAAATGTCGAAGAACTTGAAAAATACGTGAACGTTAAAAAAATCTCATGGAAAAATTAT
TAGATGTTACTAATAGTGACATTCAGTTATATCATAAAATTAATTTGTTGTTTCGATTTGGGAGG
TAAATATGCTGAAAAAGTTTGGGAAGTAGCTTAATTAAAAAAGGAGAAAGAAAGGAAGTAAAT

orf140a →

GATATGCGCATACGAAAAAGCAGATACAACCTCATATAGAAAACGTAGTTTCAACATAAGTGA
CACAAAGCGTAGGGAAATATGCAAAAAGAAAATGGAGAAAATTAGAACAAGCATTGAAAAGCTAG
ATGGTTGGTATCTATCTAGCATGAAGGATAGTGCATACAAGGATTTTGGAAAATACGAAATC
CGCTTATCAAATCATTTCAGCAGACAAATAAATATCATGACCTAGAAAATGGTCGTTTAATTTGT
TAAATGTTAAAGCAAGTAAATGAACTTCGTTGATATCATCGAAAACAACTTGATAAAAATCA
TCGAGAAGATTGATAAGCTTGATTTAGATAAGTACAGATTTATTAACGCTACTAGAATGGAG

orf140b →

CATGACATTAATGCTACTATAAAGGATTTAAGACAAAGAAAGATGTAATCTAAATATGATAA
TAAACACTGCACGAGTTGAATTAGTTTTAATGAACAAAGCTATACCAGCAAATTTATTAGAA
AGAGAGATAGGAATTAGTTCGTTTCAGCAATTAACAAGAGTTTCGGAACGGTGAGAGAAAGCTTGA
GAACCTAACCCCTTGAACCTATTATGACTATTCAAAAGTGGATAGATGAAGGAAACTATCGCT
TTAGTTACGATTTATAGTGAGCTTATCGAAGACCTTGAGGAAGATATTGCAGAAGGCCAACA
GATGAGTATATCTATGTTGTCAGAGGTCCGTATAATGAACTTTTAGAGAAATGTCCAATCAT
TGACTATTACTACACTTCCGAAGAGATTGAAGAAGGAAAATCTCGCAGAGAAGACCTTGACAG
CTTCTGTCTTGGCTGAAATGAAACAGGACAACGAAATATTTTAAACCGTTTCATTTGTACATCT
ATTAGTATGGCAGTGTCCACAAATCTGTCCACCTTTTTTGA AAAATCTACGAAAATAAATAAA
AATAAAAACTATAAAAACTTAGTAAAATTAAGTCTTTACAGCTTCATTTATTCTCAAATTTT

↙

ATATATTTTCGTTTGGACATGCACATTA AAAACGCTCGACAGTATCTTGAGCGTGAGTTTTAG
CGTAACTATTCAGCACACGTTGGTACTCAGTGGCAGTAATAGATTTTCAGCTTCTTATCGCCG
AAAATAACTCTATCTTTCGCTGACTGTTGATATATGCCTTATAAGTTATTTTCGAAACAGT
TGGCTTCTTATAAACCTCACACCCTGTTTAAAATAATCGTATAGAGTAAATATCTTCATCGA
CATTAATGTTATCTTGAAGCTTCAGTTCCATCTCAGTAGCAGCCTTGATAGCTTCAGATTTT
GTCCATAAAGCCACCCTTTGACTTTGGTTTGCCTTTCAGTAGAATCGTAATAATTAATCCG

orf132 ←

ATATTTCCACCCGTTTGGGCGTTTGCGGTATGATGCGCATTTGATTAGTCCCTTTTACGATATA
ATAAAAGGGTGGTTTACCCTTAACACCCTCAA TTGATATAGTTTAGCCAGTCGAGAGACTGGT
TTT TTA TTTGGTTGAAAAATAAATTTTCCAATTTGATACCACAATTTGTCA TTGTATATCATGC
GTAACAATGGACGATATGGGTTTTTGGTGTGAATTACAATGAATTTATCTGTGACAGTTTCG
GTTGTATATTTCCGTATCAACTTTCCTTAGCGGACACTGCACCAACCACACCCGCAACAGG
GCCAAACAGAGTCATGCCAACCAACACCTCTTGTGAGAGCACCTTCTTCTTAACCTGAACCAG

