Institut für Mikrobiologie
Forschungszentrum für Milch und Lebensmittel Weihenstephan
Technische Universität München

Functional analysis of heterologous holin proteins in a λΔSthf genetic background

Nataša Vukov

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften
(Dr. rer. nat.)

genehmigten Dissertation.

Vorsitzender:	Univ.-Prof. Dr. Berthold Hock
Prüfer der Dissertation	1. Univ.-Prof. Dr. Siegfried Scherer
	2. Univ.-Prof. Dr. Dieter Langosch

Die Dissertation wurde am 04. 06. 2002 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 08. 07. 2002 angenommen.

To my parents

Mojim roditeljima

"Scientific understanding depends on a tissue of symbols resembling a tissue of reality"

Peter Mitchell

Table of content

List of Tables

List of Figures

Summary

Holins are small hydrophobic proteins causing non-specific membrane lesions at the end of bacteriophage multiplication, to promote access of the murein hydrolase to their substrate. We have established a λΔS genetic system, which enables functional expression of holins from various phages in an isogenic phage λ background, and allows qualitative evaluation of their ability to support lysis of *Escherichia coli* cells. Synthesis of holins is under control of native λ transcription and translation initiation signals, and the temperature-sensitive CIts857 repressor. A number of different holins were tested in this study. The opposing action of phage λ S105 and S107 holin variants in lysis timing could be confirmed, whereas we found evidence for a functionally non-homologous dual translational start motif in the *Listeria* phage Hol500 holin. The largest holin known, HolTW from a *Staphylococcus aureus* phage, revealed an early lysis phenotype in the λΔSthf background, which conferred a plaque forming defect due to premature lysis. Mutant analysis revealed that an altered C-terminus and/or a V52L substitution were sufficient to delay lysis and enable plaque formation. These results suggest that the extensively charged HolTW C-teminus may be important in regulation of lysis timing. Gene 17.5 product of *E. coli* phage T7, and Gp T from T4 was found to support sudden, saltatory cell lysis in the λΔSthf background, which clearly confirms their holin character. In conclusion, λΔSthf offers a useful genetic tool for studying the structure-function relationship of the extremely heterogeneous group of holin protein orthologs. MscL, the mechanosensitive channel forming protein, which shares main structural features with holins of was unable to complement the lysis defect of λΔSthf, confirming specitivity of holin function.

The functional properties of Hol118 holin from *Listeria monocytogenes* bacteriophage A118 were analysed in detail. The gene was cloned into λΔSthf, whose CIts857 repressor allows precise estimation of the cell lysis event mediated by a cloned holin, through the possibility to heat-induce the lytic cycle in lysogenized *E. coli*. Native *hol118* caused relatively late cell lysis, beginning at 90 min after induction. Lysis could not be prematurely triggered with energy poisons, indicating that the energized membrane does not inhibit permeabilization by this holin. Immunological analyses demonstrated that Hol118 appears in the inner membrane fraction of infected cells 20 min after phage multiplication starts in induced *E. coli.* Hol118 could also be detected in A118-infected *Listeria monocytogenes* cells. Since *hol118* features a dual start, different N-terminally modified Hol118 variants were tested for differences in lytic properties. Changing the

ATGs encoding M1 or M4 into CTG had no significant influence on lysis timing, indicating that these alleles do not assume the effector/inhibitor roles described for S. Toeprinting assays of *hol118* mRNA revealed use of an additional ATG start codon at position 40, encoding M14. Using *in vitro* approaches, we were able to demonstrate that a Hol118(83) variant is actually translated from the *hol118* transcripts. This N-terminally truncated holin lacks the first predicted transmembrane domain. Although it appears in the cytoplasma membrane, it is functionally deficient and unable to complement R in λΔSthf. Changing the M14-encoding ATG into codons not used as translational starts (M14I, M14L) resulted in an accelerated, premature lysis phenomenon, pointing to an inhibitor function of Hol118(83). This hypothesis was further supported by the observation that *hol118(83)* expressed *in trans* also inhibited holin function. This suggests that the first transmembrane domain is indispensable for the permeablization process leading to pore formation. Based on our findings, we propose a new model of holin functional regulation, where the intragenic Hol118(83) acts as an functional inhibitor, and therefore constitutes a key part of the lysis clock of A118. The strict regulation and inhibition of poreforming aids to explain the long latend period of *Listeria* phage A118, where the onset of lysis under optimal conditions takes approximately 70 min, more than twice the time needed by phage λ.

The functional properties of Hol500 holin from *Listeria monocytogenes* bacteriophage A500 was also analysed, and compared to Hol118 and to Hol2438 from *Listeria innocua*. Native *hol500* caused cell lysis, beginning at 60 min after induction of λΔSthf::*hol500.* Here, lysis could be prematurely triggered with energy poisons, indicating that the energized membrane inhibits permeabilization by this holin. N-terminally modified Hol500 variants were tested for differences in lytic properties. Changing M14-encoding ATG into ATT resulted in accelerated cell lysis. Toeprinting assays on *hol500* mRNA revealed use of M14 as a translational start pointing to the synthesis of a truncated protein from this position. We have shown that Hol118(83), the intragenic inhibitor of Hol118, can also inhibit Hol500 lysis, which further supports our model for regulation of lysis timing in these very similar *Listeria* holins. Hol2438 differs from Hol500 in the reduced net charge of the C-terminal domain, due to the lack of one lysine residue at the C-terminal end. This difference had a significant influence on lysis timing, confirming the crucial role for the distal part of the C-terminus of *Listeria* holins tested in this work.

Zusammenfassung

In dieser Arbeit wurde die Struktur-Funktionsbeziehung von Holinen, phagencodierten Membranproteinen in einem einheitlichem genetischen Hintergrund untersucht. Für den zeitlichen Verlauf und die effektive intrazelluläre Lyse von phageninfizierten Bakterien ist außer den Endolysinen als Mureinhydrolasen meist noch ein zusätzliches Protein nötig, welches über Porenbildung in der Cytoplasma-Membran den Durchtritt der Endolysine an das Zellwand-Substrat ermöglicht. Diese kleinen hydrophoben Proteine werden aufgrund ihrer Funktion als Holine bezeichnet. Die Primär-Sequenzen der Holinen sind sehr heterogen; es gibt fast keine signifikanten Homologien, außer bei einzelnen Phagen von taxonomisch sehr nah verwandten Bakterien. Hier wurde ein Derivat von dem Phagen λgt11 konstruiert, λΔSthf, in dem das *S* Holin Gen vollständig deletiert wurde und eine eingeführte *Eco*RI - Schittstelle die Einklonierung einen heterologen Holin Gens erlaubte. Die Lysisgen-Kassette mit dem klonierten Holin Gen konnte mittels Prophagen-Induktion von λΔSthf durch Inaktivierung des temperatur-sensitiven Cits857 Repressors exprimiert werden. Beobachtung und Verlauf des zeitlichen Verlaufes der Lyse ermöglichte einen Vergleich der Funktion verschiedener Holine.

Als erstes wurden die in der Funktion unterschiedliche Varianten von λ S getestet. Die Expression des S105 Effektors führte zu sehr schneller, vorzeitiger Lyse, während der Inhibitor S107 deutlich schlechter lysierte. Das größte bis jetzt bekannte Holin des *Staphylococcus aureus* Phagen Twort verursachte eine vorzeitige Lyse die zu einem "Plaque Defekt" führte. Die Holine der virulenten *E. coli* Phagen T4 (gp T) und T7 (gp 17,5) führten zu einer schnellen, abrupten Lyse, die für diese in ihrer Struktur sehr unterschiedlichen Holinen fast identisch war. Außerdem wurde gezeigt das ein porenbildendes Membranprotein, MscL, welches den Holinen gemeinsame Sekundär-Strukturmerkmalen aufweist, in λΔSthf keine Lyse verursachte. Das bedeutet, das die Funktion auch bei der extermen Heterogenität der primären Holinstrukturen spezifisch ist.

Die membranpermeabilisierende Aktivität des Hol118 Holin des *Listeria monocytogenes* Bakteriophagen A118 wurde in λΔSthf getestet. λΔSthf::*hol118* lysierte *E. coli* Zellen relativ spät, 90 min nach Induktion. Die Lyse konnte auch nicht durch eine Zerstörung des Membranpotentials vorzeitig induziert werden. Zwanzig Minuten nach der λΔSthf::*hol118* Prophagen-Induktion war Hol118 in der inneren Membranfraktion von *E. coli* immunologisch nachweisbar. Außerdem wurde das Hol118 Protein in der Membran der *Listeria* Zellen nach A118 Infektion nachgewiesen. Um den potentiellen

doppelten Start zu untersuchen, wurden Mutationen im N-Terminus eingeführt und der Effekt dieser Mutationen getestet. Veränderungen des ersten und vierten ATGs hatten keinen bedeutenden Einfluß auf den zeitlichen Verlauf der Lyse. "Toeprinting" auf der *hol118* mRNS zeigte ein zusätzliches ATG Start Kodon an Nukleotid-Position 40 in *hol118*. *In vitro* Versuche zeigten daß diese Hol118(83) Variante von *hol118* tatsächlich translatiert wird. Das gekürzte Protein kann nur zwei Transmembrandomänen in der Membran bilden, ist nicht mehr funktionsfähig und kann R in λΔSthf nicht mehr komplementieren. Diese funktionellen Eigenschaften von Hol118(83) zeigten eindeutig das die erste Transmembrandomäne für die porenbildende Funktion des Holin essentiell ist. Ersatz des M14 ATG Kodons in die für die Initiation der Translation in der Regel nicht verwendeten CTG or ATT (M14L, M14I) führte zu zunehmend schnelleren Lyse. Diese Ergebnisse deuten auf eine Inhibitor-Funktion für Hol118(83) hin. Hol118(83) inhibierte auch *in trans* Hol118 induzierte Lyse. Auf Grund dieser Ergebnisse wird ein neues Modell postuliert: Hol118(83), das an Nukleotid-Position 40 des *hol118* Gens startet funktioniert als der Inhibitor der Lyse, und hat somit einen Einfluß auf die Regulation der Dauer der Latenzphase des A118 Bakteriophagen. Die Aminosäuresequenzen der Holine Hol118 und Hol500 unterscheiden sich nur in sieben Aminosäuren, haben aber unterschiedliche membranpermeabilisierende Aktivitäten in λΔSthf. λΔSthf::*hol500* lysierte die *E. coli* Zellen schneller und effizienter als Hol118. Das Membranpotential der Zelle hatte hier einen inhibitorischen Effekt auf die Lyse; die Inaktivierung des Membranpotentials führte zu einer vorzeitigen Lyse. Wie bei Hol118 wurden Mutationen in den N-Terminus eingeführt und getestet. Die Änderungen des vierten Methionins in Leucin oder Isoleucin verstärkten die lytische Aktivität, während die Inaktivierung des ersten ATGs zu einer verzögerten Lyse führte. "Toeprinting" auf der *hol500* mRNS zeigte ebenfalls ein zusätzliches intragenes ATG Start Kodon an Position 40. Die Änderung ATG → ATT hatte einen identischen funktionellen Effekt wie in *hol118*: Beschleunigung der Lyse. Exprimiert *in trans* inhibierte Hol118(83) die Hol500-induzierte Lyse. Da die Unterschiede in der Aminosäuresequenzen zwischen Hol118 und Hol500 minimal sind kann das postulierte Modell der Lyseinhibition für A118 auf den Phagen A500 erweitert werden. Hol2438 unterscheidet sich von Hol500 nur in einer Aminosäure des C-terminalen Bereichs. Das fehlende Lysin reduzierte die positive Ladung des C-Terminus was im Vergleich zu Hol500 zu einer Beschleunigung der Lyse führte.

1. Introduction

1. 1 Bacteriophages

Bacteriophages were discovered independently by Frederik W. Twort and Félix H. d`Hérelle. Félix d`Hérelle published in 1917 his findings on agents which are obligate parasite of living bacteria, named them "bacteriophages" and laid foundations for experimental phage work. Phage research was tidily connected with the birth of molecular biology, and many basic concepts in this field have been established during research on molecular aspects of phage multiplication (Ackermann and Dubow, 1987). Bacteriophages are classified according to morphological characters into six basic morphotypes (groups) (A-F) (Table 1. 1). Further classification is based on the nucleic acid type present in phage particles.

Table 1. 1 Classification of bacteriophages

Morphotype groupe	Phages	dsDNA	ssDNA	dsRNA	ssRNA
A	Contractile tail *(Myoviridae)*	**T4**, A511			
B	Long and non contractile tail *(Siphoviridae)*	**λ, A118, A500**, 187			
C	Short tail *(Podoviridae)*	**T7**			
D	Cubic	*(Corticoviridae)* PM2 (lipid containing capsid) *(Tectiviridae)* PRD1 (double capsid)	*(Microviridae)* ϕX174	*(Cystoviridae)* ϕ6 (envelope)	*(Leviviridae)* MS2
E	Filamentous		fd		
F	Phleomorphic				

Host ranges can be very different for various bacteriophages. *Listeria* phages are strictly genus specific, while phages infecting *Enterobacteria* can be polyvalent (Ackermann and Dubow, 1987). This specificity depends to some degree on receptor structures on host cells, which are recoginized by phages and used to adsorb and infect bacterial cells.

Multiplication of bacteriophages in bacterial hosts proceeds in four main steps: adsorbtion, infection, multiplication, maturation and release (Ackermann and Dubow, 1987). According to the established relationship between bacteriophages and its host they are grouped into: virulent phages (obligate lethal parasites of bacterial cells), and temperent phages, which can shuttle between the lytic cycle and lysogeny.

1. 1. 1 The lytic cycle

Phage growth cycles are usually determined using a one-step growth experiment (Ellis and Delbrück, 1939), in which the production of phage in a synchronous phage infected cell culture is measured (Fig. 1. 1).

