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**Genomic Analysis of the Lysogeny
Module
of Temperate *Streptococcus
thermophilus*
Bacteriophage TP-J34**

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1 Chapter 1:

Introduction

1.1 Bacteriophages

Bacteriophages, or phage for short, are viruses that infect bacteria. They are obligate intracellular parasites that multiply inside bacterial cells. Bacteriophage are estimated to be the most widely distributed and diverse entities in the biosphere (**Labrie *et al.*, 2010; McAuliffe *et al.*, 2007**). Actually, phages cannot be ignored at any field of bacterial applications. They play a key role in regulating bacterial populations in all sorts of environment, hence any bacterial strain can be infected by or harbors one or more phage/s (**Wuensche, 1989**). Relationships between bacteria and phages can be grouped in two types: i) lytic relationships, where the phage destroys the bacterial cell and produces new progeny, and ii) lysogenic relationships, where a temperate phage, named *prophage*, is integrated into the host chromosome.

Prophage are found in many (**Ventura *et al.*, 2002b**) or about 50% of all bacterial genomes (**Lawrence *et al.*, 2001**). It also seems possible that lytic phage are generated from temperate ancestors via multiple deletion and rearrangement events in the lysogeny module (**Delcour *et al.*, 2000; Ventura *et al.*, 2004**), hence new phage types are readily produced by recombination processes as indicated by the *modular theory* of bacteriophage evolution (**Botstein, 1980; Delcour *et al.*, 2000; Neve *et al.*, 1998**). Prophage carry genes, that are considered as extra genetic material, and that affect the phenotype of their host cells and play important roles in the evolution of bacteria (**Dobrindt *et al.*, 2010; Ventura *et al.*, 2002b**). They encode genetic functions which may be

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either of selective advantage or represent a burden to the host (leading to the loss of the prophage) (**Desiere *et al.*, 2001; Ventura *et al.*, 2002b**) e.g., i) toxigenicity in many bacteria is due to their prophages' genes, for instance some strains of *Corynebacterium diphtheria* (**Freeman, 1951**), ii) growth phenotype: prophage causes homogenous growth of the lysogen *Streptococcus thermophilus* J34 in broth medium (**Neve *et al.*, 2003**), and iii) ecological fitness: prophage also increase the ecological fitness of the host in its environment e.g. by confirming immunity against superinfection by other phage (**Capra *et al.*, 2010; Desiere *et al.*, 2001; Ptashne, 2004; Sun 2006; Ventura *et al.*, 2002b & 2004**).

Virulent phages, with respect to their economical importance, have dual effects: i) they may be useful, when they destroy harmful bacteria, or ii) harmful, when they destroy useful ones. In many cases, virulent phages can be used positively in our life, because they do not harm human or animal cells or anything that is not bacteria. They have been proven to be valuable alternatives in mankind's fight against bacterial diseases, so they were successfully used to treat dysentery (not long after their discovery), also in the 1920s and 1930s they were used for treatment of *Staphylococcus* infection, and show great promise as alternatives to traditional antimicrobials in the control of foodborne pathogens under the safety and health concerns, especially with today's emphasis on the availability of fresh foods without chemical preservations (**Walker, 2006**). In this field, U.S. Food and Drug Administration (**FDA**) gave the notification –for the first time- to bacteriophage as a direct addition to food for human consumption on 18th August, 2006, to reduce the risk of foodborne listeriosis (**71 FR 47729; August 18, 2006**). Phage could be also related to some probiotic properties like inhibition of pathogens (**Capra *et al.*, 2010**). In some cases, prophages are involved in the production of enzymes and special substances (**Wuensche, 1989**), for example *int*_{C31} of phiC31 and *int*_{TP901-1} of lactococcal phage TP901-1 have been demonstrated as gene

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therapy vector systems in human and animal models as well as in cell culture (**Keravala et al., 2009; Stoll et al., 2002; Woodard et al., 2010**). Bacteriophages have been a growing concern for dairy industry and phage model systems have been used to address a number of essential questions in evolutionary biology and have played a fundamental role in the history of molecular biology (**Czyz et al., 2001; Desiere et al., 2001; Hadas et al., 1997; Ventura et al., 2009**).

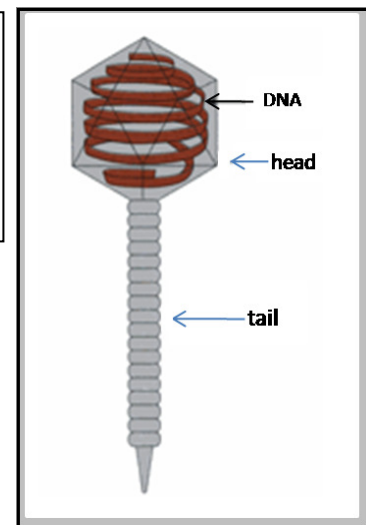
Historically, **Ernest Hankin**, a British bacteriologist, **1896**, reported that something in the water of Ganges and Jumna rivers in India revealed antimicrobial action against *Vibrio cholerae* (causes the cholera disease). This antimicrobial remained even when those waters were passed through a bacterial filter. Therefore these antimicrobial entities are smaller than any known bacteria. In **1898**, **Gamaleya**, a Russian bacteriologist, observed a similar phenomenon while working with *Bacillus subtilis*. These observations had been confirmed by several investigations on bacteriophages. **Frederick Twort**, a medical bacteriologist from England, **1915**, described virus-like particles in prokaryotic cells. The name of "bacteriophage" was proposed by **Felix d'Herelle**, a French-Canadian microbiologist at the Institute Pasteur in Paris (**Sulakvelidze et al., 2001; Wuensche, 1989**).

1.1.1 Composition and classification of bacteriophage

All bacteriophages contain one type of nucleic acid (either DNA or RNA, but not both) and proteins (**Figure 1.1**). The nucleic acid can be ssDNA, ssRNA, dsDNA or dsRNA in a linear or circular form. They often contain unusual or modified bases to protect the phage nucleic acid from the nucleases of the host cell during the infection. Phage genomes differ in size. Their number of genes ranges from 3-5 to over 100 genes (**Mayer, 2010; Walstra et al, 2006**).

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Figure 1.1. A λ particle. The λ chromosome -some 50,000 base pairs of DNA- is wrapped around a protein core in the head



The International Committee on Taxonomy of Viruses (ICTV) classified the phages according to morphology and nucleic acid content into orders. The order *Caudovirales* (dsDNA tailed phages) is the most significant one, representing about 95% of all the reported phages in the published literature. *Caudovirales* comprises the most important 3 families: i) *Myoviridae*, phages possessing long contractile tails, ii) *Podoviridae*, phages with short non-contractile tails, and iii) *Siphoviridae*, phages with long non-contractile tails. On the other hand, the tailed phages of LAB are classified according to the scheme of **Bradley, 1967** into 3 groups: i) morphotype A, with a contractile tail, ii) morphotype B, with non-contractile tails, and iii) morphotype C, with short non-contractile tails. Concerning their head shapes, they are grouped in 3 groups: i) isometric-headed, ii) short prolate-headed, and iii) long prolate-headed.

1.1.2 Life cycle of temperate phage

Briefly, temperate phage, such as λ phage can enter either a lytic or a lysogenic pathway as shown in **Figure 1.2**. The life cycle can be divided into three steps as follows:

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1.1.2.1 Adsorption

Adsorption is the first step of infection. The phage particle attaches onto the susceptible host cell by its tail fibers or by some similar structure on those phages that not have tail fibers (**Heller, 1992**). The attachment occurs between specific receptors on the bacterial cell and the host specificity protein of the phage. The bacterial receptors vary among different bacteria. For instance, in lactic acid bacteria (LAB), adsorption occurs via specific interaction between the receptor binding protein (RBP) in the distal part of the phage tail and the phage receptor on the host surface. Likewise, some proteins on the outer surface of bacterium, pili, lipopolysaccharide (LPS), and lipoprotein may be involved. These receptors on bacteria are for other purposes and phage evolved to use these receptors for infection. Firmness of attachment is demanded and is mediated by one or more components of the baseplates. While, phages that do not have baseplates or the distal part of the tail have other ways to become tightly bound to the bacterial cell. Adsorption also depends on environmental factors (**Duplessis *et al.*, 2006; Hadas *et al.*, 1997; Mayer, 2010; Ptashne, 2004; Shao & Wang, 2008**).

1.1.2.2 Penetration

Penetration of the phage genome is the next step. Irreversible attachment, results in the contraction of the sheath (phages which have a sheath) or pushing of a hollow tail fiber through the bacterial envelope (phages that lack contractile sheaths use other mechanisms e.g. some phages use enzymes to digest the bacterial envelope). Nucleic acid then passes from the head through the hollow tail and enters the bacterial cell (**Hadas *et al.*, 1997; Heller, 1992; Mayer, 2010**).

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1.1.2.3 Multiplication

Concerning the multiplication cycles, temperate phages can enter two pathways (**Figure 1.2**) as follows:

1.1.2.3.1 Lytic cycle

Simply, temperate phage, under lytic conditions (or lytic phages such as T4), take over the machinery of the cell to make phage components. They then destroy, or lyse, the cell by encoding lysis protein (lysin), which gets access to the peptidoglycan by a membrane-spanning hole-forming protein (holin). At the end of phage replication, phages are, with the host cell components, released leaving empty cell.

1.1.2.3.2 Lysogenic cycle

Temperate phages, after infection of a sensitive host, multiply in a dormant state in the lysogenic cells formed prophage. However, the prophage still has the potential to produce phage. If the cell follows the lysogenic pathway, the lytic genes of the prophage are repressed by a master regulatory protein termed, in phage λ , CI repressor. The prophage can provide additional reservoirs of genes. *Polylysogeny* is possible for a cell when it carries more than one prophage (**Birge, 2000; Little, 2007; Refardt & Rainey, 2010; McShan & Fretti, 2007; Ptashne, 2004**).

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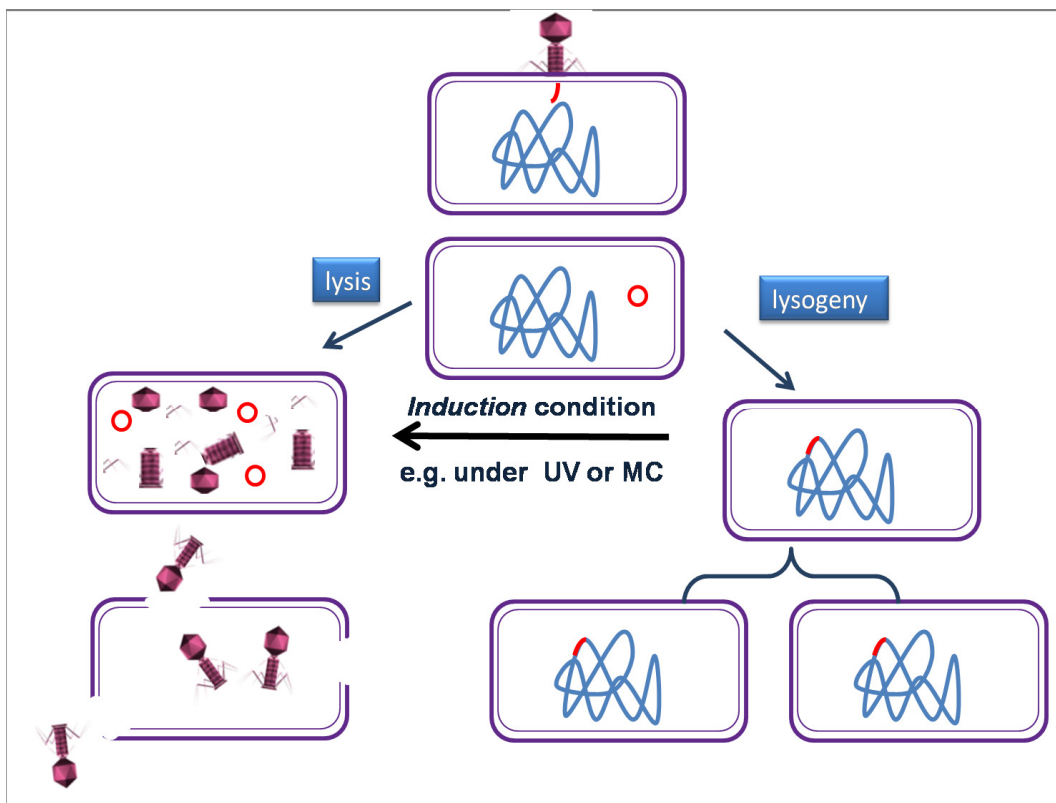


Figure 1.2. Life cycles of temperate bacteriophage

1.1.3 Gene regulation and genetic switch in phage lambda

Phage λ is a virus of *Escherichia coli* (*E. coli*), considered a classical example of temperate phage, which has been subject to decades of intensive study making it one of the best understood gene regulatory system. Phage λ can move between two different styles: as a lysogen, and as a free phage. The lysogenic state is highly stable. However, specific environmental conditions, e.g. UV irradiation and MC, elicit the cellular SOS response, triggering a highly efficient switch from lysogeny to lytic development (Atsumi & Little 2006; Hochschild & Lewis, 2009; Ptashne, 2004; Refardt & Rainey, 2010; Svenningsen *et al.*, 2005). Prophage induction arises from a cascade of events (Refardt & Rainey, 2010; Teif, 2010). Dodd *et al.*, 2007 suggesting that λ -style approach has limitations for investigating other gene regulation systems.

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The decision of a temperate phage to enter either a lysogenic or lytic pathway is controlled by a gene regulatory system termed a *genetic switch* that affects the initiation of lysogenic and lytic transcripts (**Pedersen *et al.*, 2010; Ptashne, 2004**). The maintenance of lysogeny and prophage induction is controlled by two antagonists, *ci* and *cro*. The switch comprises two positions i) in lysogeny, the *ci* repressor is “on”, but the *cro* gene is “off. In contrary, in lytic state, the *cro* is “on” and *ci* is “off”. These two regulatory genes, transcribed in opposite directions, together with RNA polymerase (RNAP) and their promoters, back to back, and operators constitute the switch (**Finzi & Dunlap 2010; Ptashne, 2004**). As shown in **Figure 1.3**, the λ phage genome contains six individual operator sites, three in each of the two control regions, O_R (comprises O_{R1} , O_{R2} , and O_{R3}) and O_L (comprises O_{L1} , O_{L2} , and O_{L3}) which are separated by 2317 bp from each other (**Finzi & Dunlap 2010**). The O_R region flanked by promoters P_R , for early lytic genes, and P_{RM} for *ci* gene. O_L region has a single promoter, P_L , which like P_R , directs transcription of early lytic genes (**Ptashne, 2004**). Ci binds as a dimer to individual operator sites, containing two structurally distinct domains region: i) the N terminal domain (NTD) contains a helix-turn-helix DNA-binding domain, and ii) the C-terminal domain (CTD), linked by a protease-sensitive linker, mediating dimer formation and also the higher-order dimer-dimer interactions that enable Ci to bind cooperatively to two (or more) operator sites (**Hochschild & Lewis, 2009**). During lysogenic pathway, Ci represses transcription of the early lytic genes by occupying sites O_{R1} and O_{R2} in O_R and O_{L1} and O_{L2} in O_L . Ci binds at O_{R2} activates transcription of the *ci* gene from P_{RM} , Ci in high concentration, has the potential to repress transcription of its own gene (negative regulation) when binding to O_{R3} (**Hochschild & Lewis, 2009; Ptashne, 2004**), thereby it mediates a DNA loop binding two distinct operator regions O_L and O_R (**Svenningsen *et al.*, 2005**). They are autoregulated to generate high enough concentrations to switch lytic genes “off” but not too high as to comprise

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the switch to lysis, thereby functioning as a repressor and an activator of transcription (**Finzi & Dunlap 2010; Hochschild & Lewis, 2009; Ptashne, 2004**). However, the role of Cro protein in induction process is still not clarified (**Teif, 2010**). Hence, **Schubert *et al.*, 2007** sustained the previous CI-Cro module and reported that Cro is needed to block synthesis of new CI which impedes lytic development, **Svenningsen *et al.*, 2005**, showed that *cro* gene of λ phage might be unimportant for the lysogenic to lytic switch during induction. Also, **Atsumi & Little, 2006** noted that Cro of λ phage is not necessary for prophage induction to occur, and concluded that repression of P_{RM} by Cro is not necessary for phage induction.

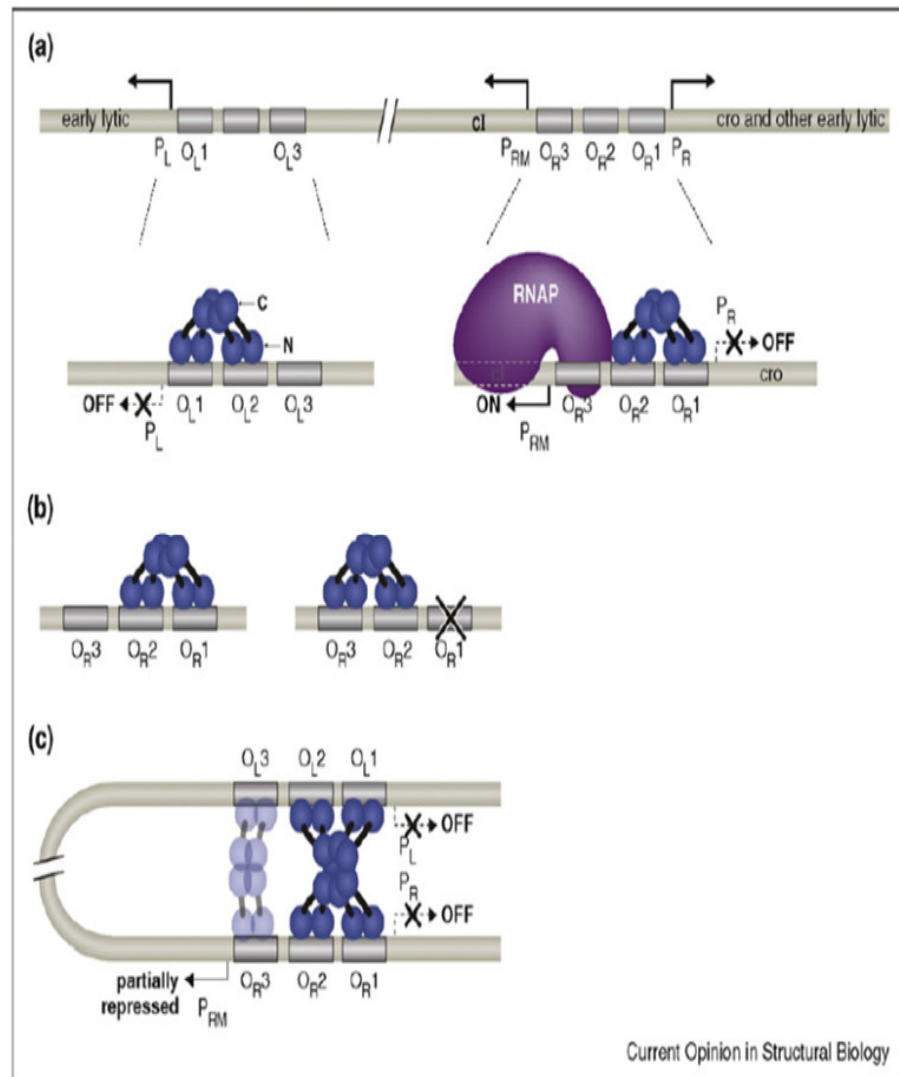


Figure 1.3. The interactions of CI at the right and left operator regions in a λ lysogen. **(a)** CI dimers bound cooperatively to adjacent operator sites in O_R and O_L . The CI dimers are shown in blue. Each subunit of the dimer consists of an N-terminal DNA-binding domain (N), a C-terminal oligomerization domain (C), and a linker region (black) connecting the two. The dimer pair bound cooperatively at O_{R1} and O_{R2} represses

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transcription from P_R and the dimer bound at O_{R2} also activates transcription from P_{RM} . The dimer pair bound cooperatively at O_{L1} and O_{L2} represses transcription from P_L . **(b)** Alternate pairwise cooperativity. No more than two adjacently bound CI dimers can interact. Ordinarily, a dimer bound at high-affinity site O_{R1} stabilizes the binding of a second dimer to O_{R2} . But if O_{R1} is inactivated by mutation, CI dimers can bind cooperatively to O_{R2} and O_{R3} . **(c)** Higher-order looped complex that facilitates negative autoregulation. The cooperatively bound CI dimer pair at O_{R1} and O_{R2} interacts with the cooperatively bound CI dimer at O_{L1} and O_{L2} , forming an octameric complex that loops 2.4 kb of intervening DNA. Formation of the looped complex juxtaposes O_{L3} and O_{R3} , allowing another pair of CI dimers (shown in pale blue) to bind cooperatively to these sites. When bound at O_{R3} , CI represses transcription from P_{RM} (negative autoregulation); however, in a lysogen O_{L3} and O_{R3} are only partially occupied. (Source **Hochschild & Lewis, 2009**).

1.2 LAB

LAB are a large group of fermenting bacteria which exist naturally in or additionally added to nutrient-rich environments where carbohydrates and proteins are usually abundant, playing an important role in many food and feed fermentations. The acidification and enzymatic processes accompanying the growth of LAB impart the key texture, flavor, and preservative qualities of a variety of fermented food and they are considered as GRAS (Generally Regarded As Safe). They are described as a heterogeneous group of low GC content gram-positive, nonsporing cocci or rods, which are related in their ability to ferment carbohydrates to lactic acid as the major end-product. The boundaries of this group have been subject to some controversy, but historically the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* form the core of this group. Based on the final products of LAB, they are divided into two groups: i) *homofermentative* LAB which convert fermentable carbohydrates almost quantitatively into lactic acid, and ii) *heterofermentative* LAB that produce lactic acid and other compounds like acetic acid, CO_2 , and ethanol as primary metabolic end-product. LAB have been increasingly recognized by the food

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industry and consumers alike in the production of most fermented foods like pickling of vegetables, baking, winemaking, curing fish, meats, sausages, and fermented milk products such as yoghurt, cheese, butter, buttermilk, kefir, koumiss, and have also been confirmed for their probiotic potencies to maintain human health. Furthermore, LAB are part of the indigenous intestinal microflora, and are also part of the microflora of a wide range of foods, milk, vegetables, wines, and meats (**Axelsson; 2004; Feord, 2002; Forsythe, 2010; Geis, 2003; Higashikawa *et al.*, 2010; Iyer *et al.*, 2010; Klaenhammer *et al.*, 2002; Makarova *et al.*, 2006; Ramchandran & Shah, 2010; Ventura *et al.*, 2002b**). LAB can be roughly classified into two groups: i) a group that comes from animal sources, like raw milk and intestines, and has been used to make cheese or yoghurt, and ii) a group clarified from plant sources, such as grasses, vegetable, and fruits, and has been used in traditional Asian foods (**Higashikawa *et al.*, 2010**). Due to their practical significance in fermentation, bioprocessing, agriculture, food and recently in medicine, LAB have been the aim of considerable research and commercial concerns over the past decade (**Klaenhammer *et al.*, 2002**).

1.3 Bacteriophage infection of LAB

Bacteriophage is the main cause of reduction or complete starter failure in the dairy industry and subsequent economic losses (**Marco *et al.*, 2010; Zinno *et al.*, 2010**), despite the extensive efforts of an array of strategies to control phages. The lysogenic stage in LAB is considered the main source of phages in the dairy environment (**Quiberoni *et al.*, 2010; Suarez *et al.*, 2008**). All LAB phages contain a linear dsDNA, which may become circular if the DNA contains cohesive ends (**Walstra *et al.*, 2006**). Lysogeny may occur in commercial bacterial starter cultures; consequently efforts should be directed towards more investigations to get more knowledge helping in the control of the virulent phage. In this

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field it is recommended to generate food-grade mutants, since the mutated bacteria do not have antibiotic-resistance genes or other plasmid-carried DNA sequences (**Baccigalupi et al., 2000**).

1.4 *Streptococcus thermophilus*

European Bioinformatics Institute, EBI, (2009) described *Streptococcus thermophilus* (*S. thermophilus*) as “a Gram-positive facultative anaerobe. It is a cytochrome-, oxidase- and catalase-negative organism that is nonmotile, non-spore forming and homofermentative. *S. thermophilus* is an alpha-hemolytic species of *viridians* group. It is also classified as LAB”. **Sherman & Stark, 1938** reported that Orla-Jensen (1919) was the first to differentiate and describe *S. thermophilus* as a distinct streptococcal species. This species possesses a relatively small genome of approximately 1.8 Mb, making it the smallest genome of all LAB and has a comparable low G+C ratio (40%) (**EBI, 2009; Klaenhammer et al., 2002; O'Sullivan & Fitzgerald, 1998**). *S. thermophilus* is considered the second most important industrial starter strain after *Lactococcus lactis* (**Mills et al., 2010**). It is used for several varieties of Argentinean soft and semi-hard cheeses and is considered the second most important industrial starter strain after *Lactococcus lactis*. It has annual value worldwide of about \$ 40 billion, making it a species that has major economic importance. As thermophilic bacteria, it has the ability to grow at elevated temperatures (up to 52 °C) (**Arena et al., 2006; Blomqvist et al., 2006; Bolotin et al., 2004; Geis, 2003; Iyer et al., 2010; Mills et al., 2010; Quiberoni et al., 2010**). It has also several functional activities like production of bacteriocins, vitamins and extracellular polysaccharides. Furthermore, it has potential as a probiotic and various health effects (**Andrighetto et al., 2002; EBI, 2009; Iyer et al., 2010; Sturino & Klaenhammer, 2007**).

*Introduction***1.5 Phages of *S. thermophilus***

All *S. thermophilus* phages appear to be derived from a common ancestor (**Le Marrec *et al.*, 1997**). Bacteriophages of *S. thermophilus* generally reveal high thermal resistance, rapid replication and large burst sizes, furthermore they may remain in the air for long periods. Also, the number of strains of *S. thermophilus* used as starter cultures is very limited, restricting the development of strategies to reduce phages infection of this species in industrial plants (**Klaenhammer *et al.*, 2002; Quiberoni *et al.*, 2010**). The vast majority of dairy streptococcal phages are virulent phages, and lysogens are uncommon in *S. thermophilus* cultures (**Brüssow *et al.*, 1994**). Although prophages have been linked to an autolytic phenotype of some *S. thermophilus* strains, the role of lysogeny in milk fermentation process is still unsettled (**Quiberoni *et al.*, 2010**). Phages of *S. thermophilus* are relatively homogeneous in relation to morphology and genomic organization, and are derived from a common ancestor. This dsDNA phages belong to *Siphoviridae* family (morphotype B1) of viruses having small isometric heads, long non-contractile tails (**Mills *et al.*, 2010; Quiberoni *et al.*, 2010; Sturino & Klaenhammer, 2007**). These phages are considered as reference phages for comparative phage genomics, sharing sequence similarity not only with phages from *Lactococcus lactis* and *Lactobacillus spp.*, but also from important human pathogens such as *S. pyogenes* (**Ventura *et al.*, 2002a**). They are divided into two groups based on the packaging mechanism of their dsDNA (*cos*- and *pac*-type) and the number of their structural proteins: hence *cos*-type phages have two major structural proteins, while *pac*-type have three of those proteins (**Mills *et al.*, 2010; Neve *et al.*, 1998; Quiberoni *et al.*, 2010; Sturino & Klaenhammer, 2007; Walstra, 2006**). Although many, perhaps several hundreds distinct *S. thermophilus* phages may survive in the environment (**Bruttin *et al.*, 1997**), they have only been investigated in detail during the last two decades (**Neve *et al.*, 2003**). So far, at least 345 virulent and temperate phages

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infecting *S. thermophilus* have been reported, mostly in North America and Europe (**Quiberoni et al., 2010**). During the last 55 years, numerous studies from Europe, Canada, US, and Argentina documented the isolation of virulent phages for *S. thermophilus* and identification of a few temperate phages (**Quiberoni et al., 2010**). Currently, 11 complete genomes of *S. thermophilus* phage are available in the public database of GeneBank (NCBI), and sequencing of as many genomes is in progress in different sequencing centers worldwide.

1.6 Temperate *S. thermophilus* phage TP-J34

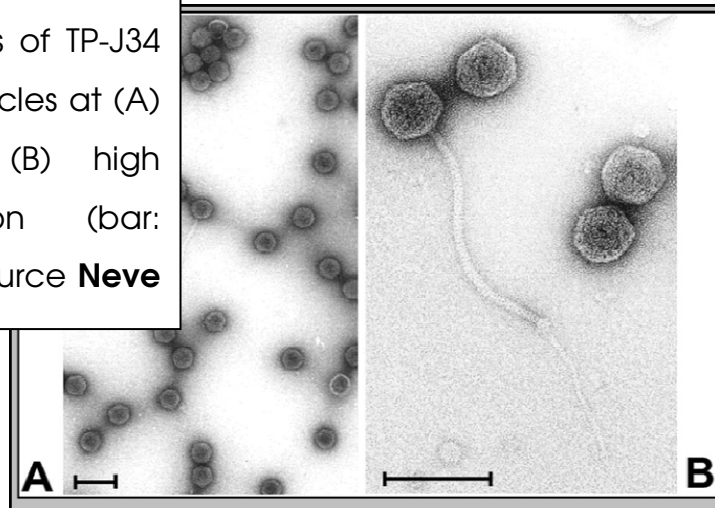
Phage TP-J34, the centerpiece of this work, (**Figure 1.4 and 1.5**) is a temperate phage in the *S. thermophilus* lysogen host strain J34. It is a *pac*-type phage, the amount of DNA contained in the viral head is always about the same, of about 45.6 kb linear DNA, comprising 61 putative *orfs* organized in a life-cycle specific order divided into two oppositely transcribed regions: i) the leftward-transcribed region encompasses 4 *orfs*, and ii) the rightward-transcribed region contains 57 *orfs*. This phage belongs to the group of small isometric-headed phages with long, flexible, non-contractile tails. The majority of phage particles produced upon induction is defective and non-infectious. TP-J34 has been induced from the lysogenic starter strain *S. thermophilus* J34 from a yoghurt sample in Germany. Some DNA sequence data and biological characteristics (**Neve et al., 1998 and 2003**) as well as some molecular function have been published (**Bockelmann, 2002; Sun, 2002; Sun et al., 2006**).

As a successful approach to determine the gene's function in prophage TP-J34, three knock out mutations in the lysogeny module have been achieved, i.e., in *orf3*, *ltp*, and *int* genes via crossing into the target gene a recombinant vector carrying an internal fragment of the gene of interest cloned into pGhost9. A mutated *orf3* derivative

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performed a crucial role in the prophage induction with mitomycin C (MC), in other words this mutation prevented prophage induction at all. The homogeneous growth is affected also by this mutant, i.e., the mutated derivative named *S. thermophilus* J34 KO-orf3 showed cell aggregation in broth instead of homogeneous growth in w.t. strain **(Bokelmann, 2003)**. *Ltp* gene was shown to mediate superinfection exclusion and its gene product is associated with the cell membrane, and plays also a remarkable role in prophage induction, hence the mutated *ltp* derivative of the lysogen named *S. thermophilus* J34 KO-ltp displayed a delayed lysis phenotype with MC **(Sun 2002; Sun et al., 2006)**. *Int* gene revealed an important role in the induction of the prophage, hence *int* knock out derivative named *S. thermophilus* J34 KO-int was noninducible with MC, some other mutation and transcriptional analysis of the lysogeny module were found out as well **(This work)**.

Figure 1.4. Electron micrographs of TP-J34 phage particles at (A) low and (B) high magnification (bar: 100 nm) (Source **Neve**



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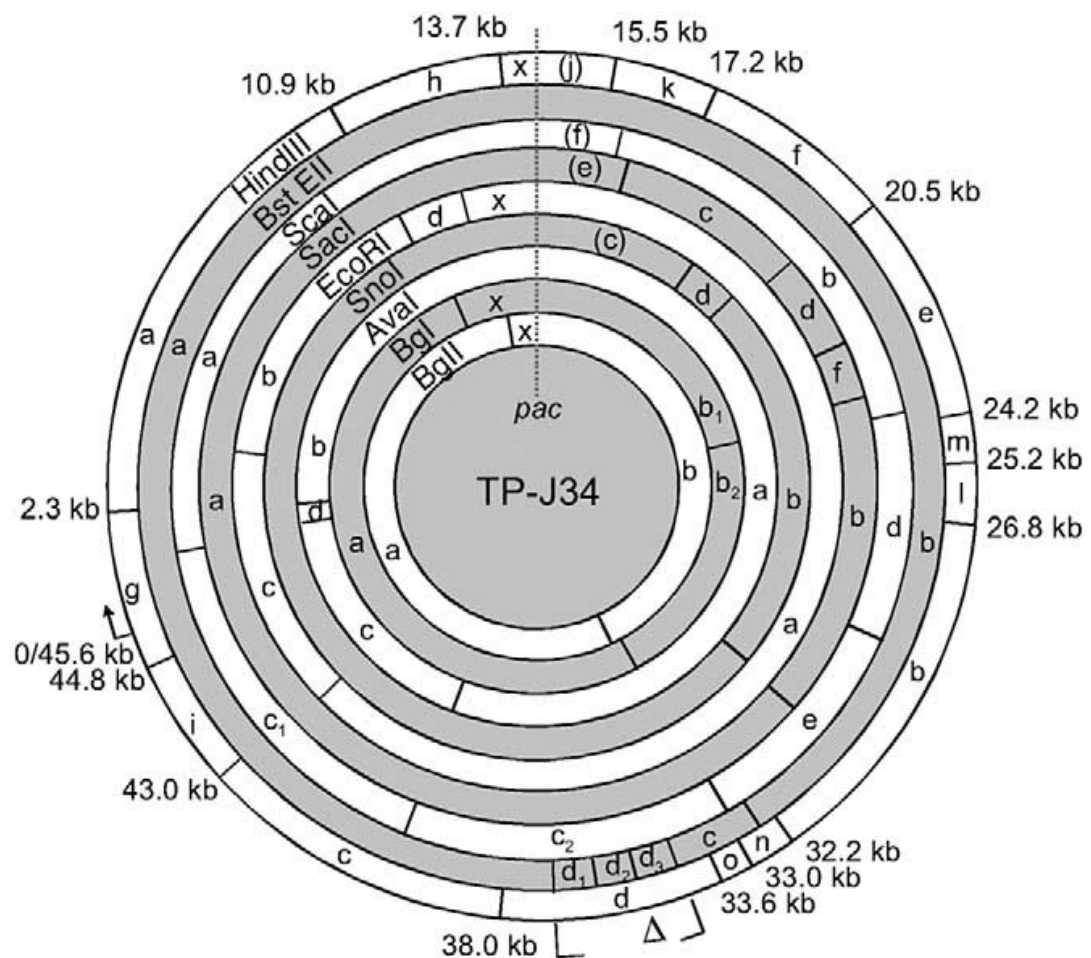


Figure 1.5. Circular restriction map of phage TP-J34 genome (Neve *et al.*, 2003). Restriction enzymes used are shown within the circles. Restriction fragments are marked in alphabetical order, starting with “a” for the largest fragment. The position of the *pac*-site (top) is indicated. Minor bands formed between the *pac*- and the first restriction site (clockwise) are shown in brackets. Minor bands not detectable on agarose gels are marked with “x”. The site-specific deletion event occurring during lytic growth of phage TP-J34 is indicated at the bottom of the map. The arrow in clockwise orientation indicates the start/end of the physical map immediately downstream of the integrase gene in the lysogeny module. The positions of the HindIII restriction sites are indicated.