TGGTAGTAGTTATCTGTCTAGTTTTCTCGTCTGTTTCGATGTTTACTATATCAGATAAAGGA
 ATTGGGATTTTTGGAGTACCAGGCAAACTAATATCTGTTTGTATCGTCGACAGAAATTCC
 AGCTCCTATTTTTCTGAGGTTACATGATTTAATGATCGCCTTATATTGTTCTCTTATAGGTT
 TTGGAATTTTAGAAAGTTGAACAGCTTGATAAACCAATACAATGGTGATGATAAATTAAGATT
 ← *orf168* |
 AATGCTAGTAG **CAT** AGAGATTACCTCTTTTCTAAAAATTTGAATTATTTTATCCAGCTTGTCA
 TTTATGGTTTTCTGGTTTTTGAATTTGTTGACGTAAGAGAGTAGCAATATCATTTGTTTCATG
 CTCTGGTTCGAAATCTAAATCAGAAGTTTCAAACCGTGGATCGATAGCAGATTTCT **TCA** CAC
 CGAAATAATCAGCAAGTTGTGGTAAACCAATTCGCCGAGCGGTTTACCTTGTTCATCTATCA
 GTTCTCATACTGTTTCAGGTTTAAATGCGAGGAGATTCCTTGGGATTTTTCGAATATCAAGCAAT
 ← *orf56* |
 GATTTTAATAAATTTCCCTAGCTTTTTCGTGAAATGATAAT **CAT** ACAGTACCTCGTTTTTTTAAA
 | *orf97* →
 ACTATTATATCAAAAAATAGAAAGGGAAACA **ATC** AACGAAATAGCACTATCAGGCAACCTTTC
 TCAAATTTGAATTTGGAATAAACACCACCAAGCAAAATGCTGGGCAATCAAATTTGGGAAATTG
 GAAGGCGGTTGAAACACGTC AAGGAAAACGACCTAACTCATGGACAGTTTTCTGATTTGGGTG
 GAACGCCAAGGAATACATATCCGAGAAGCTCAAAGAATGATGAAAAATGCTAACGAGCCTCC
 AAATACGACAAATGTTGTCGCATTTAGGAGCAACCGCTTTACACCTAATCGCAACACTTCCAG
 AGGAAGAAAAG **TAA** GAGCAAAATCGAAAAGATTGAACAAGGCGAATCACC AACGGTCAGAGAA
 TTGCAAGAGGTCAAGCGTCTCTAAAACTCAAAGACCAAGCACTGGAAGCAGTCAAGGGAGA
 GTTGGAGCGTGTCAAACAAACCAAGACCCTGAAAAGGTAAATCGAAAATTTGTGCACTTATATT
 GTATATTTCAAGACTAAAAAACCCAGTTAGATTTAGTTTCTAATTTGGG

12.2 Complete DNA sequence of the 2.7-kb region of the *attL-cro* region of *S. thermophilus* prophage TP-778

CTGTCCCTCGTGGCAGATTTTTTAATAAGTCCCTCTTAGTTAAATGGATATAACAACCTCCCTCC
 | *attL* | ← *int*
 TAAGGAGTCGTTGCTGGTTCGATTCC **GGCAGGGGACATTTTAAAGCCTTTA** ACCATGCGGTT
TTAA AGCGTTTCGTCCACATTCTGTCCACATTTGTTTTATCTTTTCGTCTGTTGCGTGATTTT
 TGCTCTTGCAATTTGATGAGCGTAAACTTCAAGTGTGATGTTTTCAGATTTTTCATGCCCTAAAAC
 TTGCGATACAGAAATCAAGTCAATATCGTGGGCAATTAATAAGCTAGCGTAAGTGTGCCCTTA
 ATGAGTGGACACGTACTTCACGACCAACGATTTTACGCAATGTCTTATTAACATGCATTTGTTG
 GATAGTGGGGTAGTATTCTACCATCATCGGTTGGTGGCAGTTGGTCAATAAAAATTTATAAA
 TTCATCATCAAGCGGTATCTCTCGGATACTGCTTTTTTGTGTTTGTGGGCATAAAATCCAGTAT
 TGTCTTTGTAGTCCCATGTTTTATTAACTGATAGCATGCCAGTATCTCGTTTGATATCATCC
 ACTGTTAAGCCCAGGCATCAGCAAAACGGATACCAGTTTTGGCGATGATATAGAGTGTCTGC
 ATAAGACGCATACCTCTGGATGCTTGTCTCGTAGATCAAATCGCTCGTATTCTTCGACCT
 CTAGGAAATTCGTTTCAAATATCACGCCCTTTGTTCTTTGCGTTTATTTTAGCAAACCTTGCAA