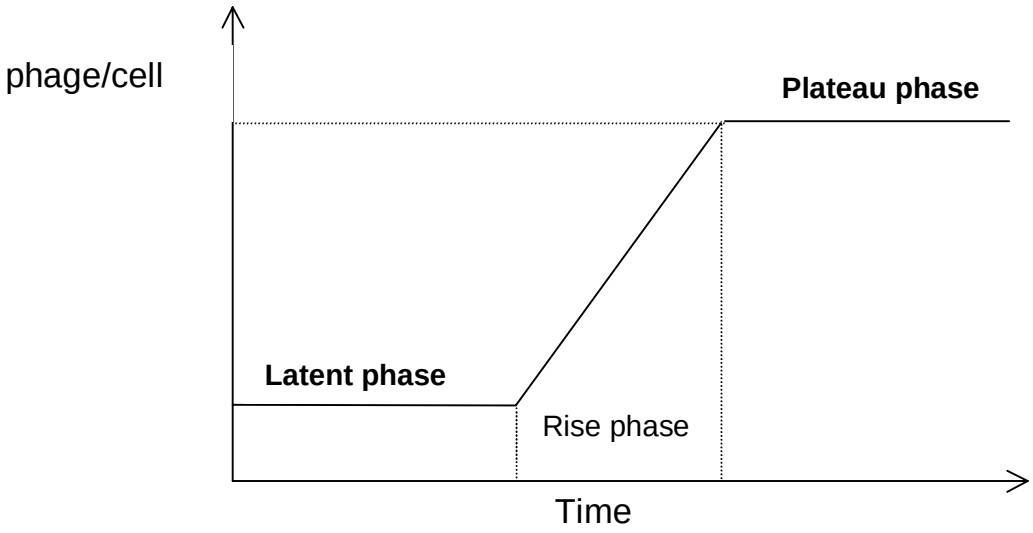


Fig. 1. 1 Schematic presentation of a one - step growth curve

A typical curve (Fig. 1. 1) resulting from an one step growth experiment defines three major time periods: (i) latent phase, during which the number of phages is not changed, (ii) rise phase, when new phage particles are liberated into the growth medium, and (iii), plateau phase. Length of the latent period, and the average number of phage particles released per infected cell, the burst size, determined as the ratio between the number of infectious centers during plateau phase and latent phase are parameters

which describe a virus-host system (Ackermann and Dubow, 1987; Birge, 1994). During the latent phase, phage progeny is synthesized and at the end of the phase, phages are released, with simultaneous cell lysis of the host. Only filamentous phages are non lytic. They replicate in harmony with the host bacterium; infected cells are not lysed but continue to grow during infection and phages extrude from the cell (Sambrook *et al.*, 1989).

The lenght of the latent phase depends on the nature and physiological condition of the host, host cell density, and composition of the medium and temperature (Ackermann and Dubow, 1987; Wang *et al.*, 1996). The timing and regulation of lysis is coordinated with lenght of the latent period for each bacteriophage.

There are two fundamentally different strategies for host cell lysis, used by bacteriophages: (i) most double stranded DNA phages synthesize an endolysin which degrades the peptidoglycan of bacterial cell wall. Additionally, holin proteins are made which, at a certain time point, allow the endolysin to access the peptidoglycan through its pore forming ability (Young, 1992); (ii) lytic phages with smaller genomes and single stranded DNA phages encode proteins which interfere with bacterial enzymes involved in the peptidoglycan biosynthesis. Cell lysis accurs through the collaps of the bacterial cell wall from the osmotic pressure from within, influenced by the impaired peptidoglycan synthesis (Bernhardt *et al.,* 2001).

1. 1. 2 Lysogeny

Phages which are able to establish a symbiotic reationship with bacterial hosts they infect are designated as temperate phages (Ackermann and Dubow, 1987). After adsorbtion and infection, the phage genome is either replicated and new particles are produced, or it intergates into the host chromosome and becomes latent, persisting as a prophage. Bacteria carrying prophages are described as lysogenic, with the potential to produce phages and eventually lyse. The equilibrium between host and phage can be destroyed, naturally through changes in environmental and physiological conditions (UV induction, host starvation) a process called induction. Events leading to the establishment of the lysogenic or lytic cycle are best known for bacteriophage λ.

When λ enters *E. coli* cell, both lytic and lysogenic pathways require expression of early phage genes. The lytic cycle is followed by expression of late genes and lysogeny is established if the synthesis of the CI repressor is established. CI binds to the operator regions of p_L (left), and p_R (right) promotors, preventing transcription of genes

responsible for entering the lytic cycle and at the same time it binds to p_{RM} promotor, supporting own continued synthesis. Prophage is induced to enter the lytic cycle if the repressor is inactivated. The control region responsible for lysogeny establishment determines the immunity of the phage. So lysogenic phages confer immunity to infection for any other phage which posess the same control region where the repressor can act. Lambdoid phages ϕ80, 21, 434 and λ all have unique immunity regions (Lewin, 1997).

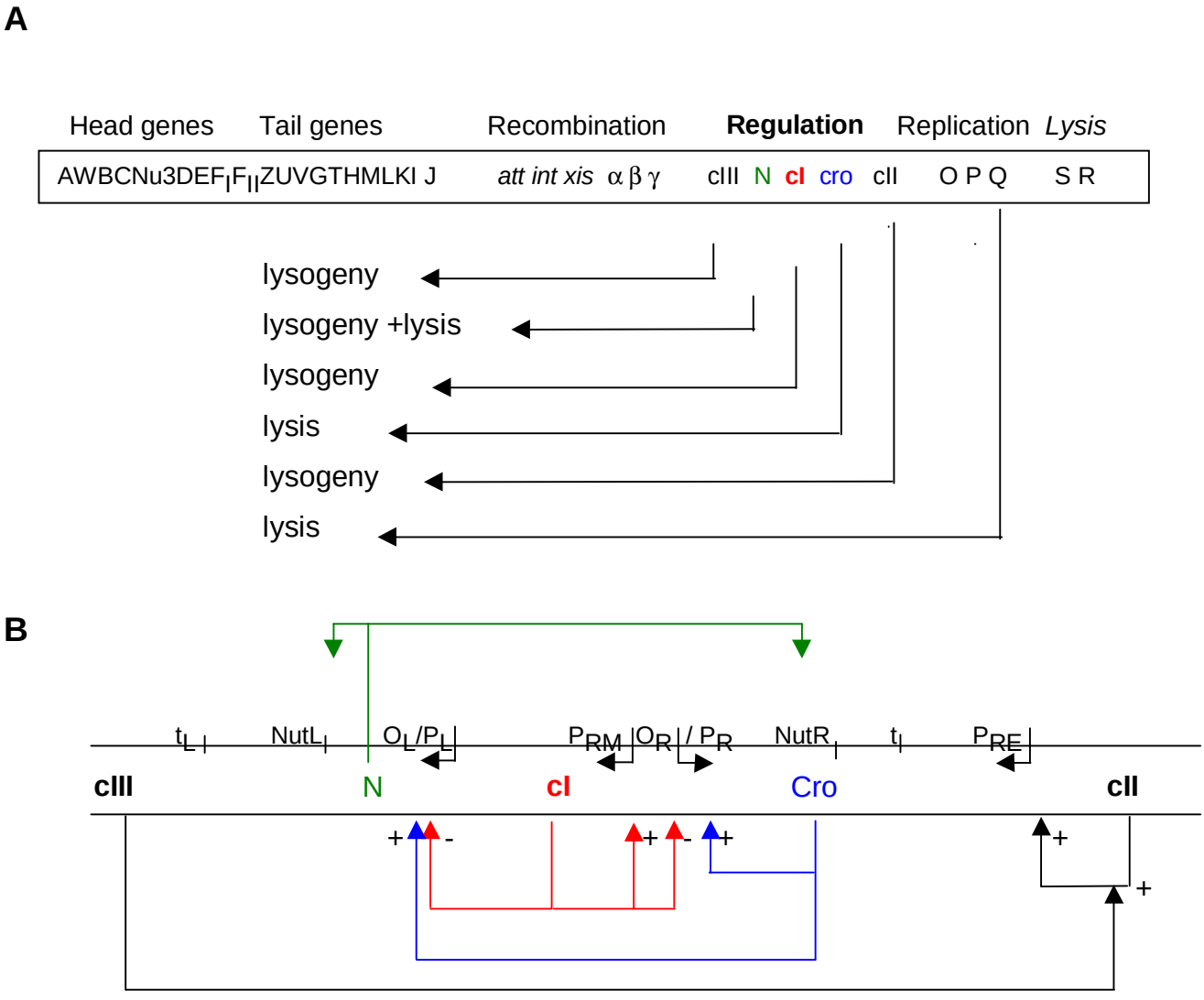


Fig. 1. 2 Regulatory, λ, genes involved in establishing lysogeny or the lytic growth mode. Panel A. Clustering of related functions on the lambda map (Lewin, 1997). The influence of regulatory genes for establishing the lytic or lysogenic mode is indicated. Panel B. Regulatory (immunity) region of λ. Binding of specific gene products are indicated by arrows (Chauthaiwale *et al.*, 1992).

1. 2 The Holin-endolysin system of phage-induced cell lysis

Bacteriophages generally end their growth cycle through host cell lysis, in order to release newly synthesized phage progeny into the environment (Young, 1992). Most tailed phages use two proteins to achieve cell lysis: a holin protein and an endolysin which actively degrades the peptidoglycan of the cell wall. Endolysins are soluble cytoplasmatic proteins with one or more different enzymatic activity cleaving the glycosidic, peptide or amide bonds of the peptidoglycan. Generally, endolysins lack secretory signal sequences, they use the permeabilization activity of a holin protein in the membrane to gain access to the murein (Young, 1992). The enzymes accumulate fully folded in the cytoplasm of the cell during latent phase, and, at a geneticaly programmed time, holins accumulated in the cell membrane start a permabilization process leading to endolysis access to the peptidoglycan. Eventually, destruction of the cell wall results in rapid lysis and liberation of phage progeny (Young, 1992). Holin/endolysin lysis systems has been found in bacteriophages infecting gram-negative and as well as gram-positive bacteria. Holin genes are often positioned upstream of the endolysin coding sequences, as it is found in the lysis region of *E. coli* lambdoid bacteriophages λ, 21, and *E. coli* P2, *Salmonella* phage P22 (Young, 1992; Ziermann *et al.*, 1994), but also in lysis regions of phages infecting various gram-positive bacteria: *Bacillus* (ϕ29); *Lactobacillus* (ϕadh, Pl-1, ϕg1e, mv1)*; Lactococcus* (Tuc2009*,* ϕUS3, c2, ϕvML3, r1t); *Staphylococcus* (Twort*,* ϕ11, 80α,187); *Streptococcus* (ϕ01205, Cp-1*; Listeria* (A118*,* A500); *Oenococcus* (fOg44); *Clostridium* (ϕ3626); and *Borrelia* (cp32) (Arendt *et al.*, 1994; Bon *et al.*, 1997; Damman *et al.*, 2000; Henrich *et al.*, 1995; Kashige *et al.*, 2000; Loessner *et al.*, 1995; Loessner *et al.*, 1999; Loessner *et al.*, 1998; Martin *et al.* 1998; Oki *et al.*, 1996; Parreira *et al.*, 1999; Tedin *et al.*, 1995; van Sinderen *et al.*, 1996; Wang *et al.*, 2000; Zimmer *et al.* 2002). A functional holin/endolysin, pair has also been found in sequences of cryptic prophages: *Bacillus sublilis* prophage PBSX, SPß, in defective prophages of *Listeria innocua* species, and also in Haemophilus somnus (Krogh *et al.*, 1998; Pontarollo *et al.*, 1997; Regamey and Karamata, 1998; Zink *et al.*, 1995). Additionally, it has been reported that phage lytic genes are involved in the secretion of several phage-encoded toxins, as the shiga-like toxin, Stx-I, produced by different *Eschericia coli* serotypes (Muniesa *et al.*, 2000; Neely and Friedman, 1998).

In *E. coli* phages P1, T7 and T4, holin and endolysisn genes were identified which are positioned at different genetic loci on phage genomes (Lu and Henning, 1992; Schmidt *et al.*, 1996; Young, 1992). Although it is generally accepted that endolysins

lack secretory signal sequences, recently secretory lysins have been indentified. It has been shown that Lys44 from fOg44 has an active N-terminal signal sequence, and can employ the GSP (general secretory pathway) for passage through the membrane bilayer. Ply21 endolysin from phage TP21, infecting *Bacillus cereus,* has also a potential N-terminal signal peptide, but its function has not been further investigated (Loessner *et al.*, 1997). In contrast to fOg44 were a holin gene has been found adjacent to Lys44, no holin genes could be found adjacent to endolysins in phages Bastille, TP21 and, 12826 of *Bacillus cereus* and adjacent to Ply511 from *Listeria* bacteriophage A511 (Loessner *et al.*, 1995; Loessner *et al.*, 1997; Sao-Jose *et al.*, 2000).

1. 3 Lysis region of bacteriophage λ

In bacteriophage λ, three lysis genes (*S, R and* R_Z) were identified which are all transcribed from the late $p_{R`}$ promotor. $P_{R`}$ is a constitutive promotor activated by the Q antitermination factor, which is a product of the delayed early genes. Activation of the promotor beginns about 8-10 min after induction of λ lysogenes. Hence, lysis proteins are synthesized long before the acctual lysis event starts, 40 min after induction of λ lysogens. λ lysis can be induced earlier by the addition of energy poissons, called premature lysis, a phenomenon also described for T even phages (Campbell and Rolfe, 1975; Doermann, 1952; Young, 1992).

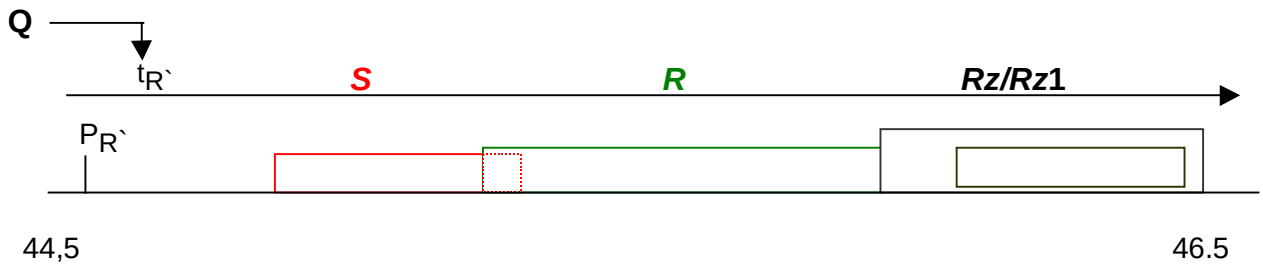


Fig. 1. 3 Map of the λ lysis region (Young, 1992). *S,* lysis control gene (holin), *R,* murein transglycosylase, R_Z, R_{Z1}, outer membrane proteins. Position of the late $p_{R`}$ promotor and $t_{R`}$ transcriptional terminator is indicated. The Q antitermination factor is acting on this region to start transcription. The arrow indicate the mRNA of the lysis genes.