1.6.1 The lysogeny module of phage TP-J34

Experimental and sequence analysis of phage TP-J34 revealed that all except four of the phage genes are transcribed in one direction. The four genes are separated from the others by a genetic switch region

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and appear to be transcribed in one operon. They encode the following functions – in the order of transcription:

- i) **Repressor**, designated *crh* hereafter, for *ci*-repressor homologue, orf 121 gene product (gp), showed significant similarity to a number of bacteriophage like repressor proteins: *c2* from lambdoid phage P22, the PBSX repressor of a defective prophage from *Bacillus subtilis* phage *g1e*, and the repressor *Lactococcus lactis* Tuc2009 (Neve *et al.*, 1998). *Crh* of TP-J34 has the same topological position as repressor genes of *S. thermophilus* phage TP-778, Sfi21, and the lactococcal phages Tuc2009, and TP901-1.
- ii) The product of **orf3** is identical with orf 122 gp of temperate *cos*-phage Sfi21. This gene is located in a comparable topological position in both phages (two genes upstream of *int*). *Orf3* of TP-J34 has the same topological position as similar genes of *S. thermophilus* phages TP-778 (Ali, 2009), and Sfi21 (Ventura *et al.*, 2002a).
- iii) **Ltp, 142 gp**, is a lipoprotein mediating superinfection exclusion (*sie*). This gene has the same topological position with that of *S. thermophilus* phages TP-778 (Ali, 2009), Sfi21 (*orf203*) (Ventura *et al.*, 2002a), and the lactococcal phage Tuc2009 (*sie₂₀₀₉*) (McGrath *et al.*, 2002), being located immediately upstream of *int* gene. Ltp of TP-J34 has high similarity to corresponding Ltp_{TP-778} gp with a significant 92% aa identity (Ali, 2009; Neve *et al.* 2003; Sun *et al.*, 2006). “Sie” is a resistance mechanism in some temperate phages which blocks the additional phages DNA ejection into the host. This mechanism was investigated and detected with Ltp of TP-J34. The *Ltp* gp of TP-J34 is associated with the cell membrane (Sun 2002; Sun *et al.*, 2006).
- iv) **Integrase (*inf*)** gene, 359 gp, encodes a putative phage integrase. It is identical to that of phage Sfi21, and demonstrates a high

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degree of similarity to integrase family of proteins including integrases from *Lactococcus lactis* phages (**Neve et al., 1998**). Phage TP-J34 integrated its genome into the tRNA^{Arg} gene of the chromosomal DNA exactly as described for ϕ Sfi21 (**Ali, 2009; Bruttin et al., 1997; Neve et al., 1998**). There are common core sequences of 40 bp for *attB*, *attR*, *attL*, and *attP* identical in both phages

In the opposite orientation to the previous four genes, the remaining genes of TP-J34 are arranged. In this work, we focus only on *cro* and *ant*:

- **Cro** (control of repressor and other genes), 67 gp, shows a significant similarity to an anonymous protein from *Methanococcus jannaschii* with 22 shared aa. *Cro* and *crh* of TP-J34 are adjacent in the DNA, but are transcribed in opposite directions. *Cro* of TP-J34 has the same topological position with that of phages TP-778 (*cro*_{TP-778}), Sfi21 (*cro*_{Sfi21}) and the lactococcal phage TP901-1 (*cro*_{TP901-1}).
- **Antirepressor (*ant*)**, orf 238 gp, shows similarity, mainly over the N-terminal half, with hypothetical protein from *Haemophilus influenza* (score = 199, $P = 10^{-16}$). Over the C-terminal half, the Orf 238 gp revealed similarity with the Ant and K1A from coliphage P1, coding respectively for the anti-repressor and phage protein lethal to the host cell. Orf 238 gp demonstrates similarity to the helix-turn-helix motif of coliphage proteins (**Neve et al., 1998**).

1.6.2 The genetic switch in TP-J34

The two regulatory proteins, *Cro* and *Crh*, together with RNAP and their promoter and operator sites constitute the switch. The analysis of the *crh/cro* intergenic region (168-bp) revealed that

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it contains two back to back putative promoters elements. P1 points rightward, while P2 points leftward. These promoters are showing high homology to *E. coli* consensus promoters. "O_{1A}" is a putative operator consisting of a 15 bp direct repeat with internal dyad symmetry (i. e., are palindromic, "mirror image" of each other) which overlaps the rightward promoter (P1) sequence. (Neve *et al.*, 1998).

1.7 The aim of this work

Chapter 3 was aimed at finding out the role of *int* gene in the induction of prophage TP-J34. This was done by disruption of this gene by crossing into its DNA vector pGhost9 derivative carrying an internal fragment of *int*, and analysis its influence on induction. Also, to understand why *S. thermophilus* J34-12 is non-inducible. The lysogeny module of this derivative (comprising *attL*, *int*, *ltp*, *orf3*, *crh*, *cro*, and *ant*) was sequenced. A mutation was detected only within *orf3*. The involvement of *orf3* in induction was further investigated by i) curing of the integrated vector from *S. thermophilus* J34 KO-*orf3*, ii) isolation of a non-inducible *S. thermophilus* J34-cu50, revealing a point mutation in *orf3*, iii) sub-cloning of intact *orf3* gene in lactococcal expression vector pMG36e and transformation into *S. thermophilus* J34 cu50 to restore the phenotype of the w.t. strain.

Chapter 4 was performed to shed some light on transcription of the gene of the TP-J34 lysogenic module under both lysogenic and induction development via employment of Northern blot and RT-PCR. Promoter analysis and SLs were also investigated. The aim was here to find out i) at which time point these genes are transcribed, ii) which genes are transcribed polycistronically and which are transcribed

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monocistronically, iii) which relationships occur between the genes which controlling the genetic switch, iv) finally, a hypothetical representation of genetic switch regulation depending on the results of Northern blot, RT-PCR and sequence analysis is presented.

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2 Chapter 2:

Materials and Methods

2.1 Bacterial cultures and growth conditions

The bacterial strains, and plasmids used in this study are listed in **Table 2.1**, and **Table 2.2**. Luria-Bertani (LB) medium, supplemented with appropriate antibiotics, was used with aeration at 37 °C for *E. coli*. While *S. thermophilus* strains were grown in LM17 medium without shaking at 40 °C.

Table 2.1. Bacterial strains used in this work

Bacterial strains	Relevant phenotype/genotype	Source
<i>E. coli</i> EC1000	Intermediate host for cloning, Km ^R , MC1000 derivative, carrying a single copy of pWV01 <i>repA</i> in <i>glgB</i> .	Leenhouts <i>et al.</i> , (1996); Sanders <i>et al.</i> , (1998)
<i>E. coli</i> strain NovaBlue	Recipient for cloning experiments	Novagen
<i>S. thermophilus</i> J34	Lysogenic host of phage TP-J34, 3.4-kb plasmid pJ34, homogenous growth.	Neve <i>et al.</i> , 1998
<i>S. thermophilus</i> J34f-2	A plasmid-cured derivative of J34, homogenous growth.	Sun, 2002
<i>S. thermophilus</i> J34-6	Prophage-cured derivative of strain J34.	Neve <i>et al.</i> , 2003
<i>S. thermophilus</i> J34-12	A J34 derivative bearing a non-inducible prophage, homogenous growth.	Neve <i>et al.</i> , 2003
<i>S. thermophilus</i> J34 KO- <i>ltp</i>	<i>ltp</i> knock out derivative of strain J34f-2, Em ^R .	Sun, 2002
<i>S. thermophilus</i> J34 KO- <i>orf3</i>	<i>orf3</i> _{TP-J34} knock out derivative of strain J34f-2, Em ^R .	Bokelmann, 2003
<i>S. thermophilus</i> J34 KO- <i>int</i>	<i>int</i> _{TP-J34} knock out derivative of the prophage of <i>S. thermophilus</i> J34, Em ^R .	This work
<i>S. thermophilus</i> J34f-2 cu50	A J34f-2 derivative with a replacement mutation within <i>orf3</i> _{TP-J34} gene.	This work

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<i>S. thermophilus</i> J34 cu50 trans1&2	<i>S. thermophilus</i> J34 cu50 was transformed by pMZorf3	This work
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- *S. thermophilus* J34 KO-ltp is named *S. thermophilus* J34f-2 int in **Sun, 2002**, *S. thermophilus* J34 KO-orf3 is named *S. thermophilus* J34 f-2 pKOint in **Bokelmann, 2003**.

Table 2.2. Plasmids used in this work

Plasmids	Description	Source
pGhost9	Thermosensitive shuttle vector; Em ^R ; 3.8 kb	Maguin <i>et al.</i>, (1996)
pMAZ2	A 518 bp internal fragment of the <i>int</i> cloned into EcoRI and HindIII sites of pG ⁺ host9; Em ^R	This work
pSTBlue-1	Blunt cloning vector; Kan ^R ; Ap ^R	Novagen
pMG36e	3.6 kb lactococcal vector, Em ^R	van de Guchte <i>et al.</i>, 1989
pMZorf3	4.1 kb, <i>orf3</i> cloned into pMG36e, Em ^R	This work

2.2 Polymerase chain reaction (PCR)

For PCR, a thermocycler (Mastercycler 5333; Eppendorf AG, Hamburg, Germany) was used. The reaction was performed in either 50 μ l or 25 μ l as a final volume. Hence, 10 μ l or 20 μ l of 2.5x PCR master mix (5 Prime GmbH, Hamburg, Germany), 3 μ l or 1.5 μ l from forward and reverse primers (10 pmol μ l⁻¹), 23 μ l or 11.5 μ l nuclease free water and 1 μ l or 0.5 μ l DNA (about 100 or 50 ng), for 50 μ l or 25 μ l final reaction, respectively. A negative control was included without DNA.

2.2.1 Colony PCR

For colony PCR, one colony was transferred from an agar plate to a 1.5 microfuge tube containing 100 μ l 1x colony PCR buffer (Fermentas GmbH, St. Leon-Rot, Germany). The cells mixed well in the buffer by vortexing. The suspension was heated at 95 °C with shaking (650 rpm) in a

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thermo block (Thermomixer 5436, Eppendorf) for 15 min. 2 μ l or 1 μ l was used for PCR reaction in 50 μ l or 25 μ l volume, respectively.

2.2.2 Primer design

The primers used in this work (**Table 2.3**) were designed by DS Gene 1.5 software (Accelrys Limited, Cambridge, England), and manufactured by Eurofins MWG Operon, Ebersberg, Germany.

Table 2.3. Primers used in this work

Primer name	primer sequence (5'→ 3')	Tm (°C)	Reference
int. F	ACT GAA TTC TTG GAT AGT GAG G	56.5	This work
int. R	CGG CGA TAA GAA GCT TAA ATC T	56.5	This work
Primer 4	GCA AGG TAA AGC TGC AC	52.8	Bruttin <i>et al.</i>, 1997
pGh9-R2	TGG GAT TCA GGA GTG GAC AG	59.4	This work
Tail-F	CCA AGC GAT GAC AAA ACC TAA G	52.2	This work
Tail-R	GCT AAA TGC TGT ACC CAA AGC	52.1	This work
YP-F	GCT CGT CTT GAA GCT ATG C	56.7	Ali, 2009
YP-R	GAT AAG AGT CAA GTG ACC GTC	57.9	Ali, 2009
int- 2	GTG ATG TTC AGC TTT TCA TGC C	58.4	This work
ltp-f	TAG CAA CAG CGT AGT CAG C	56.7	This work
ltp-R	TTT GGC TTG CTA AGC CTT TC	55.3	Ali, 2009
pri.CI.F.	CTT TGT ACC CGT TGG GAT AC	57.3	This work
pri.CI.R	AAG CAA AGA GGT AGC AGA ATC	55.9	This work
MZ. R1	TGG GGG ATT AAA TAA ATG GC	53.2	This work
MZ. R2	CTC AAG AAG AAG CTG ACT ACG	57.9	This work
MZ ant. F	ATA CAA GAG CCT CTT GTT TCC G	58.4	This work
MZ ant. R	ATA AAT ACT GCT GAC CTT TGC	54.0	This work
MZ12. F	ATA GCC GAA CTG TTC AAG CAA TTC	59.3	This work
MZ12. R	ATA GCA GAT TAT CGA ATC GGT CAG	59.3	This work
orf3-C-f	ATA TCT AGA GCA GGT TAC ATG AAT AGC AAG G	64.2	This work
orf3 HindIII. R	ATA AAG CTT AAC GTG ATT CAG TTT TGC ACG	62.7	This work
pri. MAZ-F	CAA GGA GCT AAA GAG GTC CC	59.4	This work
pri. MAZ-R	ACT TTG ACA ATC ATG AGT GCA G	56.5	This work
MZ. F1	GAT ATT CCC ACC CAT TTG AGC	57.9	This work
EcoRI-ko	TCT GAA TTC GTT TTT CAC AAA AGT CTC A	59.3	Bokelmann, 2003
ko-EcoRI	TGT GAA TTC TTA ATC GTG TGG TTT ACA T	59.3	Bokelmann, 2003

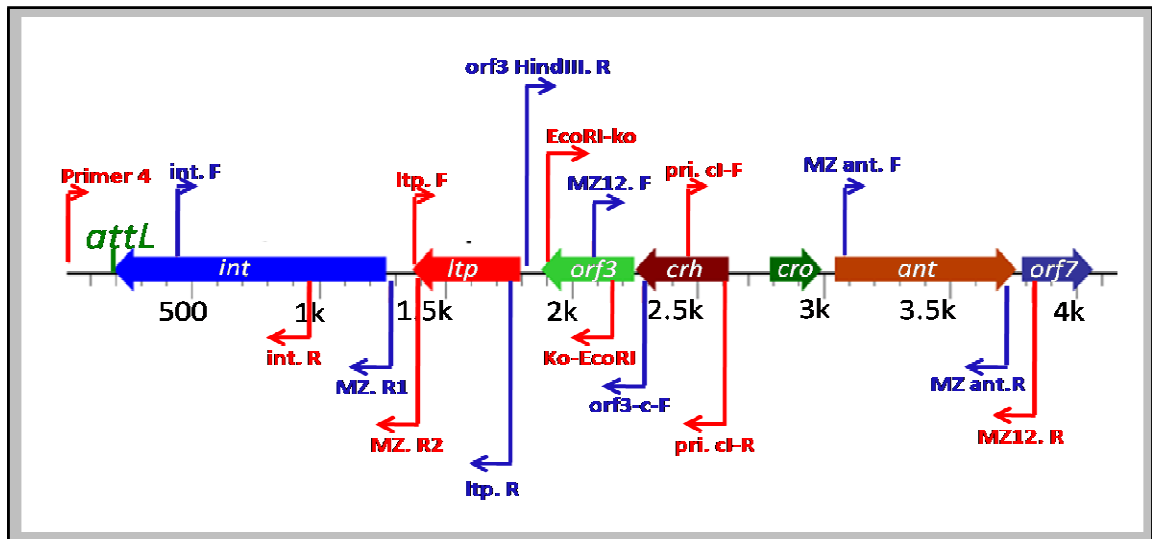


Figure 2.1. Schematic representation of the primers used in this study and their sites in the lysogeny module of *S. thermophilus* J34.

2.2.3 Designing of PCR programs

PCR programs used in this work were designed according to **Table 2. 4.**

Table 2.4. Thermal cycler program used in PCR

Step	Temperature °C	Time	Number of cycles
1	94 °C	4 min	1
2	94 °C	30 s	35
3	5 °C below Tm of primers	30 s	
4	72 °C	1 min per kb	
5	72 °C	7 min	1
6	4 °C		Hold

2.3 Induction of prophage using mitomycin C (MC)

One volume of LM17 broth was inoculated with 1% o/n culture (i.e. *S. thermophilus* J34 and/or its derivatives) and incubated at 40 °C until OD_{620nm} of 0.2. Then another one volume of LM17 liquid medium containing 0.6 µg ml⁻¹ MC (to get final concentration of 0.3 µg ml⁻¹ MC) was added and incubated at 40 °C until complete lysis. The induction of integrants *S. thermophilus* J34 KO-int (see inactivation of *int* gene) was carried out at

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relatively higher temperature, 42 °C, to insure the depressing effect of high temperature on the free vector pMAZ2, which has a temperature-sensitive replicon (Ts) in it.

2.4 Isolation of chromosomal DNA of *S. thermophilus*

2.4.1 Reagents

- DL-threonine (1 M)
- Lysozyme (50 mg ml⁻¹)
- Solution A (20% (w/v) sucrose, 10 mM Tris-HCl, (pH 8.0), 50 mM NaCl)
- SDS 10% (w/v)
- Mutanolysin (5000 U ml⁻¹)
- Proteinase K (20 mg ml⁻¹)
- RNase A (10 mg ml⁻¹)
- Phenol
- Chloroform/isoamyl alcohol (24:1)
- Na-acetate (3 M, pH 5.2)
- TE buffer (10 mM Tris, pH 8, 1 mM Na-EDTA)

2.4.2 Procedure

- 1) 3% of *S. thermophilus* overnight culture was inoculated into 10 ml of LM17 supplemented with 40 mM DL-threonine, and incubated at 40 °C till OD_{620 nm} of 0.6-0.8.
- 2) The cells were collected in 2-ml micro-tubes by centrifugation.
- 3) The cells were re-suspended in 500 µl solution A supplemented with 5 mg ml⁻¹ lysozyme and 5 µl mutanolysin.
- 4) The re-suspended cells were frozen at -20 °C for 20 min, thawed, and incubated at 55°C for 10 min.
- 5) 60 µl proteinase K were added, mixed by inversion 8 times before incubating at 60 °C for 1 h.
- 6) 250 µl deionized water were added.

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- 7) 250 μ l phenol were added and mixed by inversion about 30 times.
- 8) 250 μ l chloroform/isoamyl alcohol (24:1) were added and mixed by inversion about 30 times.
- 9) The samples were centrifuged at 14,000 rpm (Eppendorf centrifuge 5415D) for 5 min.
- 10) The aqueous phase was transferred to a new 2-ml micro tube.
- 11) The last 5 steps were repeated.
- 12) 500 μ l chloroform/isoamyl alcohol (24:1) were added and mixed by inversion about 30 times, followed by centrifugation at 14,000 rpm (Eppendorf centrifuge 5415D) for 3 min, then the aqueous phase was transferred to a new 2-ml micro tube.
- 13) 1/10 volume of 3 M Na-acetate and 1 volume of isopropanol and one volume of isopropanol were added, and mixed by inversion until chromosomal DNA was visible.
- 14) The samples were incubated at -20 °C for 1 h.
- 15) The samples were centrifuged (Eppendorf centrifuge 5417R) at 14,000 rpm at 4 °C for 15 min to collect the DNA pellets.
- 16) The supernatant was discarded. The pellets were washed by adding 1 volume of 70% (v/v) ethanol and centrifuged (Eppendorf centrifuge 5417R) at 14,000 rpm at 4 °C for 5 min.
- 17) Ethanol was discarded, with care, and the caps were completely dried at air for about 7 min.
- 18) 200 μ l TE buffer (pH 8.0) were added to dissolve the DNA pellets.
- 19) 5 to 10 μ l RNase A was added and incubated at 37 °C for 30 min.
- 20) DNA concentration and purity could be estimated by loading about 8 μ l DNA solution on 0.8 (w/v) agarose gel at 74 V for 60 min. A known concentration of DNA was used for comparison.

2.5 Agarose gel electrophoresis

2.5.1 Reagents

2.5.1.1 50x stock solution Tris Acetate EDTA buffer (TAE), (Sambrook *et al.*, 1989)

Tris base	242.0 g
Acetic acid	57.1 ml
Na-EDTA	(0.5 M, 100 ml)

filled up to 1 l with deionized water

2.5.1.2 Loading dye (6x)

25 mg bromophenol blue	(0.25% (w/v))
3.79 ml glycerol	(33.00% (v/v))

In final volume of 10 ml deionized water

2.5.1.3 Staining solution

The staining solution ($0.5 \mu\text{g ml}^{-1}$) was prepared by adding 1 ml of ethidium bromide stock solution (0.5 mg ml^{-1} , Carl Roth GmbH, Karlsruhe, Germany) to 1 l of deionized water.

2.5.2 Procedure

2.5.2.1 DNA samples preparation

DNA samples (chromosomal DNA, plasmid DNAs, and PCR product) were mixed with 6x loading dye (to get 1x final concentration)

2.5.2.2 Agarose gel electrophoresis

Agarose gels were prepared, 0.8 to 1.5% (w/v) in 1x TAE buffer pH 8.0, according to the size of DNA fragment to be analyzed. The solution was cooked in a microwave oven for 3 to 5 min. Then, the gel was incubated at 60 °C to reduce its temperature before pouring into the gel chamber. The appropriate comb was fixed on the solved agarose and waited till the agarose gel polymerized (about 20 min at RT). The comb was then removed after adding some TAE buffer. The polymerized gel was then placed in the agarose gel electrophoresis unit (Bio-Rad Laboratories GmbH, München, Germany). DNA samples were poured in the wells of the gel, and run at 76 V for 45 min or more. The agarose gel was then stained in ethidium bromide solution for 20 min and destained in H₂O for 15 min. The gel was photographed by Universal Hood II-ChemiDoc XRS unit and analyzed using Quantity One Software (Bio Rad Laboratories, Segrate (Milan), Italy).

2.6 Plasmid DNA isolation

2.6.1 Isolation from *E. coli*

- 2 ml over night culture of *E. coli* (in LB-medium supplemented with the recommended antibiotic/s, at 37 °C with shaking) was centrifuged in 2-ml micro tube to collect the pellets.
- The pellets were washed with 300 µl sterile deionized water. The plasmid was then isolated and purified from the pellets according to the Macherey & Nagel protocol (Macherey Nagel GmbH & Co. KG, Düren, Germany)
- The concentration, purity, size, and integrity of the purified plasmid could be determined or analyzed (after digesting with appropriate restriction enzyme/s depending on its genetic maps) on agarose gel in comparison to an appropriate DNA ladder.

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- Plasmid solution was stored at -20 °C for further use.

2.6.2 Isolation from *S. thermophilus***2.6.2.1 Reagents**

- NucleoSpin® Plasmid kit buffers A1, A2, A3, A4, AW, and AE.
- Lysozyme (50 mg ml⁻¹ in deionized water).
- Mutanolysin (5,000 U ml⁻¹ in deionized water).
- Proteinase K (20 mg ml⁻¹ 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 8.8))

2.6.2.2 Procedure

1% overnight culture was inoculated into 10 ml LM17 (supplemented with the recommended antibiotic/s, and 40 mM DL-threonine). The culture was then grown at 40 °C till OD_{620nm} of 0.7 - 0.8. The pellets were then collected in 2-ml micro-tubes by centrifugation, and then washed with 300 µl sterile deionized water.

The “NucleoSpin® Plasmid Kit” was used to isolate the plasmid from the pellets, according to the protocol (Macherey Nagel GmbH & Co. KG, Düren, Germany), with some modifications as follows:

- (1) 250 µl buffer A1 was added to the pellets and resuspended by micropipette tips up and down several times, and then mixed well by vigorous vortexing.
- (2) 25 µl lysozyme and 5 µl mutanolysin were added and mixed by hand some times.
- (3) The suspension was incubated at 37 °C for 60 min.
- (4) 250 µl buffer A2 was added, mixed gently by hand (about 8 times), and incubated at RT for 3 min.

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- (5) 300 μ l ice-cold buffer A3 was added, and mixed gently by hand (about 8 times), before placing on ice for 5 min.
- (6) The ice-cold samples were centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) for 12 min at 4 °C. The upper phase was transferred into a fresh 2-ml micro-tube.
- (7) 20 μ l proteinase K was added to the sample, mixed gently by hand and incubated at 37 °C for 30 min.
- (8) 400 μ l phenol was added and mixed by inverting the micro-tubes by hand (about 30 times).
- (9) 400 μ l chloroform/isoamyl alcohol was added, and mixed by inverting (about 30 times).
- (10) The samples were then centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 5 min.
- (11) The upper phase was transferred to a Nucleospin® plasmid column and centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 1 min. The filtrate in the microtube was discarded.
- (12) 500 μ l buffer AW was added to the column and centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 1 min. The filtrate in the microtube was discarded.
- (13) 700 μ l buffer A4 was added and centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 1 min. The filtrate in the micro-tube was discarded.
- (14) The silica membrane was centrifuged at 13,000 rpm (Eppendorf centrifuge 5415D) for 2 min to dry.
- (15) The dried column was placed in a 1.5-ml micro-tube, then 30 μ l AE buffer was added to the column, and incubate at RT for 3 min. Plasmid DNA was collected in the 1.5-ml micro-tube by centrifugation at 13,000 rpm (Eppendorf centrifuge 5415D) for 1 min.
- (16) The concentration, purity, size, and integrity of the purified plasmid could be determined and analyzed, after digesting with appropriate restriction enzyme/s on agarose gel in comparison.
- (17) Plasmid solution was stored at -20 °C for further use.

2.7 Purification of PCR product from agarose gel

PCR products were purified from agarose gel by electrophoresis in 0.8 % TAE gels for 1 h at 74 V. Following electrophoresis, gels were stained with ethidium bromide and the bands were visualized using UV light (for short time). Bands containing the PCR fragment were excised and purified using “NucleoSpin® Extract II” kit (Macherey-Nagel GmbH & Co KG, Düren, Germany) according to the manufacturer’s directions.

2.8 Preparation of *E. coli* EC1000 competent cells

Preparation of competent cells of *E. coli* EC1000 was done according to **Hanahan *et al.*, 1991** with some modifications, as follows:

- (1) A loopful of *E. coli* strain EC1000 culture, from -80 °C stock culture, was directly inoculated into 500 ml of pre-warmed LB medium (supplemented with 40 µg ml⁻¹ kanamycin).
- (2) The culture was then incubated at 37 °C with vigorous shaking until OD_{620nm} of 0.3-0.4.
- (3) The culture was chilled on ice for 20 min.
- (4) The cells were collected by centrifugation, in ice-cold centrifuge bottles, at 5,000 rpm in a JA10 centrifuge rotor (centrifuge model: J2-21, Beckman, USA) for 20 min at 4 °C.
- (5) One volume, to the culture, of 10% glycerol (ice-cold) was used to re-suspend the cells. The suspension was then centrifuged at 5,000 rpm in a JA10 centrifuge rotor (centrifuge model: J2-21, Beckman, USA) for 20 min at 4 °C.
- (6) The last step was repeated 3 times.
- (7) Finally, the cells were resuspended in 1 ml ice-cold 10% glycerol by gentle swirling, and then 50 µl aliquots were dispensed into ice-cold 1.5-ml micro-tubes, flash frozen in a liquid nitrogen bath, and stored at -80 °C until further use.

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- (1) 1% overnight culture of *S. thermophilus* (in Belliker broth), was inoculated in 200 ml pre-warmed Belliker broth supplemented with 40 mM DL-threonine.
- (2) The culture was grown at 40 °C until an OD_{620nm} of 0.3-0.4. and then incubated on ice for 15 min.
- (3) The pellets were harvested by centrifugation for 15 min at 5,000 g at 4 °C.
- (4) The pellet then washed two times with pre-cold electroporation buffer (272 mM sucrose, 1 mM EDTA, 7 mM HEPES, pH 6.5, and 15% (v/v) glycerol).
- (5) Finally, the cells were re-suspended in 2 ml electroporation buffer, flash frozen in liquid nitrogen (in 100- μ l aliquots) and stored at -80 °C until further use.

2.10 Cloning**2.10.1 Dephosphorylation of insert DNA**

Shrimp Alkaline Phosphatase (SAP) (Fermentas GmbH, St. Leon-Rot, Germany) was used to dephosphorylate DNA inserts before ligation process, as recommended by the supplier as follows:

DNA (PCR product)	50 μ l
10x SAP buffer	10 μ l
Enzyme SAP	5 μ l
Nuclease-free water	35 μ l
<i>Final volume</i>	<i>100 μl</i>

The reaction solution was incubated at 37 °C for 60 min, and then the reaction was stopped by incubating at 75 °C for 15 min. Finally, the reaction was purified by "NucleoSpin® Extract II" kit (Macherey-Nagel GmbH & Co KG, Düren, Germany).

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Cloning into plasmids pGhost9 and pMG36e was performed according to **Sambrook *et al.*, 1989**. Cloning into plasmid pSTBlue-1 was carried out according to the supplied protocol of “pSTBlue-1 Perfectly blunt® Giga Cloning Kit” (Novagen). Ligation mix was dialyzed for 30 min before using for transformation assay.

2.10.2 Cloning of an internal fragment of *int* into pGhost9 (pMAZ2)

The vector pGhost9 has the thermo-sensitive replicon of pWVO1, isolated after mutagenesis of pGK12 (**Maguin *et al.*, 1992 & 1996; Kok *et al.*, 1984**). Hence, this vector can replicate extrachromosomally at the permissive temperature (28°C) in the target strain, while a temperature shift to more than 35°C forces integration. The replicon of this vector functions both in gram-positive and gram-negative bacteria. *E. coli* strain EC1000 bears a single copy of the wild type (w.t.) *repA* gene from pWVO1, and hence it can act as a helper strain for providing, *in trans*, RepA protein for replication at a non-permissive temperature.

To construct a vector for *int* gene knock out, a 518 bp of an internal fragment of *int*, Δint , was amplified by PCR using primers “int. f” with EcoRI site and “int. R” with HindIII site was cloned into EcoRI/HindIII sites of pGhost9 to get a recombinant vector named pMAZ2. It was transformed into *E. coli* EC1000 (see below) to get a high copy number from this vector. It could be easily isolated and analysed to determine the intact ones before transformation into the target cell, *S. thermophilus* J34f-2.

2.10.3 Cloning of *orf3* in the expression vector pMG36e

The pMG36e is a lactococcal expression vector (**van de Guchte *et al.*, 1989**) which has been constructed on basis of the cryptic lactococcal plasmid pWVO1 and its ori (origin of replication). The vector contains a promoter, P32, and the corresponding ribosomal binding site from the *Lactococcus lactis* subsp. *cremoris* Wg2 chromosomal DNA. Downstream

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from the P32 is the MCS (multiple cloning sites) followed by a terminator. Em^R is also present on the pMG36e.

To construct an *orf3* expression vector, a 481-bp fragment, containing the native *orf3* was amplified, without its native promoter, using primer-pairs "orf3 HindIII. R" and "orf3-C-f", blunted in the 5' and digested with HindIII in the 3' end. The digested fragment-DNA was then dephosphorylated to prevent self ligation and purified, as described above and was then cloned into pMG36e Smal/HindIII site. Thereby, *orf3* was inserted in correct orientation. Resulting vector was designated as pMZorf3.

2.11 Electro-transformation of *E. coli* EC1000

- (1) 5 µl of ligation mixture of DNA was microdialysed against deionized water at RT for 20 min.
- (2) About 100 ng DNA, from ligation mixture, was mixed well with 50 µl thawed competent cells on ice.
- (3) The cells-DNA mixture was transferred directly into an ice-cold, 0.1-cm gap-width cuvette (Eurogenetec GmbH, Köln, Germany), and then placed in the Electroporator cuvette-holder.
- (4) Electro-transformation was carried out, using Electroporator 2510 apparatus (Eppendorf AG, Hamburg, Germany), at 1250 V (capacitance: 25 µF, resistance: 200 Ω)
- (5) 1 ml SOC medium, at RT, was immediately added to the cuvette and mixed well by pipetting gently up and down.
- (6) The treated culture was transferred into sterilized test tube and incubated at 37 °C with gentle shaking for 1 h.
- (7) An appropriate volume of culture was plated onto LB agar plates containing the recommended antibiotics, and incubated at 37 °C for about 24 h.

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- (8) *E. coli* transformants were analyzed by plasmid isolation, and restriction endonuclease analysis.

2.12 Electro-transformation of *S. thermophilus* J34f-2

- (1) About 100 ng of ice-cold plasmid DNA was mixed, on ice, with 100 μ l of thawed competent cells.
- (2) The cells-DNA mixture was transferred directly into an ice-cold 0.1-cm gap-width cuvette (Bio-Rad Laboratories GmbH, München, Germany), and then placed in the Electroporator cuvette-holder.
- (3) Electro-transformation was carried out, using Electroporator 2510 apparatus (Eppendorf AG, Hamburg, Germany), at 2100 V (25 μ F, resistance: 200 Ω , time constant 3.2-3.6 ms).
- (4) Immediately after pulsing, 900 μ l of HJL broth medium was added to the cuvette and mixed well by pipetting gently up and down.
- (5) The cuvette including the treated culture was then incubated at 37 $^{\circ}$ C.
- (6) The treated culture was plated onto LM17 agar plates supplemented with the recommended antibiotic, and incubated at 30 $^{\circ}$ C (in the case of pGhost9 derivative) or at 40 $^{\circ}$ C (in the case of pMG36e derivatives) for 4-6 h for about 48 h.
- (7) Several single colonies were picked from the plates into LM17 broth tubes (supplemented with 1 μ g ml⁻¹ Er), and incubated for 24 h at 30 $^{\circ}$ C (in the case of pGhost9 derivative) or at 40 $^{\circ}$ C (in the case of pMG36e derivatives).
- (8) The isolates were then analyzed via extraction of the transformed plasmid, and restriction enzyme analysis.

2.13 Integration of pMAZ2 into chromosomal DNA of *S. thermophilus* J34f-2

The intact transformant, based on plasmid isolation and analysis, named *S. thermophilus* J34f-2 (pMAZ2), was stored in LM17 medium supplemented with 1 μ g ml⁻¹ Er at -80 $^{\circ}$ C.

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- (1) 1% (v/v) of overnight culture (*S. thermophilus* J34f-2 (pMAZ2) in LM17 supplemented with 1 $\mu\text{g ml}^{-1}$ Er) at 30 °C, was inoculated into LM17 tube in the absence of Er.
- (2) The culture was incubated in water bath at 30 °C until an OD_{620nm} of 0.2-0.25.
- (3) The culture was then shifted into 42°C and incubated to an OD_{620nm} of 1.0.
- (4) The culture was diluted in LM17 broth and plated onto LM17 agar plates supplemented with 1 $\mu\text{g ml}^{-1}$ Er, and incubated at 40°C for about 48 h.
- (5) Single colonies were picked into LM17 broth tubes (supplemented with 1 $\mu\text{g ml}^{-1}$ Er).
- (6) Integration of the pMAZ2 into the prophage DNA was further verified via PCR analysis, DNA sequencing, and Southern blot hybridization.

2.14 Isolation of a mutation in *orf3*

A mutation in *orf3* was performed randomly via curing the integrated vector from the integrant *S. thermophilus* f-2 J34 KO-*orf3* (Bokelmann, 2003) as follows:

- (1) 60 independent isolates from *S. thermophilus* KO-*orf3* (everyone was a loopful from the -80 °C stock culture) were inoculated separately into LM17 broth tubes in the absence of Er, and incubated at 40 °C till the stationary phase (or overnight).
- (2) 1% (v/v) culture was re-inoculated into a new LM17 broth tube, in the absence of antibiotic, and incubated at 40 °C till the stationary phase.
- (3) The last step was repeated about 40 times.
- (4) Appropriate amounts of each of the 60 culture were plated separately onto LM17 agar plates in the absence of Er.
- (5) About 100 colonies were picked from each isolate (6000 isolates from the sixty tubes).

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- (6) Every selected colony was plated onto a cell from 52 cells of each LM17 agar plates in the presence or absence of antibiotic. Isolates which could grow only in the absence of antibiotic might be vector-cured isolates.
- (7) To confirm the excision of integrated vector from *orf3* of TP-J34, multiplex PCR was performed using: i) primer binds to the host chromosomal DNA, "Primer 4", to pair with primer binds within *int*, "int. R", (to insure the presence of prophage), and ii) primer binds within *Itp* gene "*Itp-F*" (downstream to *orf3*) to pair with "pri. cl. R" in the *crh* (upstream of *orf3*), to confirm the excision of the integrated vector.
- (8) Mutations, within *orf3* afterwards, were detected via induction with MC and sequencing of *orf3* of supposed mutant.

2.15 DNA sequencing

DNA sequencing of PCR was done at MWG Operon, Ebersberg, Germany. Sequences were analyzed using DS Gene 1.5 software (Accelrys Limited, Cambridge, England).

2.16 Analysis of mutated *orf3* gene

To determine the effect of *orf3* mutations in both *S. thermophilus* J34-12 and J34 cu50, final product, sequences of those mutated *orfs* were analysed via translating into proteins using the *Genetic code* with respect to ATG as a start codon.

2.17 Sequencing of RT-PCR products within pSTBlue-1

RT-PCR were purified from agarose gel using "NucleoSpin® Extract II" kit (Macherey-Nagel GmbH & Co KG, Düren, Germany), and blunted by T4 polymerase. The blunted bands were ligated into EcoRV site of blunt vector pSTBlue-1 (Novagen) **Figure 2.2** using T4 ligase. The ligation mixtures were transformed into *E. coli* NovaBlue competent cells (Novagen)

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according to the protocol provided by the manufacturer. The transformed cells were then plated onto agar plates selective medium of blue/white screening (LB agar supplemented with 200 $\mu\text{g ml}^{-1}$ ampicillin, 1 M IPTG (isopropyl- β -D-thiogalactopyranosid), and 50 $\mu\text{g ml}^{-1}$ X-gal), and incubated at 37°C for about 24 h. This approach, named *blue/white colony screening assay*, distinguishing recombinant colonies (white) among non-recombinant one (blue). Next, the white colonies, which include the recombinant plasmid of RT-PCR products, were isolated. Plasmids were extracted and analysed after digestion with EcoRI on agarose gel to confirm that they carried the target insert. For sequencing, the insert, i.e., RT-PCR products, the recombinant plasmid was sequenced (MWG Operon, Ebersberg, Germany) using primers flanking the insert, "T7" and "SP6".