AAGTTACGCTTGATATATCCTTCATGCACAGCCATTTCAATGCACGCCTTGACATGCACATT
 AAAACGCTCGACAGTATCTTTGAGCATGAGTTTTAGCGTAACTATTCAGCACACGTTGGTACT
 CAGTGGCAGTAAATAGATTTTAGCTTCTTATCGCCGAAAAATAACTCTATCTTGCCTGACTG
 TTGATATATGCCTTATAAGTTATTTTTGAAACAGTTGGCTTCTTATAAACCTCGCACCACTG
 TTTAAAAATAATCGTATAGAGTAAATATCTTCATCGACATTGACGTTATCTTGTAGCTTAAGTT
 CCATCTCAGCAGCAGCCTTGATAGCTTCTGATTTAGTCCTGAAACCACCTTAGTTTTTCGGT
 TTGCGTTTACCAGTAGAATCGTAATAAATTTATACGATATTCCACCCATTTGAGCGTTTACG

← *int*

GATGATGC **CAT** TTATTTAAATCCCCAAAAATATAGATTTACACAAATAACCAAAATTC TAAT
 AAAAGAACCCTGCACTCATGATTGTCAAAGTTTTAGAGTGTGGGGCTTTTTG **TTA** TTTAAG
 ATTAGCAACAGCGTAGTCAGCTTCTTCTTGTAGTCAAATTTGTCAAACCTTACCAATTGATCAC
 GTATTGCGTCTGGGGACATGGCTAAAGTGTCTTGATATTGCTTCGCTTTTTCAAGAGCCTGT
 TTGTTATAGTCAATACCAGCGTTGTCTACAGCGTAGTCAGAAGCCTCTTGAGAGTACTTTTC
 AAAACTTACTAGTTGAGAACGCAACCCCTCCTTAGACATGGGAACAGTACTAGCGTACTGCT
 TAGCTTTGCTCACC GCCGTTCTGTACTCTTTAGGCACTTTGCTCTCACTTGATTGCTCGGTT
 TTAGCCTCTGACGTTTGTAGTCTGAGAAGACGTGCTTTTGGTTTGAGAACAAGCAGCAAG

← *ltp*

AGCAATAATAGAAAGGCTTAGTAAGCCAAAAGACAAATAATTTTTT **CAT** AAAATTTCTTACCA
 GCTTTTAAACGTGATTCAGTTTTTGCACGTAGTTTTAAACTTTATAAATATCTACGACCTGTT
 TAT **TCA** GGTTTCTATTAACCTTAAGTATTCATTTTTTACAAAAGTCTCATCACAAATAGTGG
 TGAGATTGTATTTTTTTCATGAAGTGTATATAGTTAAAATTGTCCAGATTTTCGTTTTTCAAC
 AGTTCATGGATCATATTTCTATTTGCTTGAGCCTCATATTTCTCTCGCAGACGCTCATAGTG
 TTTAGGGTCGTGTTCTAAGTGCCCTAATTCGTGCAGTAGGACCTTTAAACGTTTTCGGTTG
 GTAAATCCCTATTGATGTAGACCACACGGTTAATAGGGTCTAAAAACCCATCTCGTGGCCAC

← *metalloproteinase*

TGGCTAGAGCTAAATTCACAAAGAGAAACGCCGAACGTTC AAGCAATTCACCTCTATT **CAT**
 AAAATTC **TCA** CTTTTCTTGTATTTCATGTAACCTGCAATAATACCACGGATAGCACGCTTAT
 CATCATCAGTCAATGGTTTTCCGTGGAACATCATGGCATTCTTCTATTTATTTTCATCAATGTCG
 GTTGAGATAATTTGCTGACCATCATTGTTTTCTGAGTTATCACC AAAGAGAATGTAGTCTGT
 AGTTGTTCCCAAAGCTTGAGCTAATTTTACAACTTTGTACCCGTTGGGATACTAGCTCCAC
 TCTCCCATTTTTGAAATAGTTGAATCGGATTTATACCCGAGCATTTTTGCTAATTC AAGCTGA

← *cI-repressor*

CTAATACCTTTACTTGTCTGCAAACCTTTCGATTTCTGTACCCTCTTTGCTTATTCAAATC **CAT**
 ATCTTTCTCCTTGTCTTATATCGACATTATATAGTAGACTTTACTAATTTTTCAAGTAGAT
 ATATAAAAAAAACAAAAAACCTTGAAAAAAAATCAA TAAAACTATTGACGTTGAA TTTAA TT
 CAAGTTATAATATGTTTTGTAAGTTAGTTAGAGAGGAGAAACAAAA TGACAGAAGTAGTTCCA
 AAAATTC AATCAAAGA ACTCCGAGCACGTCACAA TTGACGCAGGAGGAGTT .