R encodes for the endolysin, a muralytic transgycosylase, with no secretory signal sequence. R reaches the periplasm through the action of the S protein, which forms "holes" or pores, permeabilizing the membrane, a process which actually starts lysis. The product of the *Rz* gene is only required for lysis in the presence of divalent cations. Additionally, a protein named Rz1 is translated out of frame within the sequence of *Rz*; Rz and Rz1 are outer membrane proteins and assumed to be auxiliary lysis factors (Zhang and Young, 1999). Under standard laboratory conditions only S and R proteins are required for cell lysis. The most frequently used model system for studying lysis is the use of termosensitive λ lysogenes which allows the vegetative cycle to be initiated synchronously in an entire culture, so the lysis phenotype can be measured very precisely.

1. 4 Holins

S protein was the first membrane permeabilizing protein identified which led to a definition of a new class of membrane protein, making "holes" into the membrane, therefore designated as holins (Young, 1992). Holin proteins have two main functions in cell lysis: (i) they form "holes" through an oligomerization process in the membrane that enable the endolysin to reach the murein and, (ii) they have a timing function "programmed" into the structure of the protein that regulates the process of cell lysis to start at a defined timepoint. Because of these properties, holins are thought to be the simplest "molecular clocks". Triggering of the clock ends the latent period, a period when the phage replicates and new phage particles are assembled. All holin proteins have some structural features in common: at least one transmembrane domain, short, mostly charged, N-terminal sequence and, a highly hydrophilic, positively charged C-terminal tail. Holins are grouped into two classes, according to the nummber of potential transmembrane domains. Class I holins (90-125 aa) have the potential to form three transmembrane domains. The prototype of this class is the well studied λ S holin. Class II holins (57-185 aa) can form only two transmembrane domain, it is represented by the S^{21} holin from lambdoid bacteriophage 21. There are also holins which do not fit into these two classes, such as gpT from bacteriophage T4 having only one transmembrane domain. More than 100 known and putative holin protein constitute the most structurally diverse functional group of proteins, presently classified into 34 different families (Wang *et al.*, 2000). Most of them were identified only by primary and secondary structural features of protein sequences. Holin function is nonspecific for endolysin activity, which

enables testing of novel holins in a λ genetic background, by complementation of S mutants either from transactivation plasmids or directly, from holin genes inserted into λΔSthf phage (Vukov *et al.*, 2000; Wang *et al.*, 2000).

The timing of cell lysis is an as equally important function as the pore forming ability of a holin. The major determinant of timing is intrinsic to the structure of the transmembrane domains, as was shown at least for λ S (Johnson-Boaz *et al.*, 1994). This timing is modulated by charged amino acids in the N- and C- terminal domains, and by the expression of "inhibitors" or "antiholins" (Bläsi *et al.*, 1989; Bläsi *et al.*, 1990; Ramanculov and Young, 2001c; Steiner and Bläsi, 1993).

Some of the holin genes from classes I and II have a dual start motif which permits translation of two proteins with different N-termini and "opposite" functions (Barenboim *et al.*, 1999; Bläsi and Young, 1996; Nam *et al.*, 1990; Tedin *et al.*, 1995). The dual-start motif is found among holins from bacteriophages infecting both gram-positive and gram-negative bacteria, and which belong to class I or class II holins. In all cases were this has been investigated, the longer product was found to be an inhibitor of the shorter product, which is the main effector of lysis. Experimental evidence indicated that S can form homo-oligomers but the exact nature of the hole is still not known (Wang *et al.*, 2000; Zagotta and Wilson, 1990). There is biochemical evidence for dimerization between the effector S105 protein and the S107 inhibitor leading to heterodimer formation (Gründling *et al.*, 2000c). Apart from intragenic holin inhibitors found in S or S^{21}, separate genes are described coding for the holin and antiholin protein in P1, P2 and T4 bacteriophage (Ramanculov and Young, 2001c; Schmidt *et al.*, 1996; Ziermann *et al.*, 1994). Recently, the product of the *r*I gene was described as the specific antiholin in T4. It was shown that RI binds to T and forms heterodimers, as demonstrated for S107 and S105 (Ramanculov and Young, 2001c). It is accepted that the dual start motif represents fine-tuning regulation of holin function. Moreover, it is assumed that the effector / inhibitor ratio is actively regulated under physiological conditions of the cell, but experimental evidences for such regulation have still not been reported (Young *et al.*, 2000).

1. 4. 1 S holin

S was first defined as the control protein of the lysis process in λ infected cells. Inactivation of S leads to continuing respiration and macromolecular synthesis past the normal lysis timing, which results in virion and endolysin accumulation in the cell to very

high levels. The possiblity to complement this defect by the addition of $CHCl_3$ revealed that the acctual function of S is to permeabilize the cytoplasmic membrane (Young, 1992). Premature lysis through energy poissons in λ-infected cell is entirely dependent on S. λ S null mutants could not be triggered prematurely, so the timing of lysis has been correlated exclusively to the function of S protein (Campbell and Rolfe, 1975).

S is translated from a 107 codon sequence which posses a dual start, permitting translation od two proteins with opposite function: The S105 effector protein and S107, the intragenic inhibitor of S function (Fig. 1. 4) (Bläsi *et al.*, 1989; Bläsi *et al.*, 1990). Translation of the proteins is regulated by a secondary structure (*sdi*) positioned upstream, and a stem-loop structure, (downstream).

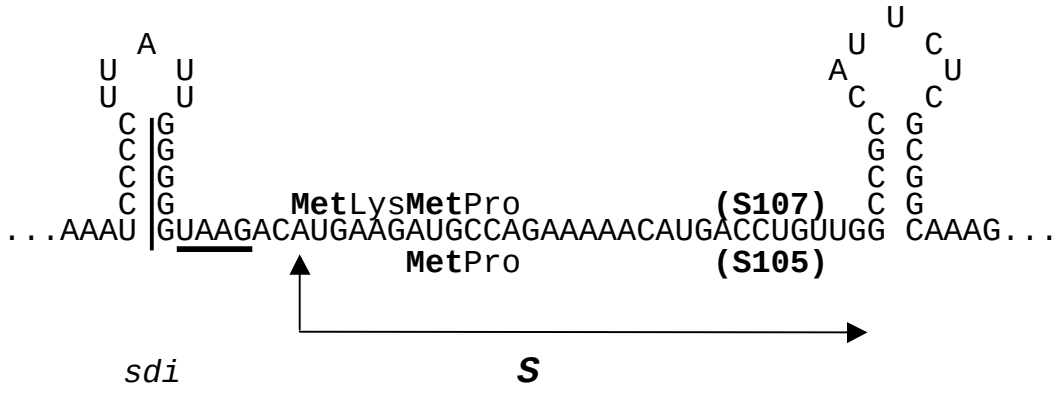


Fig. 1. 4 Translation initiation region of λ *S*. Sequence complementary to the 3´ end of the 16S rRNA is underlined. The *sdi* (structure directed initiation) is indicated. The N-terminal amino acid sequences of the two *S* gene products are given above (S107) and below (S105) the mRNA sequence (Bläsi *et al.*, 1989)

The Shine-Dalgarno sequence (GGGGG) for S107 is sequestered within the sdi structure, regulating ternary complex formation (30S ribosomal subunit, fMet-tRNA and AUG) at the first AUG codon. Translation initiation at the third AUG is about 2,5 more frequent to initiation at the first (Fig. 1. 4). In λ wt, the two S proteins are expressed in a ratio 2-3:1 in favour to S105. Mutations which destabilize the secondary structure enhance translation initiation over at first AUG, changing the S105/S107 ratio in favour to S107, which leads to lysis delay.

The major determinant of lysis timing is intrinsic in the structure of the transmembrane domains. S is a typical class I protein, and a cysteine-scanning analysis actually demonstrated the presence of three transmembrane domains in the membrane

(Gründling *et al.*, 2000b). An early lysis S variant was selected, with a A52G change in the middle of the second transmembrane domain. The mutant had a dominant effect over S wt, a finding that revealed that this position is crucial for intermolecular interactions between membrane-spanning domains of S (Johnson-Boaz *et al.*, 1994). Charged amino acids in the N - and C - terminal tails influence the lysis timing set by the intrinsic lysis clock (Bläsi *et al.*, 1999; Steiner and Bläsi, 1993). On the molecular level, charged amino acids in the N-terminal tail influence the topology of S. The holin needs the N-terminus positioned in the periplasm for pore formation (Graschopf and Bläsi, 1999b). This N_{out} - C_{in} topology of S is inhibited by an energized membrane to a different degree for S105 and S107 proteins (Fig. 1. 5). Passage of the S107 N-terminus is inhibited by an additional positive charged residue, which retards electrophoretic translocation of the N- terminus through the membrane (Graschopf and Bläsi, 1999). The inhibitory function of S107 is based on its different N-terminus and the possibility to form heterodimers with S105 (Gründling *et al.*, 2000c) (Fig. 1. 5, Fig. 1. 6) However, as soon as the membrane is depolarized, the N- terminus of S107 protein reaches the periplasm, takes on the active N_{out} - C_{in} topology, and contributes to the pool of active pore forming proteins, resulting in a very rapid onset of cell lysis.

The C-terminal domain of S is highly hydrophilic, with an overall positive net charge. Mutation analysis in this region revealed that at least one basic residue must be present to retain function (Bläsi *et al.*, 1999). Excess positive charge in the C-terminus led to lysis delay, compared to S wt. S protein which, features a deletion of its last 15 aa can still oligomerize in the cell membrane; a finding which indicated that this region is not involved in intermolecular interaction (Rietsch *et al.*, 1997). It is assumed that the C-terminus is a regulatory domain with little structural features.

The simplest currently proposed model for holin function, based on extensive genetical and biochemical analyses of λ S, is the critical-concentration model. It is assumed that a two-dimensional precipitation of holin proteins occurs in the membrane when their amount exceeds a critical level in the fluid bilayer. It has been shown for S that the precipitation event is inhibited by the energized state of the membrane (Campbell and Rolfe, 1975). The permeabilization process supposedly starts at a locus in the membrane were the mechanical integrity is locally disrupted. After the electrochemical potential collapses, all available holin would suddenly precipitate, resulting in massive membrane disruption. The model is build upon experiments that showed that the energy state of the cell is completely intact until just before the actual pore forming event takes place (Gründling *et al.*, 2001).

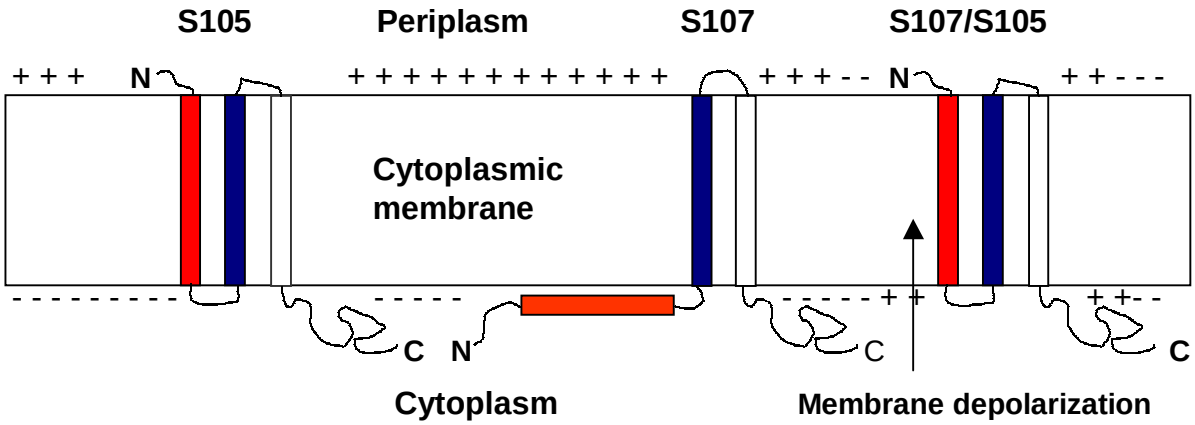


Fig. 1. 5 Membrane topologies of S variants. S107 protein assumes the N_{in}-C_{in} conformation in the cytoplasmic membrane when the membrane is energized. Upon depolarization, both proteins assume the same topology (Graschopf and Bläsi, 1999).

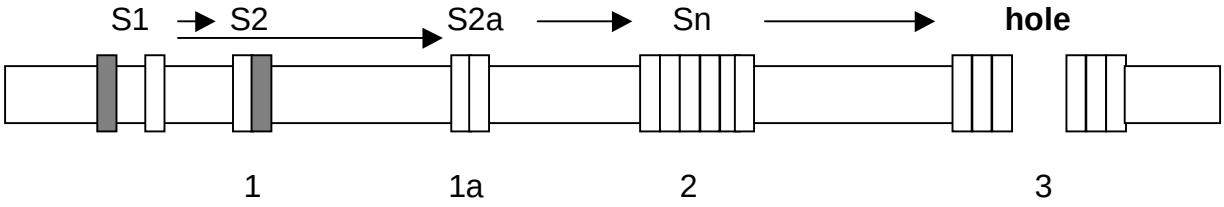


Fig. 1. 6 Model for S hole formation. At least three steps, are required for hole formation: (1) dimerization, (2) oligomerization and, (3) conformation or triggering. S monomers are depicted as open (S105) or filled (S107) bars. According to the model, S105/S107 heterodimers (1) can not lead to hole formation. The model is presented on physiological conditions when the cell membrane is energized. Upon depolarization both monomers contribute to pore formation (Gründling *et al.*, 2000a; Gründling *et al.*, 2000c)

2. Material and Methods

2. 1 Bacterial strains, phages, plasmids, and culture conditions

All bacterial strains used in this work are listed in Table 2. 1. Plasmids and phages constructed are listed in Tables 2. 2 and Table 2. 3. *Escherichia coli* strains were grown in Luria Bertani, LB (10 g Tryptone, 5 g Yeast extract, 5 g NaCl for 1 l) medium at 37°C. For propagation of the λ phages, media were supplemented with 0,2% maltose and 10 mM $MgSO_4$. XL1-Blue and DH5ΔMCR were used for plasmid propagation. HB101 was used for plating of *λ*gt11. LE392 was used as a general strain for propagation and lysogenization with λΔSthf. For efficient lysogenization in recombination experiments, C600*hfl* was used. Plasmid-bearing cells were selected on LB medium supplemented with 80 or 100 μg ml^{-1} ampicilin, 7 μg ml^{-1} chloramphenicol, 12 μg ml^{-1} tetracyclin, depending on the plasmid. *E. coli* lysogenized with λΔSthf was selected on LB supplemented with 30 μg ml^{-1} amplicilin or 30 μg ml^{-1} kanamycin, were appropriate. For the destrution of the energized state of the cell membrane CCCP (carbonyl cyanide m-chlorophenyl-hydrazone) was added to induced cultures to a final concentration of 50μM.