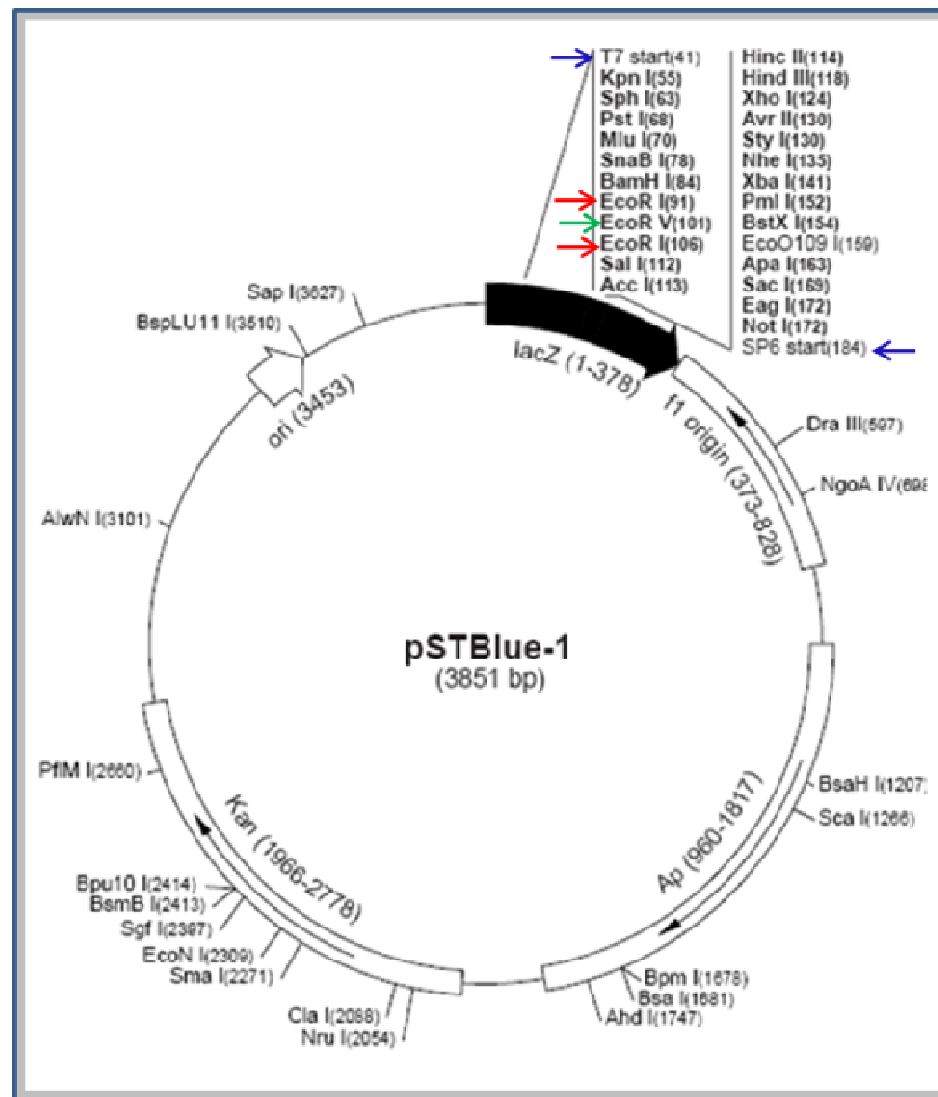


Figure 2.2. Map of the cloning vector pSTBlue-1 (Novagen). **EcoRI** sites are indicated by red arrows; **EcoRV**, used as a cloning site marked by green arrow; The primers of sequencing, "T7" and "SP6", are labelled by blue arrows.

2.18 Southern blot analysis

Southern blot has been one of the cornerstones of DNA analysis. It is a method routinely used for detection of a specific DNA sequence in DNA samples. DNA fragments from an agarose electrophoresis gel are first transferred onto a membrane and then detected by a labeled "probe". In other words, it comprises: i) transfer of electrophoresis-separated DNA

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fragments to a filter membrane, and ii) subsequent fragment detection by probe hybridization.

2.18.1 Procedure

2.18.1.1 Gel electrophoresis

- (1) Appropriate restriction enzymes were used to cut DNA into smaller fragments.
- (2) Restriction digested DNA samples were prepared (mixed with the loading dye) and run on 1% gel for 1 h at 74 V.
- (3) The gel was stained in an ethidium bromide bath for 20 min to visualize DNA fragments, and was then submerged in distilled water for 15 min.
- (4) The gel was photographed under UV light.

2.18.1.2 Blotting

- (1) The gel was immersed in 250 mM HCl for 5 min with slightly shaking at RT.
- (2) The gel was washed in autoclaved distilled water for 1 min.
- (3) The gel was submerged in denaturation solution 2 times for 15 min each at RT with gentle shaking. This treatment denatures the double-stranded DNA into single-stranded accessible for hybridization.
- (4) The gel was washed with distilled water for 1 min.
- (5) Neutralization was done, to the gel, using neutralization solution 2x 15 min at RT with gently shaking.
- (6) Blotting from the agarose gel to positively charged nylon membrane, was performed via *downward transfer assembly* (**Chomczynski, 1992**) as shown in **Figure 2.5** for 4 h using 20x SSC buffer.

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2.18.1.3 Fixation

The membrane was washed with 2x SSC for 1 min, dried on filter paper and fixed by baking in a hot air oven at 120 °C for 30 min. The membrane then can be stored at 4 °C until further use.

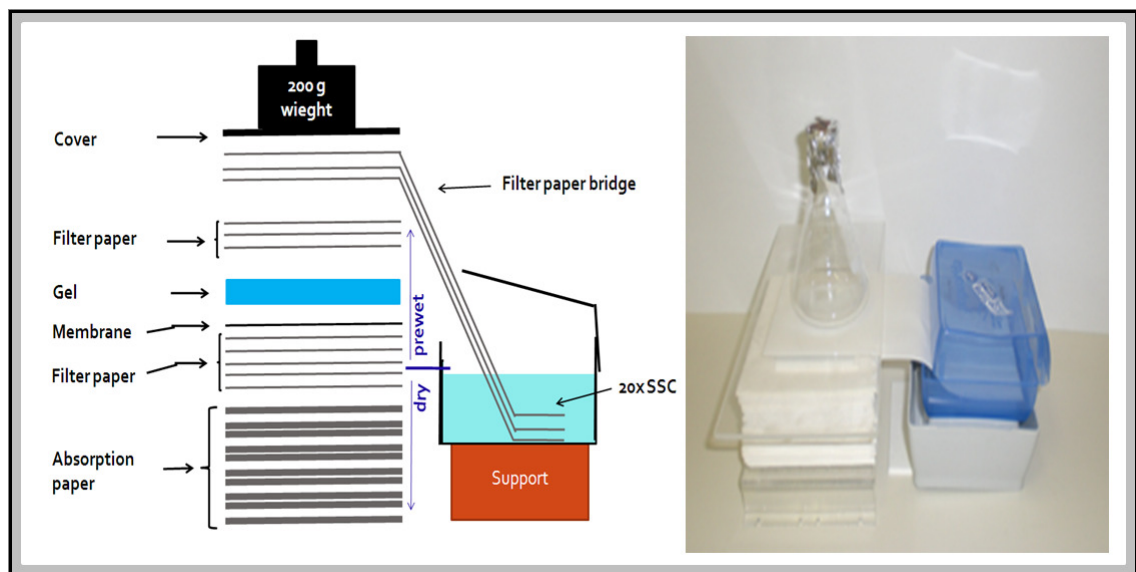


Figure 2.5. Downward transfer assembly (Chomczynski, 1992).

2.18.1.4 Pre-hybridization and hybridization

Pre-hybridization is important to lower background, and was done via blocking non-specific nucleic acid-binding sites on the membrane.

For prehybridization, 20 ml pre-warmed DIG-Easy Hyb solution (Roche) was filtered through 0.45 μm Millipore membranes, and used in a hybridization glass tube (Biometra GmbH, Göttingen, Germany) with gentle agitation at 42 °C for 2 h. Consequently, the solution was poured off. Immediately, hybridization was done, hence pre-warmed probe-DIG Easy Hyb solution (see below) was added to the membrane, and incubated overnight at 42 °C with gentle agitation in a hybridization oven (Biometra OV 3; Biometra GmbH, Göttingen, Germany).

2.18.1.5 Detection

- (1) The membrane was removed from the hybridization tube and washed twice for 5 min using washing buffer 1 (2x SSC, 0.1% SDS) with vigorous shaking at RT.
- (2) The membrane was washed twice using wash buffer 2 (0.1% SSC, 0.1% SDS) at 68 °C in water bath, for 15 min with shaking.
- (3) The membrane was submerged for 1 min in buffer 1 containing 0.3% Tween20 to equilibrate the membrane.
- (4) Blocking was performed by submerging the membrane in buffer 2 with gently shaking for 1 h.
- (5) The membrane was submerged in antibody solution (4 µl with 40 buffer 2) with gently shaking for 30 min.
- (6) The membrane was washed twice for 15 min each, using buffer 1 containing 0.3% Tween 20 with vigorous shaking to remove the excess of antibody.
- (7) The membrane was equilibrated by submerging in buffer 3 for 5 min.
- (8) The membrane was placed in between of transparent plastic sheet, and immediately fresh SCPD solution (5 µl SCPD mixed well with 2ml buffer 3) was dropped in the surface of the membrane, and incubated at RT between the pair of the transparency sheets for 5 min.
- (9) Finally, the membrane was dried on filter paper, sealed between a pair of transparency sheets and incubated at 37 °C for 15 min.
- (10) The membrane was exposed to an X-ray film (Fuji photo film GmbH, Düsseldorf, Germany) in dark for 30 min or more.
- (11) The film was developed by shaking in developing solution (Tetenal AG & Co. KG, Norderstadt, Germany) until bands appeared.
- (12) The film was submerged in distilled water for 1 min.

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(13) The film was fixed by shaking in a fixing solution (Adefo-Chemie GmbH, Nürberg, Germany) for 3 min.

(14) Finally, the film was washed with tap water and dried to be ready.

2.18.1.6 Stripping and reprobing of membrane

The membrane was washed in sterilized deionized water for 1 min, and then incubated twice for 10 min in alkaline probe-stripping solution (see below) at 37 °C. This incubation removes the alkali DIG probe. The membrane was finally rinsed thoroughly in 2x SSC. To confirm the stripping process, the membrane was exposed to X-ray film for 24 h, before developing.

2.18.2 Reagents**2.18.2.1 250 mM HCl solution**

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

2.18.2.2 Denaturation solution (1 l)

NaOH	20.00 g
NaCl	87.66 g

2.18.2.3 Neutralization solution (L)

Tris-Cl	66.55 g
NaCl	175.32 g
pH 7.5	

2.18.2.4 Transfer buffer (20x SSC-buffer (1 l))

NaCl	175 g
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Trisodium citrate 88 g
pH 7

Sterilized by autoclaving at 121 °C for 20 min.

2.18.2.5 20% (w/v) SDS

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

2.18.2.6 2x SSC

100 ml 20x SSC with 900 ml sterile deionized water.

2.18.2.7 RT washing buffer (2x SSC, 0.1% SDS)

5 ml of 20% SDS was added to 100 ml of 20x SSC and the volume made up to 1000 ml with sterile deionized water.

2.18.2.8 4 M lithium chloride (LiCl)

16.96 g LiCl was dissolved in a final volume of 100 ml deionized water and autoclaved at 121 °C for 20 min.

2.18.2.9 200 mM Na-EDTA

7.45 g Na-EDTA was dissolved in 80 ml deionized water. The pH was adjusted to 8.0 and the volume was filled up to 100 ml, and autoclaved at 121 °C for 20 min.

2.18.2.10 Buffer 1 (10x)

0.1 M malic acid, 0.15 M NaCl, and pH 7.5. Hence, 116.07 g malic acid, and 87.66 g NaCl were dissolved in 800 ml deionized water. The pH was adjusted to 7.5 with NaOH pellets. The volume is made up to 1000 ml deionized water and autoclaved at 121 °C for 20 min.

2.18.2.11 Buffer 1 (1x)

100 ml buffer1 (10) was deluted with 900 ml deionized water.

2.18.2.12 Washing buffer (1x buffer 1, 0.3% Tween 20)

3 ml Tween 20 was filled up to 1000 ml with Buffer 1 (1x).

2.18.2.13 Buffer 2 (10x)

50 g blocking powder was dissolved in 450 ml buffer 1 (1x) at 70 °C for 1 h. The buffer was divided into 100-ml aliquots and autoclaved at 121 °C for 20 min and stored at -20 °C until use.

2.18.2.14 Buffer 2 (1x)

One volume of buffer 2 (10x) was added to nine volume of buffer 1 (1x).

2.18.2.15 Buffer 3

100 ml of 1 M NaCl was mixed with 1 M Tris (pH 9.5) as follows.

- 1 M NaCl: 14.61 g was dissolved in final volume of 250 ml deionized water and autoclaved at 121 °C for 20 min.
- 1 M Tris: 60.55 g was dissolved in 400 ml 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 at 121 °C for 20 min.

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NaOH	8.0 g
SDS 20%	5.0 ml

2.18.2.17 Anti-Digoxigenin-AP

From (Roche Diagnostics GmbH, Mannheim, Germany):

Polyclonal sheep anti-digoxigenin, Fab-fragments, conjugated to alkaline phosphatase.

2.18.2.18 CSPD

(Roche Diagnostics GmbH, Mannheim, Germany).

2.18.2.19 DIG-DNA labeling kit

(Roche Diagnostics GmbH, Mannheim, Germany)

- Hexanucleotide mix (10x concentrated)
- DIG DNA labelling mix (10x concentrated)
- Klenow enzyme

2.18.2.20 DIG Easy Hybridization Solution

(Roche Diagnostics GmbH, Mannheim, Germany)

2.19 RNA manipulations

All mRNAs are an unstable molecules due to ribonucleases (RNases). RNases are very stable, do not require cofactors, are effective in very small quantities and are difficult to inactivate. Moreover, they are everywhere. Therefore, isolation and manipulation of RNA require specialized techniques and great care should be taken. Above and beyond, genomic DNA contamination in RNA solutions cause real problems in downstream applications such as Northern blot and RT-PCR.

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Presence of DNA residues in RNA solution can be detected by standard PCR. Hence, getting PCR product from appropriate primer-pairs (with RNA template) reveals that this sample is contaminated with DNA which is not only an obvious cause of complete failure in Northern blot due to DNA residues, but also they are one of the main reasons of RT-PCR false results. Thus, genomic DNA contamination in RNA samples must be eliminated before any of downstream applications.

RNAs were isolated, and purified using "RNase-free DNase set" from QIAGEN (Qiagen GmbH, Hilden, Germany). Its purity and integrity were confirmed as shown below. Northern blot hybridizations and RT-PCR were done under both lytic and lysogenic conditions under RNase-free conditions (see below).

2.19.1 Calculation of the amount of bacterial culture for RNA isolation

In order to obtain optimal yield and purity of RNA samples (by RNeasy® Mini Kit), the equation: **0.6/OD_{620nm} of *S. thermophilus* culture** was used, i.e. the amount of culture, at a specific time of bacterial growth, was determined via the last equation. For simplicity, one ml was taken from the culture at OD_{620nm} of 0.6.

2.19.2 RNase Decontamination

Northern blots were done under RNase-free condition such as solutions, equipment and the environment, as described by **Wilkinson, 1991** with some modification as follows:

- 0.1% (v/v) diethyl pyrocarbonate (DEPC) (AppliChem, Darmstadt, Germany) was added with care, under a fume hood, into deionized water or other solutions, incubated overnight at 37° C to destroy RNase, and finally autoclaved at 121 °C for 20 min to destroy the DEPC which converted into ethanol and CO₂.

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- Solutions containing Tris cannot be effectively treated with DEPC, because Tris inactivates DEPC. These solutions could be prepared using RNase-free water.
- Cleaned glassware and glass beads were baked at 200 °C for 12 h in hot air oven.
- Using sterile disposable polypropylene is recommended. These are generally RNase-free and hence can be used directly without prior treatment.
- Microfuge tubes and pipette tips were transferred from their original container to RNase-free containers, without directly handling, and autoclaved at 121 °C for 20 min.
- To decontaminate laboratory apparatus, surfaces, and equipment, RNase-ExitusPlus™ solution (AppliChem, Darmstadt, Germany) was used. The solution was added directly to surfaces, wiped thoroughly with a paper towel, for one min, rinsed with RNase-free water and dried with a clean paper towel. Apparatus were treated with a pre-wetted paper towel with that solution.

2.19.3 Protection of cellular RNA prior to isolation

This step was done prior to RNA isolation. **RNAprotect™ Bacteria Reagent** was used to protect RNA from either enzymatical degradation during handling and processing of bacterial cells.

1 volume of bacterial culture was added to two volume of 2x RNAprotect™ Bacteria Reagent (Qiagen GmbH, Hilden, Germany). The mixture was mixed well by vortexing for 10 s, and incubated at RT for 5 min. The cells then could be collected in 2-ml micro-tube by centrifugation at 14,000 rpm for 10 at RT. The collected cells were flash frozen in liquid nitrogen and stored at -20 °C (for up to 2 weeks, or at -70 °C for up to 4 weeks).

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2.19.4 Isolation of RNA

Total RNA was isolated from *S. thermophilus* J34 and its derivatives which were grown in LM17 in the presence or absence of 0.3 $\mu\text{g ml}^{-1}$ of MC at 40 °C using **RNeasy® Mini Kit** (Qiagen GmbH, Hilden, Germany) via enzymatic lysis, proteinase K digestion, and mechanical disruption of bacteria

2.19.4.1 Equipments and Reagents

- RNase-free water
- TE buffer (30 mM Tris-HCl, 1 mM EDTA, pH 8) containing 15 mg ml⁻¹ lysozyme.
- Ethanol (96-100%) and ethanol (80%)
- RNeasy mini kit buffer: RLT; RW1; RPE and. RNeasy Mini Spin Column.
- 14.3 M β -Mercaptoethanol
- 2-ml Safe-lock tubes.
- Cell Disruptor
- Acid-washed glass beads (see **2.19.9**)
- Proteinase K (>600 mAU/ml, solution)
- RNase-Free DNase Kit (Qiagen) includes RNase-free DNase I and RNase-free buffer RDD)
- Disposable gloves.
- RNase-free pipette tips
- Microcentrifuge
- Vortexer
- Incubator

2.19.4.2 Procedure

Total RNAs of *S. thermophilus* J34 and its derivatives were isolated using the supplier protocol with RNeasy mini kit: “Enzymatic lysis, Proteinase K Digestion, and Mechanical Disruption of Bacteria” with some modifications as follows:

- (1) 20 μl proteinase K was mixed with 100 μl TE buffer containing lysozyme. The mixture was added to the pellet. Carefully, the pellets were resuspended by pipetting up and down.

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- (2) The suspension was vortexed for 10 s and incubated at RT for 20 min. During incubation, vortexing was repeated every 2 min for 10 s.
- (3) 800 μ l buffer RLT (including β -mercaptoethanol 10 μ l per ml RLT buffer) was added and mixed by vortexing for 10 s.
- (4) The suspension was transferred into 2-ml Safe-Lock tube containing 50 mg acid-washed glass beads (see 2.19.9).
- (5) The cells were disrupted in the FP120 Fastprep Cell Disruptor (Bio 101 Savant Instruments, Inc. USA) for 30 s, which was repeated 10 times with 1-min intervals.
- (6) The suspension was centrifuged at maximum speed for 10 s, and the supernatant (about 760 μ l) was transferred into a new 2-ml tube.
- (7) To the supernatant, 600 μ l ethanol (80%) was added, and mixed well by pipetting.

2.19.5 Purification of RNA

- (1) Up to 700 μ l lysate was transferred to an RNeasy Mini Kit spin column placed in a 2-ml collection tube, and centrifuged for 15s at maximum speed. The flow through was discarded. This step was repeated with the rest amount of lysate.
- (2) 700 μ l buffer RW1 was added to the spin column, and centrifuged for 15 s maximum speed to wash the spin column membrane. Discard effluent.
- (3) RNeasy spin column was placed in a new 2-ml collection tube, and then 500 μ l buffer RPE was added to the spin column. The spin was centrifuged for 15 s to wash the membrane. Discard effluent.
- (4) 500 μ l buffer RPE was added to the spin column, and centrifuged for 2 min at maximum speed. Discard effluent.
- (5) RNeasy mini spin column was placed in a new 1.5 ml collection tube, 50 μ l RNase-free water was added directly to the spin column membrane and centrifuged for 15 s at maximum speed. Discard effluent.

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- (6) To get more concentration of RNA solution, the last step was repeated by re-adding its eluate to the column and centrifuged. Discard effluent.
- (7) The integrity and purity of RNA solution were detected by running on agarose formaldehyde gel and performing standard PCR, respectively (as described **2.19.7**). Discard effluent.
- (8) RNA solutions were stored at -80 °C until further use.

2.19.6 Removing contaminating genomic DNA

To remove genomic DNA, RNase-free DNase set from QIAGEN (Qiagen GmbH, Hilden, Germany) was used as recommended by supplier with some modifications as follows:

- (1) 50 µl RNA solution (contaminated with genomic DNA) was mixed with 35 µl buffer RDD, and 15 µl DNase I stock solution and diluted for 200 µl final volume. The mixture mixed by gently inverting the tube by hand several times with care, and centrifuge briefly to collect residual liquid from the sides of the tube.
- (2) The mixture was incubated at 25 °C for 10 min.
- (3) 350 µl of buffer RLT was added and mixed well.
- (4) 250 µl ethanol (96-100%) was added and mixed well by pipetting.
- (5) 700 µl of sample was transferred to an RNeasy Mini spin column placed in a 2 ml collection tube, and centrifuged for 15 s at maximum speed. Discard effluent.
- (6) 500 µl buffer RPE was added to the spin column, and centrifuged for 15 s at maximum speed to wash the spin column membrane. Discard effluent.
- (7) 500 µl buffer RPE was added to the spin column, and centrifuged for 2 min at maximum speed to wash the spin column membrane. Discard effluent.

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- (8) The RNeasy spin column was placed in a new 1.5 ml collection tube. 50 μ l RNase-free water was added and centrifuged for 1 min at maximum speed to elute the RNA.

2.19.7 Detection of RNA integrity and purity

RNA integrity was assessed. Intact RNA forms two sharp bands (23 S rRNA = 2904-bp long and 16 S = 1542-bp long) on agarose formaldehyde gel (**Figure 2.6/A** left-hand). The purity from DNA genome contamination was determined by performing standard PCR with primer-pair binding within the genome. Pure RNA solution appears negative with PCR as shown in **Figure 2.6/A** right-hand. For detection of integrity, RNAs samples were mixed well with the same volume of 2x RNA loading dye solution (Fermentas GmbH, St. Leon-Rot, Germany). The samples were then treated as described below in Northern blot. RNA purity from DNA contamination was detected by performing standard PCR using RNA solution as template. PCR products were

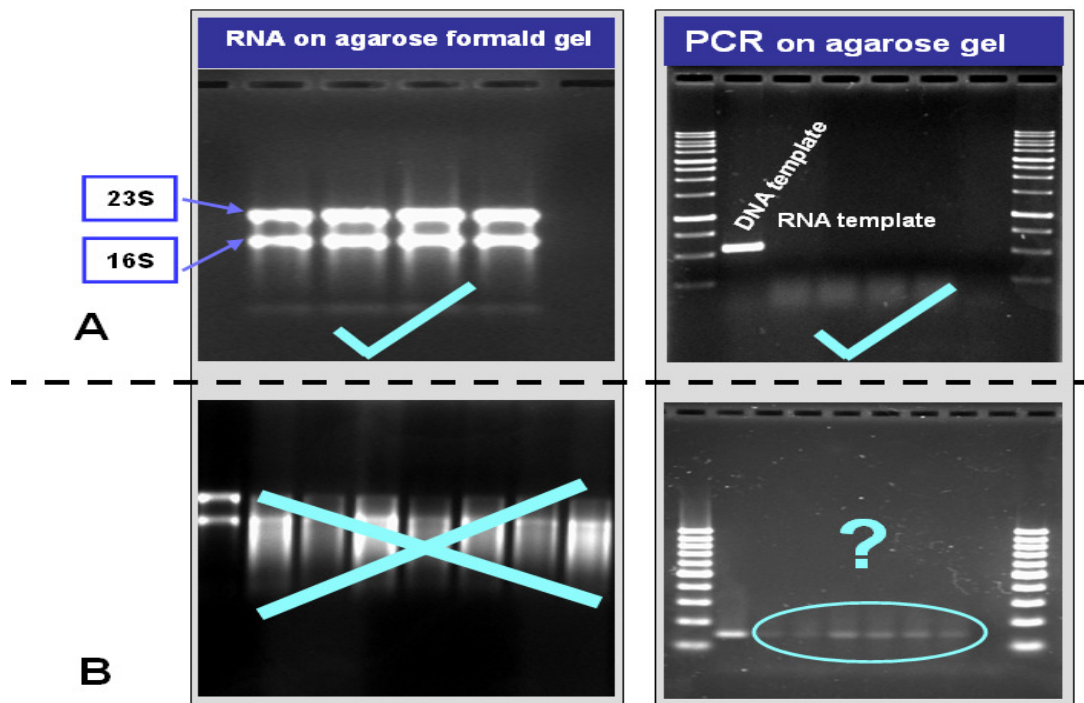


Figure 2.6. The integrity and purity of RNAs solutions. Section A: Top-left, intact RNA samples in agarose formaldehyde gel (16S and 23S are clear and sharp); Top right, agarose gel of standard PCR products using RNA as template. Absence of PCR product with RNA samples reveals that they

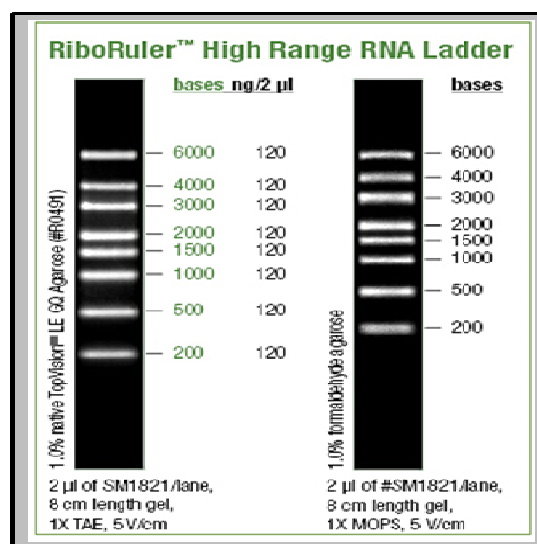
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are pure from DNA contamination; Section B, comprises both degraded RNA (left), and contaminated RNA with DNA (right).

2.19.8 Quantification of RNA solutions

RNA quantification was done using “RiboRuler™ RNA Ladder, High Range” (Fermentas, Germany). The samples were loaded in parallel with a defined amount of RNA Ladder on 1% agarose formaldehyde gel (as recommended by the supplier). By comparison, the ladder could be used for determination of RNA amounts (**Figure 2.7**).

Figure 2.7. RiboRuler™ RNA Ladder, High Range” (Fermentas, Germany), provides accurate values of RNA concentration in each ladder band.



2.19.9 Preparation of acid washed glass beads (for RNA isolation)

150–600 µm-diameter glass beads were prepared to use in RNA isolation (see **2.19.4**) from *S. thermophilus* as follows:

- The glass beads were submerged in acid solution (2:1 (v/v) HCl 37% to deionized water).
- The acid solution was filtered from the beads.
- The beads were washed with deionized water and filtered (repeated 40 times), and then submerged in deionized water for 24 h.
- The beads were filtered, dried on air and finally sterilized at 200 °C for 12 h in a hot air oven.

*Materials & Methods***2.20 Northern blot****2.20.1 Reagents****2x RNA loading dye solution** (Fermentas GmbH, St. Leon-Rot, Germany)

95% formamide	0.025% SDS
0.025% bromophenol blue	0.025% xylene cyanol FF
0.025% ethidium bromide	0.5 mM EDTA

2.20.1.1 MOPS 10x (1 l)

MOPS	83.71 g
Sodium acetate	0.82 g
EDTA	3.72 g
pH 7.0	
Autoclaved at 121°C for 20 min and stored in dark	

Running buffer for Northern blot (MOPS 1x)

100 ml MOPS (1x) mixed in 900 ml DEPC-H₂O

1% agarose formaldehyde gel (100 ml)

Agarose	1 g
DEPC-H ₂ O	72 ml
MOPS 10x	10 ml
Formaldehyde (37%)	18 ml

2.20.2 Procedure**2.20.2.1 Gel preparation**

- Agarose was mixed in 1x MOPS solution and melted in a microwave oven until completely dissolved. The dissolved mixture was cooled down to 60-70 °C before adding 18 ml formaldehyde. The agarose

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formaldehyde gel was then poured into a gel tray in a fume hood, and an appropriate comb was placed on the melted agarose.

- After solidification of the gel, a thin layer of 1x running buffer was poured over the surface before removing the comb to get intact and smooth wells.

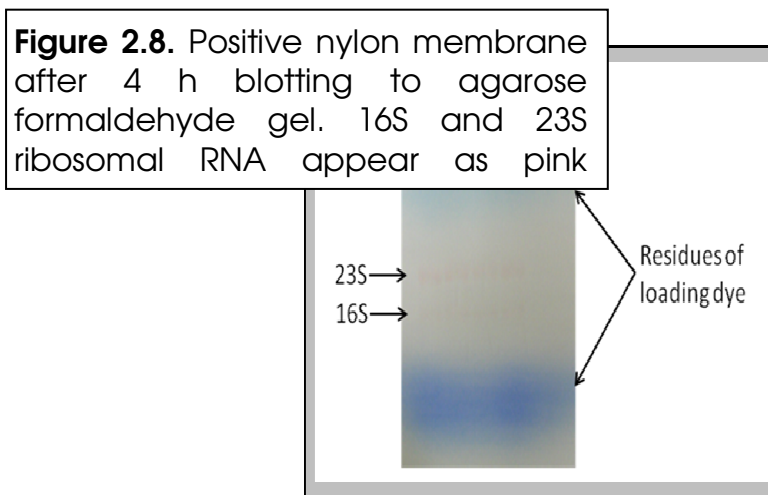
2.20.2.2 Preparation RNA samples for running, gel run and photographing

- (1) About 1 µg of total RNA sample was mixed well with equal volumes of 2x RNA loading dye solution (Fermentas GmbH, St. Leon-Rot, Germany), vortexed briefly and spanned down to collect the mixture.
- (2) The mixture was heated at 70°C in a thermo block (Thermomixer 5436, Eppendorf) for 10 min.
- (3) The samples were chilled quickly on ice and loaded on gel.
- (4) The gel was run in fume hood at 74 V for 90 min, using MOPS 1x as a running buffer.
- (5) The gel was soaked 3 times for 20 min each in DEPC-water (to remove formaldehyde)
- (6) The gel was photographed alongside with a fluorescent ruler for identification of band positions in subsequent X-ray film.

2.20.2.3 Blotting, baking, and detection

Blotting was performed for 4 h using the downward transfer assembly (**Chomczynski, 1992**), as shown in Fig. 2.5.

- Before removing the membrane from agarose formaldehyde gel, the wells positions of the gel were marked on the membrane using a pencil (so that the wells position could be seen later on the X-ray film)
- The membrane was pulled off the gel, in one motion, using forceps.

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- The membrane was rinsed in 2x SSC for one min to remove any agarose, at this step the two bands of rRNA (23- and 16S) can be observed as pink bands as shown in **Figure 2.8**.
- The membrane was baked, pre-hybridized, hybridized with a target probe, and treated as the steps of Southern blot (see **1.18.1.2-5**), but under RNase free condition.

2.21 Double-stranded DNA probe**2.21.1 DIG labeling DNA probes**

Probes were prepared by the random priming method. Labeling of target DNA to be used as a probe in hybridization experiments, either for Southern or Northern hybridization, was performed, using DIG DNA labeling Mix (Roche Diagnostics GmbH, Mannheim, Germany), as follows:

- (1) 1 μg DNA was denatured, by boiling, in water bath for 10 min.
- (2) Rapidly, DNA solution was chilled on ice/NaCl mixture.
- (3) 10 μl Hexanucleotide mix (10 x), 10 μl DIG DNA labelling mix (10 x), and 5 μl Klenow enzyme were added, on ice, to the denatured DNA, and the volume was completed up to 100 μl using deionized water.
- (4) The contents were mixed well and collected from the tube's wall by centrifugation for some seconds.

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- (5) The solution was incubated for about 20 h at 37 °C.
- (6) 10 µl of 200 mM Na-EDTA (pH 8.0) was added to stop the reaction.
- (7) 10 µl LiCl was added, followed by adding 300 µl of pure ice-cold ethanol.
- (8) The sample was incubated either at -70 °C for 30 min or at -20 °C for 2 h.
- (9) The sample was centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) at 4 °C for 15 min.
- (10) The supernatant was discarded, and 500 µl of 70% ethanol was added to wash the pellet.
- (11) The sample was centrifuged at 14,000 rpm (Eppendorf centrifuge 5417R) at 4 °C for 15 min.
- (12) The supernatant was discarded, and the DNA pellet was air-dried for about 10 min.
- (13) 50 µl of TE buffer was added to dissolve the labelled DNA.
- (14) The labelling efficiency was determined.

2.21.2 Determination of labeling efficiency of probes

1. The efficiency of the probe labeling was determined by comparison with a DIG labeled control DNA. Hence a series of dilutions prepared from the DIG-labeled probe, in TE buffer, pH 8.5, were used (see **Table 2.5**).
2. These serial dilutions were spotted, 1 µl from each, on a piece of positively charged nylon membrane. In parallel to these spots, on the same membrane, 1 µl from known concentrations (A-F) of DIG-labeled DNA control were spotted. The DNA was fixed by baking at 120 °C for 30 min in a hot air oven.
3. The membrane was incubated for one min in buffer 1 containing 0.3% Tween 20 at RT.
4. The membrane was submerged in buffer 2 with shaking at RT for 10 min (blocking).

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5. Antibody treatment: the membrane was submerged in an antibody solution, 1 μ l of anti-digoxigenin in 5 ml of buffer 2, in a Petri dish and manually shaking for 10 min.
6. The excess antibody was removed by shaking the membrane vigorously twice for 5 min in 50 ml buffer 1 containing 0.3% Tween 20.
7. The membrane was incubated for 1 min in buffer 3.
8. The membrane was laid inside a pair of rectangular transparent sheets, larger size than the membrane.
9. CSPD solution treatment: 5 μ l CSPD (Leitz®, 0.08 mm/colorless: Esselte Leitz GmbH & Co KG, Stuttgart, Germany) mixed in 500 μ l buffer 3, was poured on the membrane in a transparent plastic sheet, and incubated at RT for 5 min.
10. The membrane was dried on a filter paper (not completely dry).
11. The membrane was sealed between two transparency sheets.
12. The membrane was incubated at 37 °C for 15 min.
13. The membrane was exposed to X-ray film (Fuji Photo Film GmbH, Düsseldorf Germany) and left in dark for 30-60 min.
14. X-ray film was developed by shaking in a developing solution (Tetenal AG & Co. KG, Norderstedt, Germany) for about 5 min, washed in deionized water for one min and finally fixed by shaking in a fixing solution (Adefo-Chemie GmbH, Nürnberg, Germany) for about 5 min. The X-ray film was then washed with tap water and dried on air.

15. **Table 2.5.** Serial dilutions of probe and control DNA

Serial	Dilution	final concentration
A	2 μ l DNA + 38 μ l TE (1:20)	0.25 0 ng
B	5 μ l A + 45 μ l TE (1:200)	25.0000 pg
C	5 μ l B + 45 μ l TE (1:2000)	2.5000 pg
D	5 μ l C + 45 μ l TE (1:20 000)	0.2500 pg
E	5 μ l D + 45 μ l TE (1:200 000)	0.0250 pg
F	5 μ l E + 45 μ l TE (1:2000 000)	0.0025 pg

2.21.3 Preparation of probe solution for use

Probe solution was prepared for use by boiling the double-stranded DNA probe at 100 °C for 10 min in water bath, and then rapidly chilled on ice mixed with NaCl for 3-5 min. The desired amount of probe (about 200 ng probe DNA) was mixed with 10-ml pre-warmed DIG-Easy Hyb solution, and used in the hybridization.

2.21.4 Storage and reuse of hybridization solution

The probe was reused several times; hence the hybridization solution still contained large amounts of unannealed DIG-labeled probe. So, the solution was poured into a plastic tube and stored at -20 °C till further use.

2.22 Reverse transcription PCR (RT-PCR)

RT-PCR was performed using a one-step RT-PCR kit from QIAGEN (Qiagen GmbH, Hilden, Germany), and appropriate primer-pairs. All steps and reactions were under RNase-free condition. As a positive control, the efficiency of primer-pairs was determined using an appropriate standard PCR programs, with DNA as a template.

2.22.1 Reactions solution

(all set up on ice)

5x QIAGEN OneStep RT-PCR Buffer	5.0 μ l
dNTP Mix (containing 10 mM of each dNTP)	1.0 μ l
Primer A (10 pmol μ l ⁻¹)	1.5 μ l
Primer B (10 pmol μ l ⁻¹)	1.5 μ l
QIAGEN OneStep RT-PCR Enzyme Mix	1.0 μ l
RNA (or DNA as a positive control)	0.5 μ l
RNase-free water	14.5 μ l
<i>Final volume</i>	<i>25.0 μl</i>

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2.22.2 RT-PCR programs

Programs of RT-PCR were performed in a Thermocycler (Mastercycler 5333; Eppendorf AG, Hamburg, Germany). The Thermocycler was preheated to 50 °C before placing samples in it as in **Table 2.6**.

Table 2.6. Thermal cycler program used in RT-PCR

Step	Temperature	Time (min)	Number of cycles
1	50 °C	30	1
2	95 °C	15	1
3	94 °C	1	30
4	5 °C below T _m of primers	1	
5	72 °C	1	
6	72 °C	10	1
7	4 °C	Hold	

2.23 Media and buffers

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

Peptone	1.0% (w/v)
Yeast extract	0.5% (w/v)
NaCl	1.0% (w/v)
pH 7.0	

SOB (Hanahan, 1985)

Tryptone	2.0%	(
		w
		/
		v
)
Yeast extract	0.5%	(
		w

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		/
		v
)
NaCl	10.0 mM	
KCl	2.5 mM	
MgCl ₂	10.0 mM	
pH 6.8-7.0		

SOC

SOC medium is SOB medium supplemented with 20 mM glucose.