12.3 Complete DNA sequence of the 1.4-kb region of the *S. thermophilus*

TP-778L-*attP* flanking region

orf140b
 AATTACAAGAGTTCGGAACGGTGAGAGAAAGCCTTGAGAACCCTAACCCCTTGAAACTATTATGAC
 CTATTTCAAAGGTGGATAGATGAAGGAAACTATCGCTTTAGTTACGATTATAGTGAGCCTTATC
 GAAGACCTTGAGGAAGATATTGCAGAAGGCCAACAGATGAGTATATCTATGTTGTCAGAGG
 TCCGTATAAATGAACCTTTAGAGAAATGTCCAATCATTGACTATTACTACACTTCCGAAGAGA
 TTGAAGAAGGAAATCTCGCAGAGAAGACCTTGACAGCTTCTGTCTTGGCTGAAATGAAACAG

orf140b
 GACAACGAAATATTTTAAACCGTTCAATTTGTACATCTATTAGTATGGCAGTGTCACAAATTCT
 GTCCACCTTTTTTTGAAAACTACGAAAAATAAATAAAAAATAAAAACTATAAAAACTTAGTAAA
 ATTAAGTCTTTACAGCTTCATTTATTCTCAAATTTTATATATTTTTCGTTTTGACATGCACAT
 TAAAACGCTCGACAGTATCTTGAGCGTGAGTTTTAGCGTAACTATTCAGCACACGTTGGTAC
 TCAGTGGCAGTAATAGATTTTCAGCTTCTTATCGCCGAAAAATAACTCTATCTTGCCTGACT
 GTTGATATATGCCTTATAAGTTATTTTTCGAAACAGTTGGCTTCTTATAAACTTCAACCACT

 46-bp core sequence
 GTTTAAAAATAATCGTATAGAGTAATATCTTCATCGACATTGACGTTATCTTGTAGCTTAAGT
 TCCATCTCAGCAGCAGCCTTGATAGCTTCTGATTTAGTCCTGAAACCACCCCTTAGTTTTTCGG
 TTTGCGTTTACCAGTAGAATCGTAATAATTTATACGATATTCCACCCATTTGAGCGTTTAC
 GGTATGATGCCATTTATTTAATCCCCAAAAATATAGATTTACACAATAACCAAAATTCCTAA

 TAAAAGAACCCCTGCACTCATGATTGTCAAAGTTTTAGAGTGTGGGGCTTTTTGTTTAA
 GATTAGCAACAGCGTAGTCAGCTTCTTCTTGAGTGAATTTGTCAAACCTTACCAATTGATCA
 CGTATTGCGTCTGGGGACATGGCTAAAGTGTCTTGAATTTGCTTCGTTTTTCAAGAGCCTG
 TTTGTTATAGTCAATACCAGCGTTGTCTACAGCGTAGTCAGAAGCCCTTTGAGAGTACTTTT
 CAAAACCTTACTAGTTGAGAACGCAACCCCTCCTTAGACATGGGAACAGTACTAGCGTACTGC
 TTAGCTTTGCTCACCGCCGTTCTGTACTCTTTAGGCACCTTTGCTCTCACTTGATTGCTCGGT
 TTTAGCCTCTGACGCTTGTCTAGTCTGAGAAGACGTGCTTTTGGTTTGAGAACAAGCAGCAA
 GAGCAAT

ltp
ltp

12.4 Complete DNA sequence of a 6.2-kb region of *S. thermophilus* virulent phage P738 coding for the large subunit of terminase and structural proteins of the head morphogenesis