Strain CC118 ($phoA^-$), was used for detection of alkaline phosphatase activity on LB media supplemented with the chromogenic substrate XP (5-bromo-4chloro-3-indolyl phosphate, Sigma) at the final concentration of 50 μg ml^{-1}. Beta- galatosidase activity was selected on the same media supplemented with X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactosidase, Sigma) the chromogenic substrate for beta-galactosidase at the final concentration of 40 μgml^{-1}.

Listeria monocytogenes WSLC 1001 and *Listeria* monocytogenes WSLC 1042 were grown in TEB (20 g Tryptose, 1 g glucose, 5 g NaCl, 0.005 g Thiamine for 1 l, ph 7.3-7.4) medium at 30°C. The strains were used for propagation of A118 and A500 phages, respectively.

2. 2 Phage plating

Concentration of phages in suspentions were determined by phage plating, determing the number of plaques formed on plates with host bacteria. In order to perform plaque assays serial dilutions of bateriophage was performed in SM buffer (5.8 g NaCl, 2 g $MgSO_4$ x 7 H_2O, 50 ml 1 M Tris-HCl (pH 7.5) in 1L). Aliquots (0.1 ml) of each

dilution were mixed with plating bacteria (0.1 ml) in 3 ml molten (0.6%) top agar. The entire mixture was poured onto a agar plate surface. Plates were incubated overnight, (42°C) for λΔSthf and (30°C) for *Listeria* bacteriophages.

Table 2. 1 Bacterial strains

Strain	Genotype	Source or Reference
Listeria monocytogenes WSLC 1042	Wild type, serovar 4b	ATCC 23074
Listeria monocytogenes WSLC 1001	Wild type, serovar 1/2 c	ATCC 19112
E. coli DH5ΔMCR	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA*1 *endA*1 *supE*44 *thi*-1 *gyrA*96 *relA*1	In vitrogen
E. coli C600*hfl*	*sup*E44, *hsd*R, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *hfl*A150[*chr* :: Tn10(*tet^r*)]	Laboratory stock
E. coli XL1-Blue	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac* F´[*pro*AB$^+$ *lac*I^q *lac*ZΔM15 Tn10(*tet*)	Stratagene
E. coli LE392	RF$^-$ *hsd*R574(r_k^-, m_k^+)*sup*E44 *sup*F58 *lac*Y1 *gal*K2 *gal*T22 *met*B1 *trp*R55 (phage host; permissive for λgt11)	Promega
E. coli HB101	F$^-$ *hsd*S20 *sup*E44 *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl*-1 (phage host; non-permissive for λgt11)	Promega
E. coli Y1090	F$^-$ Δ(*lac*U169), *pro*A$^+$, Δ(*lon*), *ara*D139,*str*A, *sup*F, [*trp*C22:Tn10(*tet^r*)], (pMC9), *hsd*R(r_k^-, m_k^+)	Promega
E. coli CC118	*ara D 139* Δ(*ara-leu*) 7697 Δ(*lac*) *X74* Δ(*phoA)* 20 *galE galK thi rpsE rpoB argE(Am) recA1*	(Manoil and Beckwith, 1985)

Table 2. 2 Plasmids

Plasmid	Genotype or relevant features	Source or Reference
pBR322	4.6kb cloning vector, amp^r, tet^r	(Bolivar *et al.*, 1977)
pBluescript II	3.0 kb cloning vector, amp^r	Stratagene
pPHO7	5.5 kb vector, ampr, promotorless *pho*A	(Gutierrez and Devedjian, 1989)
pMC1871	7.476 kb fusion vector, tet^r, promororless *lac*Z	Pharmacia
pSP72	2.46 cloning vector, amp^r, T7 promotor	Promega
pCK1	5.5 kb *Lactococcus* - *E. coli* shuttle vector, cam^r, kan^r	(Gasson and Anderson, 1985)
pBR322*E*	4.6kb cloning vector, amp^r, tet^r EcoRI site inactivated	This work
pBS*E*1	pBluescript II, 3.0kb cloning vector, amp^r, *Eco*RV-*Sma*I deletion in the MCS	This work
pUC4K	3.9 kb, kan^r, amp^r	Pharmacia
pBS-λΔS	1865 bp PCR product (*Bam*HI fragment with *S* gene deletion) cloned into pBluescript II	This work
pCK-λΔS	1865 bp PCR product (*Bam*HI fragment with *S* gene deletion) cloned into pCK1	This work
pCK-λΔSK	Kan^r gene from pUC4K cloned into the *Eco*RI site in plasmid pCK-λΔS	This work
pBS*E*-λΔS1	1865 bp PCR product (*Bam*HI fragment with *S* gene deletion) cloned into *Bam*HI site of pBS*E*1	This work
pBS*E*-λΔS*hol500*	*hol500* cloned as an *Eco*RI PCR fragment into pBSE-λΔS	This work
pBS*E*-λΔS*hol118*	*hol118* cloned as an *Eco*RI PCR fragment into pBSE-λΔS	This work
pSPhol118 (wt)	*hol118* cloned as a *Bgl*II/*Eco*RI PCR fragment in pSP72	This work
pSPhol118 (96)	*hol118* (encoding Hol118 M4L) cloned as a *Bgl*II/*Eco*RI PCR fragment in pSP72	This work
pSPhol118 (93)	*hol118* (encoding Hol118-93) cloned as a *Bgl*II/*Eco*RI PCR fragment in pSP72	This work

Table 2. 2 Plasmids (continued)

Plasmid	Genotype or relevant features	Source or Reference
pSPhol118 Δ(M1-Y13)	*hol118 (*encoding Hol118-83) cloned as a *Bgl*II/*Eco*RI PCR fragment in pSP72	This work
pSP-λΔS*hol118*	412 bp, *Bgl*II-*Hin*dIII PCR, fragment containing the upstream region and *hol118* from λΔSthf::*hol118,* cloned into pSP72	This work
pSP-λΔS*hol500*	412 bp, *Bgl*II-*Hin*dIII PCR, fragment containing the upstream region and *hol500* from λΔSthf::*hol500,* cloned into pSP72	This work
pBRT	1865 bp *Bam*HI fragment with *S* gene deletion cloned into the *Bam*HI site of pBR322*E,* $p_{R`}$ promotor	This work
pBRT-*hol118 (83)*	*hol118(83)* gene variant of *hol118* cloned into the *Eco*RI restriction site of pBR*E*-λΔS	This work
pBSH32	130 bp *Xba*I/*Bam*HI PCR fragment containing an optimal RBS and codons for the first 33 aa of Hol118 inserted into the same sites of Bluescript	This work
pBSH32-*phoA*	2600 bp *Bam*HI/*Pst*I *phoA* fragment from pPHO7, inserted into the *Bam*HI/*Pst*I sites of pBSH32	This work
pBSH32-*lacZ*	3117 bp *Bam*HI *lacZ* fragment from pMC1871 inserted into the *Bam*HI site of pBSH33	This work

Table 2. 3 Bacteriophages

Bacteriophage	Genotype or relevant features	Source or Reference
λ (wt)	B1 Siphovirus for *E. coli,* temperate	ATCC23724-B2
T4	Myovirus for *E. coli,* wt	ATCC11303-B4
T7	C1 Podovirus for *E. coli*, virulent	ATCC11303-B7
Twort	A1 Myovirus for *Staphylococcus aureus*, virulent	(Ackermann and DuBow, 1987)

Table 2. 3 Bacteriophages (continued)

Bacteriophage	Genotype or relevant features	Source or Reference
A500	B1 Siphovirus for *Listeria monocytogenes* serovar 4b strain, temperate	ATCC B-23074
A118	Siphovirus for *Listeria monocytogenes* serovar 1/2 strain, temperate	(Loessner, 1991)
A511	Virulent, *Listeria* genus specific	(Loessner and Busse, 1990)
λgt11	cIts857, Sam100, *lac* promoter for expression of cloned gene	Promega
λgt11::*bla*	*bla* gene inserted into *Eco*RI site of λgt11, amp^r	This work
λgt11ΔSkan	λΔS, kan^r, amp^r, *c*Its857	This work
λΔSthf	λgt11ΔS, single *E*coRI site, amp^r, *c*Its857,	This work
λΔSthf::*S*	native *S* inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*S105*	mutated *S* (encoding S105) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*S105*L62P	mutated *S* (encoding S105 L62P) spontaneous mutant of λΔSthf::*S105*	This work
λΔSthf::*S105*K43E	mutated *S* (encoding S105 K43E) spontaneous mutant of λΔSthf::*S105*	This work
λΔSthf::*S105* F94S	mutated *S* (encoding S105 K43E) spontaneous mutant of λΔSthf::*S105*	This work
λΔSthf::*S107*	mutated *S* (encoding S107-M3L) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*S107-M3K*	mutated *S* (encoding S107-M3K) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol500*	A500 holin gene (*hol500*) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol500(93)*	modified *hol500* (encoding Hol500-93) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol500(96)*	mutated *hol500* (encoding Hol500-M4L) inserted into *E*coRI site of λΔSthf	This work

Table 2. 3 Bacteriophages (continued)

Bacteriophage	Genotype or relevant features	Source or Reference
λΔSthf::*hol500M1L*	*hol500* (encoding Hol500 ΔM1) spontaneous mutant	This work
λΔSthf::*hol500M1L2*	*hol500* (encoding Hol500 ΔM1 and M4I) spontaneous mutant	This work
λΔSthf::*hol500ΔM1*	*hol500* (encoding Hol500ΔM1) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol500ΔMF81L*	spontaneous mutant of λΔSthf::*hol500*ΔM1, (Hol500 ΔM1,F81L)	This work
λΔSthf::*hol500M14I*	*hol500* (encoding Hol500 M14I) inserted into *E*coRI site of λΔSthf	This work
λΔSthf*::hol500(96)-M2K*	mutated *hol500* (encoding Hol500-96-M2K) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*holTW*	Twort holin gene (*holTW)* inserted into the *E*coRI site of λΔSthf	This work
λΔSthf::*holTW-M*	holTW holin gene with additional cytosine at nucleotide position 552 causes an altered C-terminus and a G⟶ C exchange at nucleotide position 154 causes a V52L sustitution (see Fig. 3. 7).	This work
λΔSthf:::*T4-t*	*T* holin gene from bacteriophage T4 inserted into the *E*coRI site of λΔSthf	This work
λΔSthf::*T7-17.5*	T7 holin gene (*17.5*) inserted into the *E*coRI site of λΔSthf	This work
λΔSthf::*hol2438*	*hol2438 from* monocin producing strain *Listeria innocua* inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*orf2*	*orf2* from *Listeria monocytogenes* bacteriophage A511 cloned into the *EcoRI* site of λΔSthf	This work
λΔSthf::*mscL*	*mscL* gene from *E. coli* cloned into *E*coRI site of λΔSthf	This work
λΔSthf::*hol118 wt*	*hol118* inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol118 (96)*	*hol118* (encoding Hol118-96) inserted into *E*coRI site of λΔSthf	This work

Table 2. 3 Bacteriophages (continued)

Bacteriophage	Genotype or relevant features	Source or Reference
λΔSthf::*hol118 (93)*	*hol118* (encoding Hol118-93) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol118M14I*	*hol118* (encoding Hol118 M14I) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol118M14L*	*hol118* (encoding Hol118 M14L) inserted into *E*coRI site of λΔSthf	This work
λΔSthf::*hol118M14V*	spontaneous mutant phage, A→ G transition causing M14V substitution in Hol118	This work
λΔSthf::*hol118M1L4*	*hol118* (encoding Hol118 M1L, F84S) holin variant, spontaneous mutant	This work
λ*imm*434*cI* S_{wt}	cIts, Swt	(Bläsi *et al.*, 1990)
λ*imm*434*cI*::*hol118*	recombinant λ*imm*434*cI*ts phage, *S* recombinated for *hol118* from pBSE-λΔS*hol118*	This work
λ*imm*434*cI*::*hol500*	recombinant λ*imm*434*cI*ts phage, *S* recombinated for *hol500* from pBSE-λΔS*hol500*	This work

2. 3 Standard DNA manipulations

2. 3. 1 Isolation of λ DNA

λ DNA was isolated from λΔSthf LE392 lysogens. 500 ml *E. coli* liquid culture was grown to an OD600 about 0.5 (32°C). Phages were induced (30 min, 42°C), and the culture further incubated (60 min, 37°C). Cells were disrupted, treating the culture with 20 ml of $CHCl_3$. The obtained lysate was either filtrated through a 0.2 µm pore filter, or treated with DNAse and RNAse (1µg/ml). After centrifugation (2 X 15 min, 9600 x g at RT, JA-14, Beckman), phages obtained in the supernatant were precipitated with polyethylene glycol (PEG 8000, 15%) and NaCl (1M), over/night (4°C). Precipitated phages were recoverd by centrifugation (20 min, 9600 x g RT, JA-14, Beckman). Bacteriophage pellets were resuspended in 5 ml SM buffer (per 1 l: 5.8 g NaCl, 2 g $MgSO_4$ x 7 H_2O and 50 ml 1 M Tris HCl (pH 7.5)). Phages were purified by using stopped CsCl gradient centrifugation (Sambrock *et al.,* 1989). A discontinuous CsCl gradient was formed (from the bottom to the top of the centrifugation tube: 2ml CsCl ρ= 2.0; 2ml CsCl ρ= 1.50; 2ml CsCl ρ=1.45; 2ml CsCl ρ=1.40; 2ml CsCl ρ=1.30; 2.5ml CsCl ρ=1.20), and the phage particle solution layed on top. After centrifugation (15 hours, 90.000 x g_{max}, 4°C, SW-28.1, Beckman), a bluish band of bacteriophage particles was visible. The particles were collected by puncturing the sides of centrifugation tubes with a needle, and stored on 4°C. The CsCl suspension of phage particle was diluted with SM buffer and centrifuged (90 min, 150.000 x g at 15°C, SW-60Ti, Beckman). Phage pellet was resuspended in EPS buffer (20 mM EDTA,

50 $\mu g ml^{-1}$ Proteinase K and 0.5% SDS), and the suspension incubated (60 min, 50°C) for digestion of phage proteins. DNA was further extracted with Phenol-Chlorophorm, repeating the procedure several times. After extraction, the DNA was precipitated with 1/10 Vol 3M NaAc and 2 Vol Ethanol.