2.23.2 Belliker broth

Tryptone	2.00% (w/v)
Yeast extract	0.50% (w/v)
Gelatin	0.25% (w/v)
Dextrose	0.50% (w/v)
Sucrose	0.50% (w/v)
Lactose	0.50% (w/v)
Ascorbic acid	0.05% (w/v)
Beef extract	1.00% (w/v)

HJL-broth (Stingele & Mollet 1996)

Tryptone	3.0%
	(w/v)
Yeast extract	1.0%
	(w/v)
KH ₂ PO ₄	0.5%
	(w/v)
Beef extract	0.2%
	(w/v)
Lactose	1.0%
	(w/v)
pH 6.5	

LM17 (for *S. thermophilus*) (Krusch *et al.*, 1987)

Phytone peptone	0.50%
	(w/v)
Polypeptone	0.50%
	(w/v)
Beef extract	0.50%
	(w/v)
Yeast extract	0.25%

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Ascorbic acid	(w/v)
	0.05%
MgSO ₄ . 7H ₂ O	(w/v)
	0.05%
Na-β-glycerophosphate	(w/v)
	1.90%
Lactose	(w/v)
	0.50%
	(w/v)

All media were sterilized by autoclaving at 121 °C for 20 min.

2.23.3 □□ strength Ringer's solution

1 Ringer's tablet (Merck) was dissolved in 500 ml distilled water, and sterilized in the autoclave at 121 °C for 15 min.

Antibiotics and other additives used for the media**Table 2.7.** Antibiotics and other additives used with the media

Antibiotics or additives	Final concentration in medium		Dissolving agent/ Storage temperature
	<i>S. thermophilus</i>	<i>E. coli</i>	
Ap (20 mg ml ⁻¹)	-	200 ìg ml ⁻¹	Deionized water/-20 °C
Er (10 mg ml ⁻¹)	1-2 ìg ml ⁻¹	150 ìg ml ⁻¹	Ethanol 99.9 % /-20 °C
Kan (10 mg ml ⁻¹)	-	40 ìg ml ⁻¹	Deionized water/-20 °C
MC (20 µg ml ⁻¹)	0.2-0.6µg ml ⁻¹		Deionized water/-20 °C
IPTG (200 mg ml ⁻¹)	-	250-350 µg ml ⁻¹	Deionized water/-20 °C
X-Gal (100 mg ml ⁻¹)	-	50 µg ml ⁻¹	100% dimethylformamide (DMF)/-20 °C

Materials & Methods**2.23.4 Electroporation buffer for *S. thermophilus* (Sun, 2002)**

Sucrose	272 mM
EDTA	1 mM
Hepes	7 mM
Glycerol	15%
	(v/v)
pH 6.5	

PCR buffer 10x (Fermentas GmbH, St. Leon-Rot, Germany)

Tris-Cl	200 mM (pH 8.8 at 25 °C)
(NH ₄) ₂ SO ₄	160 mM
MgCl ₂	15 mM

The buffer was autoclaved for 20 min at 120 °C and stored at -20 °C in 1-ml aliquots

3 Chapter 3: Results

Mutations in TP-J34 lysogeny module

3.1 Abstract

Inactivation of genes is an important step to determine their function. Integrase (*int*) is one of the lysogeny module genes of temperate *S. thermophilus* phage TP-J34. As well known, integrase plays a role in the integration process of phage DNA into the host genome. To find out whether the *int* gene of TP-J34 is needed also in the excision process, the gene was inactivated by integration of a pGhost9 derivative (carrying an internal fragment of *int* gene) into the chromosome of the lysogenic strain *S. thermophilus* J34f-2. Integration was verified by: i) Southern blot analysis, ii) PCR, and iii) sequencing. The results revealed that knock out of *int* resulted in: i) prevention of induction by MC, this finding evidenced that *int* gene is required in induction of prophage TP-J34, and ii) reduction of growth rate of the host culture.

Next, the molecular events leading to the inability of mutant TP-J34-12 prophage to excise from the host chromosome have not yet been clarified (**Neve *et al.*, 2003**). The lysogeny module of this prophage comprising *attL*, *int*, *ltp*, *orf3*, *crh*, *cro*, and *ant* was amplified by PCR and sequenced. The alignment of this region of TP-J34-12 with that of w.t. TP-J34 showed identity except for just one single point-mutation within *orf3*, a gene for a putative metalloproteinase. It results in a frame-shift causing premature termination of translation (TGA) after 43 codons instead of the 122 in the intact gene. This supported the earlier finding of **Bockelmann, 2003** that knock out of *orf3* resulted in loss of induction. Further support is provided in this chapter by: i) curing of the integrated vector from *S. thermophilus* J34 KO-*orf3* which restored induction (as in w.t. strain), ii)

isolation of a non-inducible *S. thermophilus* J34 cu50, revealing a point mutation in *orf3*, resulting in the amino acid replacement D29A, and iii) sub-cloning of intact *orf3* gene in lactococcal expression vector pMG36e and transformation into *S. thermophilus* J34 cu50, converting its phenotype from noninducible to inducible. All results clearly verify the pivotal role of Orf3 in lytic development of prophage TP-J34. It was supposed that Orf3 may exert its function by binding to *cro* to start lytic development. The results revealed that Orf3 of TP-J34 affected the growth type, aggregation or homogeneous, of the host cell in liquid medium. However, not all Orf3 mutants have the same effect. Although, mutant *S. thermophilus* J34-12 grows homogeneously more than the w.t. strain, both mutant *S. thermophilus* J34 KO-*orf3* and *S. thermophilus* J34 cu50 revealed more aggregation than the w.t.

3.2 Introduction

Sun, 2002 revealed that knock out of *ltp* gene of prophage TP-J34 resulted in delayed lysis phenotype and might be due to a polar effect of the *ltp* disruption. Also, **Bokelmann, 2003** found that knock out of *orf3* of prophage TP-J34 caused prevention of induction with MC, and the cause might be due to a polar effect of the integrated vector which blocks P2 promoter from *int* gene. Moreover, **Neve et al., 2003** pointed out that mutant *S. thermophilus* J34-12 is a noninducible derivative for unknown reason. Thus, this chapter was employed to find out two points i) the relationship between *int* gene of TP-J34 and induction process, and ii) to analyse mutations of *S. thermophilus* J34-12 lysogeny module which convert this derivative to be non-inducible.

Integrases are enzymes that mediate unidirectional site-specific recombination between the phage attachment site (*attP*) and the bacterial attachment site (*attB*). Attachment sites are generally stretches

lysogeny module

of almost identical bases in the genomes of the phage and its host bacteria, respectively. After integration, the prophage will be flanked by two hybrid sites, left (*attL*) and right (*attR*), each consisting of half of the *attP* and half of the *attB* sequence. However, in excisive recombination, which can be induced by various environmental factors, these two hybrid sequences are used together with integrase and/or another phage protein called excisionase (**Figure 3.1**). Phage DNA is then released from the host chromosome and lytic development can start. Some integrases act autonomously, while others require help of other phage proteins and/or bacterially encoded host factors. Concerning their site-specificities, integrases can be grouped into two major families: i) *tyrosine* recombinases utilize catalytic tyrosine to mediate strand cleavage. They tend to recognize longer *attP* sequences and require other proteins encoded by the phage or the host bacteria. λ and coliphage P2 integrases are examples for this family. Phage λ integration needs in addition to the core *att* sites of 21 bp, additional flanking regions of the *attP* site of ca. 200-bp (**Landy, 1989; Sylwan, et al., 2010**), and ii) *serine* recombinases use a catalytic serine for strand cleavage. They recognize shorter *attP* sequences, of about 40 bp, and do not require host factors. Integrase of phages phiC31, TG1 and TP901-1 are examples of this family (**Frumerie et al., 2008; Keravala et al., 2009; Groth & Calos, 2004; Morita et al., 2009a & b**). Int of TP-J34 seems to be related to use serine recombinase because it recognizes shorter *attP* sequence of 40 bp (exactly with that of *attB*). Some investigations have shown that integrase also carries out the excision of the prophage from the host genome (**Fadeev et al., 2009; Groth & Calos, 2004; Sentchilo et al., 2003**). The properties of integrases, i.e., site-specificity and efficiency, make them excellent tools for the manipulation of DNA in many areas like gene therapy. Hence, numerous studies have demonstrated the effectiveness of phiC31 integrase as a gene therapy vector system in human and animal models as well as a useful tool for genome engineering in plants

(Kempe *et al.*, 2010; Keravala *et al.*, 2009; Thomson *et al.*, 2010; Wang *et al.*, 2010; Woodard *et al.*, 2010). The same finding was observed with the integrase gene of the lactococcal phage TP901-1 (Stoll *et al.*, 2002) and with the phage P2 integrase (Frumerie *et al.*, 2008).

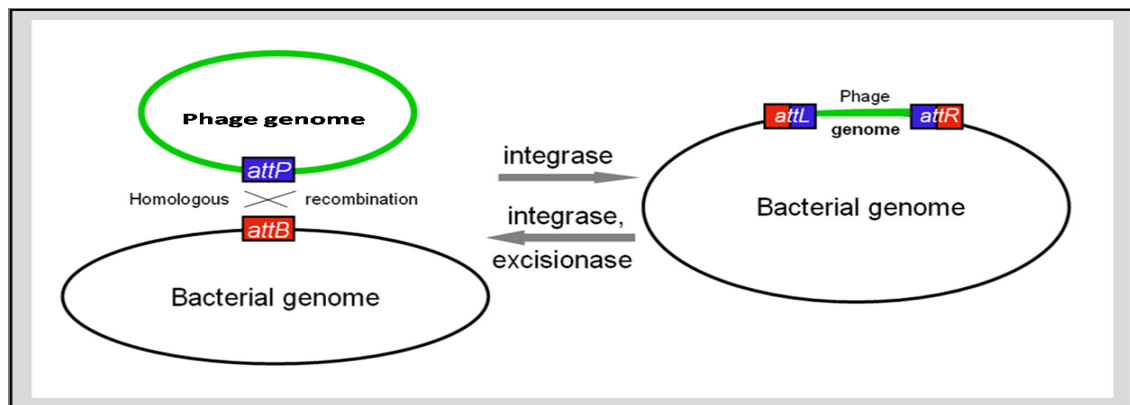


Figure 3.1 Schematic representation of integration/excision reaction. The attachment sites of both, *attP*, blue box, and of bacterium, *attB*, red box are shown. The hybrid *attL* and *attR* (blue/red boxes) flank the integrated prophage. Integration requires both *att* attachment sites and integrase. However, excision may additionally require excisionase (modified from Groth & Calos, 2004).

The *int* gene of TP-J34 is identical to that of phage Sfi21, and reveals a high degree of similarity to integrases of *Lactococcus lactis* phages (Neve *et al.*, 1998). Phage TP-J34 integrates its genome into the tRNA^{Arg} gene of the chromosomal DNA, exactly as described for phage Sfi21 (Ali, 2009; Bruttin *et al.*, 1997; Neve *et al.*, 1998).

Three knock out mutations in the lysogeny module genes of prophage TP-J34 were constructed via crossing-in of recombinant vectors (an internal fragment of a gene of interest was cloned into pGhost9) into the genes of interest, i.e., *int* (this study), *Itp* (Sun, 2002), and *orf3* (Bokelmann, 2003) as summarized in Figure 3.2. Knock out of either *int*- or *orf3*- genes caused non-inducibility of its derivatives, named *S. thermophilus* KO-*int* (This work) and *S. thermophilus* KO-*orf3* (Bokelmann, 2003), respectively. However, knock out of *Itp* gene derivative, *S. thermophilus* J34 KO-*Itp*, displayed a delayed lysis phenotype upon induction with mitomycin C (Sun, 2002). Relationship

lysogeny module

between prophage TP-J34 and growth shape of the host strain *S. thermophilus* was investigated by Neve *et al.*, 2003 who revealed that prophage caused homogenous growth of the lysogen *S. thermophilus* J34 in the broth medium. Otherwise, Bokelmann, 2003 pointed that knock out of *orf3* gene of prophage TP-J34 resulted in aggregation growth in the liquid medium. However, our results revealed that not all mutations in *orf3* have the same effect on growth phenotype of the host culture.

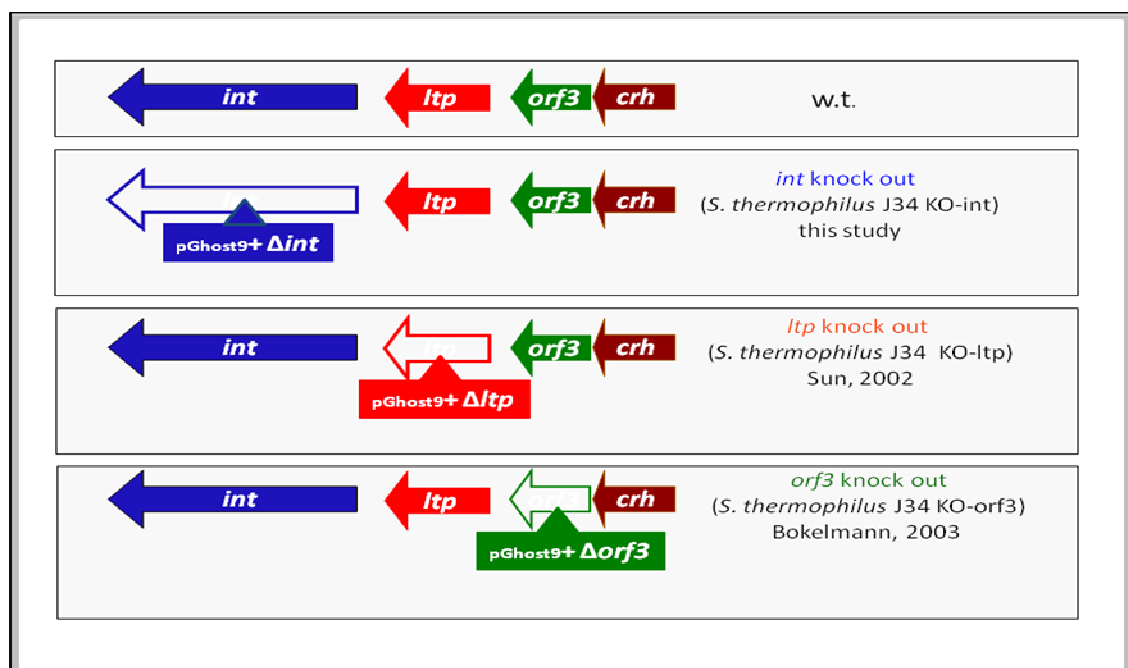


Figure 3.2. Schematic representation of constructed mutations in prophage TP-J34 lysogeny module, performed by crossing-in recombinant plasmids (an internal fragment, Δ , of the gene of interest cloned into pGhost9). The disrupted gene in each mutant is shown as unfilled arrows.

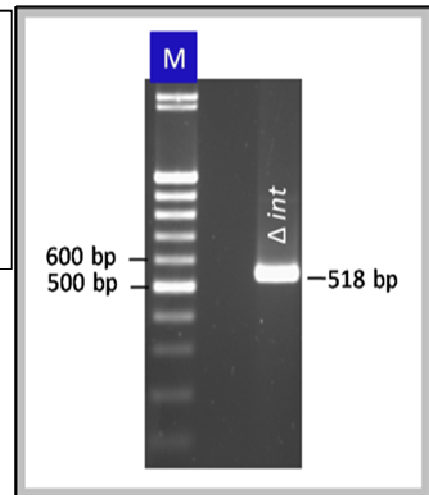
3.3 Inactivation of *int* gene

3.3.1 Construction of the integration vector pMAZ2

A 518-bp internal fragment of the *int* gene (Δ *int*) flanked by HindIII and EcoRI restriction sites, was amplified by PCR (**Figure 3.3**) using primer-pairs "int. F" and "int. R" (**Figure 3.4**). PCR primers and programs are

described in Materials & Methods. The recombinant vector, named pMAZ2, was constructed by ligating Δint into the EcoRI/HindIII sites of pGhost9 (**Figure 3.4**) and the ligated product was transformed into *E. coli* EC1000. Subsequently, plasmid DNA was isolated from 14 independent *E. coli* colonies, and analyzed on agarose gel. For unknown reason/s, deletions of various sizes in the vector DNA were observed in 13 out of 14 analyzed transformants. However, the remaining plasmid, pMAZ2, was found to harbour the correct Δint fragment (**Figure 3.5**).

Figure 3.3. PCR product of Δint , 518-bp, using primers "int. F" and "int. R". Sizes are noted in bp; M, 100-bp size marker (AppliChem, Darmstadt,



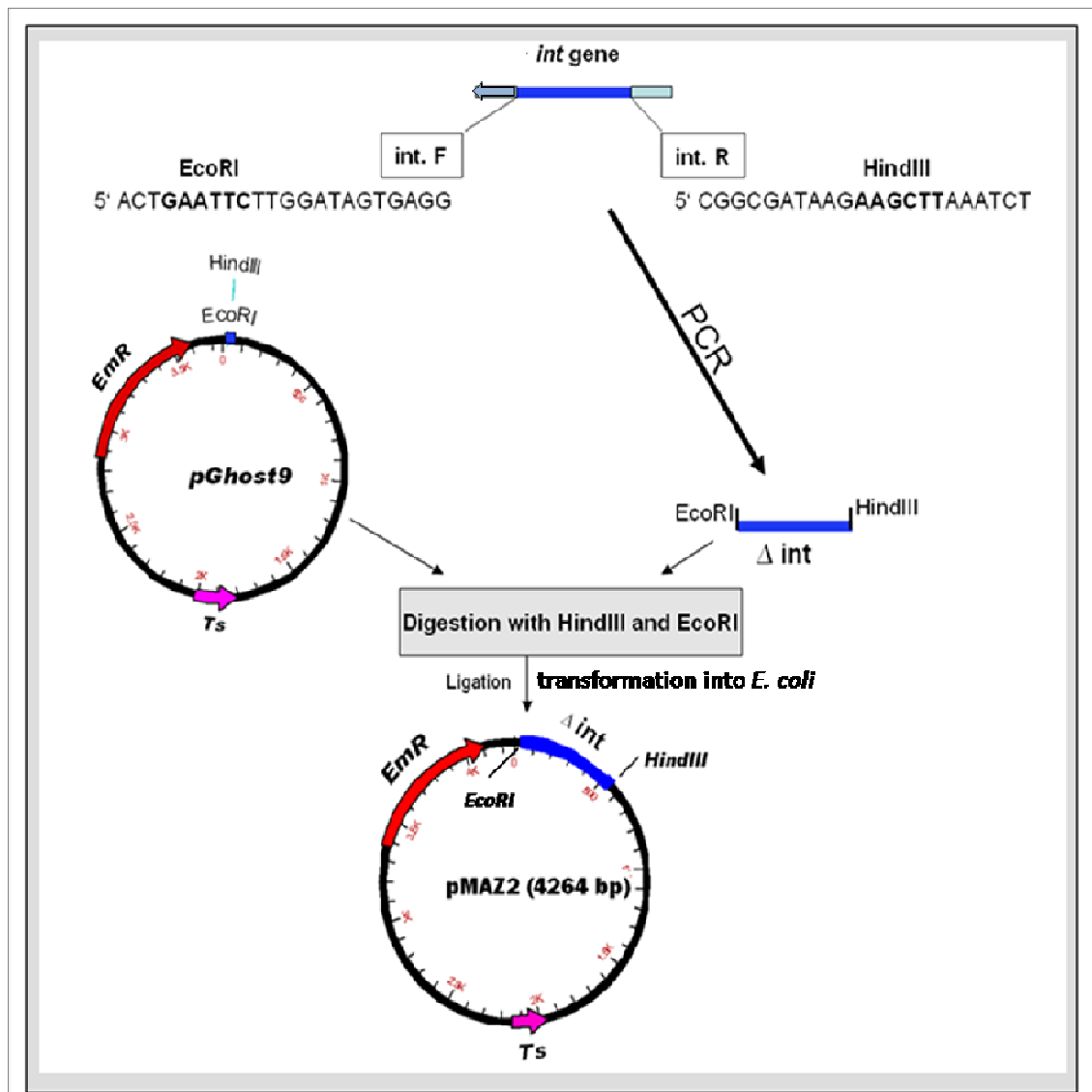


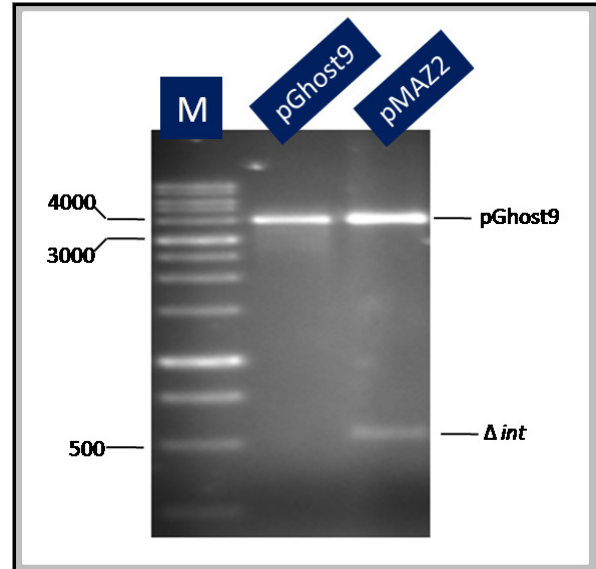
Figure 3.4. Construction of the recombinant plasmid pMAZ2. An internal fragment of *int* gene, Δint , was amplified by PCR using primer-pair, "int. F" with EcoRI site (noted), and "int. R", with HindIII (noted). The HindIII/EcoRI digested PCR product was cloned into the same site of pGhost9.

3.3.1 Electro-transformation of *S. thermophilus* J34f-2 and integration of pMAZ2

Intact pMAZ2 was successfully electro-transformed into *S. thermophilus* J34f-2 competent cells as described in Materials & Methods. The treated cells were plated on LM17 agar plates supplemented with $1 \mu\text{g ml}^{-1}$ Er and incubated at $30 \text{ }^\circ\text{C}$ for 48 h. Subsequently, plasmid DNA was isolated from 4 independent transformants and analyzed on agarose

gel before and after digesting with EcoRI/HindIII. In contrast to *E. coli*, all plasmids analysed were of correct size.

Figure 3.5. Vector pMAZ2 digested with EcoRI and HindIII; M, 1-kb size ladder (AppliChem, Darmstadt, Germany). Sizes of interested bands in bp are noted on the



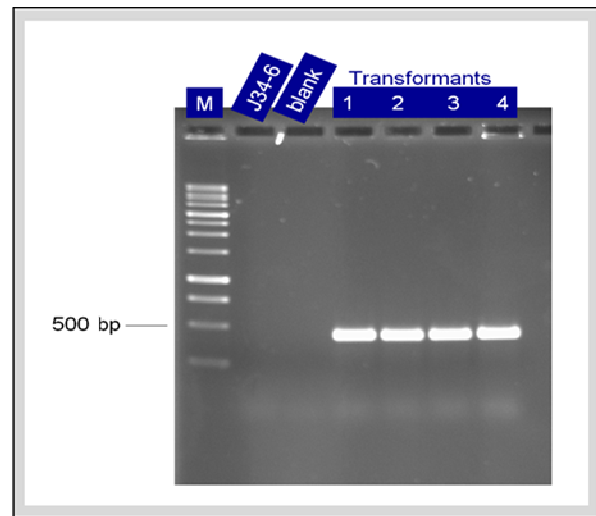
3.3.2 Verification of the lysogenic state of the transformants

Confirming the lysogenic state is needed prior to integration experiment. **Ali, 2009** reported that the lysogenic state in *S. thermophilus* could be detected rapidly and accurately by colony PCR using primers designed from the conserved sequence of major head protein. These primers, "YP-F" and "YP-R", generate a 432-bp PCR-product in the case of lysogeny with a *pac*-type phage.

The results of the PCR (described in Materials & Methods) of the 4 pMAZ2 transformants of *S. thermophilus* J34 f-2 revealed that they still were in lysogenic state, producing the expected PCR product of 432 bp, as shown in **Figure 3.6**.

lysogeny module

Figure 3.6. Colony PCR-products from 4 independent transformants 1-4, *S. thermophilus* f-2 (pMAZ2), using primer-pairs of *pac*-type major head protein, "YP-F" and "YP-R", described in Materials & Methods. *S. thermophilus* J34-6 was used as a prophage-cured derivative. M: 1-kb marker



3.3.3 Integration of pMAZ2 into *S. thermophilus* J34 chromosome

Integration of pMAZ2 into the prophage *int*-gene was performed successfully as described in Materials & Methods and outlined in **Figure 3.7**.

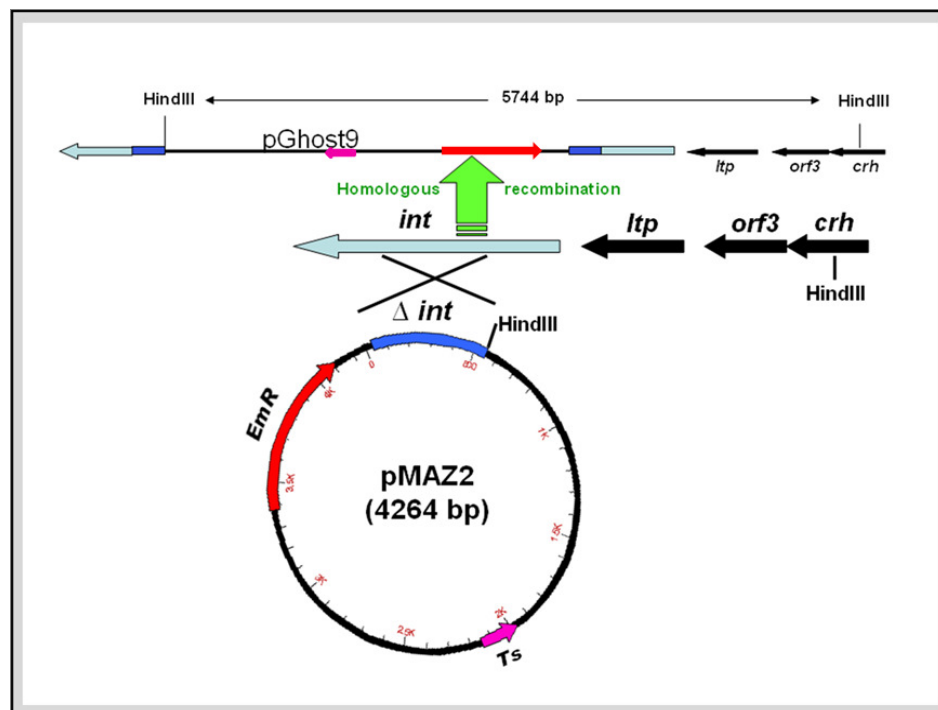


Figure 3.7. Recombinant plasmid pMAZ2, carrying an internal fragment of *int* cloned into thermo-sensitive vector pGhost9, was introduced into *S. thermophilus* J34f-2 at a permissive temperature of 30 °C. The culture was

grown at 30 °C till OD_{620nm} of 0.2, and then shifted to 42 °C for integration of the vector into the prophage DNA.

3.3.4 Verification of integration

Integration of pMAZ2 into the *int* gene of the prophage was verified by three independent approaches: Southern blot analysis, PCR, and DNA sequencing.

3.3.4.1 Verification by Southern blot analysis

Southern blot analysis is routinely used to detect a specific DNA sequence in DNA samples. In this work, DNAs, digested with HindIII, were transferred to a nylon membrane (Roche Diagnostics GmbH, Mannheim, Germany) by a downward transfer assembly (see **Figure 2.5**), and hybridized with Digoxigenin-labeled DNA probe (DIG DNA labeling kit, Roche Diagnostics GmbH, Mannheim, Germany). As a probe, pGhost9 was digested with HindIII, labeled, hybridized, and detected according to the protocol provided by the manufacturer with some modifications as described in Materials & Methods.

Figure 3.8 shows that integration had occurred. Since a HindIII fragment identified by hybridization with pGhost9 showed the expected size of 5.7 kb as outlined in **Figure 3.7**. A selected integrant was named hereafter *S. thermophilus* J34 KO-int.

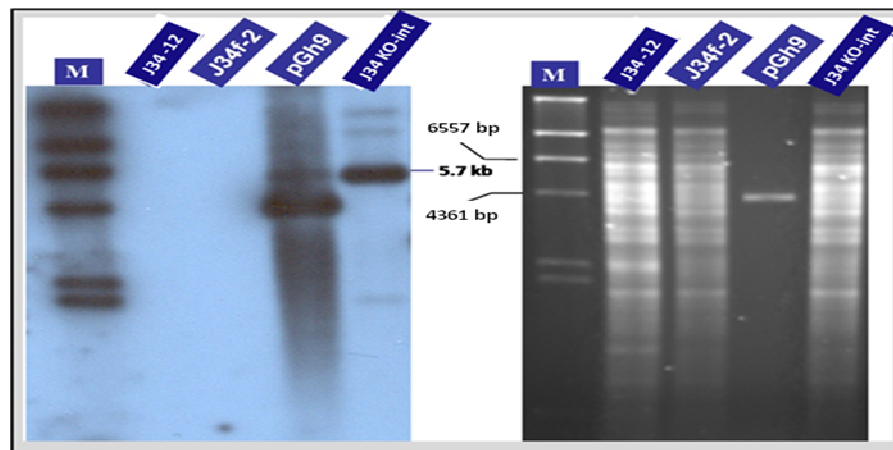


Figure 3.8. Agarose gel (0.8%) electrophoresis (right) and Southern blot hybridization (left) of HindIII-digested chromosomal DNA using pGhost9 digested with HindIII as a probe. Abbreviations, M: phage λ HindIII DIG labeled; J34-12, *S. thermophilus* J34-12; J34f-2, *S. thermophilus* J34f-2; pGh9, pGhost9; J34 KO-int, *S. thermophilus* J34f KO-int.

3.3.4.2 PCR approach

By the approach, integration should have resulted in introduction of pGhost9 into the *int* of the prophage (within the bacterial chromosome). PCR was applied to investigate that integration had occurred in the prophage (not in other parts of the genome). The idea as outlined in **Figure 3.9**, involved analysis of the fusion sites of the prophage linking the host chromosome on one hand and of pGhost9 with the TP-J34 genome on the other. Two independent PCR programs were used with primers: i) "Primer 4" (binds to sequence in the chromosomal DNA) and "pGh9-R2" (binds to the vector DNA) to confirm both prophage state and integration of pGhost9, and ii) "Pri-MAZ-F" binding within the vector DNA, and "Pri-MAZ-R" binds in the *int/ltp* intergenic region. The PCR results shown in **Figure 3.9** confirm the correct integration of pMAZ2 within *int*, producing the expected fragments of 1443 bp and 974 bp, as shown.

3.3.4.3 Verification by sequencing

Sequence analysis revealed that integration of pMAZ2 had occurred as expected. Hence, sequence analysis of the right-hand and left-hand PCR products (**Figure 3.9**), i.e., a 974-bp and 1443-bp fragments, respectively showed identity with the expected sequences. **Figure 3.10** shows a part of the left-hand product including a part of chromosomal DNA connected to the 3' end of *int* gene, and the *attL* site (40 bp core region identical in *attP* and *attB*).

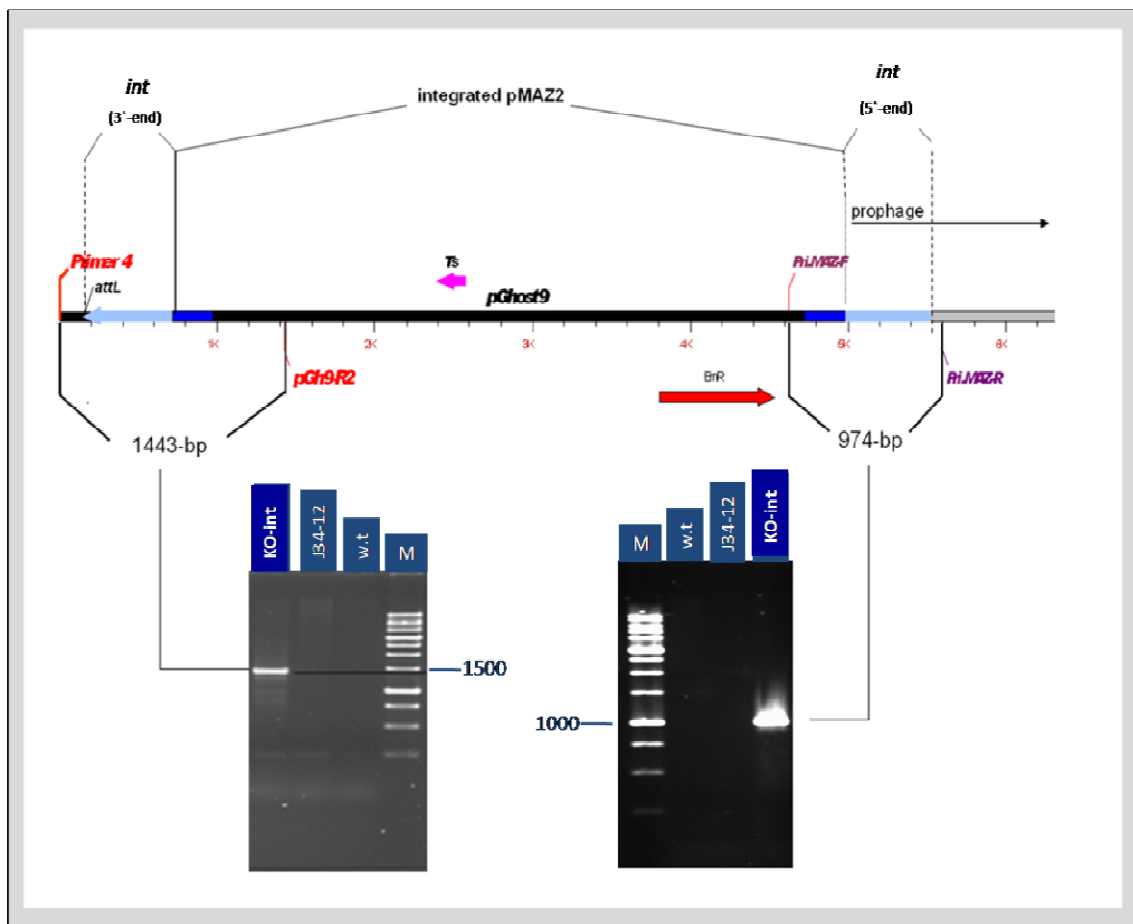


Figure 3.9. Top, schematic representation of the integration of pMAZ2 into *int* of the prophage TP-J34 within *S. thermophilus* J34 KO-int. Primers binding sites are noted. The size of the product of each primer-pair is indicated and the PCR products are shown in the gels (bottom). M, 1-kb size marker (AppliChem, Darmstadt, Germany); *S. thermophilus* J34f-2 and J34-12 were used as w.t. strain and non-inducible control, respectively.

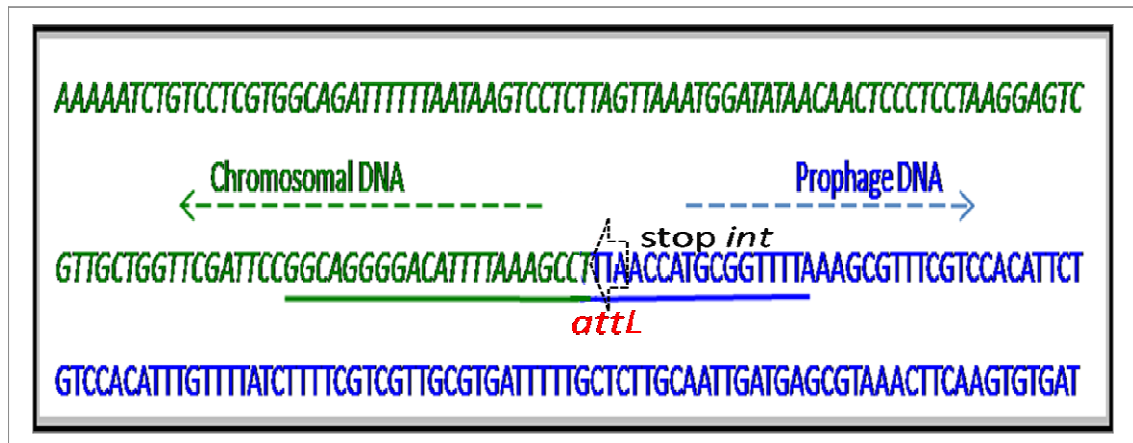


Figure 3.10. Sequence analysis of *S. thermophilus* J34 KO-int comprising *attL* using primer-pairs "Primer 4" and "pGh9-R2". The **green sequence** is chromosomal DNA; the **blue sequence** is prophage DNA; *Int* stop codon (complementary to TAA) is shown; Site specific integration (40 bp core region identical in *attP* and *attB*) is underlined. The sequence was done at MWG Operon, Ebersberg, Germany.