orf425 →

AGATATCAATATTGAAAGCGTGGTGTTTAAAGATGACATTGATTAGACTAAGTCAGTTGCTG
 CCGACTAAATTCACAGCGTATGGAAAGCTTCAAAGTCTAAAGAGATTCTTAACGTTGTGTG
 TTCTGGCGGCCGTGGTTCCTGGTAAATCTTCCAATGTGGCCCATATTTATCACACAGCTTCTTA
 TGAGGTATCCAGTCAACGCCGTTGGTATTTCGTAAGATTGACAATACACTTGAACAATCTATT
 TATGAGCAAATGAAGTGGGCTATTGAAGTTCAGAACGTTACTCACCTTGTGTTGAATTCAATAA
 GAGCCCGTTGAGAATTACATACCTTGCCCGTGGTAACTACATGGTATTCCGTGGGGCGCAGT
 ATCCGGAGCGAATTAAGTCGCTGAAAGACAGCCGTTATCCCTTTTGTCTATTGCTTGGGTGGAA
 GAGCTTGCTGAAATTTAGAACAGAAGATGAAGTGACAACAATTACAAACTCATTGCTTCGTGG
 TGAGTTAGATGAAGGTTTGCAATACAAATTTTTCTTCACGTATAAACC GCCAAAAAGAAAAC
 AAAGCTGGGTCAATAAAAAATACGGAAACAAATTTGTAAGTGGTAAACATTTGTACACCAT
 TCAACGTACTTAGATAAACCCTTCATCGCGAAGGAGTTTATCAAAGAAGCCGAAGAGACTAA
 GAAGAAAAACGAAAACAAATATCGCTGGGAAACATGGGTGAAGCTATCGGTTCTGGTATTA
 CGCCATTCATAACTTGGCAATTCAGAGAAATACAGATGAAGAAGTCGCGAACTTCGATAAT
 ATCCGTAACGCGAACGACTTCGGTTACGCAACTGACCCGAACGCCCTTTGTTTCGTGTTCAATTA
 CGATAAGAAGAAGAATGGTATCTATATCTTTGATGAATTAACAAGCAGCAACTATCTAACA
 GAAAAATTAGCTGACTGGCTGAAAGAAAAAGGTTATGATAATGATATAATCTTCGCAGACAGC
 GCAGAACC TAAGTCAATTCGCTGAATTTGAAGAACGACTTCGGTATTAGTAAGATTAAGGTGT
 AAAAAAGGCCAGATTCAGTAGAGTTTGGCGAACGCTGGTTAGATGACTTGGACTTCATCT
 GTATCGACCCAAAACGTACACCAAAAAACAGCTTTTTGAATTTGAGAAATATCGACTATCAAACC
 GACCGAGATGGTAACCCAAAACCTCGCCTAAATGATAAAGATAACCACGCTATCGACGCTGT
 TCGCTATGCAATGAGTGAAGATATGAGAAGTAACAAAACGAAAGTTAAAACATTTAGAGGTA
 GTTTTTAAATGCGATTTTACAAAACAAATGGACGCTTCAACAGAGTTCAGTACAACATTAATCC
 AAAAAACGATTTGCAAAAACCAAAGAAATATATCCAAGATTACAAGAATTACAAAACCTCTAC
 CTTGGTAACCACGCAATCTTGTTCGCAAACAGCAAAAAGAACAATACAAACCAGATAACCGACT
 GGTTCGTTAACTTCGCTAAGTACATTTGTGGACACATTC AATGGCTACTTTATCGGTAACCC TG
 TAAGTATGTTCCATGAGAACAAAACAGTGAACGATTTATCTTGCCTATATTGACGGATACAAT
 GACCAATCGGATAACAACGCAGAACTTTCTAAAATCTGTTCCATTTACGGACACGGCTTTGA
 GTTGGTGTTC AATGATGAAAAATCTGAAATTTGGTATCACCTTACATGACACCGATTGACGGTT
 TTATTATTTACGATAAACACGATTCAGAACAAGCCGCTATTCGCTTGTCACTATGGTTTGAAT
 GATGACAACAAAGAAGTTGGATACCTTCTATACAAAAGATTCATATCTATCAAATTTGAAGCTTC
 TAACGGTAGTTATACAAATTTGTAAGAAGAAACGCCAAAACGTATTTGGAGATAATCCAATGATTG
 AATATCTTGAGAACGAAGAACGACAATCAAATTTTTGAAAACGTAAAAACGTTGATTAATGCT
 TTTAACAAGGCGCTGTCTGAAAAAGCGAACGACGTAGAATAATTACGCGGACGCTTATCTTAA
 AGTTCTCGGTGCTGAAAATGATGAATCAACATTAACAATCGTTACGCGATAGCCGATTTATCA