Alternatively, DNA was isolated from λΔSthf phages using the Qiagen Lambda Mini Kit (Qiagen). The clear lysate, obtained after liberation of phages from cells through $CHCl_3$ and centrifugation, was treated with DNAse and RNAse. 10 ml of this lysate was taken as the starting volume to isolate DNA following the steps of instructions given by the manufacturer. DNA was dissolved in TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0).

2. 3. 2 Plasmid isolation, PCR, agarose electrophoresis, and ligation reactions

Plasmid DNA was isolated from plasmid-carrying *E. coli* cultures, after overnight incubation in liquid culture. Three to five ml aliquotes were taken for isolation of plasmid DNA and further proceeded according to instructions for Plasmid Mini-Prep kit (Qiagen) or GeneElute^{TM} Plasmid Mini-prep Kit (Sigma).

All PCR reactions were performed using the Taq DNA polymerase (Qiagen), and the corresponding reaction buffer with the addition of a dNTP mix containing eqimolar concentration of all four nucleotides (2 mM). The following PCR conditions were used: (1X [120s, 94°C], (30X [90s, 52°C (or different anealing temperature depending on the primer set), 50s, 72°C, 60s, 94°C], 1X [120s, 52°C], 1X [5 min, 72°C]). All PCR products used for further manipulations were purified using the QIAquick PCR Purification Kit (Qiagen).

DNA molecules isolated from λ phages, plasmids, PCR products, and DNA fragments generated after digestions with restriction enzymes were separated by agarose gel electropheresis. Agarose gels varying in concentration between 0.7%-1.3% (SeaKem LE, FMC, BioProducts) were run in 1 X TAE buffer (0.04 M Tris-acetat, 0.001 M EDTA). After electrophoresis, gels were stained with ethidium-bromide EtBr (0.5 μg ml^{-1}), and visualized by UV-transilumination using the ImageMaster® VDS (Pharmacia). In order to extract DNA fragments from agarose gels, the QIAquick Gel Extraction Kit (Quiagen) was used

Ligation reactions of different DNA restriction fragments with plasmid or phage were performed using T4 DNA ligase (Boeringer), and the corresponding T4 ligation buffer. Ligation reactions were incubated over night (8°C-16°C), and desalted prior to transformation into *E. coli* cells by electroporation.

2. 3. 3 DNA sequencing

All holin genes cloned into λΔSthf phages were sequenced, in a region 105 bp upstream and 40 bp downstream of the insert. The fragment to be sequenced was first amplified using For2 (5'-GCCCGTGCATATCGGTCACG-3') and Rev2 (5'-ACCACGCCAGCATATCGAGG-3') primers. The obtained PCR fragments were sequenced with the identical IRD-800 labeled primers. Reactions were performed using the *SequiTherm EXEL^{TM}* II DNA Sequencing Kit-LC (for 66-cm gels), sequencing in the

presence of dideoxy termination nucleotides GTP, ATP, TTP and CTP. The mixes were resolved on a 7.5% PAA gel, in a LI-COR automated DNA sequencer.

2. 3. 4 Preparation of electrocompetent E. coli cells

500 ml LB medium was inoculated with 1ml over night growing cultures of *E. coli* cells. The culture was grown to an OD between 0.5-0.6. Cells were collected by centrifugation (15 min, 9600 x g, 4°C, JA-14). The obtained cell pellet was washed twice with pure water, with subsequent centrifugation under the conditions mentioned above. Finally, cells were washed in 10% glycerol and, after centrifugation and resuspension in 1/100 Vol., stored in 40 µl aliquotes at -70°C.

2. 3. 5 Electrotransformation

Transformation of *E. coli* cells was carryied out by electroporation using electrocompetent cell (Dower *et al.*, 1988). A BioRad Gene Pulser was used, and 2-mm electroporation cuvettes (EquiBio) under the following conditions: 200 Ω Resistance, 25 µFD capacitance, 12.000 V/cm fiels strenght. After regeneration in LB medium, transformants were selected on medium supplemented with the appropriate antibiotic, and further tested by PCR or plasmid isolation.

2. 4 Construction of λΔSthf

λΔSthf phage was constructed by recombination between λgt11 in the lysis region of the phage and a fragment carring the ΔS deletion on plasmid. First the ampicillin resistance gene (*bla*) was inserted into λgt11. The ampicillin resistance gene (*bla*) was amplified using primer (*Mun*I sites are underlined, start and stop codons are in boldface): bla-5' (5'-ATAT<u>CAATTG</u>TAAAGGAGATTTATT**ATG**AGTATTCAACATTTCCGTGTCG CCCTT-3') and bla-3' (5'-ATAT<u>CAATTG</u>**TTA**CCAATGCTTAATCAGT GAGGCACCTA-3'), using pBluescript as a template. The 860 bp product was cloned into the *Eco*RI site within *lacZ* in λgt11, and thereby destroyed this recognition sequence. The ligation mixture was packaged into λ particles according to the instruction of the manufacturer (Packagene system, Stratagene), and aliquots containing the recombinant phages plated

on Y1090. Phage clones containing *bla* gave colorless plaques in a softagar overlayer supplemented with ampicillin, X-Gal, and IPTG.

Deletion of the *S* gene and introduction of a new *Eco*RI site upstream of *R* in place of *S* in the intermediate construct λgt11::*bla* was achieved by homologous recombination of this region with a corresponding fragment on a plasmid. For this purpose, two λ DNA fragments were amplified using λgt11 DNA as a template: (1) a 1053 bp fragment (nuleotide coordinates 44129 to 45182 on wt λ DNA) using primer pair A (5'-ATATGGATCCGTGGTGTGGCAAAGCTTGAAG-3') and B (5'-ATATGAATTC TTCCCCCCC*C*AATAAGGGGATTTGCTCTATTTAATTAG-3´) and (2), a 812 bp fragment (coordinates 45482-46294) using primer pair C (5'-ATATGAATTC *A*GGAGTAGAAG**ATG**GTAGAAATCAA-3´) and D (5'-ATATGGATCCCCGGAGGC GGTG GTGGCTTCACGCA-3'). *Bam*HI and *E*coRI sites are underlined, changed nucleotides are shown in italics, and the start codon is shown boldfaced. After *Eco*RI digestion, both fragments were ligated to yield a 1865 bp fragment with *Bam*HI sites on each end and an *Eco*RI site in the middle. For easier handling, this construct was initially cloned into *Bam*HI-digested pBluescript, to yield pBS-λΔS. Then, the entire fragment was excised and subcloned into the *Bam*HI site of pCK1 (Gasson and Anderson, 1985), a vector that replicates through a rolling-circle (sigma) mechanism, and is well suited for homologous recombination. pCK-λΔS was electroporated into *E. coli* LE392, and recombinants selected by chloramphenicol. In order to introduce an additional selective marker for recombination into gt11::*bla*, the kanamycin resistance gene *kan* was obtained (on an *Eco*RI fragment) from the pUC4K vector, and ligated into the unique *Eco*RI site replacing S within the λ DNA fragment. Homologous recombination was performed as follows: LE392 (pCK-λΔSK) cells were infected with λgt11::*bla* in a volume of 10 ml and at a multiplicity of infection (m.o.i.) of approximately 10, and grown for 1 h at 37°C. In the absence of S, R-mediated lysis was induced by addition of a few drops of $CHCl_3$, and, after 15 min, debris removed by centrifugation at 6000 x g for 10 min. The resulting lysate containing the recombinant phages was used to lysogenize *E. coli* C600*hfl* cells, and, in oder to enrich for double-crossover mutants, the infected culture immediately plated on agar containing both ampicillin and chloramphenicol. Following incubation, amp^r/cam^r colonies were picked and pooled, grown at 30°C, thermally induced (42°C, 20 min), and aerated at 37°C for 1 h. Phages from the cleared lysate were again used to lysogenize C600*hfl* cells, followed by plating on medium containing

kanamycin and ampicillin. Approximately 100 clones (i.e., colonies) were then tested for chloramphenicol sensitivity, to exclude plasmid co-integrates resulting from single-crossover events. Using PCR, kan^r/cam^s clones were checked for presence of *kan* in the *Eco*RI site. Then, the lysogenic clones were tested for lytic competence after temperature shift, and a single clone with a non-lytic phenotype was selected. Phage λgt11ΔS*kan* particles were isolated from the cells by chloroform-induced lysis as described above. Following purification of the virus particles by banding in CsCl, their DNA was extracted (Sambrook *et al.*, 1989), and digested with *E*coRI to remove the *kan* fragment. The generated arms (approximate sizes 40.7 and 3.0 kbp) were separated by agarose gel electrophoresis, recovered from the gel, and religated. After packaging, aliquots containing the recombinant viruses were again used to lysogenize C600*hfl* cells, and plated on LB plates containing ampicillin. The correct sequence of the altered holin gene region in λΔSthf was confirmed by nucleotide sequencing using an automated DNA sequencer (Li-Cor model 4200) and the primers For2 (5'-GCCCGTGCATATCGGTCACG-3') and Rev2 (5'-ACCACGCCAGCATATCGAGG-3'). Finally, high-titre phage stocks were prepared as decribed above, and λΔSthf DNA was extracted and purified according to standard methods (Sambrook *et al.*, 1989).

2. 5 *Growth and lysis kinetics of individual λΔSthf::hol lysogens*

In order the test holins inserted into λΔSthf, LE392 cells were lysogenized with different λΔSthf::*hol* phages. Phages were mixed with cells grown to an OD600 ~ 0.2 and, after incubation (60 min, RT), the cell/phage mixture was poured on LB plates supplemented with 30 μg ml^{-1} ampicilin, selecting for colonies of λΔSthf lysogens.

The permeabilization feature of each inserted holin or membrane protein in λΔSthf was tested in liquid culture, measuring the lysis kinetics, after inducing the phages to lytic growth cycle. Lysogens carrying λΔSthf::*hol* were grown to OD600 0.150- 0.250 at preinduction temperature (32°C), induced (20 min, 42°C) and further grown (37°C) for the rest of the experiment. The OD600 was measured in intervals of 10 min or less to follow cell lysis.

2. 6. Cloning heterologous holins into λΔSthf

λΔSthf DNA was digested with *E*coRI, and two fragments (arms) treated with shrimp alkaline phosphatase (United States Biochemical). DNA fragments encoding various native and mutated holin genes were amplified from intact phage DNA by PCR, using the primers listed in Table 2. 4. The products were digested with *E*coRI, and directly ligated into the λSthf arms (approximately 0.2 µg). After phage packaging, (Packagene system, Stratagene), phage particles were plated on LE392 cells. Individual plaques from the cell lawn were picked and phages eluted. Aliquots containing recombinant phages were then used to lysogenize LE392 cells. Lysogenic clones could be directly selected by their resistance to ampicillin. Presence and identity of individual holin genes was checked by PCR amplification and nucleotide sequencing.

Table 2. 4 Primer used for amplification of holins, holin variants, and membrane proteins tested in λΔSthf

Primer	Sequence (5´ → 3´)[a]
S-5´	ATCAGAATTC**ATG**AAG**ATG**CCAGAAAAACATGAC
S105-5´	ATCAGAATTC**ATG**CCAGAAAAACATGACCTGTTG
S107-5´	ATCAGAATTC**ATG**AAG*C***TG**CCAGAAAAACATGAC
S107-M3K-5´	ATCAGAATTC**ATG**AAG**A***A***G**CCAGAAAAACATG
S-3´	ATCAGAATTC**TTA**TTGATTTCTACCATCTTCTACTCC
Hol500-5´	ATCAGAATTC**ATGATG**AAA**ATG**GAGTTTGGAAAAGAG
Hol500M1L	ATCAGAATTC*C***TGATG**AAA**ATG**GAGTTTGGAAAAGAG
Hol500ΔM1	ATCAGAATTC**ATG**AAA**ATG**GAGTTTGGAAAAGAG
Hol500-96-5´	ATACGAATTC**ATGATG**AAA*C***TG**GAGTTTGGAAAAGAG
Hol500-93-5´	ATCAGAATTC**ATG**GAGTTTGGAAAAGAGTTACTAGTT
Hol500-M2K-5´	ATCAGAATTC**ATGA***A***G**AAA*C***TG**GAGTTTGGAAAAGAG
Hol500-3´	ATCAGAATTC**TTA**TTTATCATCCTTTCCATATTTTTTAGC
HolTw-5´	ATCAGAATTC**ATG**GATAAAAAAGATAAAACACCTACA
HolTw-3´	ATCAGAATTC**TTA**ATGTAGGACTCTGCTTGT
T7 gp17.5-5´	ATCAGAATTC**ATG**CTATCATTAGACTTTAAC
T7 gp17.5-3´	ATCAGAATTC**TCA**CTCCTTATTGGCTTT
T4R	ATCAGAATTC**TTA**TTTAGCCCTTCCTAATATTCTG
T4F	ATCAGAATTC**ATG**GCAGCACCTAGAATA

Table 2. 4 Primer used for amplification of holins, holin variants, and membrane proteins tested in λΔSthf (continued)

Primer	Sequence (5´ → 3´)[a]
Li2438R	ATCAGAATTC**TTA**ATCATCCTTTCCATATTTTTTAGC
Hol118F	ATCAGAATTC**ATG**ATAGAA**ATG**GAGTTTGGAAAA
Hol118(96)-5´	ATCAGAATTC**ATG**ATAGAA*C***TG**GAGTTTGGAAAA
Hol118(93)-5´	ATCAGAATTC**ATG**GAGTTTGGAAAA
Hol118H-3´	ATCAAAGCTT**TTA**TTTATCATCCTCTC
Hol118ΔM14-5´	ATCAGAATTC**ATG**ACATTTTTAGTAGTTGTAACACCTGTG
Hol118M14I-5´	ATACGAATTC**ATG**ATAGAA**ATG**GAGTTTGGAAAAGAGTTACTAGTTT AC***AT****T*ACATTTTTAGTA
Hol118M14L-5´	ATACGAATTCATGATAGAAATGGAGTTTGGAAAAGAGTTACTAGTTT AC*C***TG**ACATTTTTAGTA
Hol500M14I-5´	ATACGAATTC**ATGATG**AAA**ATG**GAGTTTGGAAAAGAGTTACTAGTTT AT**AT**TACATT
orf2F	ATACGAATTC**ATG**CACGATAATGAATTTGAA
orf2R	ATACGAATTC**TTA**TTTTATTACACTATTTAC
mscLF	ATATGAATTC**ATG**AGCATTATTAAAGAATTTCGCGAA
mscLR	ATATGAATTC**TTA**AGAGCGGTTATTCTGCTCTTTCAG

a Start codons are indicated in bold letters and introduced mutations are underlined.