3.3.5 Lysis phenotype of *S. thermophilus* J34 KO-int

As shown in **Figure 3.11**, inactivation of *int* prevented lysis induction with MC and reduced the growth rate of *S. thermophilus* J34 KO-int compared to the control *S. thermophilus* J34-12, the final optical density reached was reduced by a factor of almost 2. Induction experiments were done using $0.3 \mu\text{g ml}^{-1}$ MC at $42 \text{ }^\circ\text{C}$ in the absence of Er (to eliminate any side effects in the induction process).

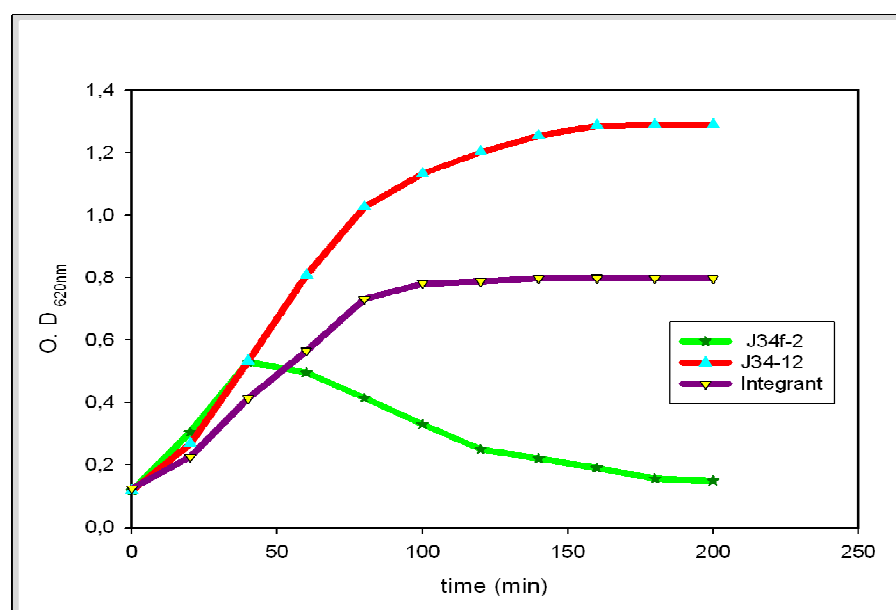


Figure 3.11. Induction of integrant *S. thermophilus* J34 KO-int in LM17 broth supplemented with $0.3 \mu\text{g ml}^{-1}$ MC at $42 \text{ }^\circ\text{C}$. J34f-2 (w.t.) and J34-12 were used as inducible and non-inducible controls, respectively.

3.4 Mutations in TP-J34-12 lysogeny module

3.4.1 Sequencing of TP-J34-12 lysogeny module

The lysogeny module of this derivative was investigated to detect the cause of its non-inducibility via sequencing and alignment with the w.t. TP-J34. Hence, the region of interest is encompassing the host chromosomal DNA ("Primer 4") through *attL*, *int*, *ltp*, *orf3*, *crh*, *cro* and *ant* into the 5'-end of *orf7* (primer "MZ12. R") as outlined in **Figure 3.12**. The alignment between w.t. *S. thermophilus* J34 and *S. thermophilus* J34-12 revealed an unexpected result. It showed identity in the whole sequenced region, except for a frame-shift mutation in *orf3* of TP-J34-12 causing premature termination of translation as shown in **Figure 3.13** and **Figure 3.14**. This insertion of an "A" after 122 bases from the start of this gene results in a premature stop codon (TGA) after 43 aa out of 122 aa total gene product in the w.t. strain, leaving the remaining codons untranslated. Thus, non-inducibility of *S. thermophilus* J34-12 is evidently a

lysogeny module

consequence of this mutation in *orf3*, whose product, Orf3 protein, must be necessary for induction.

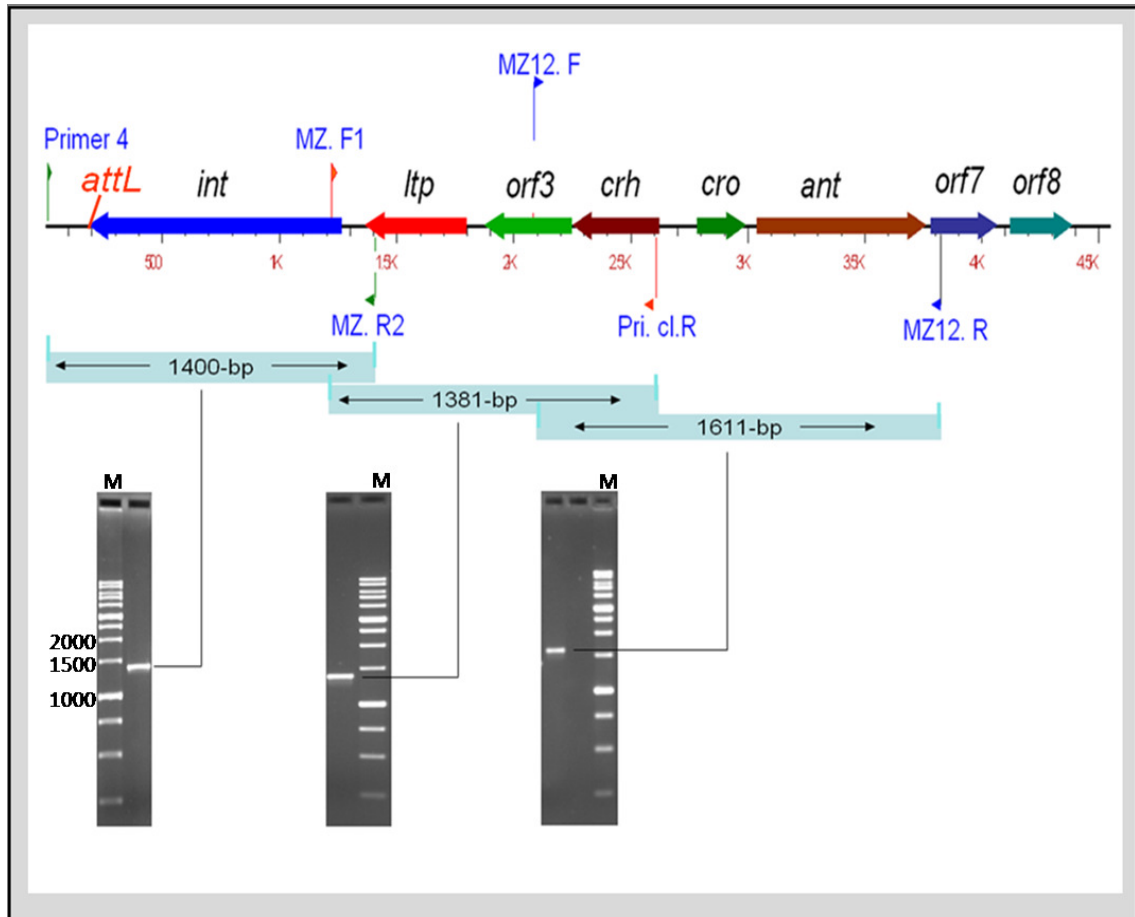


Figure 3.12. PCR amplification of lysogeny module of TP-J34-12. The module was amplified in three independent products overlapping each other. Binding sites of PCR primers are noted in the sequence. M: 1-kb marker (AppliChem, Darmstadt, Germany).

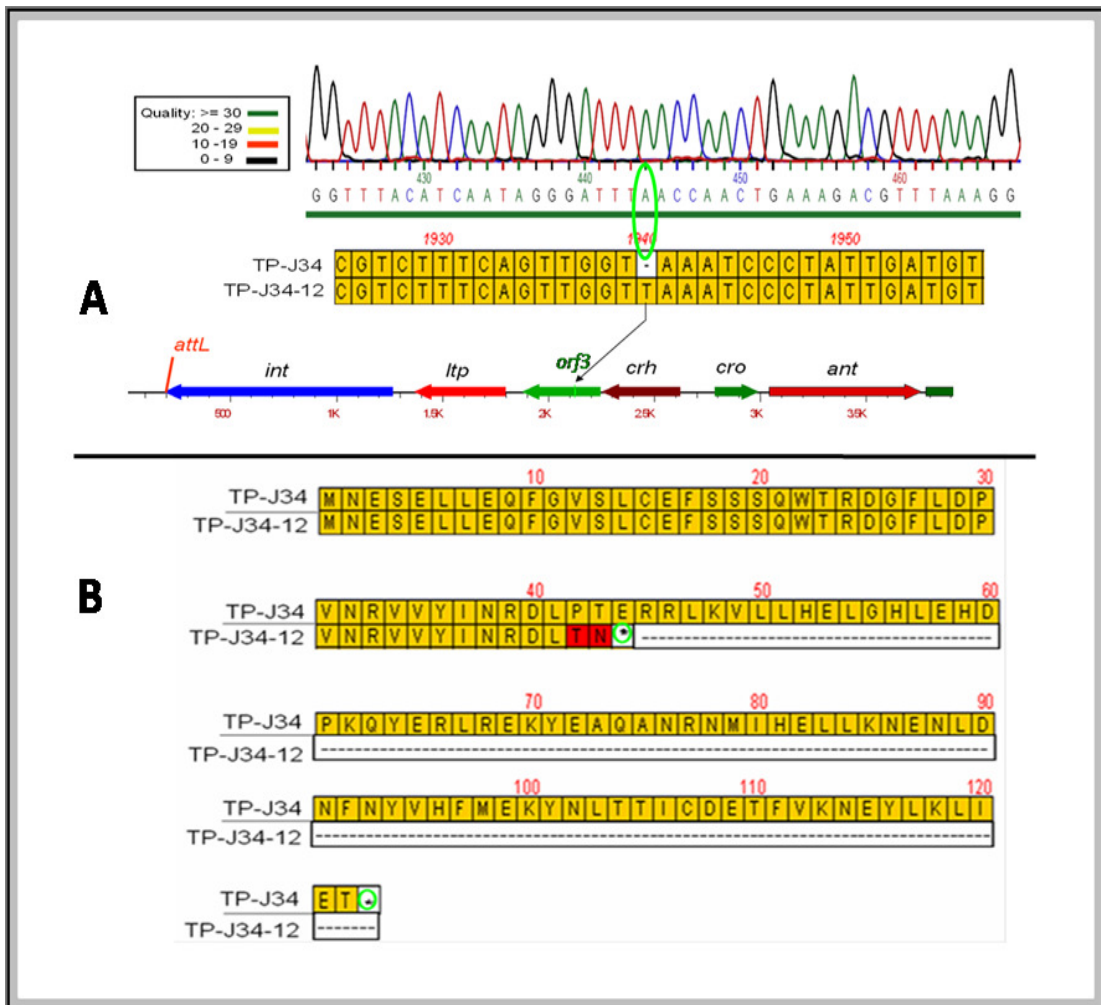


Figure 3.13. The insertion mutation in *orf3* of TP-J34-12. A, the sequence of bases of the complementary strand is shown (revealing an insertion of T); B, alignment of amino acids of both w.t. (TP-J34) and TP-J34-12. The highlighted red cells indicate the changed amino acids. The circled star shows the stop codons.

```

ATG.AAT GAG AGT GAA TTG CTT GAA CAG TTC GGC GTT TCT CTT TGT
GAA TTT AGC TCT AGC CAG TGG ACT CGA GAT GGG TTT CTA GAC CCT
GTT AAT CGT GTG GTT TAC ATC AAT AGG GAT TTA ACC AAC TGA AAG
ACG TTT AAA GGT GCT ACT GCA CGA ATT AGG TCA CTT AGA ACA CGA
CCC TAA ACA ATA TGA GCG TCT GCG AGA GAA ATA CGA GGC TCA AGC
AAA TAG GAA TAT GAT CCA TGA ACT GCT TAA AAA CGA AAA CCT AGA
TAA TTT TAA CTA CGT CCA TTT TAT GGA AAA ATA TAA CCT CAC CAC
TAT.TTG TGA TGA GAC TTT TGT GAA AAA CGA ATA CTT AAA GTT AAT
AGA AAC TTG A
    
```

Figure 3.14. *Orf3* of TP-J34-12. The inserted base, **A**, is marked in red and the premature stop codon (**TGA**) is highlighted in green. Untranslated region is underlined.

3.5 Isolation and verification of a new mutation in *orf3* of prophage TP-J34

In order to further confirm the involvement of *orf3* in induction, a new mutation in *orf3* was expected to be created by imprecise excision of pGhost9 from *orf3* of *S. thermophilus* J34 KO-*orf3*.

3.5.1 Curing of the integrated plasmid from *S. thermophilus* J34 KO-*orf3*

Integrated plasmid of *S. thermophilus* J34 KO-*orf3* was cured by culturing this integrant for 40 passages in LM17 broth in the absence of Er as described in Materials & Methods. **Figure 3.15** shows that the cured isolates grew only in the absence of Er. 74 out of 6000 tested isolates were Er sensitive and were named *S. thermophilus* J34 cu1-74.

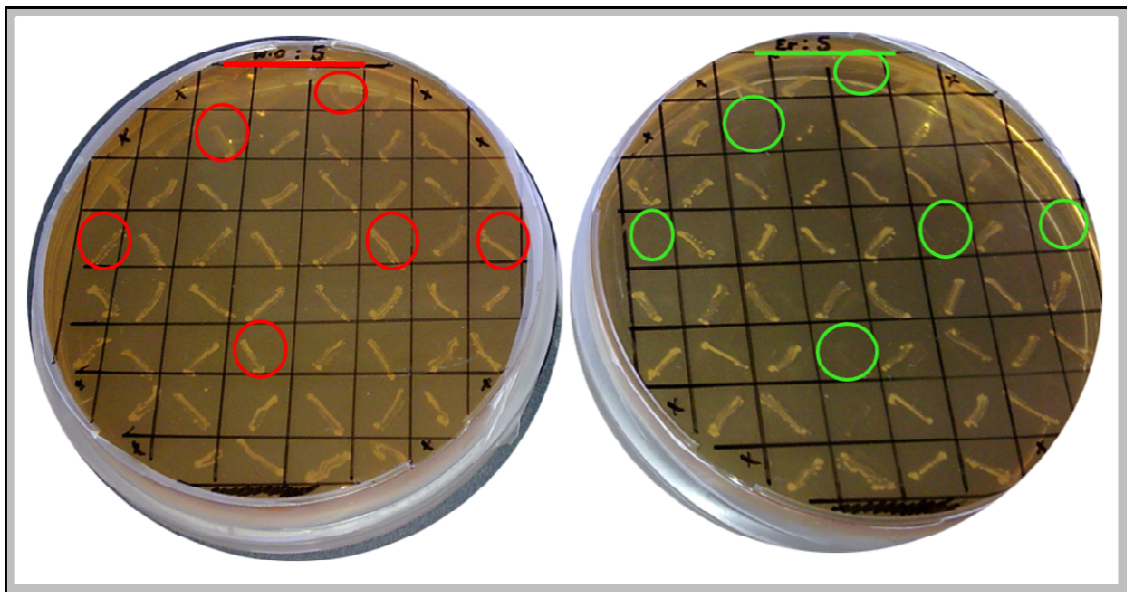


Figure 3.15. LM17 agar plates with Er (right) and without (left) to screen plasmid-cured isolates of *S. thermophilus* J34 KO-*orf3*, as described in Materials & Methods. Both plates are divided into 52 cells. Isolates that appeared only in the absence of Er, circled in red color, were considered as plasmid-cured isolates.

3.5.2 Verification of curing by PCR and isolation of *orf3* mutant

The 74 cured isolates were analysed by PCR to: i) confirm the excision of the integrated vector from *orf3* via primer-pairs binding to the flanked *orf3* (i.e., primer-pairs "ltp. f" and "pri. cl-R", produce 1226 bp PCR product in w.t. strain), and ii) verify the lysogenic state of the cured isolates via a primer binding in the host DNA to pair with a primer binding within *int* gene (i.e., primer-pairs "Primer 4" and "int. R", to get 966 bp in the lysogenic state) as outlined in Figure **Figure 3.16**.

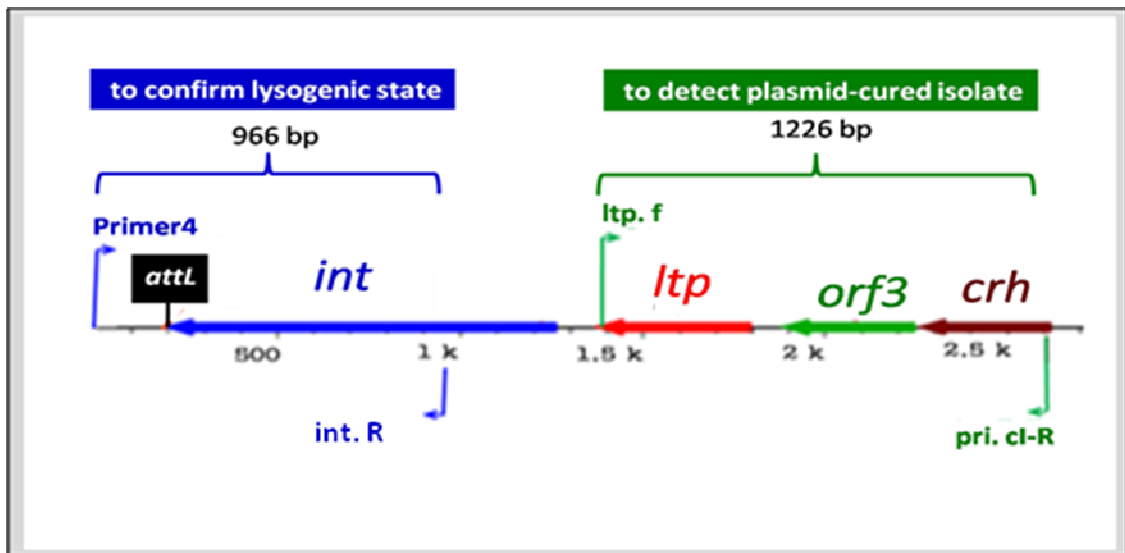
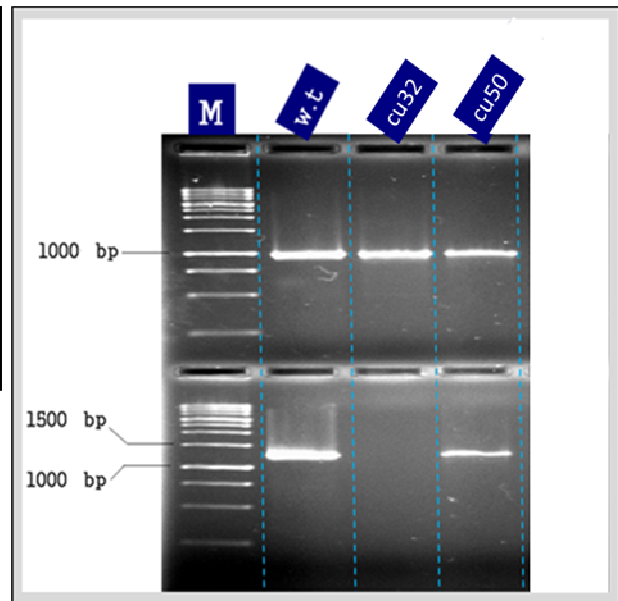


Figure 3.16. Schematic representation of primers sites which were used to detect the excision of integrated vector from *S. thermophilus* J34 KO-*orf3* and confirmed the lysogenic ste. The cured isolates produce two bands (1226 bp and 966 bp), while the integrants produce one (966 bp)

PCR results revealed that all of the 74 isolates were plasmid cured isolates except isolate No 32 as shown in **Figure 3.17**.

Figure 3.17. PCR-analysis of two cured isolates using primers described in Materials & Methods; w.t., *S. thermophilus* J34f-2; cu32, *S. thermophilus* J34 cu32; cu50, *S. thermophilus* J34 cu50; M, 1-kb



3.5.3 Sequencing analysis of *orf3* of *S. thermophilus* J34 cu50

Based on induction experiments, one of the cured mutants, *S. thermophilus* J34 cu50, was further analysed. The alignment of the DNA sequence of *orf3* between w.t. and *S. thermophilus* J34 cu50 revealed a mutation in the *orf3* of *S. thermophilus* J34 cu50. It resulted in an amino acid replacement D29A (replacement of aspartic acid of w.t. with alanine in the mutant Orf3) known as a missense mutation, as shown in **Figure 3.18**.

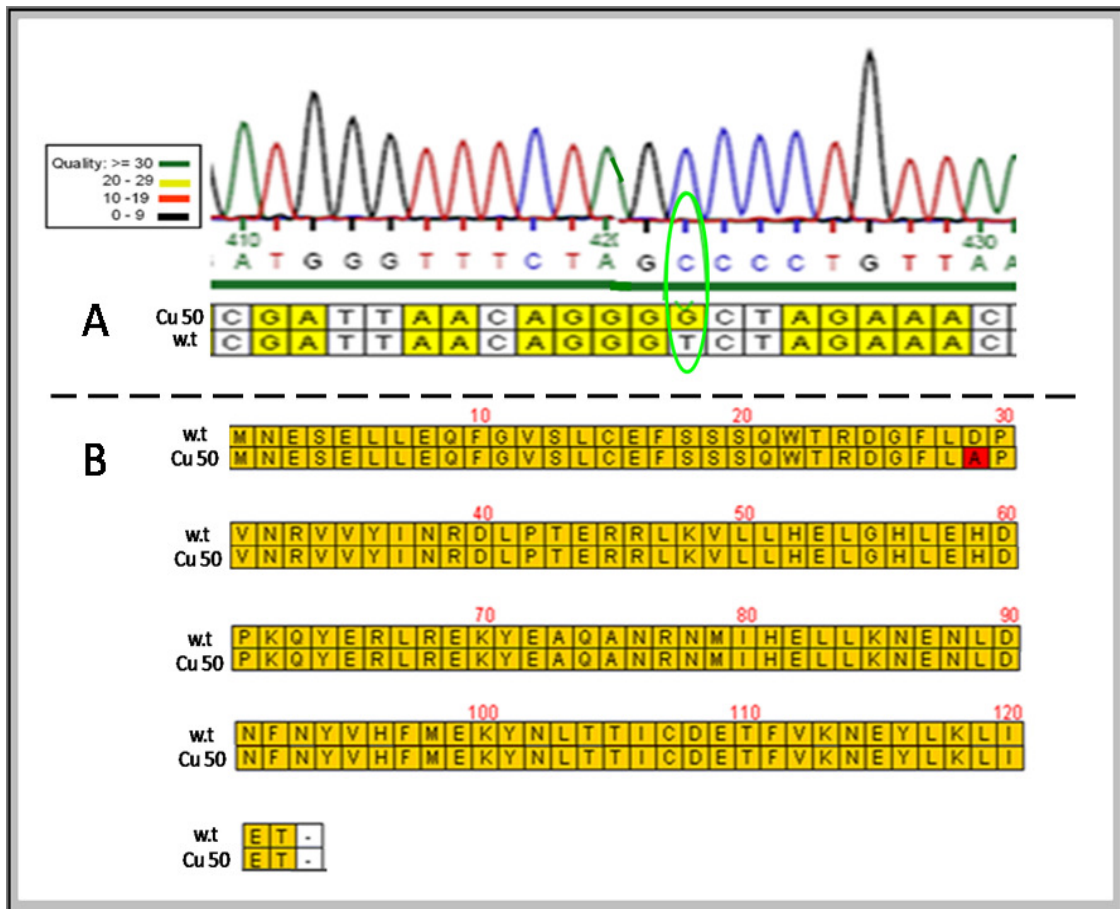


Figure 3.18. Replacement mutation in *orf3* of *S. thermophilus* J34 cu50 is circled in the sequence (A), and pointed at the subsequent amino acid alignment (B). Abbreviations: cu50 = *S. thermophilus* J34 cu50, and w.t. = *S. thermophilus* J34.

3.5.4 Secondary structure of the mutated Orf3

Secondary structure analysis (performed by program available at <http://bioinf.cs.ucl.ac.uk/psipred/>) of mutated Orf3 of *S. thermophilus* J34 cu50, and that of the w.t. revealed no difference as shown in **Figure 3.19**.

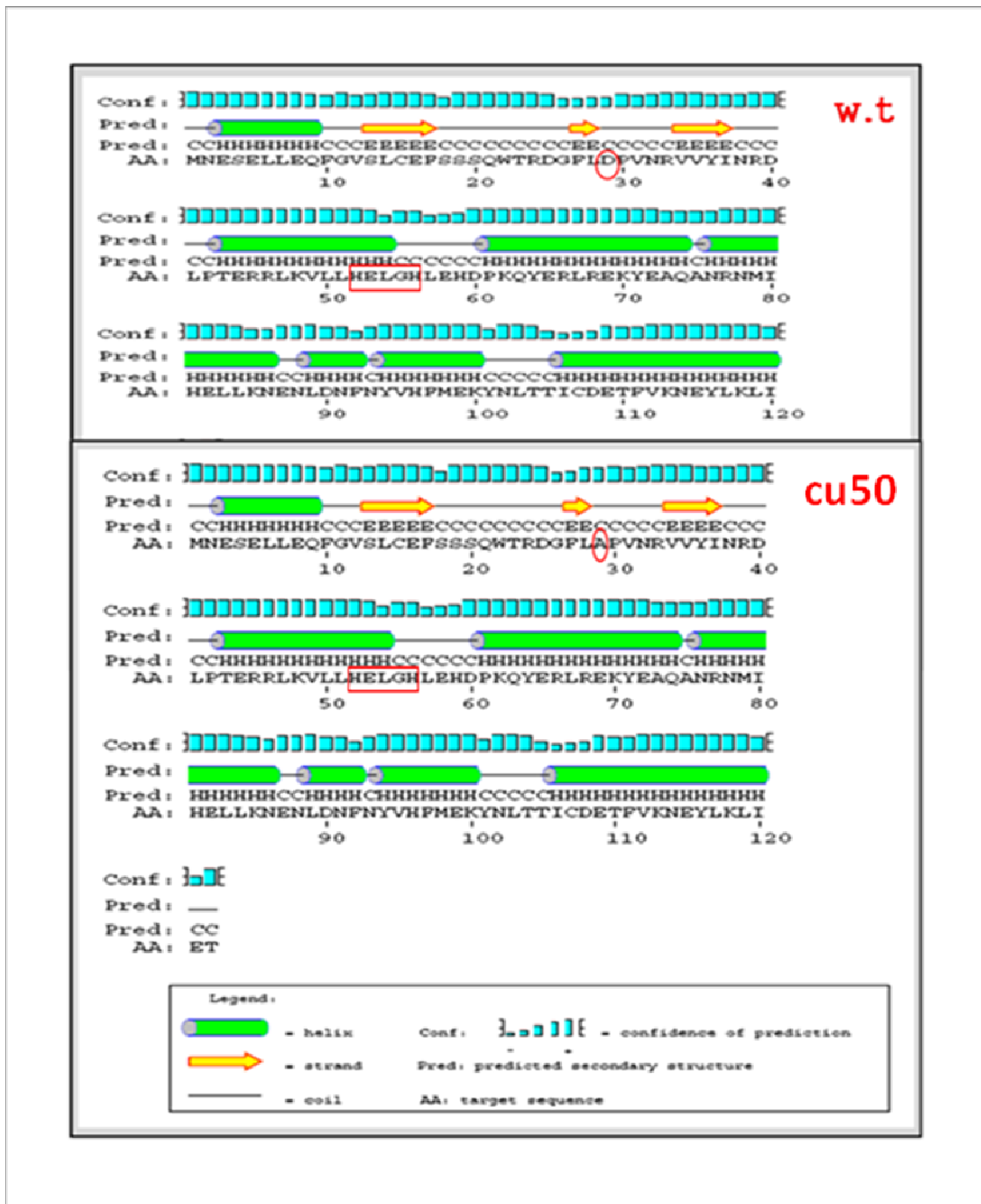


Figure 3.19. Secondary structure of Orf3 protein of both *S. thermophilus* J34, w.t. (top) and mutated *S. thermophilus* J34 cu50 (bottom) using program available at: <http://bioinf.cs.ucl.ac.uk/psipred/>. The replaced amino acid is circled in red; Zinc-binding site of metalloproteases, as described by Rawlings & Barrett, 1995, is noted in red rectangle

3.5.5 Induction of cured isolates with MC

The 74 cured isolates were tested for induction with MC. 72 isolates were inducible, only two isolates were non-inducible, i.e., cu32 (still containing the integrated vector) and cu50. So, it can be concluded that mutated *orf3* converted the host *S. thermophilus* J34 cu50 to non-inducibility (**Figure 3.20**).

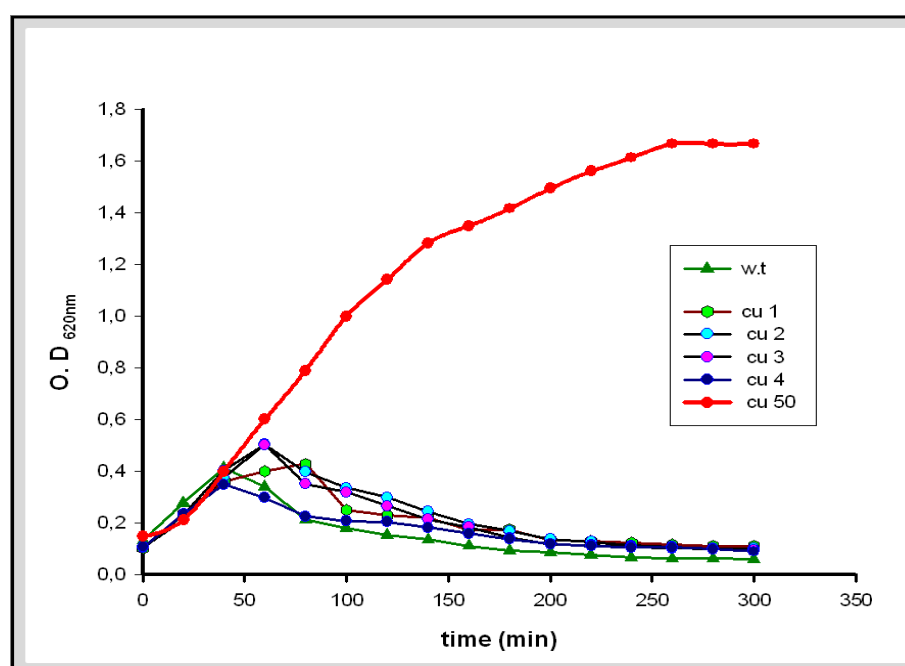


Figure 3.20. Induction of cured isolates *S. thermophilus* J34 cu1, 2, 3, 4, and cu50 with $0.3 \mu\text{g ml}^{-1}$ MC in LM17 at 40°C ; w.t., *S. thermophilus* J34.

3.5.6 Growth type of *orf3* mutants

The results revealed that Orf3 of TP-J34 affected the growth type, aggregation or homogeneous, of the host cells in liquid medium. **Figure 3.21** shows that *S. thermophilus* J34-12 cells grew homogeneously. In contrast, both the knock out *S. thermophilus* J34 KO-*orf3*, and the mutant *S. thermophilus* cu50 revealed an aggregation phenotype when incubated in Ringer's solution.

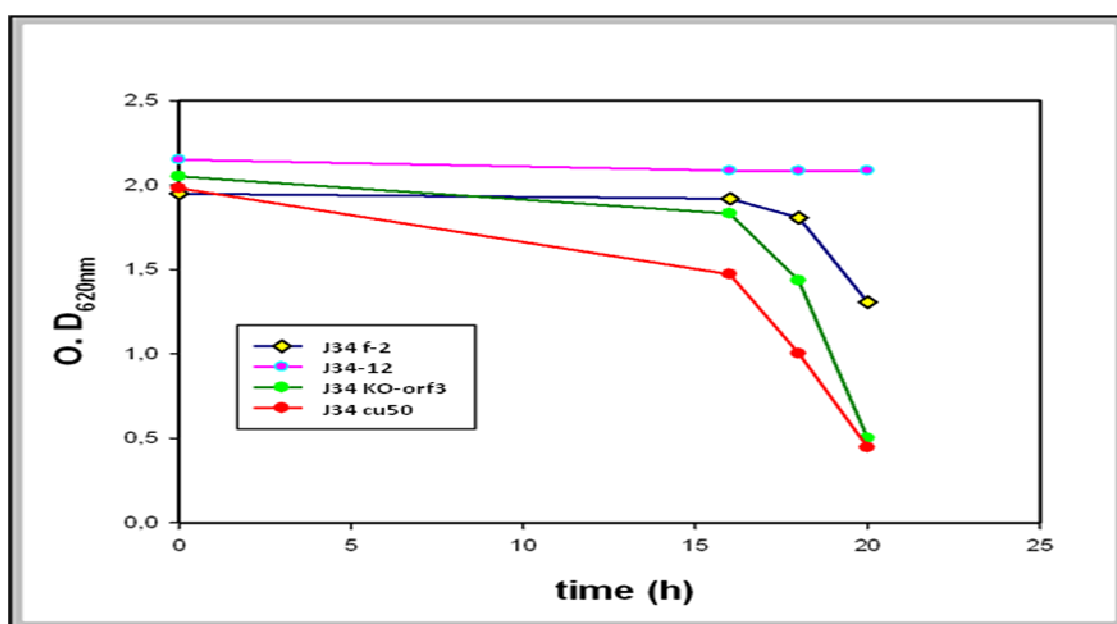


Figure 3.21. Aggregation of *S. thermophilus* J34 and its derivatives (i.e., J34-12, J34 KO-orf3 and J34 cu50) in Ringer's solution. The cultures were grown in LM17 at 40 °C for 24 h. Subsequently, the cells were washed in autoclaved water and re-suspended in Ringer's solution (as described in Materials & Methods). The ODs were measured afterward.

3.5.7 Introducing of an intact *orf3* into its mutant derivative, *S. thermophilus* J34 cu50

To insure that the non-inducibility of *S. thermophilus* J34 cu50 was due to its *orf3* mutation, intact *orf3* was cloned into an expression vector, pMG36e (**Figure 3.22**) and a complementation assay was performed.

3.5.7.1 Construction of pMZ-*orf3*

As shown in **Figure 3.22 & 3.23** a PCR product of 481-bp, covering *orf3* was amplified by PCR using primer: i) "orf3-C-f" binding upstream in the *crh* gene, and primer ii) "orf3 HindIII. R" binding downstream in *orf3/ltp* intergenic region and producing a HindIII site in the 3'-end (see **Table 2.3**). As outlined in **Figure 3.22 & 23**, a 481 bp fragment, containing the native *orf3* was amplified, without its native promoter, blunted in the 5'-end with T4-polymerase and digested with HindIII in the 3'-end. This

lysogeny module

fragment was cloned into pMG36e, digested with *Sma*I and *Hind*III. Thereby, *orf3* was inserted in the correct orientation. The resulting vector was designated pMZorf3.