orf430 →

ACGTGTCTGGAGATGACGCTTTGACAGTTGAAATTTATGACCAAACCAAGCGCAGATGGTACA
 CAAGAAAACCTTCTAGAGCGTTTGCAAAAAC TAATCTTTGAAATTAGTATGGTTCGCAAAATAT
 TTCAGATGAGAACTTTGCGAAATCAAGTGGTGTCTGCTCTTAGATACAAAATTGCAATCAATGG
 ACAATCTAGCCAAAGGCAAAGAACGCAAGTTCCAATCTGGTATGACAAGACGTTATCGACTA
 ATCAGCAACTTCCCAACTTCTAAAAATTGGTGCAGATGAATGGGTAGGTATCAAGTATAAAAT
 CTCTCGCAACGTACCAGCCAAC TTGGCCGAAGAAGCTGATATTTGCTCAAAACACTTAGCGGAA
 TCGTATCAGAAGAAACACAGGTAGGAATTCATCAATTTGTTCAAAATGCACGTGAAGAAGTT
 CAACGAAAAAACGAGGAA TTAGAAGGTTCTGATTCATTTACAAGAACAGAAGAGGGA TAA TG

orf271

AATG GCAGACGTTTTTAAAGAA TACTGGT CAGATTTAGAAGAAGATGAGAAGGAACTCTTCG
 AGGAAATGAGTGAGATTTATTGAAGATAACTGGATTGCAGTAGCTTCTGTAACTCTTTCTTC
 TATCTTTACAACACCAAAGACAACACAATTAATAATAATACCTTTATGCAACGGGCTAGCAA
 TCAAGAAAGGCGATATGTTAAAAAGCTCATGAATAAATACGGCAAGTCAAGTTTGTATAAAA
 ACAGTGATATCAAAGGGAGTAAGATTAATGTATTTAAGATTGCCGTAGATATCACAGAAGAA
 GTTGGCGCAAAAGGTATGAGCGCAGCACTTGAAAAGCACCTTTAGTGAAATGGCCGTAAAGT
 TGATAATTTTATCAAGAA TAAATACAACATTAAGCAGACGGCAAGGTAAAAC TTCCGAA TA
 TTTACGATAACCAGCTAGCAAAC TCTCAAAGGTTTCAAATAAATTTGTTTGAAGAGCTTAGG
 AATGGTATTTATCAAAGGTGAAAGATACGAAGATATTTCTAAAGTATTGAAAGAAGGATATGA
 AAAACACTCTCTTTCTGATATTAAGAAGATTGTTTCGTACAGAAGGAACGGCAATCACAAACG
 CTGTCCGGCTTGAAATGTTTGAAGAAGAAGGATATACAGAGTACAAAATATAGCTCTGTTATT
 GATTCTAGAACAACAGAGATATGCAGGAACTTAGACGGAGAGGTTTTTAAAAATCTCACAAAGC
 AGAACGTGGAATCAATTTCCACCTATGCACGTGAA TTGCAGAAGCGGATTTGAAATTTGTCT
 TGGAGAAA TAA CATGTGGATAGTACAATATAAAGATGGAAGCGGATTTTATTATAGCGAAGC
 CAAAACAAGCCGTTTAGCGTTAATTTTCGACTTGGTAAATGATGGTTTTGATAGAA TTAATT
 CCGATACATATAAATTTAAGGAATATGTAACCGCAACTCTAATCAAGTTGTAGTGAATTAATT
 ACCACTTGACTTCCCAATGTCGTAAAAC TTGGGCGACTTCCGTCGGTGGACGTAAAAACAC

orf195

AAAAATATTCGGGTAACAGCGTAACTGTTGAAAGGAAATAAAATG GAAGAAGAAAACAAAAA
 CGTTGAAGCTTCTACTGAAGAAGTGGAATCAAAGCTAAGGAAGCCGAAGGAGCGAAAAAAG
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orf302 →

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 AGCAGGACAAGAAGTCCAGAAAGCT TAA CTAAAAATCCAA TAGCC TAGCCACTGGGCTAGG
 TTC TTTTATAGAAAGAGGTGTAATATGGCTTATAAAGTTATCGCTAGTTTTAAAGATAAGA
 AAGACGATAAAATATCTTTATAAAAATCGGCGACACATTCCCACGCAAAGGAAAACGGCCAACT

orf108 →

AAGGCGCTAAAGAAGAGTTGGCCAGCAAAGGATTC TTTGAAAGAGGTGGAATA ATG GCAATT
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orf103 →

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 AAGTCGGGAGTTGATAAAGCAGTTGCTGA.

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