2. 7 Membrane protein preparation

Membrane protein samples were isolated by a modification of a procedure described earlier (Chang *et al.*, 1995). *E. coli* LE392 lysogenized with λΔSthf::*hol118* or λΔSthf::*hol500,* and a control strain lysogenized with λΔSthf, were grown at 32°C to an OD600 of 0.2, induced for 20 min (42°C), and further grown at 37°C. The sample aliquots (25 ml) were taken at various times after induction, as indicated in the legends of the figures. Samples were centrifuged, (10.300 x g, 4°C) and cells disrupted in 1 ml FP buffer (0.1 M Sodium phosphate pH 7.0, 0.1 M KCL, 5 mM EDTA, 1 mM DTT, 1 mM PMSF) by sonication with 3 pulses, 30 s each, 25% power setting (Sonoplus; Bandelin). Membrane fractions were collected by ultracentrifugation (100.000 x g, 60 min, 18°C). Membrane pellets were solubilized in 1/100 Vol ME buffer (20 mM Tris-HCl, 1% Triton-X, 10% glycerol, 0.5 M NaCl, 35 mM $MgCl_2$), overnight at room temperature, with agitation.

Detergent-insoluble material was removed by centrifugation (50.000 x g, 20°C, 60 min,) to obtain a soluble preparation of inner membrane proteins. Protein concentration was determined using a modified Bradford assay (Nanoquant; Roth).
Infection of log-phase *Listeria monocytogenes* strain 1001 (OD 600, 0.150) with phage A118 was done at a m. o. i. of 10, in a total volume of 70 ml. Phages were allowed to adsorb to cells for 15 min at room temperature. Infected *Listeria* cells were incubated at 30°C, without shaking. A sample (20 ml) was taken 90 min after incubation for isolation of membrane protein fraction, mixed with 5 ml 4X FP buffer (1X FP buffer: 0,1M Sodium phosphate pH 7.0, 0,1 M KCL, 5 mM EDTA, 1 mM DTT, 1 mM PMSF), and 300 µl recombinant Ply118 endolysin (Loessner *et al.*, 1996). Cells were incubated with the enzyme 30 min at room temperature and centrifuged (12.000 x g, 5 min). The supernatant was further processed as described above for isolation of membrane proteins from *E. coli.*

2. 8 SDS-PAGE electrophoresis and Immunoblotting

In order to separate and detect holin proteins in the isolated inner membrane fractions, SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and immunoblotting was performed. For SDS-PAGE, membrane fractions were diluted 1:2 with sample buffer (62.5 mM Tris-Hcl, pH6,8, 2% SDS, 25% glycerol, 0.01% Bromophenole Blue, 350 mM DTT), and boiled at 100°C for 10 min. Protein samples were resolved on a 16.5% Tris/Tricin precast gel, (Criterion system, Biorad), which is especially suited for separation of proteins below 20 kDa (Schägger and von Jagow, 1987). Electrophoresis was performed at a constant voltage (125 V), at a starting current of 140 mA in Tris/Tricin buffer (100 mM Tris, 100 mM Tricin, 0.1% SDS). After electrophoresis, proteins were transfered to a 0.45 µm PVDF (polyvinylidene di-flouride) membrane (Gelman), using a semidry-blotting method applying a discontinuous buffer system (Anode I (0.3 M Tris, 20% (v/v) methanol), Anode II (25 mM Tris 20% (v/v) methanol), cathode (40 mM 6-aminohexanoic acid, 0.01% SDS (g/v), 20% (v/v) methanol) (Kyhse-Andersen, 1984). In order to detect Hol118, antibodies were raised in rabbits against a synthetic 23 aa peptide of the C-terminal tail of the protein (AGGTGLFEQFTNRSKKYGEDDK). A cysteine residue was added to the N-terminal end, and the peptide conjugated to KLH (keyhole limpet hemocyanin). The conjugate was used to immunize rabbits in a standard 70 day protocol, and serum from the final bleed was harvested used for immunological detection (Genosys, Biotechnologies). The

serum was used at a dilution of 1:500, and the secondary antibody (anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, Pierce) was used at a dilution of 1:5000. After incubation, blocking, and washing procedures, signal detection was performed using the chemiluminescent substrate for HRP according to the manufactures instructions (Chemiluminescent Western Blotting Kit; Roth).

2. 9 In vitro gene expression

An *E.* coli T7 S30 extract system for circular DNA (Promega) for coupled *in vitro* transcription and translation reactions was used to obtain translational products from *hol118*. Reactions were carried out according to the manufacturer`s instructions, using different *hol118* variants cloned into *Bgl*II/*Eco*RI sites of pSP72 (pSPhol118 (wt), pSPhol118 (96), pSPhol118 (93) and pSPhol118(ΔM1-Y13). Five µl aliquots of the *in vitro* reactions were mixed with 20 µl ice-cold aceton and the proteins were precipitated, the pellets dried and mixed with SDS-PAGE sample buffer. Then, samples were loaded on a 16.5% Tris/Tricin gel and, after electrophoresis, immunoblotted as described above.

Table 2. 5 Primers used for amplification of hol118 variants tested in vitro

Primer	Sequence (5´⟶ 3´)
Hol118IV-5´	ATACAGATCTGAGGAGTTTTATT**ATG**ATAGAA**ATG**GAGTTTGGAA AAGAGTTACTAG
Hol118IV-96-5´	ATACAGATCTGAGGAGTTTTATT**ATG**ATAGAAC**TG**GAGTTTGGAA AAGAGTTACTAG
Hol118IV-93-5´	ATACAGATCTGAGGAGTTTTATT**ATG**GAGTTTGGAAAAGAGTTAC TAGTTTACATG
Hol118-3´	ATCAGAATTC**TTA**TTTATCATCCTCTCC
Hol118IVM14-5´	ATACAGATCTGAGGAGTTTTATT**ATG**ACATTTTTAGTAGTTGTAAC ACCTGTGTTTG

a Start codons are indicated in bold letters and introduced mutations are underlined.

2. 10 Primer extention inhibition analysis (toeprinting)

In order to analyse translation initiation sites on *hol118* and *hol500* genes in λΔSthf, plasmids were constructed which comprise the translational region of λΔSthf and the corresponding holin genes. Plasmids were constructed by inserting *Bgl*II/*Hin*dIII PCR

fragments with the upstream translational region of λΔSthf phage and *hol118* or *hol500* gene into pSP72. Primers used in the construction of pSP-λΔS*hol118* and pSP-λΔS*hol500* are listed in Table 2. 6. pSP-λΔS*hol118* and pSP-λΔS*hol500* served as a source for mRNA preparation. After linearization with HindIII, *in vitro* transcription with T7 RNA polymerase was performed, and the run-off transcripts were used for *in vitro* toeprinting studies. The ^{32}P-5´-end-labeled primer K16 5´-CCGCCAGCTCCTGC-3´, which is complementary to *hol118* and *hol500* mRNA sequences downstream of the start codon, was used in the toeprinting reactions performed essentially as described earlier (Tedin et al., 1997). After annealing of the primer, 0.04 pmol mRNA were incubated with 4 pmol 30S ribosomes and 20 pmol fMet-tRNA for 10´ at 37°C, in a final volume of 10 µl VD-buffer (10 mM Tris-Cl pH 7.3, 60 mM NH_4Cl, 10 mM MgAc, 6 mM ß-Mercaptoethanol), to allow formation of the ternary complex. Then, 16 units MMLV reverse transcriptase premixed with dNTPs were added to each reaction, and cDNA sythesis was performed by incubation of the mix for 15´ on 37°C. Reactions were stopped by the addition of the loading dye (10 M urea, 10xTBE, traces of bromphenol blue and xylencyanol). After heating the samples for 5´at 95°C, aliquots were loaded on a 8%PAA-8 M urea gel. The gel was dried under vacuum (65°C), and exposed to a radiographic film for detection followed by exposure to a Phosphoimager screen Molecular Dynamics

Table 2. 6 Primers used in construction of pSP-λΔShol118 and pSP-λΔShol500

Primer	Sequence (5` ⟶ 3`)
LDSFT-5´	ACAGATCTCCCGTGCATATCGGTCACG
Hol118-3´	ATCAGAATTC**TTA**TTTATCATCCTCTCC
Hol500-3´	ATCAGAATTC**TTA**TTTATCATCCTTTCCATATTTTTTAGC

a Stop codons are indicated in bold letters

2. 11 Construction of λimm434hol118 and λimm434hol500

The λimm434hol118/λimm434hol500 phages were constructed by recombination within the lysis region between plasmid pBS*E*-λΔS*hol118 or* pBS*E*-λΔS*hol500,* and phage λ*imm*434*cI* S_{wt}. First, *hol118* or *hol500* were inserted into the *Eco*RI site of pBS*E*-λΔS1. Orientation of the product and integrity were confirmed by sequencing.

Recombination occured in C600 *hfl* cells carrying pBS*E*-λΔS*hol118* or pBS*E*-λΔS*hol500*: cells were infected with the phages (m. o. i. ≈ 1) and after 15 min of adsorption ot room temperature, incubation temperature was rised to 42°C for 20 min in order to induce phage replication, followed by incubating for 60 min at 37°C. Liberated phages were used to lysogenize LE392 cells. Ampicilin-resistant clones were selected, which carried recombinant phages originated from a single recombination event between the plasmid and phage. A second recombination event was provoked through induction of the lysogenized phages. Liberated phages were again used to lysogenize LE392 cells. Ampicilin-sensitive LE392 lysogens were selected, and checked by PCR and DNA sequencing for the presence of *hol118* or *hol500* in λimm434*hol118* and λimm434*hol500.*

2. 12 Construction of the transactivation plasmid pBRT

pBRT was constructed in a pBR322*E* backbone. The single *Eco*RI site was inactivated through restriction digestion, blunt-end filling with the Klenow fragment (Boehringer), and ligation. A 1865 bp *Bam*HI fragment with the S gene deletion from pBS-λΔS was cloned into the *Bam*HI site of pBR322*E,* forming pBRT. The plasmid has a single *Eco*RI restriction site within the λ sequence, which can be used for inserting holin genes.

2. 13 Construction of pBSH32- phoA and pBSH32-lacZ plasmids

The gene fragment comprising the first 32 amino acids of Hol118 was amplified using 5´HolF1 (5´-ATATTCTAGAATAAGAGGAGTTTTATT**ATG**ATAGAA**ATG**G AGTTTGGAAAAGAGTTA-3´) and 3´HolF1 (5´-ATATGGATCCTCGTCTTCTTAA TCGCCTGAACAAACAG-3´) primers. The product was inserted into *Xba*I/*Bam*HI of pBluescript, forming pBSH32. *LacZ* and *phoA* genes were fused to the holin fragment at the *Bam*HI site in pBSH32 plasmid generating pBSH32-*lac*Z and pBSH32- *pho*A, respectively. The fusion genes were expressed under the *lac* promotor upon induction with 5 mM IPTG on agar plates.

3. Results

3. 1 Construction of λΔSthf phage

In order to compare the functional properties of different holin protein in the same genetic background, we have established the λΔSthf lysis deficient phage vector (Fig. 3. 1), whose lytic cycle can be induced by inactivating the temperature-sensitive CIts857 repressor. Using homologous recombination, the holin gene *S* was removed in phage λgt11, and substituted by a single *E*coRI restriction site placed immediately downstream of the ribosome binding site initiating *S* translation (Fig. 3. 1, Fig. 3. 2). After insertion of heterologous holin genes, the cloned genes are expressed under control of the native λ p_R late promoter, and the translation signals provided on the cloned fragments. In λ wt, the translational stop signal for the holin overlaps the translational start region of the R endolysin gene. In the λΔSthf phage, the translational start for the R gene is independent of the translation of the cloned holin gene (Fig. 3. 2). Within the region of the *lacZ* gene in λgt11, an ampicilin resistance gene (*bla*) was cloned into the *Eco*RI site thereby inactivating this site. The presence of the ampicilin resistance marker on the phage allows convenient selection of *E. coli* cells lysogenized with the phage, by selecting for ampicilin resistant *E. coli* cells.

3. 2 Complementation of the λΔSthf lysis defect with λ S alleles

The S holin gene encodes two protein starting at two ATG codons separated by a codon endoding lysine. The presence of the additional positive charge determines different lytic characteristics in the process of pore formation, S107 and S105 (Fig. 3. 1) (Bläsi *et al.*, 1989; Bläsi *et al.*, 1990). To test whether λΔSthf can be used as a model system for functional analysis of holins, different λ *S* alleles with lytic properties that have been previously decribed were evaluated in the λΔSthf background (Table 3. 1). The S105 effector and the S107 inhibitor were tested for lytic competence in λΔSthf. Both alleles were amplified using PCR from the λ(wt) phage. Additionally, an allele was tested which has one more positive charge on the N-terminus of S.