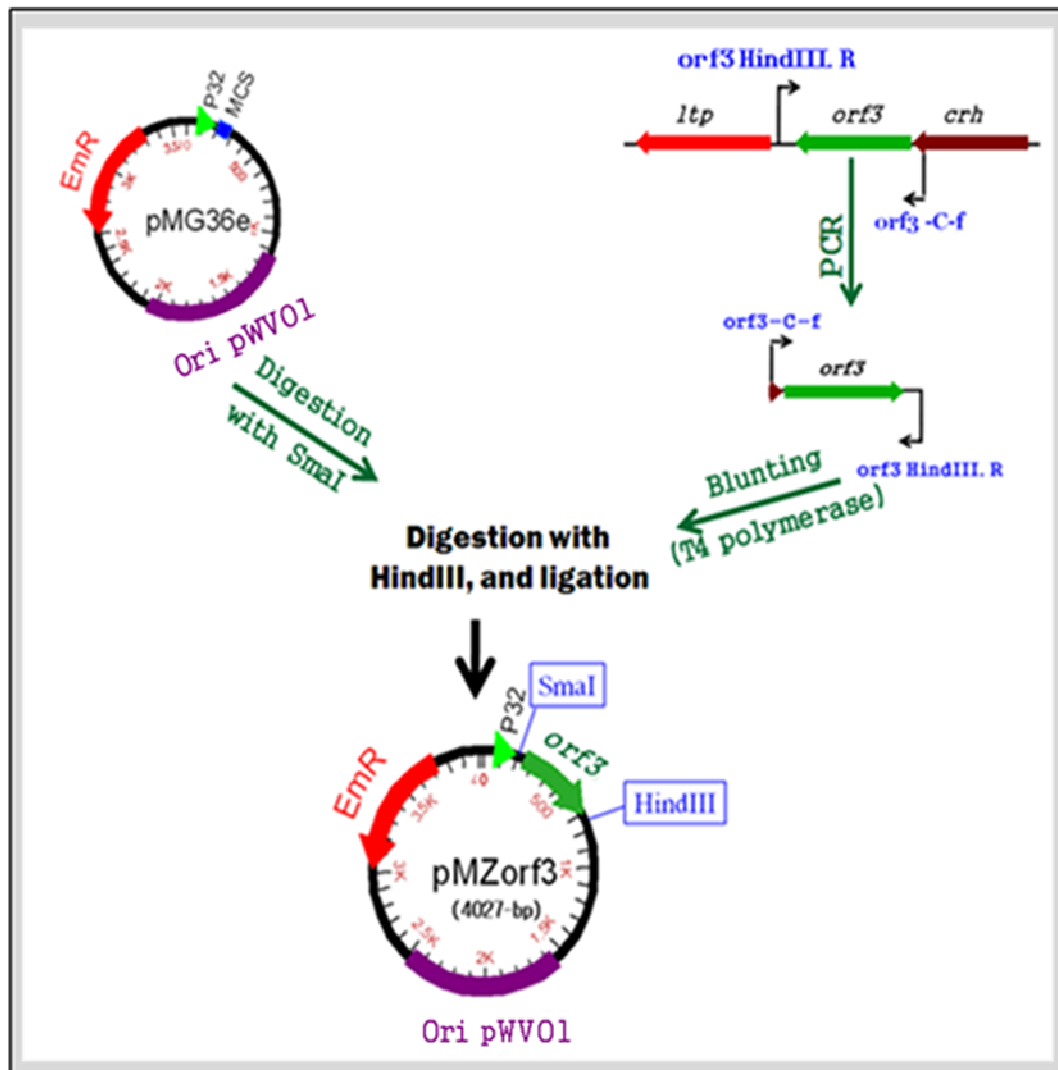
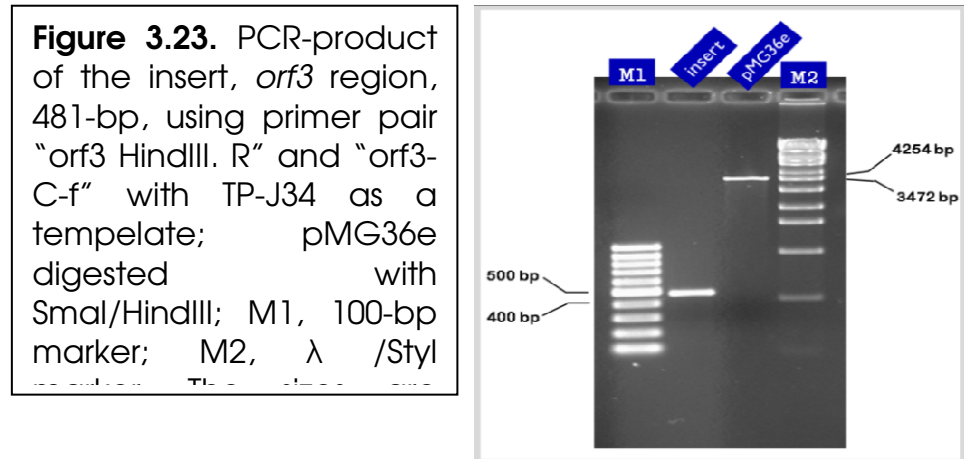


Figure 3.22. Construction of the recombinant expression vector pMZorf3. A 481 bp fragment covering *orf3* was amplified by PCR using primer-pair "orf3 HindIII, R" and "orf3-C-f" as described in Materials & Methods. The fragment was then cloned into pMG36e.



3.5.7.2 Transformation of pMZorf3 in *E. coli* EC1000

The ligation mixture of the previous step, **3.5.7.1**, was successfully used for transformation of *E. coli* EC1000 competent cells as described in Materials & Methods.

As shown in **Figure 3.24/A**, PCR analysis of 3 independent transformants of *E. coli* were positive when PCR with primers, i.e. "orf3 HindIII. R", and "orf3-C-f". The vector was isolated from those three transformants (as described in Materials & Methods) and confirmed by analysing on agarose gel after digesting with HindIII/SacI (**Figure 3.24/B**).

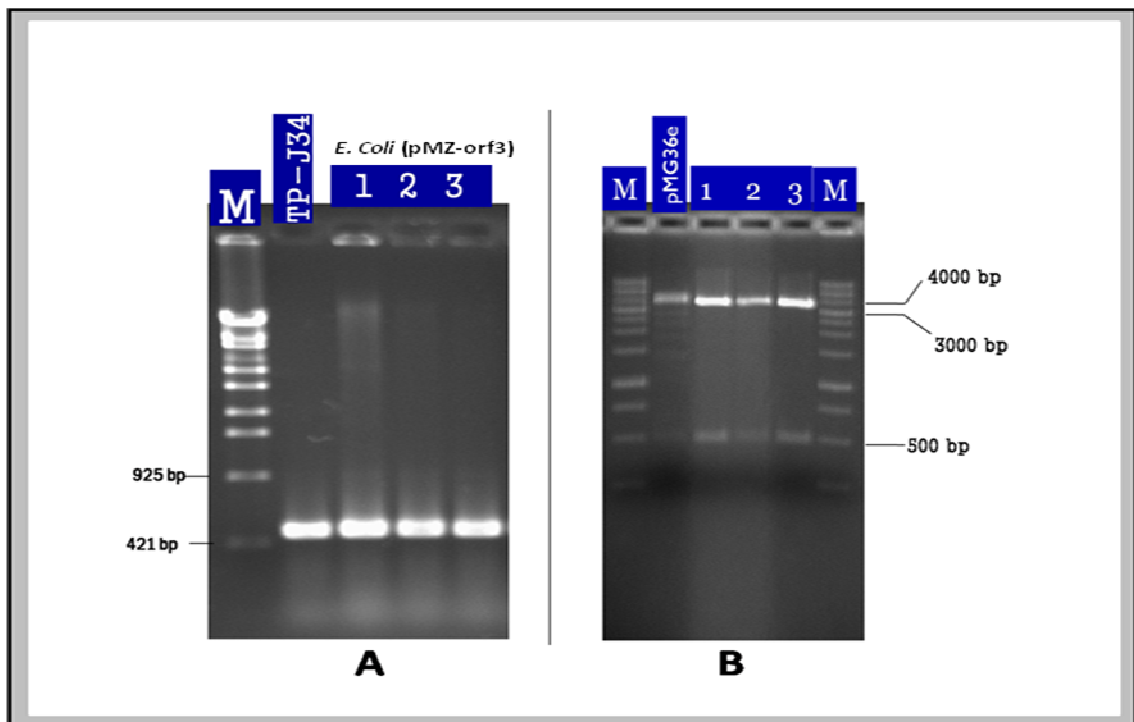


Figure 3.24. Agarose gel analysis of the transformants *E. coli* (pMZorf3, 1-3). **A**, PCR-products from colony PCR using primer-pair "orf3 HindIII. R" and "orf3-C-f". *S. thermophilus* J34f-2 was used as a positive control for TP-J34; **B**, vectors isolated from the last 3 transformants of gel A, i.e. *E. coli* (1, 2, and 3) digested with HindIII/SacI; M, DNA marker (λ /Styl in gel A, and 1-kb (AppliChem, Darmstadt, Germany) in gel B). The sizes are noted on the margins.

3.5.7.3 Transformation of *S. thermophilus* J34 cu50 with pMZorf3 DNA and behavior of induction

Recombinant plasmid pMZorf3 was introduced into *S. thermophilus* J34 cu50, as described in Materials & Methods. Transformants were selected as erythromycin resistant and two were named *S. thermophilus* J34 cu50 trans 1 & trans 2.

The results shown in **Figure 3.25** confirmed that intact *orf3* in the expression vector pMG36e conferred w.t. phenotype (lysis upon addition of MC) to non-inducible derivative *S. thermophilus* cu50.

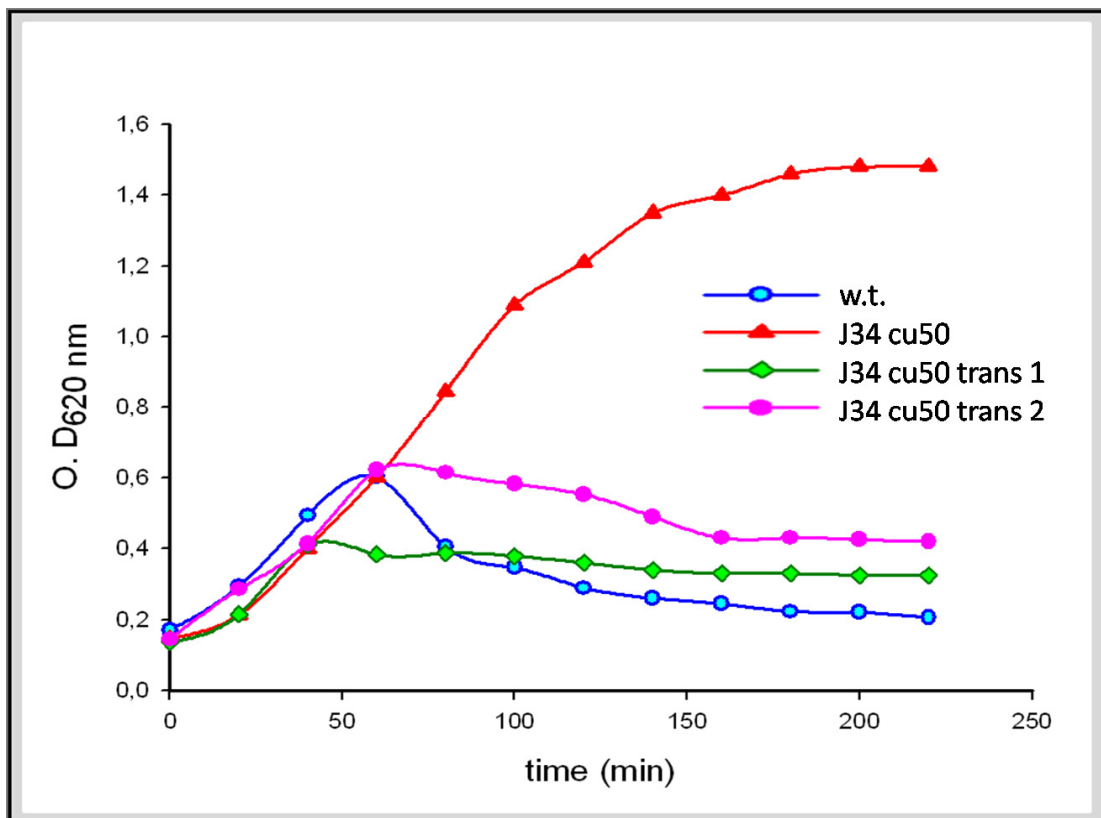


Figure 3.25. Induction experiment of *S. thermophilus* J34 cu50 transes 1 & 2 using $0.3 \mu\text{g ml}^{-1}$ MC as a final concentration in LM17 broth medium at 40°C . $\text{ODs}_{620\text{nm}}$ were determined at 20 min intervals (for more detail, see Materials & Methods).

3.6 Discussion and conclusion

Concerning the site specificity, integrase gene of TP-J34 seems to be related to *serine* recombinase family, since there are common core sequences of 40 bp for *attB* and *attP* (see introduction of this chapter). Several references have shown that integrase also carried out the excision of the prophage from the host genome (**Fadeev *et al.*, 2009**; **Groth & Calos, 2004**; **Sentchilo *et al.*, 2003**). In some temperate phages, such as phage λ , the excision reaction occurs via interaction between *int* and *xis* in addition to some environmental factors, but the mechanism in TP-J34 is different. Sequence analysis revealed that there is no *xis* gene in TP-J34. The role of *int* encoding a protein of 360 aa, in induction of the prophage was investigated. So, *Int*-deficient mutant of the lysogen *S.*

lysogeny module

thermophilus J34 KO-int was successfully obtained by direct single-crossover homologous recombination with a thermo-sensitive vector pGhost9 containing an internal fragment of *int* gene. Inactivation of this gene was verified via Southern blot, PCR, and sequencing. Inactivation of *int* gene prevented the induction with MC and reduced the growth rate of *S. thermophilus* J34 KO-int compared to the noninducible control *S. thermophilus* J34-12.

It is suggested that the Int protein is needed in the excision process. An excisionase does not seem to be involved, since DNA sequencing of the TP-J34 genome (**Neve *et al.*, unpublished results**) did not identify any gene capable of expressing an excisionase. The effect on growth rate and optical density reached in stationary phase of the integrant may be explained as follows. Induction of the prophage results in expression of all functions needed for lytic development. Thus, replication of prophage DNA may be initiated. However, due to lack of excision, this replication may well interfere with the normal replication of the host genome and/or with expression of proteins needed for regular growth of the host cells, which may lead to premature inhibition of growth. The fact that no lysis was observed may be due to the absence of multiple copies of the lysis genes, which in normal induction would have been produced by excision followed by replication. It does not seem to be appropriate to consider the extra amount of DNA of the integrated vector as a burden for the host, resulting in reduction of the growth rate by a factor of almost two. Compared to the bacterial host genome of about 1.8 Mb, the size of the vector of 4.2 kb appears to be too small to account for such a large effect.

The second aim of this chapter was to understand the non-inducible phenotype of *S. thermophilus* J34-12 lysogeny module which converts this derivative to be non-inducible. The protein sequence of *orf3* shows a zinc-binding motif **HExxH** characteristically found in

lysogeny module

metalloproteinase, which constitutes the core of one of the two zinc-binding sites in thermolysin where two histidines participate in coordination of zinc atom, while the conserved glutamic acid is part of the active site (**Jongeneel *et al.*, 1989**). This aa sequence exists as HELGH from aa position 52-55 in both w.t. and its mutated derivative *S. thermophilus* J34 cu50. Moreover they were in total agreement with the more stringent model of metalloproteases as defined by **Rawlings & Barrett, 1995** as in **Table 3.1**.

Table 3.1. Alignment between the putative aa sequence in metalloproteases (**Rawling & Barrett, 1995**) and that of orf3 of TP-J34.

Motif sequence	a	b	X	H	E	b	b	H	b	c
Orf3 of TP-J34	V	L	L	H	E	L	G	H	L	R

Where, a = almost often valine or threonine; b = uncharged residue; c = hydrophobic residue; proline is never found in this site.

Based on the zinc-binding site, metalloendopeptidases are classified into five distinct families: thermolysin, astacin, serratia, matrixin, and snake venom metalloproteinases. Two histidines and one glutamate are zinc-ligands in the thermolysin family while, three histidines and one tyrosine are zinc ligands in the other four families, which are further distinguished by the identity of the residue following the third histidine and the aminoacids surrounding the tyrosine (**Jiang & Bond, 1992**). By this way it may be considered that Orf3 is related to the thermolysin family of metalloproteinases. These proteineinases can modulate cell function in many normal physiological processes, such as cell growth, and regulation of innate immunity as the case of Matrix metalloproteinases (MMPs) (**Dean & Overall, 2007**).

In this chapter the mutation of *orf3* in *S. thermophilus* J34-12 was detected. This mutation leads to lack of more than 60% of the aa of that gene (79 out of 122). This, in turn, affects the activity of this protein as a regulator or a proteolytic protein. Hence, the conformation of the

lysogeny module

proteins within the cell could be affected, which influences the phenotype. The case of mutant *S. thermophilus* J34 cu50 is different, since the whole gene is translated into protein in the mutated *orf3* (122 aa). It is only just one missense mutation resulting in the amino acid replacement D29A. This mutation does not appear to affect the secondary structure of that protein as well. Thus, the effect of this mutation could be due to the lack of the negative charge at that position of the protein. Here two possibilities are supposed: i) D in position 29 is involved in stabilizing the conformation of Orf3 by binding to a positively charged counterpart within the protein, or ii) it is exposed on the surface of Orf3 and could thus play a crucial role in the interaction with other protein partners.

What Orf3 does in the growth phenotype of the lysogen is still confused alike. Although, mutated *S. thermophilus* J34-12 grows homogeneously, it lacks the majority of Orf3 aa. However, *S. thermophilus* J34 cu50 has almost the same aa, but grows in aggregation phenotype. Is the reason of homogeneous growth due to either the prophage as revealed by **Neve et al., 2003** or to a specific gene like *orf3* as shown by **Bokelmann, 2003**?

I suppose that *orf3* of TP-J34 acts as a master regulator protein in the lysogeny module of TP-J34. Hence, the data presented in chapter 4 evidence that. For example, mutated *orf3* derivative *S. thermophilus* J34 KO-*orf3* cannot express i) *crh*-operon (comprising *crh*, *orf3*, and *ltp*), and ii) *cro*-operon (comprising *ant*, *orf7* and *orf8*), and affects negatively in the transcription of both *int* and *ltp* (monocistronic species of *ltp*) (see **Table 4.1**). The differences in phenotypes among different *orf3* mutant derivatives may be due to the difference in the mutation sites within that gene.

Transcriptional analysis of TP-J34 lysogeny module

3.7 Abstract

Knowledge on regulation of the genetic switch of bacteriophages of LAB is limited when compared to the information available on the well studied phage λ of *E. coli*. This Chapter produces the first experimental insight in transcription of phage TP-J34. Based on experimental observations (i.e., RT-PCR and Northern blot), and genome analysis of TP-J34 lysogeny module, it can be concluded that: Firstly, the *int* gene is transcribed from its own promoter, P_{int} , located in the intergenic region between *int* and *ltp*. A potential promoter is presented. This gene is expressed in lysis induction and lysogenic state. Thus, the *int* gene of TP-J34 appears to be also involved in the excision of the prophage from its host chromosome. Secondly, *ltp* is transcribed in two different transcripts: i) a less abundant polycistronic mRNA, comprising genes *crh*, *orf3*, and *ltp*, transcribed from P_2 , and ii) a majority of monocistronic transcript mRNA comprising just *ltp*. Both transcripts were detected both in presence or absence of mitomycin C (i.e. in both lytic and lysogenic cycles). The promoter of the *ltp* monocistronic transcript, P_{ltp} , appeared to be located in the intergenic region between *ltp* and *orf3*. Thirdly, *orf3* is needed for transcription of both *ltp* and *int* as well as *cro*. Fourthly, *crh* is expressed in both lytic and lysogenic cycles. Fifthly, *cro* and *ant* are transcribed together as a polycistronic mRNA together with *orf7* and *orf8*, the transcript is detected only in the lytic development. Sixthly, there appear to be two stable SLs in the module of interest, one downstream of *ltp*, and the other one about 200 nt downstream in the 3' untranslated region (UTR) of *orf8*. The two SLs which may act as terminators.

3.8 Introduction

Transcription is the process in which a coding DNA strand is copied by an RNA polymerase (RNAP) to produce a complementary RNA. So, it is an essential process required for genetic information to become expressed. It is a crucial step in gene regulation and its understanding has been one of the main interests in molecular and cellular biology. Cell types and functions differ because of qualitative and quantitative differences in their gene expression. Consequently, control of gene expression is at the heart of differentiation and development of organisms (**Brown & Brown, 2002; Gibney *et al.*, 2010**). In bacterial chromosomes, transcription units (TUs) comprise the following genetic entities: regulatory region, transcription start site, one or more *orfs*, and transcription termination site. A TU with more than one *orf* is named polycistronic. Otherwise, it is called monocistronic. Some genes may be transcribed by several promoters, thus TUs may overlap. The group of overlapping TUs constitutes an operon which always contains a promoter that transcribes the whole set of genes conforming its TU character (**Balleza *et al.*, 2009**).

Transcription regulation emerges from the interaction between *trans* acting factors (Latin for "far side of") that bind to *cis* acting elements (Latin for "this side of") in the context of a particular chromosome structure. *Cis* elements are all those DNA regions in the vicinity of a gene. The *trans* factors, however, are all the diffusible cellular molecules that are able to bind to those regions. Regulatory regions, up to 400 bp in prokaryotes contain *cis* elements like promoter and transcription factor-binding sites (TFBS), where transcription factors (TFs) bind to modulate the binding of the RNAP. (**Balleza *et al.*, 2009; Dekhtyar *et al.*, 2008; Colladovides *et al.*, 1991**). Generally, negative regulators bind to the promoter, interfering directly with RNAP, while

positive regulators bind to the promoter's upstream region, helping to recruit the polymerase and start transcription.

The aim of the experiments described in this chapter was to find out how the genes of the lysogeny module of TP-J34 are transcribed. Transcription was analysed during both lysogenic and lytic growth cycles using RT-PCR and Northern blot as complimentary approaches. Some sequence analysis was used to detect putative promoters, SLSs, and operators.

3.9 Northern blot hybridization of the rightward genes of the lysogeny module

Northern blotting serves two objectives: i) demonstration of presence or absence of transcript, and ii) determination of the size and quantity of transcript. Hence, RNA is size fractionated in a denaturing gel, which separates the RNA species. Subsequently, transfer and immobilization on a membrane occurs in such manner that the relative positions of the RNA molecules are maintained. The membrane is then hybridized with a labeled probe complementary to the mRNA of interest. Size and abundance of the target RNA can be determined from the generated signal which providing a good reflection of gene-expression.

Figure 4.1 shows growth curves of *S. thermophilus* J34 under 3 different concentrations of induction by MC (i.e., 0.1, 0.2, and 0.3 $\mu\text{g ml}^{-1}$ LM17) introduced at 3 different growth stages of the culture ($\text{OD}_{620\text{nm}}$ of 0.1, or 0.2 or 0.3). Based on this experiment, it was concluded that the best conditions to be used were 0.3 $\mu\text{g ml}^{-1}$ MC as a final concentration at $\text{OD}_{620\text{nm}}$ of 0.3 for RNA isolation. RNAs were isolated within the first 80 min, i.e., prior to the onset of lysis.

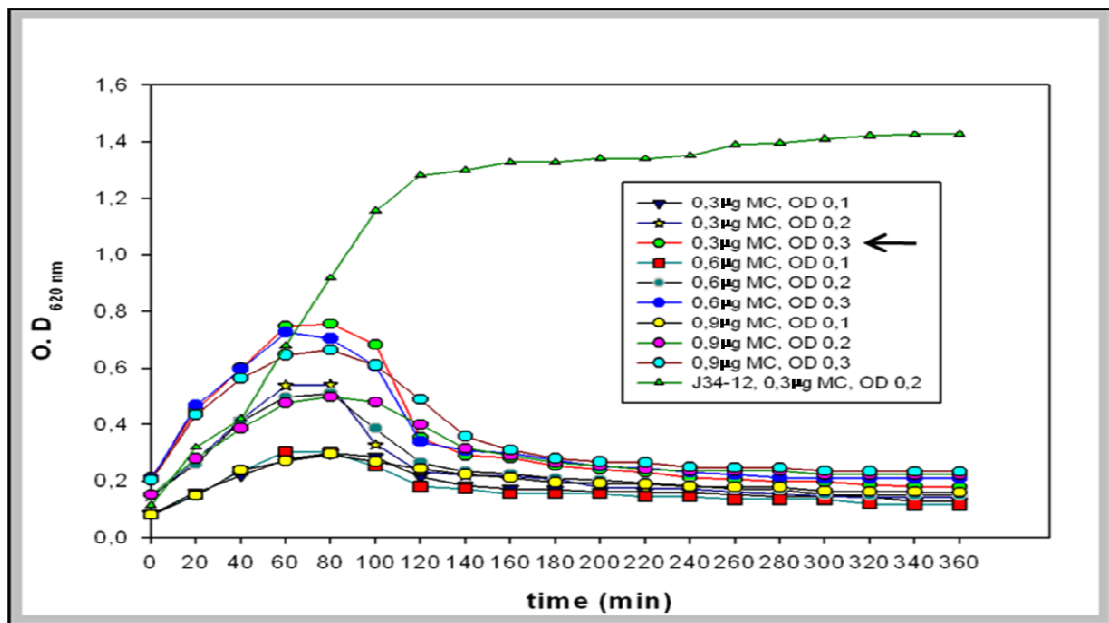
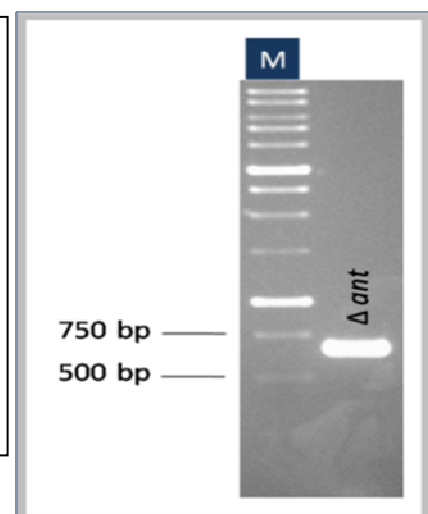


Figure 4.1. Growth curves of *S. thermophilus* J34 under different concentrations of MC added to different growth stages of *S. thermophilus* J34 in LM17 broth medium at 40 °C. OD_{620nm} was determined at 20 min intervals; J34-12 is the non-inducible control *S. thermophilus* J34-12. The arrow points to the optimal condition.

The rightward genes of the module of interest comprise *cro*, *ant*, *orf7* and *orf8*. For Northern hybridization, a 641-bp internal fragment of *ant* gene, Δant , was amplified (**Figure 4.2**) using primer-pairs "MZ ant. F", and "MZ ant. R", as described in Materials & Methods. The PCR product served as probe.

Figure 4.2. A 641-bp internal fragment PCR product for *ant* probe amplified by PCR using primer pair: "MZ ant. F", and "MZ ant. R" with *S. thermophilus* J34 as a template. The probe was labeled as described in Materials & Methods; M, 1-kb marker (AppliChem, Darmstadt, Germany); sizes



lysogeny module

Northern blot hybridization of *S. thermophilus* J34 using *ant* probe revealed that *cro*, *ant*, *orf7*, and *orf8* of TP-J34 were transcribed together as a polycistronic mRNA of about 1800 nt (**Figure 4.3**). Thus, the transcript most likely is generated from putative promoter P1. It seems to be terminated by a stable SLS, about 200 nt-long downstream of *orf8* as observed from sequence analysis using software available at <http://mfold.rna.albany.edu/?q=DINAMelt> (**Figure 4.4**).

As expected, the transcript appeared only in the lytic state (after induction with MC). It was visible already 20 min after adding MC and its amount increased with time. No transcript could be observed in the lysogenic, non-induced state. The non-inducible derivatives, J34-12 and J34 KO-*orf3*, revealed no transcript after addition of MC (**Figures 4.3 & 4.5**). This makes sense because both *cro* and *ant* are required only in the lytic pathway.

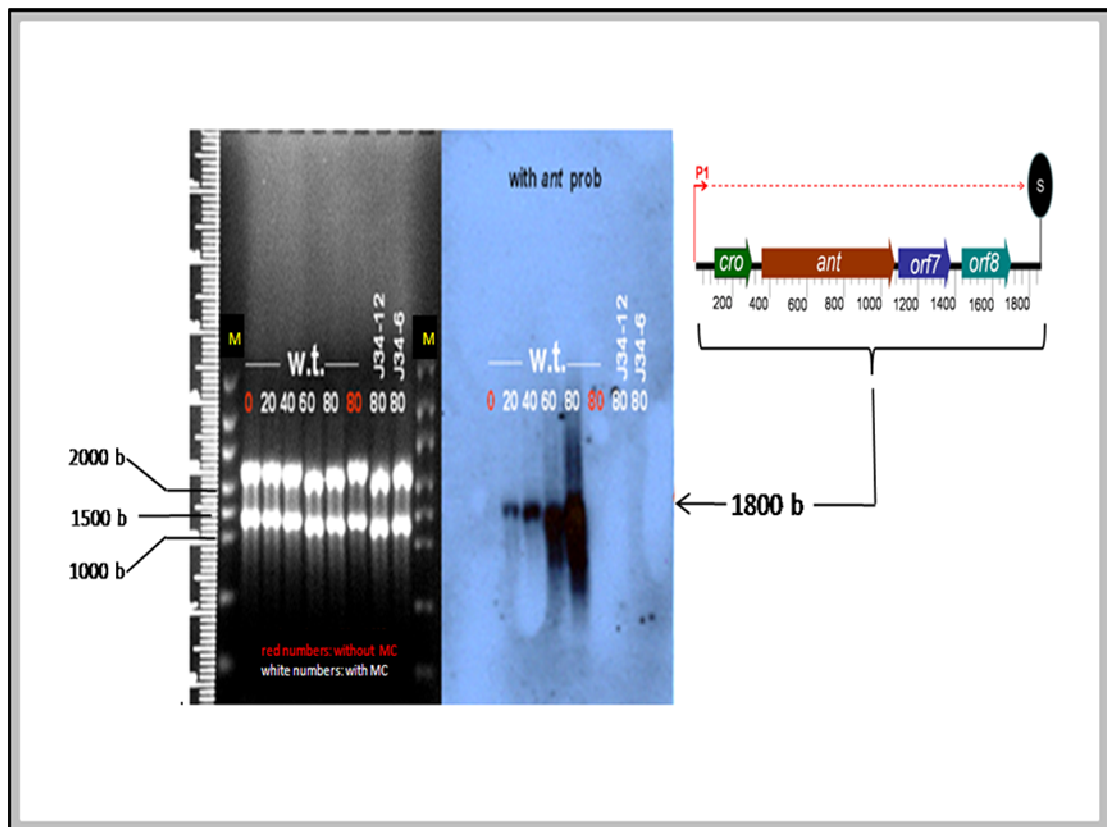


Figure 4.3. Northern blot hybridization of the w.t. strain J34 with *ant* probe. Right-hand, a schematic representation of the genes is shown. Left-hand agarose formaldehyde gel and its corresponding X-ray film are seen. A signal of about 1800 nt-long mRNA species corresponds to genes *cro*, *ant*, *orf7*, and *orf8* (polycistronic mRNA); *S. thermophilus* J34-12, and *S. thermophilus* J34-6 were used as non-inducible and phage-cured controls. Symbol S in highlighted black circle indicates a SLS (**Figure 4.4**). Total RNAs were isolated at the indicated times in min (top of lanes); M, RNA size marker (see **Figure 2.7**).

lysogeny module

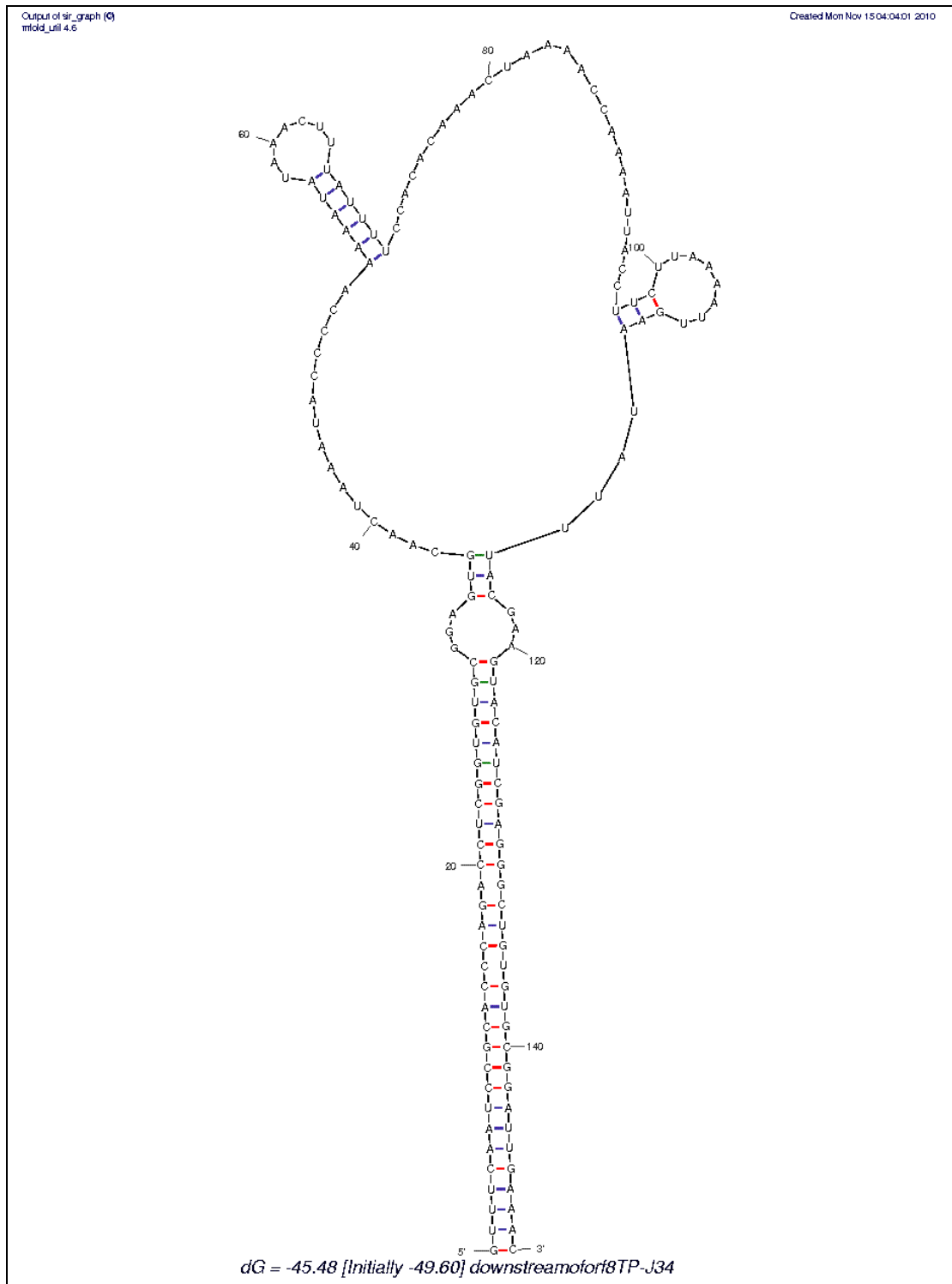


Figure 4.4. SLs located in the 3' UTR of *orf8* using software "DINAMelt" available at <http://mfold.na.albany.edu/?q=DINAMelt>

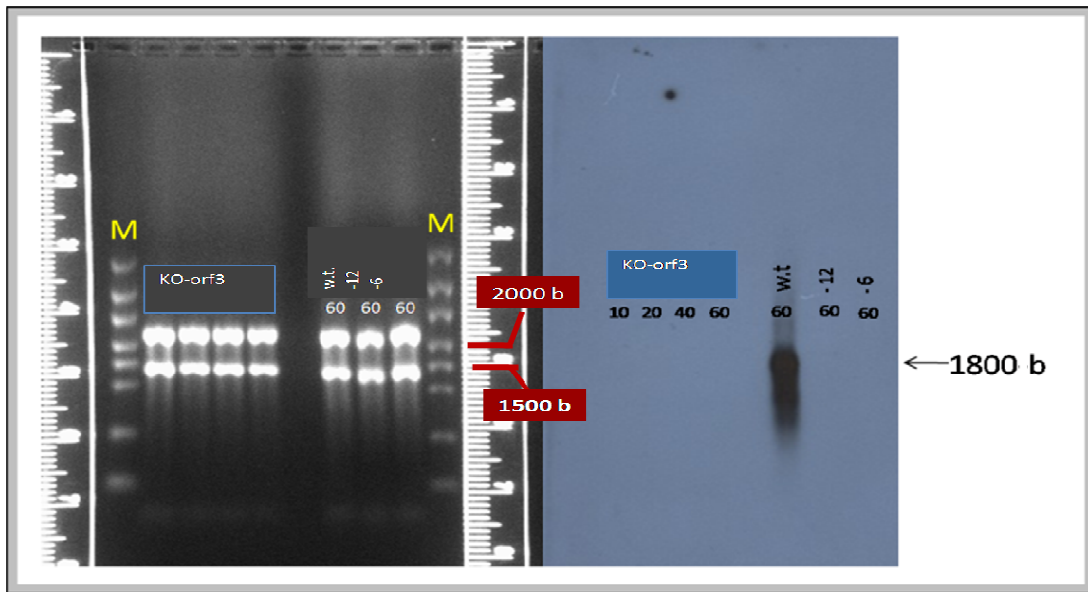


Figure 4.5. Northern blot hybridization using *ant* probe with *S. thermophilus* J34 and its derivatives (KO-orf3, -12 & -6), in the presence of MC. In the left hand agarose formaldehyde gel of total RNAs corresponding to the hybridized signal in the right hand X-ray film. The signal was observed only in the lytic state (the case of w.t. with MC). Total RNAs were isolated at indicated times in min (top of lanes). M, RNA size marker (see **Figure 2.7**). Sizes of the bands of interest are noted on the right margin. Abbreviations: w.t.=*S. thermophilus* J34; KO-orf3=*S. thermophilus* J34 KO-orf3; -12=*S. thermophilus* J34-12; -6=*S. thermophilus* J34-6.

3.10 Transcriptional units of the leftward genes

RT-PCR analysis was applied to identify the units of transcriptions of the lysogeny module, i. e., which genes are transcribed together (polycistronically) and which are transcribed independently (monocistronically). RT-PCR was employed for RNA analysis by using several primer-pairs binding to different parts of the leftward genes of the module of interest (i.e., *crh*, *orf3*, *ltp*, and *int*) (**Figure 4. 6**). Production by RT-PCR of a product comprising several genes reveals that these genes are transcribed as one unit (polycistronic). *Vice versa*, the absence of a product reveals that these genes are transcribed as different transcripts.

The results of the RT-PCR experiments are outlined in **Figure 4.6** and can be summarized as follows:

- a) Bands 1 & 2 (within *int* and *ltp*, respectively) were control bands confirming both the integrity of RNA samples and efficiency of the RT-PCR program.
- b) Band 3, 966 bp, was a product extending from *int* (primer "int. R") to downstream beyond *attL* in the host chromosomal DNA (primer "Primer 4"), revealing that there is no transcription terminator downstream of *int*.
- c) Band 4, 1226 bp, was a product extending from *ltp* (primer "ltp. F") to *crh* (primer "pri.cl-R"), indicating that *crh*, *orf3*, and *ltp* are transcribed together as a polycistronic mRNA (*cro*-operon).
- d) Band 5, 1343 bp, was a product extending from *int* to *ltp*. It was only seen in conventional PCR, but not in RT-PCR, indicating that such a transcript does not exist. Thus, the *int* gene is transcribed independently from the other genes (i.e., *ltp*, *orf3* and *crh*).