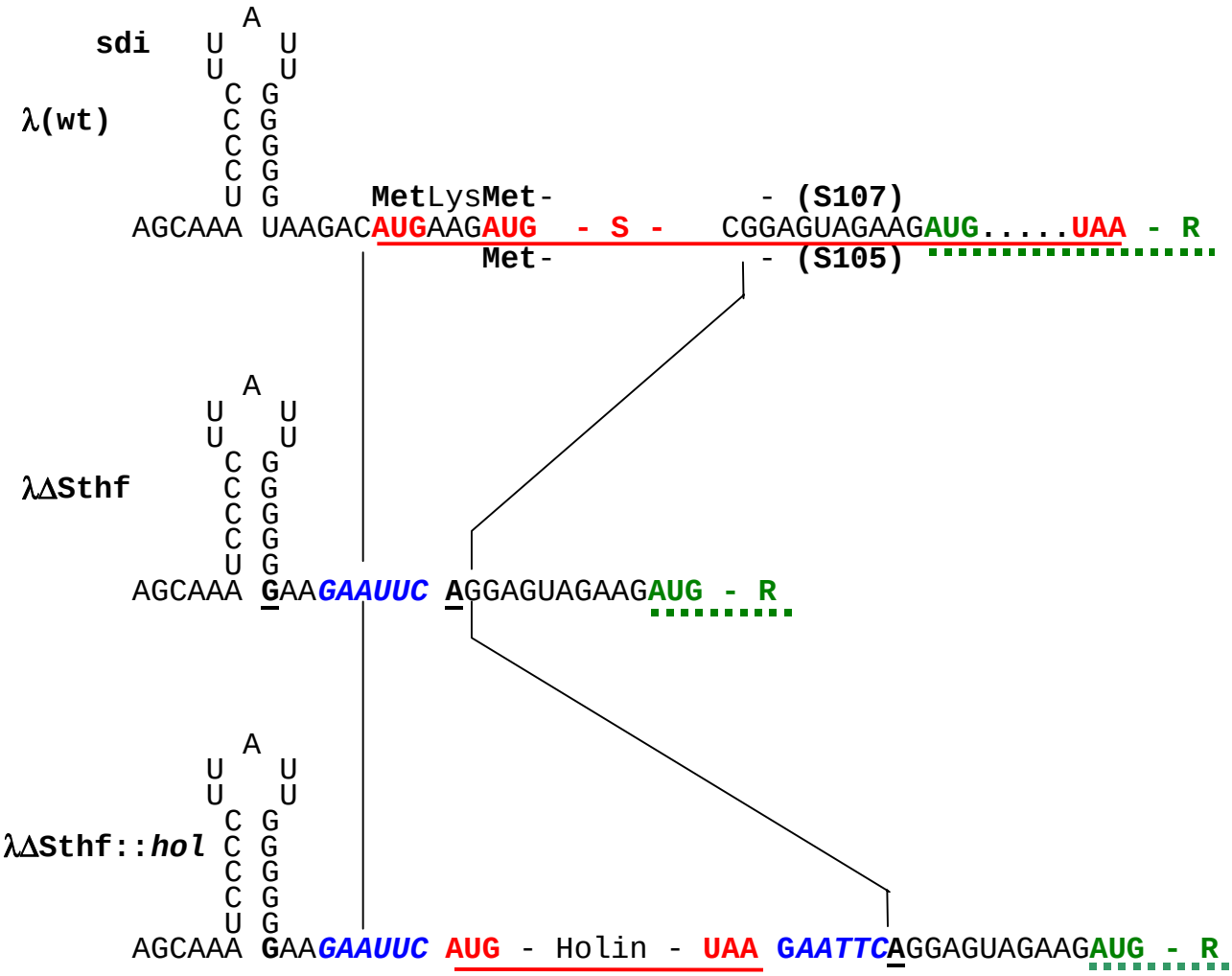


Fig. 3. 1. Schematic presentation of the construction of λΔSthf phage, and the 5´- mRNA sequence of the translation initiation region of *S* and *R* . The *S* holin and the R endolysin genes in λ wildtype, λΔSthf, and λΔSthf::*hol* are underlined. The bent vertical lines indicate the changes introduced by deletions and insertions. Altered nucleotides are underlined, and the single *Eco*RI site in λΔSthf is shown in italics. Sdi denotes the secondary structure involved in regulation of translation initiation (Bläsi *et al.*, 1989). The N-terminal amino acid sequences of the two S variants are given above (S107) and below (S105) the nucleotide sequence of λ (wt).

Fig. 3. 2 Nucletide sequence of the upstream and downstream region of the S deletion in λΔ*Sthf* phage. The single *Eco*RI restriction site is shown in italics and the ATG start codon for *R,* endolysin gene is underlined The arrows indicate the position of For2 and Rev2 primers, which were used for amplification and sequencing and LDSFT-5´ primer, used for amplification of fragments used in toeprinting assays.

Table 3. 1 Alleles and mutants of λ S tested in this study

Holin	N-terminal amino acid sequences[a]
S	+ - + MK**M**PEK
S105	- + - - **M**PEK
S107	+ -+ **M**K*L*PEK
S107-M3K	+ + - + **M**K*K*PEK

[a] Translation start codons for λΔS are indicated by bold letters, charged amino-acids by + and - above the sequence, and mutated amino acids are underlined

Induction of λΔSthf::*S* (wt) resulted in lysis beginning at 45 min after induction (Fig. 3. 3A). Expression of only *S105* led to accelerated, saltatory lysis which was completed in only 30 min, whereas the *S107* allele substantially delayed the course of cell lysis (starting at 50 min). The mutated *S107-M3K* holin resulted in a completely non-lytic phenotype showing no signs of growth inhibition. These results confirm that the functional difference between effector and inhibitor can be detected in the λΔSthf vector used, and that λΔSthf::*S* (wt) behaves essentially as in the λ wt (Raab *et al.,* 1988).

A

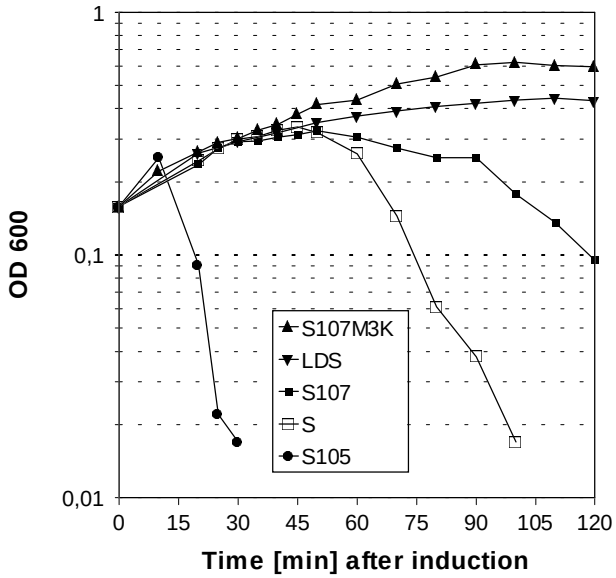


Fig. 3. 3 Panel A. **Permeabilization features of S variants tested in λΔSthf**. Panel A. Lysis profiles following thermal induction of *E. coli* LE392 lysogenized with λΔSthf carrying different alleles of S holin as indicated in the legend. λΔSthf, negative control is shown as LDS.

Additionally, three different S105 mutant were analysed. All were isolated as spontaneous S105 mutants (Fig. 3. 3B). The F94S and K43E changes influenced lysis timing, delaying the onset of lysis for 20 min compared to S105 wt, while L62P did not result in a significantly different lysis behaviour.

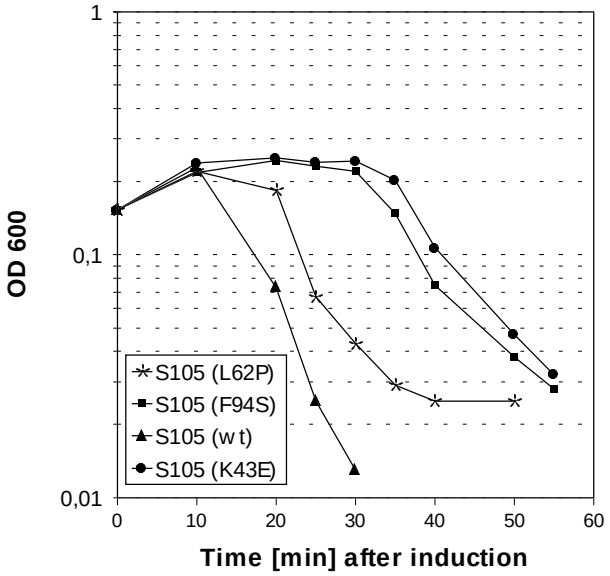


Fig. 3. 3

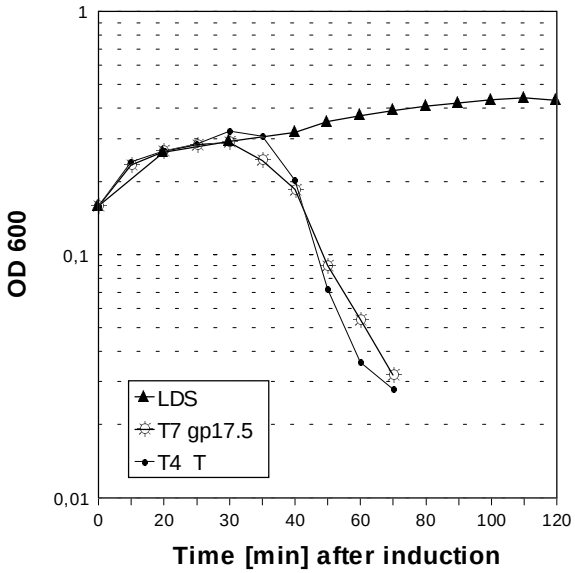


Fig. 3. 4

Fig. 3. 3 Panel B. Lysis profiles following thermal induction of *E. coli* LE392 lysogenized with λΔSthf carrying variants of S105 causing lysis delay.

Fig. 3. 4 T and Gp 17, 5 have similar lytic features tested in λΔSthf. Lysis profile observed following thermal induction of *E. coli* LE392 lysogenized with λΔSthf carrying gp T holin from bacteriophage T4 and Gp17,5 holin from bacteriophage T7, λΔSthf, negative control is shown as LDS.

3. 3. Complementation of the λΔSthf lysis defect with holins from T4 and T7 bacteriophages

Two very dissimilar holins (T and Gp17.5) were tested for their properties to complement the lysis defect of λΔSthf (Fig. 3. 4). Lysogens carrying λΔSthf::*17,5* showed a sudden, saltatory onset of cell lysis at about 30 min after induction (Fig. 3. 4). Within 60-70 min, the culture was completely clear. An almost indentical lysis onset was observed for λΔSthf::*t*. For both holins, the rate of lysis is very similar to that observed when the native λ S allele is expressed in this system (compare to curve in Fig. 3. 3A). Apparently, these two holin protein have very similar functional features, despite their very different structural characteristics (Fig. 3. 5). Both proteins have a high probability to form only one transmembrane domain in the cytoplasmic membrane, according to the prediction made by TMHMM 2. 0 (Fig. 3. 5).

λ S

```
  +  -+  -      +-               + +       +   -           +-  -                      -      ++   ++   -- +
MKMPEKHDLLAAILAAKEQGIGAILAFAMAYLRGRYNGGAFTKTVIDATMCAIIAWFIRDLLDFAGLSSNLAYITSVFIGYIGTDSIGSLIKRFAAKKAGVEDGRNQ
                                                                                          ****************
  S107
   S105
```

T7 gp17,5

```
    -   -  +          -   +        -                 +  -+  - ++  +-
MLSLDFNNELIKAAPIVGTGVADVSARLFFGLSLNEWFYVAAIAYTVVQIGAKVVDKMIDWKKANKE
                                    ***************
```

T4 T

```
    +      -      -+  +-    +    +               + -   -  +  + -   -  -+-+  + -    -         - -       + +
MAAPRISFSPSDILFGVLDRLFKDNATGKVLASRVAVVILLFIMAIVWYRGDSFFEYYKQSKYETYSEIIEKERTARFESVALEQLQIVHISSEADFSAVYSFRPKNLNY

  -    - +      -+       -+  --       +      +      ++   -            -            + -    -+ -       +   + +
FVDIIAYEGKLPSTISEKSLGGYPVDKTMDEYTVHLNGRHYYSNSKFAFLPTKKPTPEINYMYSCPYFNLDNIYAGTITMYWYRNDHISNDRLESICAQAARILGRAK
```

Fig. 3. 5 Amino acid sequences and secondary, domain structures of λ S, T7, Gp 17,5, and T4 T holins. Charged residues are indicated by + and - signs above the sequence, potential transmembrane domains, according to predictions made by TMHMM 2.0, are underlined. The double translational start motiv for S107 and S105 of λ S holin is indicated by boldface letters.

3. 4 Complementation of the λΔSthf lysis defect by HolTw from bacteriophage Twort

The *Staphylococcus aureus* phage Twort *holTW* gene specifies the largest class II holin known to date (Loessner *et al.*, 1998). λΔSthf::*holTW* lysogens showed an early lysis phenotype similar to that observed with λ allele *S105*, i.e., cells exhibited saltatory lysis at about 10 min after induction. This premature lysis also resulted in formation of very small, barely visible plaques in the plating assay. Among the different phage clones, one was found that produced significantly larger plaques and was further analysed. Nucleotide sequencing of the inserted gene revealed an insertion of a single cytosine residue immediately after cytosine-552 in the coding sequence of *holTW* gene, resulting in a minus-1 frameshift mutation. The shifted reading frame at the 5'-end of *holTW-M* specifies an altered C-terminal sequence of 7 amino acids, five residues longer than the wilde type, but with no change in net charge. The mutant gene also features a C→G missense mutation at nucleotide position 154, which results in a V52L change. Induced λΔSthf::*holTW-M* lysogens showed no premature, early lysis. Instead, onset of lysis was substantially delayed, and the rate of decrease in culture density was more gradual as compared to the wildtype. However, the HolTW-M holin was able to be triggered through the distruction of the membrane potential treating the liquid culture with CCCP (Fig. 3. 6).

3. 5 Orf2 from Listeria monocytogenes bacteriophage A511 does not complement the λΔSthf lysis defect

The product of orf2 from *Listeria monocytogenes* bacteriophage A511 was grouped as a separated holin type in the literature (Wang *et al.*, 2000) (Fig. 3. 6). In order to test whether orf2 functions as a holin or not, it was tested for plaque forming ability, in λΔSthf. Surprisingly, after inserting into λΔSthf and packaging, no plaque forming function could be selected. Therefore, the orf 2 could not be further considered as a functional holin.