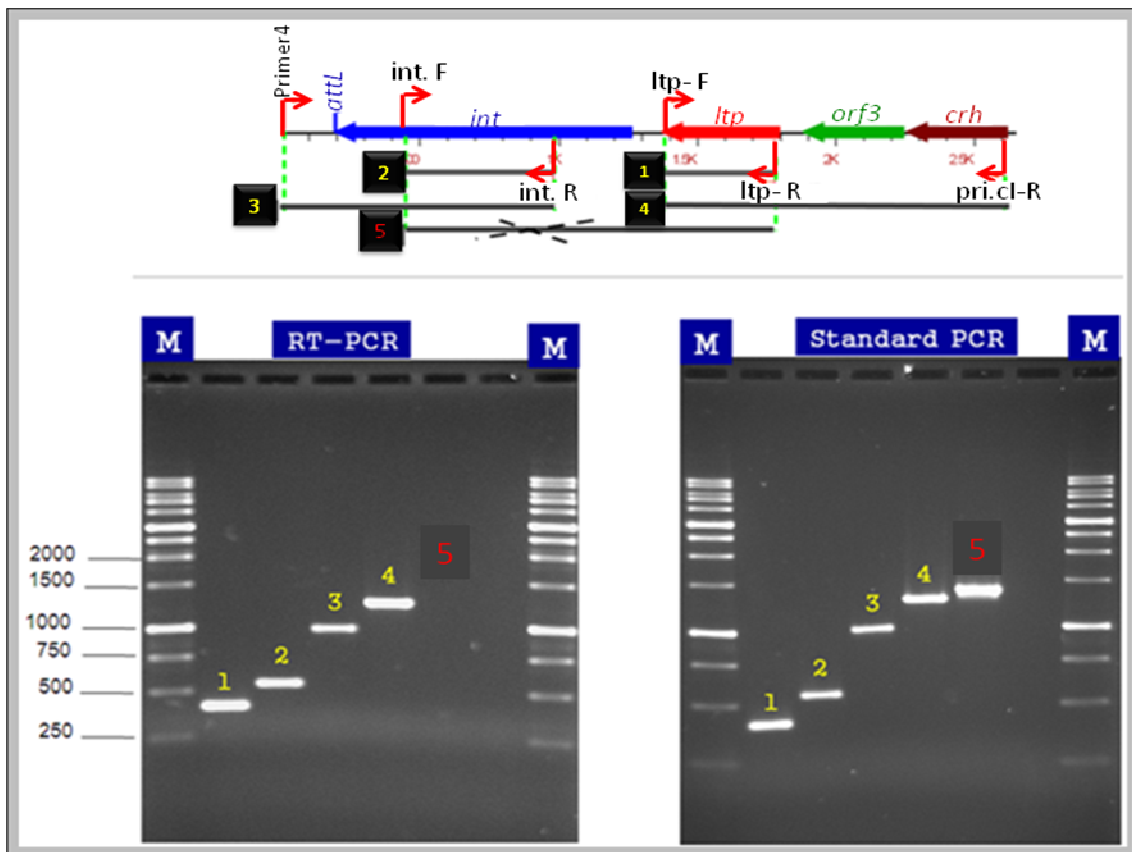


Figure 4.6. Transcriptional analysis by conventional (standard) and RT-PCR of the lysogeny module in the prophage state. The numbers above the bands on agarose gels correspond to the numbers of the transcripts shown above. Band no. 5 is only seen in conventional PCR but not in RT-PCR, indicating that such a transcript does not exist; M, 1-kb marker (AppliChem, Darmstadt, Germany), the sizes are noted on the margin.

3.11 Polar effect of *crh*-operon on *int* transcription

To find out if there was a polar effect of *orf3* knock out on the transcription of *int*, RT-PCR was performed to *S. thermophilus* J34 KO-*orf3* using primer-pairs "Primer 4" and "int. R" binding downstream of *crh*-operon in *int* gene and chromosomal DNA, respectively (**Figure 4.7**). The idea was that the existence of such an RT-PCR product (transcript of *int*) would indicate that *int* gene was transcribed in the absence of P2. The integrated vector, pGhost9, blocks the promoter upstream of this gene, and hence blocks their transcriptions (polar

lysogeny module

effect). The results show that *int* gene is transcribed in *S. thermophilus* J34 KO-orf3, confirming that it is transcribed independently from promoter P2. However, the quantity of product was weaker than that of w.t. This may be due to a regulation effect of Orf3 on the transcription of *int* gene.

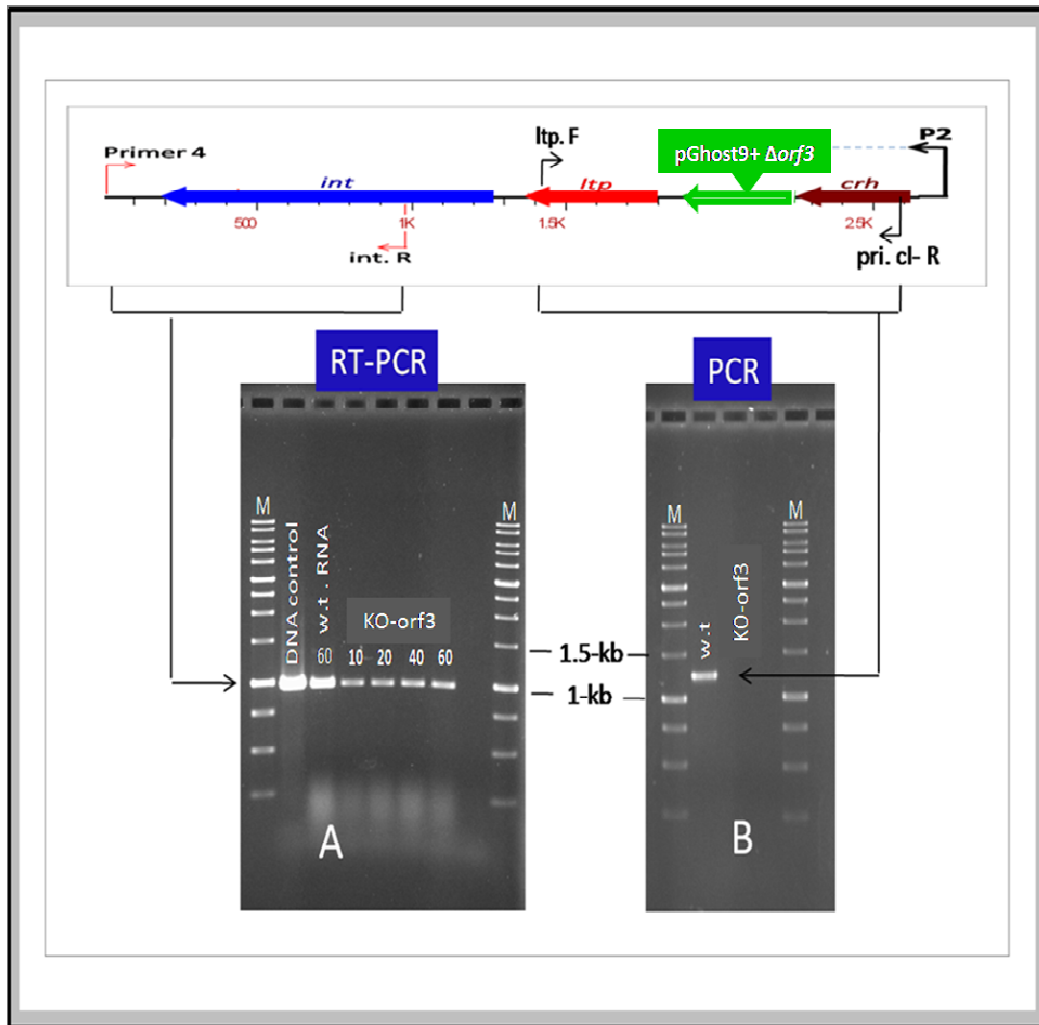
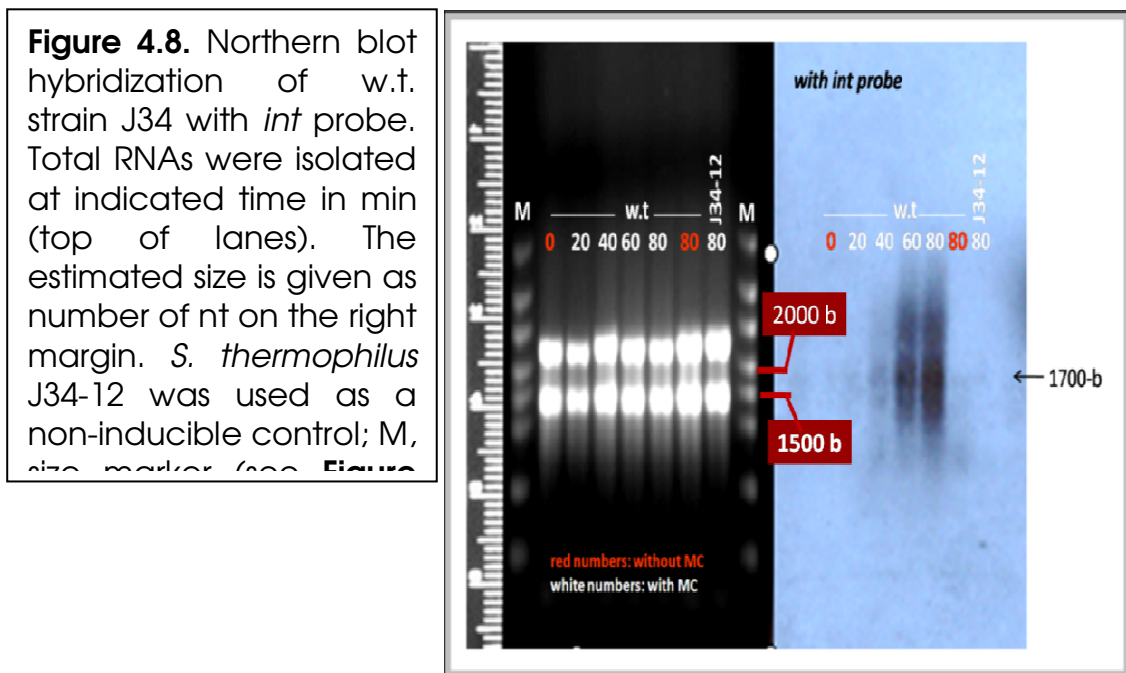


Figure 4.7. Transcription of *int* gene of *S. thermophilus* J34 KO-orf3. Left-hand gel, RT-PCR products, from primer-pair: “Primer 4” and “int. R”. Expression of *int* confirming that this gene transcribed independently from other genes. The amount of transcript in *S. thermophilus* J34 KO-orf3 was weaker than the w.t. Standard PCR (right-hand gel) was performed to confirm the inactivation of *orf3* by using primer-pair flanking this gene. PCR product observed only in the w.t. and disappeared in the knock out. M, 1-kb marker (AppliChem, Darmstadt, Germany).

3.12 Transcription of *int*

3.12.1 Northern blot analysis

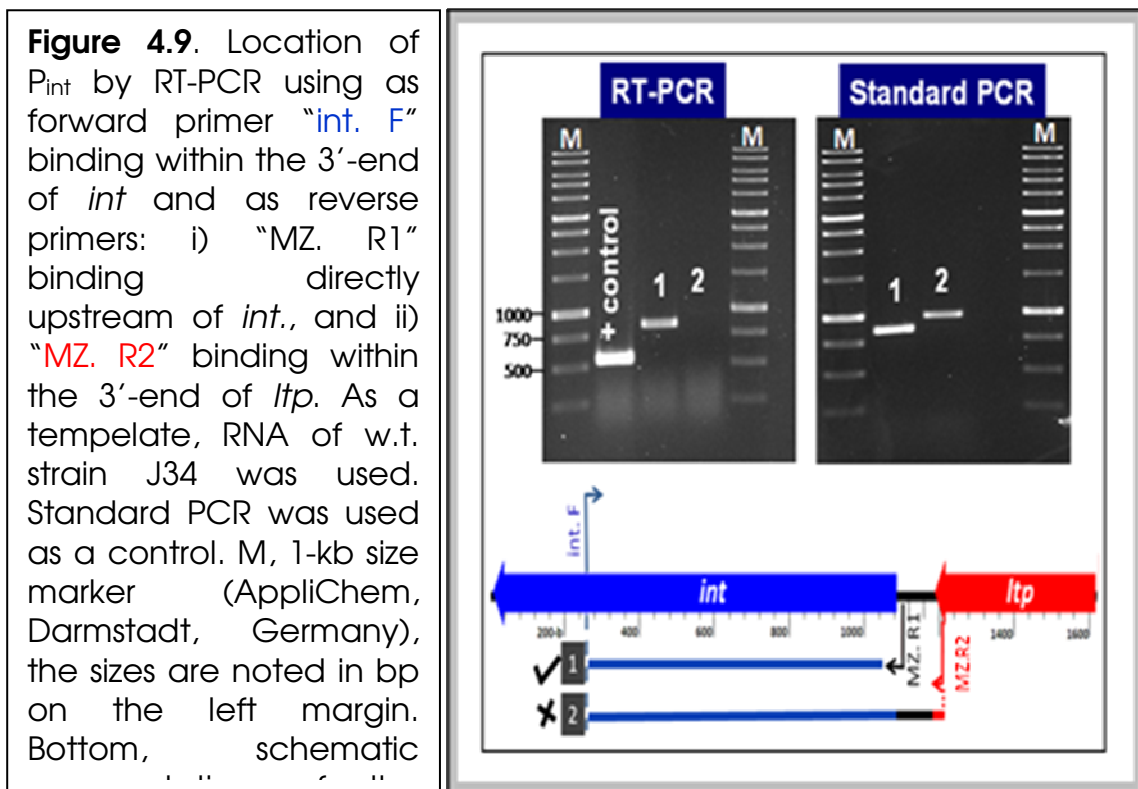
Northern blot hybridization of the *int* gene was performed for lysogenic *S. thermophilus* J34 and its non-inducible derivative J34-12 under lysogenic (-MC) and lytic (+MC) conditions. An internal fragment of *int* gene, Δint , was amplified by PCR using primer-pair "int. F" and "int. R" (see **Figure 3.3**) and labeled as described in Materials & Methods. **Figure 4.8** shows the *int* transcript as a weak transcript ≥ 1.7 kb and it was detected in the lytic state, 20 min after adding MC. The transcript of *int* has been shown to be transcribed independently of the other genes of this module. Thus, it should be transcribed from its own promoter, putative P_{int} .



3.12.2 Location of P_{int}

3.12.2.1 By RT-PCR

To find out whether P_{int} is located in the upstream *ltp/int* intergenic region, RT-PCR was carried out using as forward primer "int. F" binding within the 3'-end of *int* and as reverse primers: i) "MZ. R2" binding within the 3'-end of *ltp*, and ii) "MZ. R1" binding directly upstream of *int* (**Figure 4.9**). For more details about the primers see Materials & Methods. An RT-PCR product was detected only with primer "MZ. R1". This result confirmed that the *int* gene is transcribed from its own promoter, P_{int} , which is located directly upstream in the *ltp/int* intergenic region.



3.12.2.2 By sequence analysis

The intergenic region between *int* and *ltp* was screened for putative promoters using the "Promoter Predictor of Prokaryotes" program available at http://www.fruitfly.org/seq_tools/promoter.html.

lysogeny module

As outlined in **Figure 4.10**, a potential promoter was predicted to start at nt number 52 and end at nt number 97 within the 103 nucleotides of *ltp/int* intergenic region. The score of this promoter is not completely perfect, 0.82, and this might explain why the transcript of this gene is weak. The general conclusion here is that *int* gene is transcribed monocistronically as a weak transcript of ≥ 1.7 kb-long.

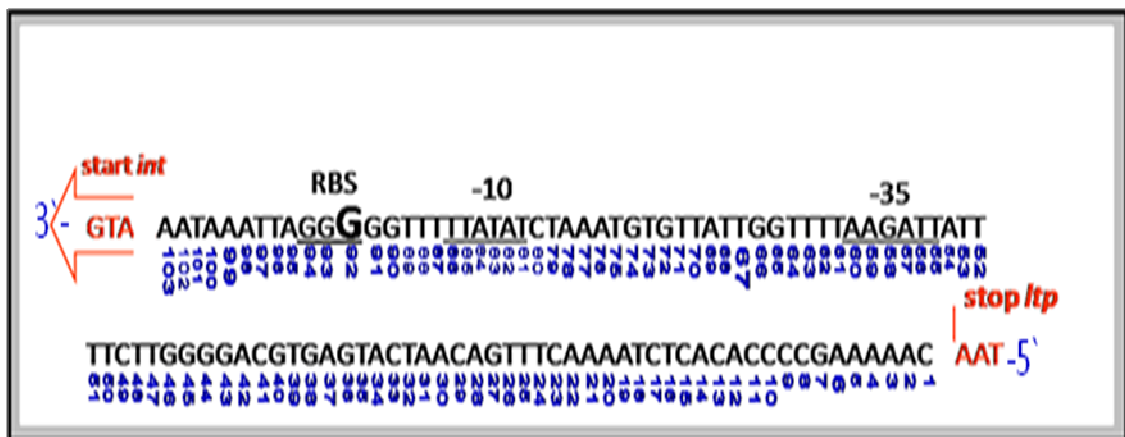


Figure 4.10. Promoter prediction within the intergenic region between *ltp* and *int* of TP-J34 using program available at: http://www.fruitfly.org/seq_tools/promoter.html ; **G** in large font, start of transcription. The score of the promoter is 0.82. RBS was determined as described by **Oliveira et al., 2004**. Note that the DNA sequence is shown from 3`- to 5`-end.

3.12.3 *Int* gene is transcribed in both lytic and lysogenic state

3.12.3.1 RT-PCR analysis

Multiplex RT-PCR was employed to detect transcription of *int* and *orf44*, a tail gene (as a control) using primers: i) "Primer 4" and "int. R" to detect *int* transcription, and ii) "Tail-F" and "Tail-R" to detect tail gene transcription. Tail gene primers were used to verify the efficiency of RT-PCR, as a late gene is expected to be detected only late in the lytic growth cycle. As shown in **Figure 4.11**, *int* transcripts were detected in both lytic and lysogenic state.

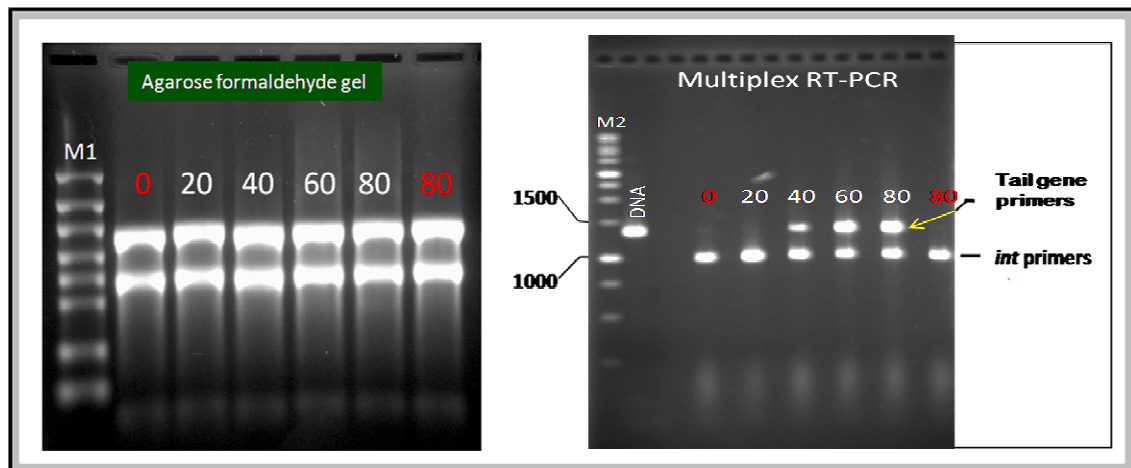


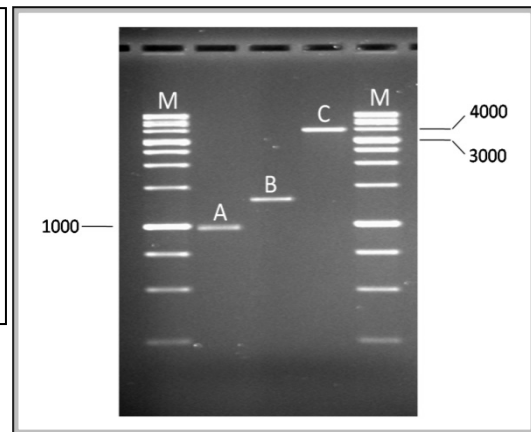
Figure 4.11. Transcription of *int* in both lytic- and lysogenic developments. Left-hand, RNAs samples of *S. thermophilus* J34f-2 on agarose formaldehyde gel. Right-hand, multiplex RT-PCR products of the same samples using primer-pair: for *int*, "Primer 4" and "int. R" and for *orf44*, "Tail-F" and "Tail-R". DNA sample was used with tail primers to confirm the efficiency of RT-PCR primers and program. Total RNAs were isolated at the indicated times in min (top of lanes). Red numbers without MC and white numbers with MC. M1 and M2, size marker for RNA (see **Figure 2.7**) and DNA 1-kb marker (AppliChem, Darmstadt, Germany), respectively.

3.12.3.2 Sequence analysis of RT-PCR product

To ensure that the RT-PCR products (**Figure 4.11**) were due to transcription of *int* and *orf44*, both fragments were isolated from the gel, cloned into pSTBlue-1 (**Figure 4.12**) and sequenced as described in Materials & Methods.

The results revealed that these two fragments were identical to the genes of interest, i.e., either *int* or *orf44*, confirming that *int* is transcribed in both lytic and lysogenic life cycles. As expected, transcription of tail gene was detected late in the lytic cycle.

Figure 4.12. Agarose gel of *int* RT-PCR product (A); tail gene product (B), and pSTBlue-1 digested with EcoRV (C). Sizes of interested bands are noted on the margins. M, 1-kb size marker (AppliChem, Darmstadt, Germany).



3.13 Transcription of *Itp*

3.13.1 Northern blot

As a probe for Northern blot hybridization, an internal fragment of *Itp* gene was amplified by PCR using primer-pairs "Itp. F" and "Itp. R" (see primer details in Materials & Methods), and labeled as described in Materials & Methods.

Northern blot hybridization of *S. thermophilus* J34 with *Itp* probe in **Figure 4.13** showed 2 transcripts: i) a majority of monocistronic transcripts comprising just *Itp* and ii) very few polycistronic transcripts comprising genes *crh*, *orf3* and *Itp* (called hereafter *crh*-operon). Both transcripts were detectable with and without addition of MC. The *crh*-operon transcript had been confirmed by RT-PCR.

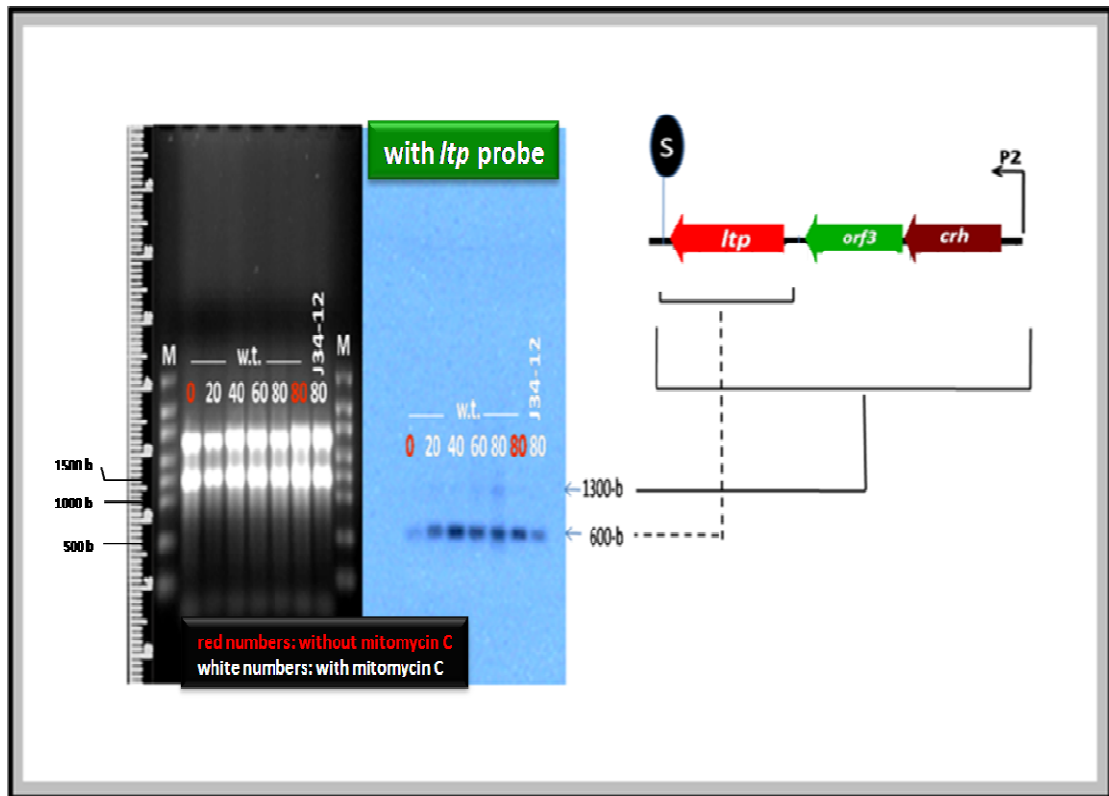


Figure 4.13. Northern blot hybridization for w.t. and its derivative J34-12 with *ltp* probe. In the right-hand, genes analysed are shown; Circled S, SLS; P2, putative promoter reads toward leftward genes. In the left-hand, a gel and its hybridization on X-ray film. Total RNAs were isolated at the indicated times in min after adding MC in white color; or without MC in red color (top of lanes). *S. thermophilus* J34-12 was used as a non-inducible control; M, RNA size marker (see **Figure 2.7**).

To clarify whether *ltp* is transcribed from its own promoter, Northern blot hybridization and RT-PCR with *S. thermophilus* J34 KO-*orf3* were performed. For RT-PCR analysis, primer-pair of *ltp*, "ltp. F" and "ltp. R", was used.

As shown in **Figure 4.14**, an RT-PCR product was detected. Northern blot hybridization detected the 550 b signal as a very weak signal and a 1300 b signal was not seen. This confirmed that inactivation of *orf3*, upstream of *ltp*, prevented transcription of the polycistronic mRNA (*crh*-operon), but not of the monocistronic one. Sequence analysis of *orf/ltp* intergenic region, using the previous

predictor promoter program, detected a weak promoter structure of score 0.49 (Figure 4.15).

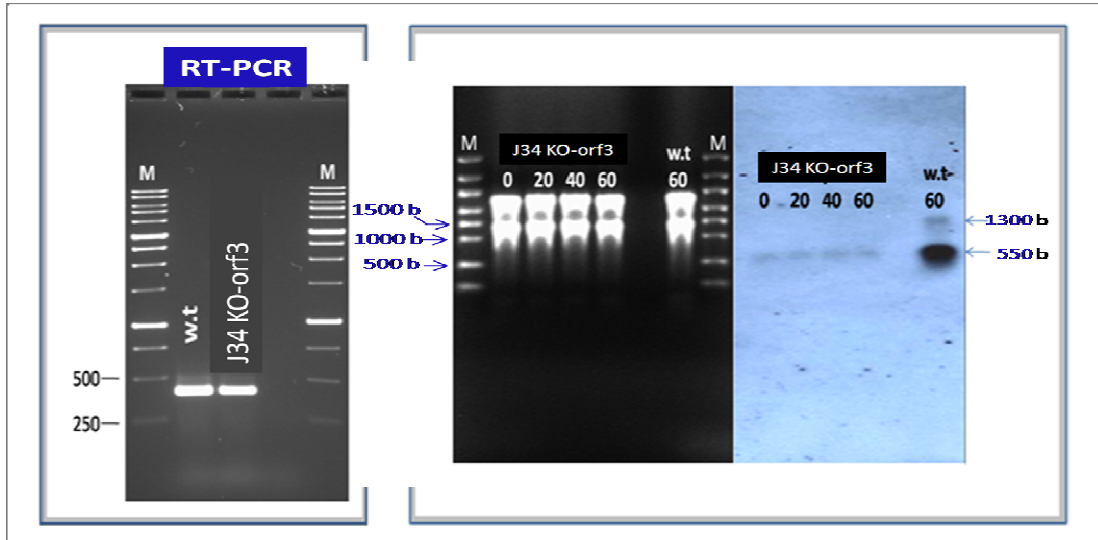


Figure 4.14. *ltp* transcription of *S. thermophilus* KO-orf3. Left-hand, RT-PCR using primer-pairs of *ltp* ("ltp. F" and "ltp. R"); Right-hand, Northern blot hybridization with *ltp* probe. Total RNAs were isolated at the indicated times in min after adding MC (top of lanes); w.t. was used as control; M, Size marker; sizes are indicated in the margins.

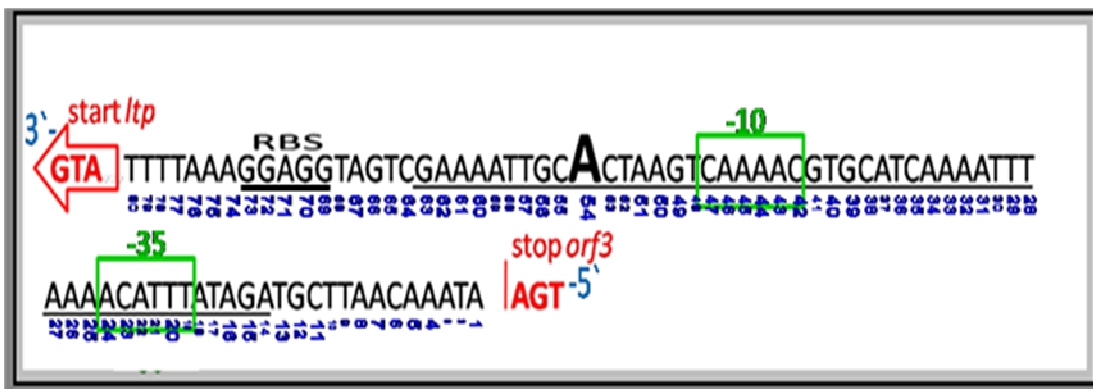


Figure 4.15. The predicted promoter of *ltp* (underlined) in *orf3/ltp* intergenic region (80 b-long) as described by "Promoter Predictor of Prokaryotes" program available at:

http://www.fruitfly.org/seq_tools/promoter.html "A" at large font is the transcription start; -10 and -35 regions are indicated in green boxes. RBS is noted and determined as characterized by **Oliveira et al., 2004**. The score of the promoter is 0.49. Note that the DNA sequence is shown from 3`- to 5`-end.

3.13.2 Stem-loop structure (SLS) downstream of *ltp*

Sequence analysis of TP-J34 revealed that there is a strong SLS downstream of *ltp* gene (**Figure 4.16**), which may act as a terminator. When taking into account the potential *ltp* promoter, P_{ltp} , (with its indicated transcription start point) and the potential terminator structure, a transcript of about 550 nt would be formed. This is well in agreement with the *ltp* transcript as analysed by Northern blotting.

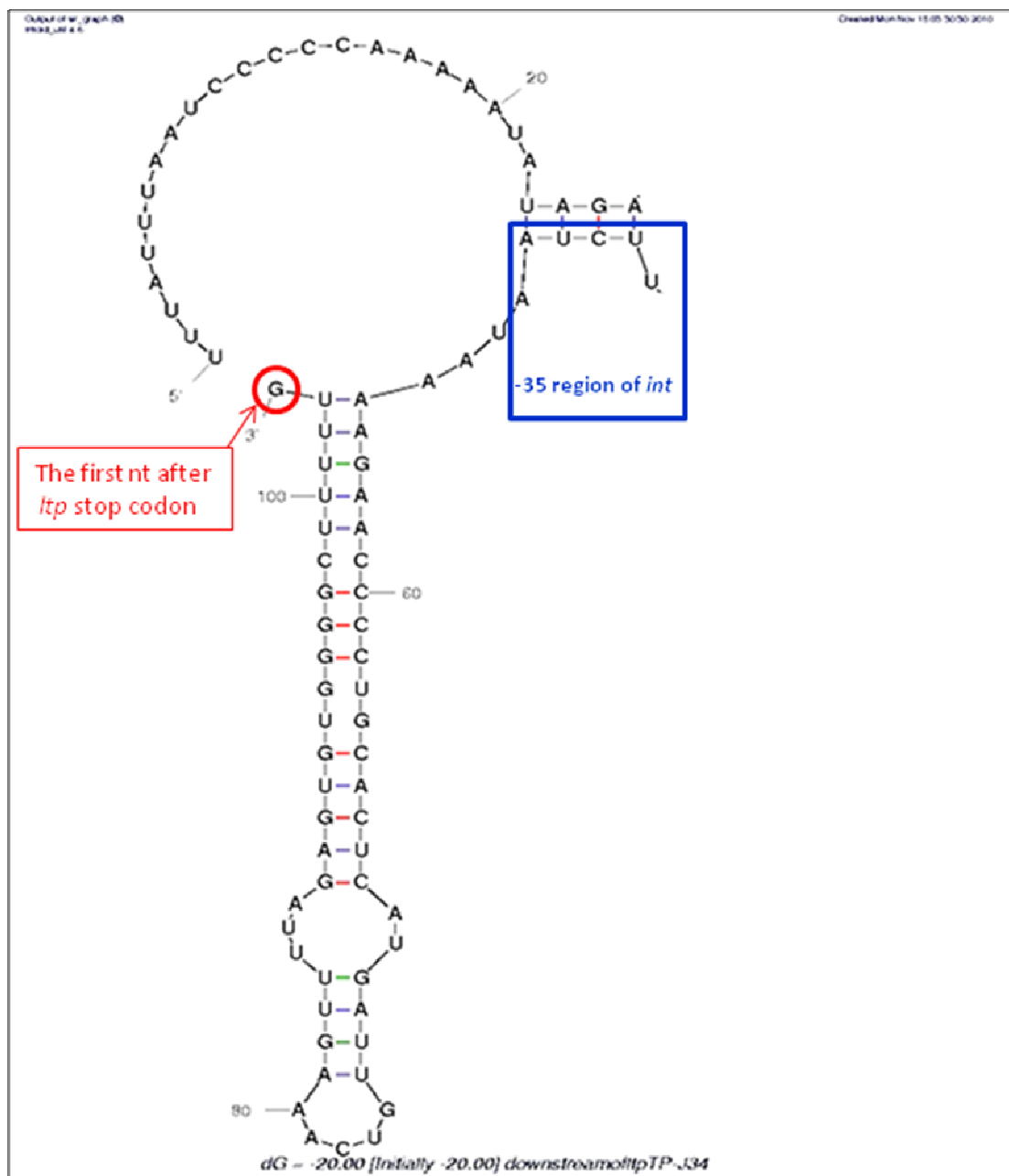
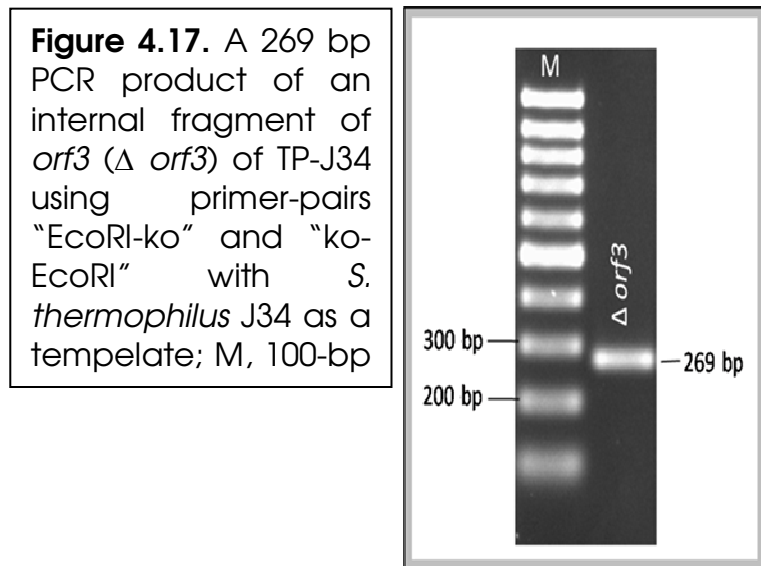


Figure 4.16. SLS in the *Itp/int* intergenic region using DINAMelt software available at <http://mfold.rna.albany.edu/?q=DINAMelt> Compared to **Figure 4.10**, the complementary strand is shown. The G (circled in red) is the first nt after the *Itp* stop codon; the -35 region is boxed in blue.

3.14 Northern blot analysis of *orf3*

As a probe for Northern blot hybridization of *orf3* gene, an internal fragment of *orf3*, Δ *orf3*, 269 bp, was amplified by PCR using primer-pairs "EcoRI-ko" and "ko-EcoRI" (**Figure 4.17**). The fragment was labeled as described in Materials & Methods.



As expected, Northern blot hybridization using *orf3* probe produced a signal of about 1300 nt-long (**Figure 4.18**), corresponding to the polycistronic mRNA of *crh*-operon. This transcript was detected also with *Itp* probe in the w.t. (**Figure 4.13**), but disappeared in the mutated *orf3* derivative, *S. thermophilus* J34 KO-*orf3* (**Figure 4.14**). Thereby, it could be confirmed that i) *crh*, *orf3*, and *Itp* are transcribed as a 1300 nt-long polycistronic mRNA, ii) inactivation of *orf3* prevented the transcription of their polycistronic mRNA, and iii) *Itp* is transcribed from two independent putative promoters, P2 and P_{Itp}.

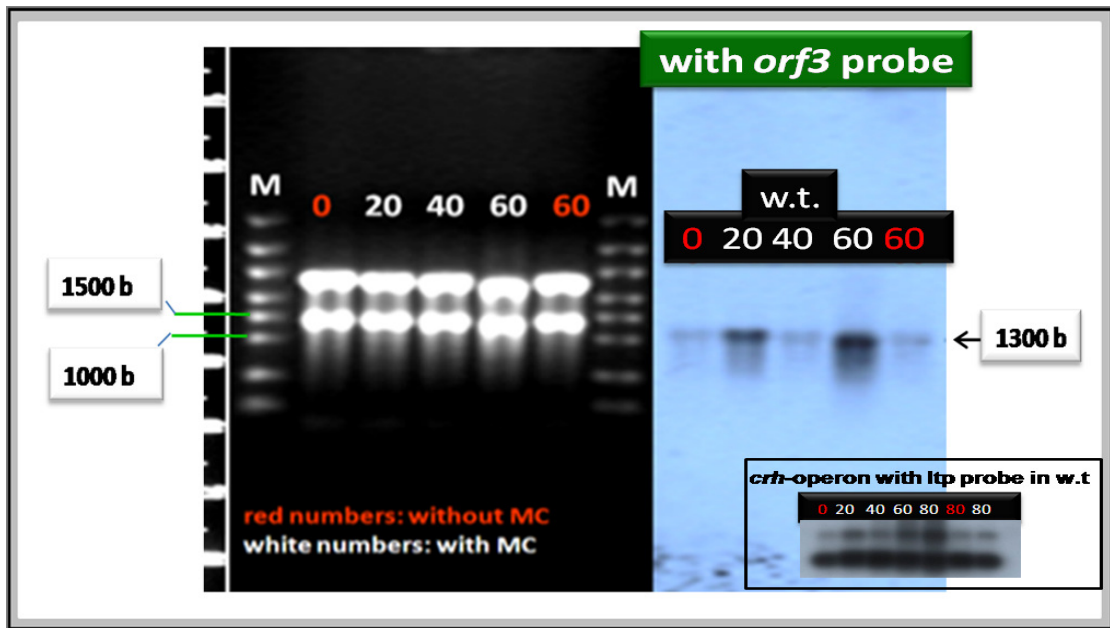


Figure 4.18. Northern blot hybridization of w.t. strain J34 with *orf3* probe. Total RNAs were isolated after adding or in the absence of MC at the indicated times in min (top of lanes). M, Size marker; the size in b is indicated in the right margin. As a comparison the polycistronic transcript of *ltp* with MC (time in min) is shown. Transcripts appear alternatively weak and strong over time after adding MC.

Northern hybridization with *orf3* probe produced alternatively weak and strong signals over time after addition of MC (**Figure 4.18**). It should be noted that this alternation appeared to be visible also when the heavily exposed Northern blot shown in **Figure 4.13** was analysed, when the *ltp* probe was applied. One may speculate that this alternation was due to fluctuations in the amount of the Crh protein, which may act for this operon (*crh*-operon) as either an activator or a repressor in low or high concentration, respectively. See scenarios 1-3 of the genetic switch region in **Figure 4.22**, for further explanation.

3.15 Discussion and Conclusion

Phage are useful tools for the molecular analysis of transcriptional regulation mechanism. Their model systems have been used to address

a number of essential questions in evolutionary biology (**Desiere *et al.*, 2001; Ventura & Brüssow, 2004**) and have played a fundamental role in the history of molecular biology (**Hadas *et al.*, 1997**). Temperate bacteriophage have two lifestyles, lytic and lysogenic. The decision of a temperate phage to enter either lysogeny or lysis is controlled by a gene regulatory system termed *genetic switch*, which affects the initiation of lysogenic and lytic transcripts (**Pedersen *et al.*, 2010; Ptashne, 2004**). In the lysogenic state, the only regulatory protein produced is the repressor, which blocks the lytic pathway until external signals (e.g. UV irradiation) command to switch from lysogeny to lytic development (**Ptashne, 2004; Teif 2010**). In the lytic pathway, many genes needed for efficient phage multiplication are turned on. *Cro* and *ant* genes switch the lysogenic genes off at the beginning of lytic development (**Desiere *et al.*, 2001; Ventura *et al.*, 2002b**). Nevertheless, even for the most extensively studied phage λ , many aspects about genetic switch and gene regulation are still matters of controversy (**Atsumi & Little, 2006; Schubert *et al.*, 2007; Svenningsen *et al.*, 2005; Teif, 2010**).

Based on experimental observations made by Northern blotting, RT-PCR, and sequence analysis of prophage TP-J34, the following conclusions are drawn:

- i) The *int* gene, with a size of 1080 bp, is transcribed as a weak transcript of ≥ 1.7 -kb, from its own promoter located in the intergenic region between *int* and *Itp*. There appears to be no terminator downstream of *int*, resulting in runaway transcription into the host genome. This result is in agreement with that of both phages O1205 and Sfi21. However, unlike TP-J34, the 5'-ends of those transcripts are located within the upstream gene, *sie* (**Ventura *et al.*, 2002b**). Likewise, *int* of the temperate lactococcal phi LC3 is expressed from its own

promoter, P_{int} , as a 1.2 kb transcript, which is only detected in the lytic state (Blatny *et al.*, 2003). Having a score of 0.82, P_{int} of TP-J34 is not completely perfect in relation to the consensus promoter, which may explain its weak transcription. This result is in harmony with that of Ventura *et al.*, 2002b, who concluded that an *int*-specific probe yielded only a weak hybridization signal in all *S. thermophilus* lysogens.

RT-PCR analysis was employed as an accurate approach to detect transcription of this gene in both lifestyles, lytic and lysogenic. By this approach, the *int* transcript could be detected in both pathways. To confirm the efficiency of RT-PCR, *orf44*, a tail protein gene transcribed only late in the lytic cycle, was analysed as a control. As expected, an *orf44* transcript was only detected late in the lytic pathway and was not detectable in the lysogenic state.

- ii) *Ltp* is transcribed in two different transcripts: a) a few copies of polycistronic mRNA comprising genes *crh*, *orf3* and *ltp* (also detected by an *orf3* probe, *crh*-operon), and b) a majority of monocistronic transcripts comprising just *ltp*. The promoter of the monocistronic transcript appears to be located in the intergenic region between *ltp* and *orf3*. This result is similar with the superinfection exclusion gene (*sie*) transcript of *cos*-site phage Sfi21 (Ventura *et al.*, 2002b). Within its lysogenic module, two transcripts were detected: i) a 1.6-kb-long mRNA starting at the promoter of the phage repressor gene and covering the next two genes, including *sie* and *orf122*, encoding a protein with a metalloprotease motif, and ii) a transcript of about 1 kb, initiating at the promoter of the *sie* gene. Also, *ltp* of TP-J34 has a transcription pattern very similar with *sie* of the *S. thermophilus* lysogen containing the *pac*-site

phage O1205 (Ventura, *et al.*, 2002b). This result is in harmony with that of Balleza *et al.*, 2009 & Ptashne, 2004 who pointed out that some genes may be transcribed by several promoters. P_{ltp} of TP-J34 appears to be very weak, score 0.49, and thus should require an activator. Orf3 may either act by itself or regulate other unknown function as an activator for *ltp* transcript. This may explain why *ltp* is transcribed only very weakly in the *orf3* knock out derivative *S. thermophilus* J34 KO-*orf3*.

- iii) Transcripts of *crh* were detected in both lytic and lysogenic developments and disappeared in the *orf3* mutant derivative *S. thermophilus* J34 KO-*orf3*. Transcripts appeared to alternate between weak and strong over time after addition of MC. This may be caused by self regulation of Crh as in the case of CI repressor of phage λ (see "Gene regulation and genetic switch in phage lambda" in the general introduction).
- iv) Based on our data from Northern blot, RT-PCR, and sequence analysis, two alternative scenarios regarding the genetic switch of TP-J34 are suggested as shown in **Figures 4.19 & 22**:

- 1) This scenario is based on two regulatory proteins, Cro and Crh, and their promoter and operator sites on the DNA constituting the genetic switch, together with RNAP. The *crh/cro* intergenic region of TP-J34 (168-bp) contains two back to back putative promoter elements, P1 for rightward transcription and P2 for leftward (**Figure 4.20**). These promoters show high homology to *E. coli* consensus promoters. "O_{1A}" is a putative operator consisting of a 15-bp inverted repeat with internal dyad symmetry, which overlaps with the rightward promoter (P1) sequence. The other operator "O_{1B}" is identical to the first one and is located in the *cro/ant* intergenic region (**Neve *et al.***,

1998). The *crh*-operon is transcribed in both pathways. Crh is apparently needed – as the λ repressor - in the lysogenic pathway as a repressor for lytic genes, whereas in lytic development, Crh may be digested by some kind of protease (such as Rec-A upon induction of the SOS system). Cro by itself cannot switch the lytic development on, and it seems to need Orf3 to do the switch, as outlined in **Figure 4.19**. Likewise, for phage TP901-1, **Nakanishi *et al.*, 2009** concluded that the lytic promoter P_L is repressed by Cl_{TP901} repressor, whereas repression of the lysogenic promoter P_R requires the presence of both the antagonistic regulator proteins, the modulator of repression designated MOR_{TP901} , and Cl_{TP901} repressor. MOR_{TP901} does not exhibit repression of either promoter.

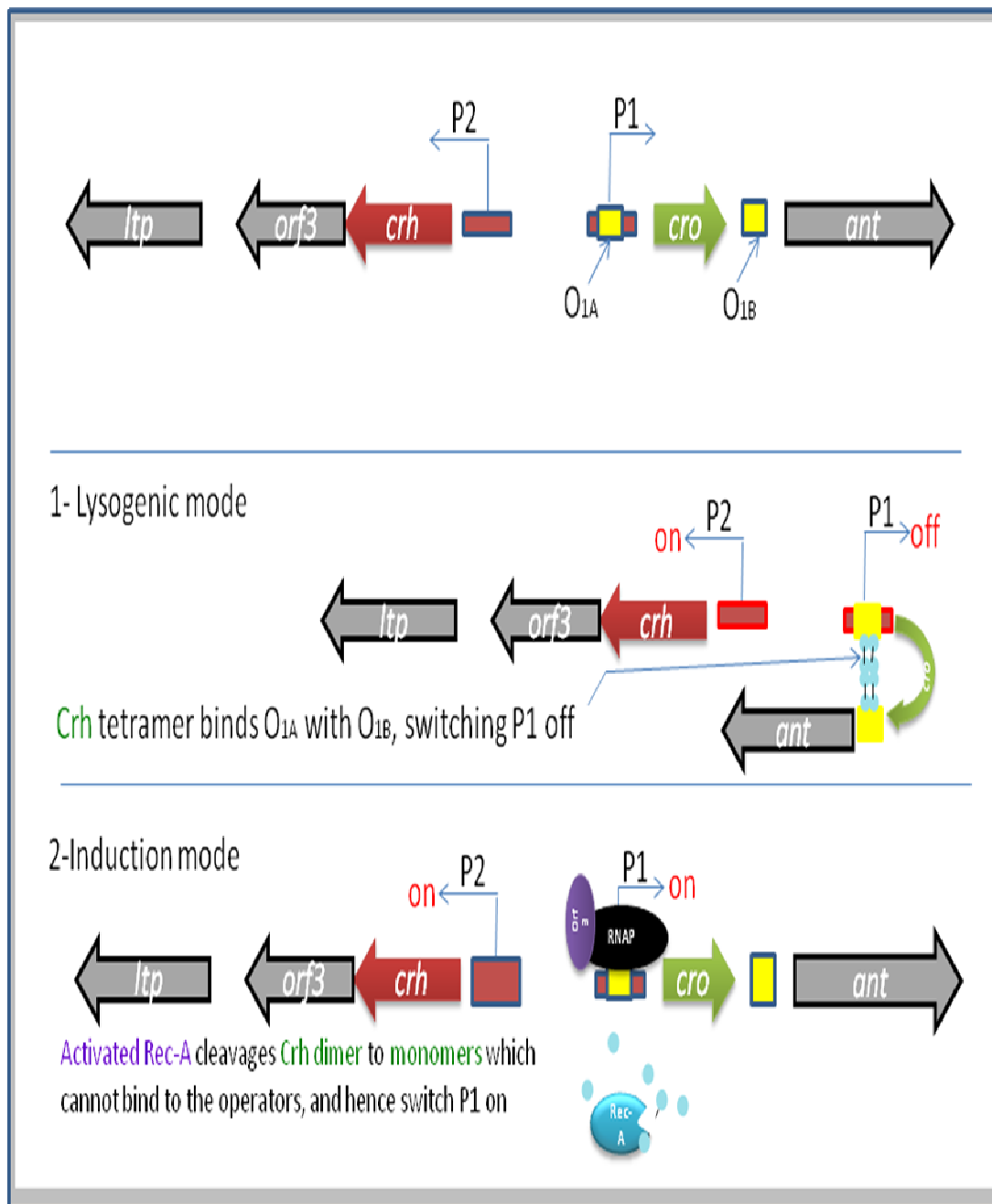


Figure 4.19. A hypothetical schematic representation to explain transcription of *crh*- and *cro*-operon (Scenario 1). (1) In the lysogenic state, *crh*-operon is "on", while *cro*-operon is "off" because Crh binds O_{A1} (Crh can form tetramers to bridge O_{1A} to a similarity organized O_{1B} operator by about 250 bp apart) and blocks P1; (2) In the induction mode, both operons are active, Crh is produced, but it is cleaved as well by activated Rec-A. So it can not block P1. P1, in turns, needs an intact Orf3 to read *cro*-operon. It is assumed that P1 is repressed by Crh dimer binding to the operator O_{1A} since the operator has two inverted repeat sequence.

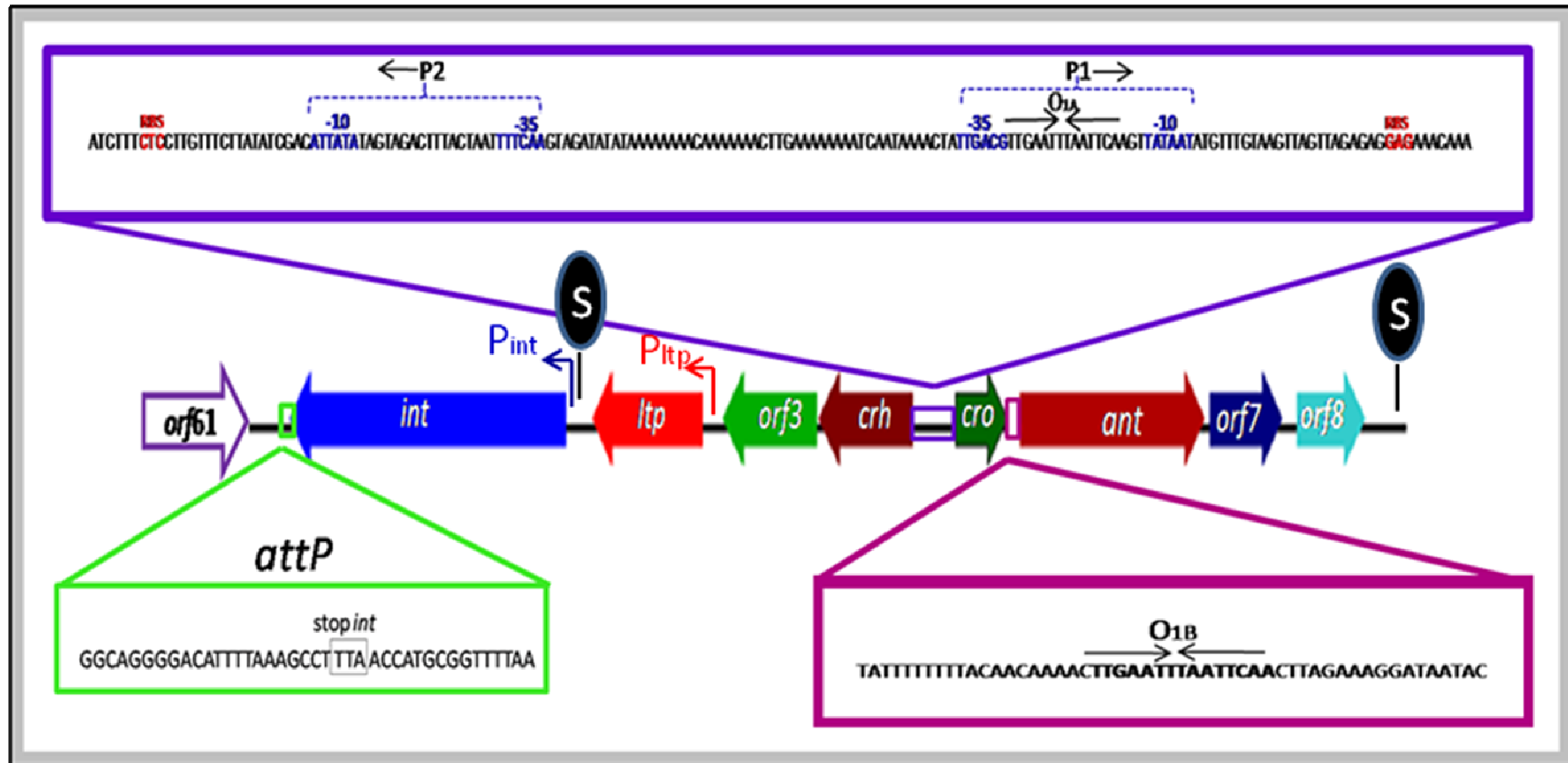


Figure 4.20. Schematic presentation of the localization of the putative genetic switch- and *attP*- region of phage TP-J34. The predicted genes are given in the highlighted arrows. Possible ribosomal binding sites (RBS), promoter sequences (-10 and -35), and operator sequences (O_{1A} and O_{1B}) are noted above the sequence. The sequence of *crh/cro* intergene region (168-bp) is mentioned. The site specific integration (40-bp core region) is identified by *attP* and *attB* to show as to the location of the *attP* site. O_{1A} and O_{1B} are also indicated.

2) The other scenario suspects that there are three operator sites in the *cro/crh* intergenic region named O1, O2, and O3 as shown in **Figure 4.21**. If one assumes a descending order of operator specificities for Crh and co-operativity between O1 and O2, then O3 - blocking *crh* transcription - would theoretically act on the opposite strand. This scenario, would be in agreement with that of phage λ (see the general introduction), where Crh repressor acts in self regulating.

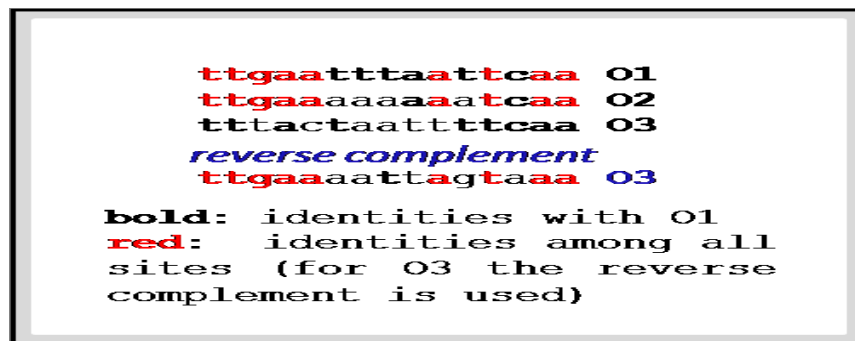


Figure 4.21. Putative operators in the *cro/crh* intergenic region

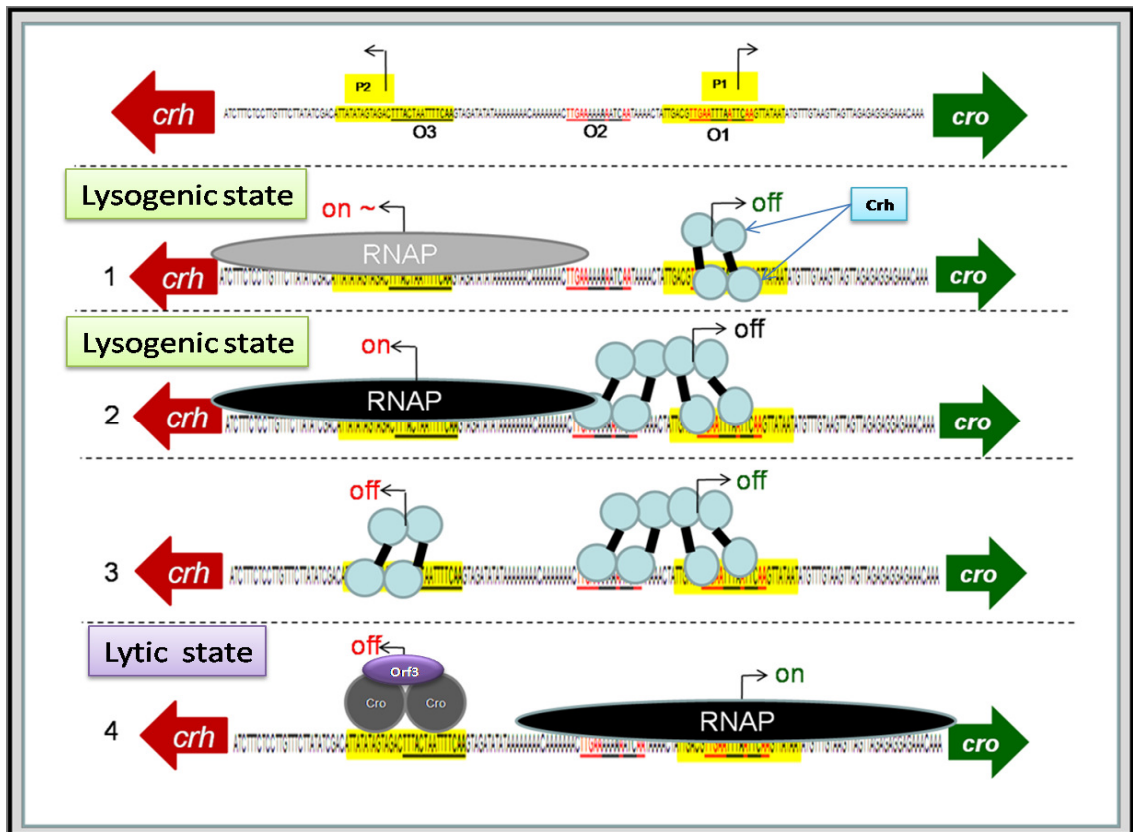


Figure 4.22. A hypothetical schematic representation to explain transcription of *crh*- and *cro*-operon (scenario 2). 1, *cro* is off and *crh* is semi active because Crh occupied O1 which is needed for P1; 2, Crh is bound in O1, O2 activating RNAP to transcribe *crh* on one side and block *cro* on the other side by covering O1 which overlaps with P1; 3, more Crh leads to repression of its own gene by covering its promoter; 4, under lytic condition proteinase such as Rec-A cleavages Crh, Cro in combination with Orf3 bound to O3 and hence block P2 and switch on *cro*-operon.

v) There are two SLs with high stability ($dG < -10$ Kcal mol⁻¹ is classified as higher stability, **Petrillo *et al.*, 2006**) in the module of interest, one downstream of *ltp* and the other about 200 nt in the 3' UTR of *orf8*. They appear to act as terminators, since the transcripts terminated are of rather well defined lengths.

vi) *Orf3* appears to affect transcription of three transcripts: 1) For the *ltp* monocistronic transcript it appears to act as positive regulator, since this transcript was weaker in *S. thermophilus* KO-*orf3* than in the w.t. in the presence of MC; 2) Transcripts of

the *crh*- and the *cro*-operon, respectively, seem to depend on Orf3, since they disappeared in *S. thermophilus* KO-orf3.

- vii) All mutations of *orf3* prevented prophage induction, even if *int*, *ltp*, *crh*, *cro* and *ant* were intact (as appeared from the sequence of TP-J34-12, chapter 3 of this work). Apparently, RNAP cannot initiate transcription of the *cro*-operon in the absence of Orf3.
- viii) *Cro*, *ant*, *orf7*, and *orf8* are transcribed together as a polycistronic mRNA (*cro*-operon) of about 1300 nt length. This transcript is detected in *S. thermophilus* J34 only during lytic development. It is not detected in the non-inducible derivative *S. thermophilus* J34-12. These results are in agreement with **Ventura *et al.*, 2002b**, who concluded that neither *cro* nor *ant* genes are transcribed in lysogenic cells of *S. thermophilus* containing *cos*-site prophage Sfi21.
- ix) Crh prevents transcription of the *cro*-operon, but transcription of the *cro*-operon cannot block transcription of the *crh*-operon. It is supposed that Crh is expressed and cleaved by activated Rec-A under conditions of induction. Orf3 appears to act as a critical positive regulator for initiation of the lytic mode, or for transcription of the *cro*-operon.
- x) **Neve *et al.*, 1998**, reported that the DNA sequence of the lysogeny module of TP-J34 showed extensive similarity to that of well characterized *cos*-type phage Sfi21. Our experimental observations based on RT-PCR and Northern blotting confirm the sequence analysis (**Ventura, *et al.*, 2002a & 2002b**).

Table 4.1. Transcript units of genes of lysogeny module of w.t. and its derivative *S. thermophilus* J34 KO-orf3

Gene	w.t. (J34)		J34 KO-orf3 in the lytic state
	lysogenic state	lytic state	

lysogeny module

<i>int</i>	(+) RT-PCR product p (-)	m (+) 1.7- kb p (-)	(+) RT-PCR product
<i>ltp</i>	m (+) 0.6-kb p (+) 1.3-kb	m (+) 0.6- kb p (+) 1.3-kb	m (+) 0.6-kb p (-)
<i>orf3</i>	m (-) p (+) 1.3-kb	m (-) p (+) 1.3-kb	m (-) p (-)
<i>crh</i>	m (-) p (+) 1.3-kb	m (-) p (+) 1.8- kb	m (-) p (-)
<i>cro</i>	m (-) p (-)	m (-) p (+) 1.8- kb	m (-) p (-)
<i>ant</i>	m (-) p (-)	m (-) p (+) 1.8- kb	m (-) p (-)
<i>orf7</i>	m (-) p (-)	m (-) p (+) 1.8- kb	m (-) p (-)
<i>orf8</i>	m (-) p (-)	m (-) p (+) 1.8- kb	m (-) p (-)

Where: m, monocistronic mRNA; p, polycistronic mRNA; (+), detected with its size; (-), undetected; (+) RT-PCR product, transcript detected by RT-PCR. The same background color comprises genes which are transcribed together.

3.16 Outlook

Many questions in the lysogeny module of TP-J34 remain unanswered and require more investigation. For instance by construction of mutations within the putative operator sites, (O_{1A}, O_{1B}) and (O₁, O₂, and O₃) of hypothetical scenarios, 1 & 2, respectively,

lysogeny module

their roles in the switch may be elucidated. More sequence- and experimental analysis are needed to find out the relationship between these operator sites and other parts of DNA.

Furthermore, the role of *crh*-repressor in the switch is also unclear, an experiment should be designed to find out why this gene is expressed in the lytic development.

The putative promoters P_{int} and P_{ftp} , P1 and P2 should be experimentally investigated in relation to their activities

The role of *orf3* in the growth phenotype of the host culture still remains unclear, so it is suggested to create an *orf3* replacement mutation and investigate the consequence reaction on the growth type and induction process as well.

4 Summary

Analysis of the DNA sequence of TP-J34 suggested that all except four of the phage genes are transcribed in one direction. The four genes are separated from the others by a genetic switch region and appear to be transcribed in one operon. They encode the following functions – in the order of transcription: repressor (*crh*), potential metalloproteinase (*orf3*), lipoprotein mediating superinfection exclusion (*ltp*), and integrase (*int*).

To elucidate the roles of Orf3 and Int in induction, I performed gene knock out and transcriptional analysis by Northern blot and RT-PCR. In addition, a non-inducible TP-J34 derivative (TP-J34-12) and an isolated mutant was analysed, which harbored just one mutation within the lysogeny module: a frame-shift mutation in *orf3*, causing premature termination of translation. TP-J34-Cu50 harbored a missense mutation causing replacement of an aspartic acid for an alanine in Orf3. Knock-out of *int* and *orf3* in TP-J34, respectively, resulted in prevention of induction by mitomycin C. Transcription of *int* was detected in both lytic- and lysogenic cells as a monocistronic mRNA. RT-PCR analysis showed that *int* was exclusively transcribed from its own promoter, located in the intergenic region between *ltp* and *int*, and sequence analysis detected this promoter as well as it revealed some polar or activation effect of *orf3* on transcription of *int*. For *ltp*, Northern blots revealed two transcripts: i) a few polycistronic transcripts comprising genes *crh*, *orf3* and *ltp* (also detected by an *orf3* probe), and ii) a majority of monocistronic transcripts comprising just *ltp*. The latter transcript was not affected by addition of mitomycin C. In KO-*orf3*, only one relatively weak monocistronic mRNA was detected, revealing that Orf3 affects the transcription of *ltp*. Sequence analysis revealed that a weak promoter, P_{ltp} , in the *ltp/int* intergenic region. It is suggested that it needs Orf3 to

Summary

work well. A very stable stem-loop structure was detected downstream of *Itp* and seems to act as a transcription terminator. *Crh* is transcribed polycistronically with *orf3* and *Itp* and was detected in both lytic and lysogenic growth cycles.

Cro is transcribed only in the lytic cycle as a polycistronic mRNA comprising *ant*-encoding the antirepressor-, *orf7*, and *orf8*. In non-inducible *S. thermophilus* J34-12 transcript of *cro* was not detected. And sequence analysis revealed a very stable stem-loop structure about 200-nt downstream of *orf8* and seems to act as a strong transcription terminator, which explains the size of the *cro* transcript as determined by Northern Blotting.

From these results the following conclusions are drawn: i) the integrase is also involved in excision of prophage DNA; ii) Orf3 plays a role in induction, possibly by binding to Cro for initiation of lytic development, since Cro alone does not appear to carry out this function alone; iii) Crh is able to block transcription of the *cro* operon, while Cro alone cannot prevent of *crh*.

5 Zusammenfassung

Die Analyse der DNA Sequenz des Phagen TP-J34 zeigt, dass alle außer vier Genen des Lysogenie-Moduls in eine Richtung transkribiert werden. Die vier Gene sind von den anderen Genen durch eine genetische Schalterregion getrennt und scheinen als Operon transkribiert zu werden. Sie kodieren – in der Reihenfolge der Transkription – für die folgenden Funktionen: Repressor (*crh*), potentielle Metalloproteinase (*orf3*), Superinfektionshemmung vermitteltes Lipoprotein (*ltp*) und Integrase (*int*).

Zur Aufklärung der Bedeutung von *orf3* und *int* für die Induktion, wurden Versuche zum Gen „knock out“ und zur Transkriptionsanalyse mittels Northern Blot und „reverse transcription“ PCR (RT-PCR) durchgeführt. Zusätzlich wurde eine nicht-induzierbare Mutante (TP-J34-12) und eine isolierte Mutante (TP-J34-cu50) analysiert. TP-J34-12 trägt gegenüber dem Wildtyp TP-J34 eine „frame-shift“ Mutation im *orf3* des Lysogeniemoduls, welche zu einem vorzeitigen Abbruch der Translation führt. TP-J34-cu50 trägt eine Missence-Mutation, die zu einem Austausch von Asparaginsäure gegen Alanin in *orf3* führt. „Knock outs“ der Gene *int* und *orf3* führten beide zum Verlust der Induktion durch MitomycinC. Transkription von *int* als monocistronische mRNA wurde sowohl während der lytischen Entwicklung als auch in lysogenen Zustand nachgewiesen. RT-PCR zeigte, dass *int* ausschließlich von seinem in der intergenen Region zwischen *ltp* und *int* transkribiert wurde. Zusätzlich konnte gezeigt werden, dass *orf3* einen aktivierenden Effekt auf die Transkription von *int* ausübte. Für das *ltp* Gen konnten durch Northern Blot zwei Transkripte nachgewiesen werden: i) ein in geringer Kopienzahl vorliegendes polycistronisches Transkript, welches die Gene *crh*, *orf3* und *ltp* umfasste, und ii) ein in hoher Kopienzahl vorliegendes monocistronisches Transkript, welches ausschließlich aus *ltp* gebildet wurde. Letzteres Transkript wurde

Zusammenfassung

durch die Induktion mittels MitomycinC nicht beeinflusst. In KO-*orf3* wurden nach Zugabe von MitomycinC nur sehr wenige monocistronische *ltp* Transkripte nachgewiesen. Dieses zeigte, dass das *orf3* Genprodukt Einfluss auf die Expression von *ltp* ausübte. Durch DNA-Sequenzanalyse wurde ein möglicher Promoter als schwacher Promoter in der *orf3-ltp* intergenen Region erkannt, welcher möglicherweise Orf3 zur vollen Transkriptionsaktivität benötigt. In der intergenen *ltp-int* Region wurde eine stabile „stem-loop“ Struktur erkannt, die wahrscheinlich als Terminator fungiert. Das polycistronische *crh-orf3-ltp* Transkript wurde sowohl bei der lytischen Entwicklung als auch im lysogenen Zustand nachgewiesen.

Die Transkription von *cro* wurde in Form einer polycistronischen mRNA (*cro.ant-orf7-orf8*). Ein *cro* Transkript wurde in TP-J34-12 bei Zugabe von MitomycinC nicht entdeckt. Durch Sequenzanalyse wurde stromabwärts von *orf8* eine sehr stabile „stem-loop“ Struktur entdeckt, die wahrscheinlich als Terminator fungiert, da über diesen Terminator die Größe des Transkripts erklärbar ist.

Aufgrund der Ergebnisse lassen sich folgende Schlüsse ziehen: i) die Integrase ist auch an der Excision der Prophagen-DNA während der Induktion beteiligt; ii) Orf3 spielt eine Rolle in der Induktion, möglicherweise durch Bindung an Cro, um die lytische Entwicklung einzuleiten, da Cro alleine dazu nicht in der Lage ist; iii) Crh kann die Transkription des Cro Operons unterbinden, während Cro alleine die Transkription von *crh* nicht unterbinden kann.

6 Curriculum vitae

Name:	Mazhar Desouki Ali Mohamed
Nationality	Egyptian
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Marital status	Married, 3 children

Education

1990 - 1994	Bachelor Degree in Environmental Agricultural Sciences from Faculty of Environmental Agricultural Sciences, Suez Canal University. Egypt
2000 – 20004	Master Degree in Agricultural microbiology, Menia Univ. Egypt.
2006 – now	PhD student in Institute of Microbiology und Biotechnology, Max Rubner-Institut, Kiel, Germany

Professional titles

- 1995-1996 A Mission for Master degree from Academy of Scientific Research and Technology (Cairo, Egypt) in:
- i) National Authority for Remote Sensing and Space Sciences (*NARSS*)
 - ii) Desert Research Center (*DRC*)
 - iii) Agricultural Research Center (*ARC*)
- 1996-2004 A demonstrator in Microbiology Dept. Fac. Agric. Sohag Univ. Egypt
- 2004-now Assistant Lecturer in Microbiology Dept. Fac. Agric. Sohag Univ. Egypt

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Abbreviations

8 Abbreviations

aa	amino acid
Ap	ampicilin
Ap ^R	ampicilin resistance gene
b	base (s)
bp	base pair(s)
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease enzyme
ds	double-stranded
EBI	europcan Bioinformatics Institute
EDTA	ethylenediaminetetraacetic acid
Em ^R	erythromycin resistance gene
Er	erythromycin
gp	gene product
h	hour
IPTG	isopropyl-Thio-2-D-Galactopyranoside
Kan	kanamycin
Kb	kilo base
LAB	lactic acid bacteria
M	molar
mA	milliampere
MC	mitomycin C
ml	milliliter
mM	millimolar
MOPS	3-Morpholinopropanesulfonic acid
mRNA	messenger RNA
ms	millisecond
nt	nucleotide (s)
OD _{620nm}	optical Density
o/n	overnight
<i>orf</i>	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAP	RNA polymerase
RNase	ribonuclease enzyme
rpm	revolution per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulfate
s	second
SLS	stem-loop structure
ss	single stranded

Abbreviations

TAE	tris-acetate-EDTA
TE	tris-EDTA
tRNA	transfer RNA
TU	transcription unit
UTR	untranslated region
UV	ultra violet
V	volt
v/v	volume to volume
Vol	volume
w/v	weight to volume
μM	micromolar
Φ	phage

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