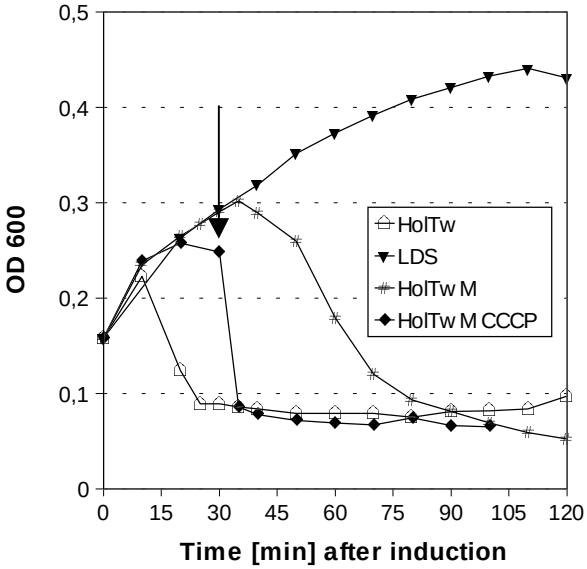


Fig. 3. 6 HolTw causes extremly early lysis in λΔSthf. Lysis profiles observed following thermal induction of *E. coli* LE392 lysogenized with λΔSthf::*holTw* and λΔSthf::*holTw-M*. Lysis of λΔSthf::*holTw-M* lysogens was induced prematurely with CCCP, indicated by an arrow on the panel.

3. 6 Analysis of Hol118 from the Listeria monocytogenes bacteriophage A118

Listeria monocytogenes bacteriophage A118 causes cell lysis by a combined action of the Hol118 holin and the Ply118 endolysin, having a unique L-alanoyl-D-glutamate specificity (Loessner *et al.*, 1995). The gene coding for Hol118 precedes the endolysin gene in a different reading frame with a overlapping stop codon for the holin and the start codon for endolysin. Hol118 belongs to the class I holin proteins which have three transmembrane domains. (Fig 3. 7). In order to test its permeabilization features the λΔSthf vector was employed, by insertion of *hol118* into the *Eco*RI site immediately upstream of *R.* N- terminally different Hol118 variants were tested for lysis timing, in order to detect possible functional similarities to the λ S. During this study, toeprinting assays, *in vitro* translation, and immunological methods were used to provide evidence for synthesis of a truncated protein starting at M14 codon in *hol118.* Site directed mutagenesis of this codon in *hol118* and *in trans* expression of Hol118(83) protein were performed to reveal the exact function of the protein.

Fig. 3. 7 **Amino acid sequences and secondary domain structures of tested holins from phages infecting Gram-positive bacteria**, **and tested proteins for holin function**. Charged residues are indicated by +and - signs above the sequence, potential transmembrane domains, according to the prediction made by TMHMM 2.0, are underlined. The position for the start for Hol118(96) and Hol118(83) are indicated. The proximal positively charged C-terminal domain is underlined by asteriskes. (A) Holins: HolTw (*S. aureus* Twort) (Loessner *et al.*, 1998). Altered amino acids in the mutant HolTW-M are indicated by bold letters. Hol118, Hol500 (*L. monocytogenes* A118, A500), and Hol2438 (*L. innocua* monocine producing strain) (Loessner *et al.*, 1995; Zink *et al.*, 1995). Hol3626 (*C. perfringens* ϕ3626), (Zimmer *et al.,* 2002). Different amino acid positions in Hol500 in respect to Hol118 are indicted in bold letters. (B) Potential holin: Orf 2 (*L. monocytogenes* A511). (C) MscI *E. coli* (Chang et al., 1998)

A

HolTw

```
-++-+             + + +                       -     -                    -   +  +-         - +- +- -+
MDKKDKTPTVVGGINLSTRFKSKTFWVATVSALAVFTNQLTGAFGLDYSSQVEQGVNIVGSILTLLAGLGILVDNNTKGIKDSVIVQTDYHEPRDSKDPEK
HolTw-M  -------------------------------------     L
```

HolTw

```
     -   + --   -  +  +-   ---  - -    - ---  +-  +      --  -  +  - +--   --  -  +
ALTWEHNHNKVEDNVVDNTKTPKEYGTDEDFSDVDPHVYDADEEPFKDQAKPYGVNWDETVDTTKNIDTKEEGVIDENPETSRVLH
HolTw-M ------------------------------------------------------------                PTLRIQE
```

Hol118

```
  - -  +-                   ++ -    +                    -                       -    + ++  ---+
MIEMEFGKELLVYMTFLVVVTPVFVQAIKKTELVPSKWLPTVSILIGAILGALATFLDGSGSLATMIWAGALAGAGGTGLFEQFTNRSKKYGEDDK
└► Hol118(96) └► Hol118(83)                                                           ***************
```

Hol500

```
  + -  +-                   ++ -    +                    -                       -    + ++  +--+
MMKMEFGKELLVYMTFLVVVTPVFVQAIKKTELIPSKWLPTVSILVGAILGALATSLDGSGSLATMIWAGALAGAGGTGLFEQFTNRAKKYGKDDK
└►Hol500(96)  └► Hol500(83)                                                            ***************
```

Hol2438

```
  + -  +-                    ++ -    +                     -                          -    + ++  +--
MMKMEFGKELLVYMTFLVVVTPVFVQAIKKTELIPSKWLPTVSILVGAILGALATSLDGSGSLATMIWAGALAGAGGTGLFEQFTNRAKKYGKDD.
                                                                                          ************
```

Hol3626

```
  +   -            +  -      +  + +  + ++ +-   +   -          -     +             +-     -   -   -  -
MFKFIPEVISWLLVLYIGFKIIDMILGVLKTIKNKNYRSRKMRDGIIRWVAELMAIAFVLILDMFLGLKFTVIGVTLALFAYKEAGSIVENLGECGVELPE
   -+ -   +  + +-   ++-
IVSEKLEVLNKNNKNKEGFNKKEN
```

B

Orf 2

```
  - - -  -          -  -+ +-      +   -+ +   -    +-   +   +  --   -     +   - ++  +     + -+  -+ +-+
MHDNEFEVPESVINASATPFEAPDRTKDTYQSSLKNWLDRVKSVGDAGMSREFVIRIPAKALEENNPEATLFLKQCNEIKRNPKCNLPIKIEKTVEKYKDK
+    -       + - -      +
KHLMLDLVFTYSNKTDIDAVNSVIK
```

C

MscL

```
    +- +-   +    -            +       -             - +      +-   -                -           +   +
MSIIKEFREFAMRGNVVDLAVGVIIGAAFGKIVSSLVADIIMPPLGLLIGGIDFKQFAVTLRDAQGDIPAVVMHYGVFIQNVFDFLIVAFAIFMAIKLINK

  +++--        +--    - +-  +-   +
LNRKKEEPAAAPAPTKEEVLLTEIRDLLKEQNNRS
```

3. 6. 1 Hol118 causes late cell lysis in λΔSthf phage

Hol118 variants, *hol118 wt*, (starting from ATG(1)), *hol118 (93)* (starting from ATG(2)), and *hol118 (96)* (starting from ATG(1), with ATG(2) mutated into CTG) were inserted into λΔSthf and tested for lytic competence (Fig. 3. 8 and Fig. 3. 9A). λΔSthf::*hol118 wt*, λΔSthf::*hol118 (96)* and λΔSthf:*hol118 (93)* were induced over lysogenic LE392 *E. coli* cultures (Fig. 3. 9A), and lysis profiles monitored in time. Hol118 wt caused relatively late cell lysis, 90 min after induction, whereas Hol118 (96) and Hol118 (93) variants, resulted in growth inhibition rather then sudden lysis (Fig. 3. 9A). It was of interest whether Hol118 could be triggered prematurely by the destruction of the intact membrane potential by addition of CCCP to induced cultures at different time points (Fig. 3. 9B) As shown in Fig. 9B, a slight effect can be seen when the cultures were treated 70 min after thermal induction. In contrast, treating the culture with $CHCl_3$, 30 min and 50 min after induction resulted in immediate lysis, and indicated that sufficient cytoplasmatic endolysin was present for degradation of the peptidoglycan. Hol118 could not be triggered prematurely with energy poisons in the manner demonstrated for the λ S holin. These results suggested that the energized membrane does not play a similar role in inhibiting the permeabilization of Hol118 holin.

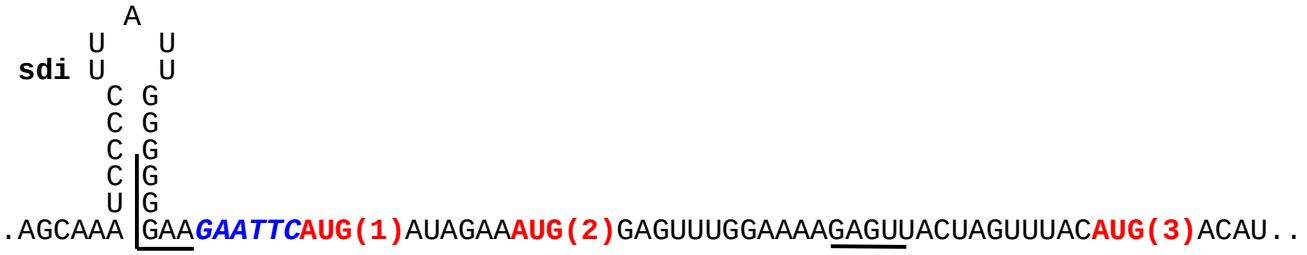
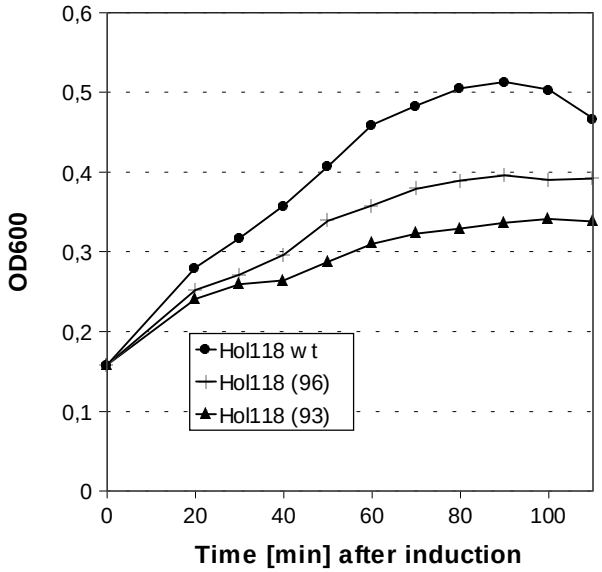


Fig. 3. 8 Translation control region of λΔSthf::*hol118*. mRNA region governing the translation of the *hol118* gene in λΔSthf. Ribosome binding region experimentally shown for λ wt (Bläsi *et al.*, 1989) and a putative RBS in front of the AUG(3) are underlined.

A



B

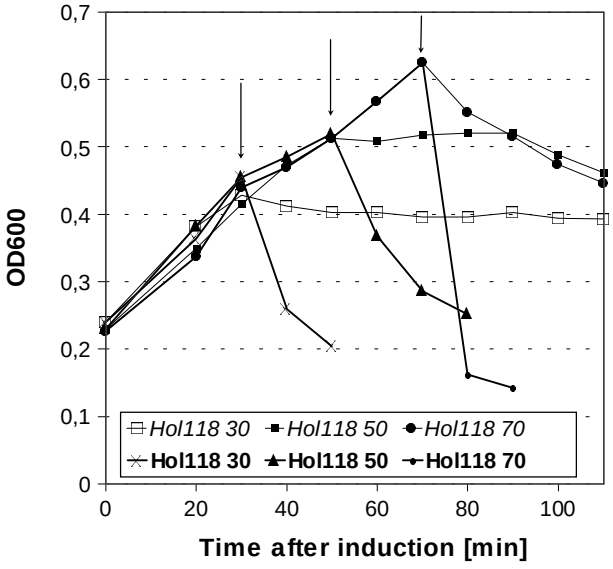


Fig. 3. 9 Lysis profiles of N-terminal variants of Hol118. Panel A. Lysis profiles of Hol118 wt, Hol118 (93), and Hol118 (96) inserted into λΔSthf. Panel B. Lysis profile of λΔSthf::*hol118* following dissipation of membrane potential, or disruption of membranes. CCCP was added to the cells 30, 50, 70 min after induction. At the same time points, $CHCl_3$ was added to different cultures.

3. 6. 2 Hol118 inserts into the E. coli membrane

The observed late cell lysis caused by the *Listeria* phage holin in the λΔSthf genetic background claimed for the inspection whether Hol118 actually inserts into the membrane of *E. coli.* Inner membrane fractions of *E. coli* cells were isolated after induction of λΔSthf phages with three N-terminal different Hol118 variants (Fig. 3. 10A) All samples were taken 55 min after induction (Fig. 3. 10A). After SDS-PAGE and immunoblotting specific Hol118 protein bands were detected for Hol118 wt, Hol118 (96) and Hol118 (93). Our results indicate that Hol118 wt and Hol118 (96) insert the *E. coli* membrane efficiently in contrast to Hol118 (93). The observed lysis curve of Hol118 (93) may therefore result from insufficient insertion of the protein into the membrane (Fig. 3. 10A).

Further, membrane fractions of *E.coli* cells were isolated from cell culture after induction of lysogenic λΔSthf::*hol118* wt phages, were aliquots were taken at different time points after induction (Fig. 3. 10B). The Hol118 specific band can be detected in the inner membrane fraction from 20 min following induction, and from the band intensities an increase in holin concentration over time was observed. In the Western Blot experiments performed on *Listeria* holin analysed, a faint band below the specific Hol118 (marked by blocked arrows on Fig. 3. 10A and Fig. 3. 10B) is observed. This band is most distinct in the lane corresponding the the culture sample taken 20 min after induction of λΔSthf::*hol118* phage (Fig. 3. 10B), and corresponds to Hol118(83).

3. 6. 3 The first ß-Turn of Ho118 resides in the cytoplasm

In order to experimentally elucidate the position of the N-terminal portion of Hol118, two fusions were constructed containing the first 32 amino acids of Hol118 and alkaline phosphatase or ß-glactosidase reporter molecules (Fig. 3. 7A). The approach is based on a reciprocal activity of the two proteins in the individual cell compartments. LacZ is active only if the protein remains in the cytoplasm; in contrast to alkaline phosphatase which is active only in the periplasm (Manoil, 1990). In pBSH32-*phoA* and pBSH32-*lacZ* plasmids, Hol32-phoA and Hol32-lacZ fusion proteins were synthesized after IPTG induction, and the activity of the reporter enzymes tested on agar plates supplemented with chromogenic substrates. *E. coli* cells expressing Hol32-lacZ fusion were blue coloured on plates with X-gal, whereas alkaline phoshatase activity could not be detected.

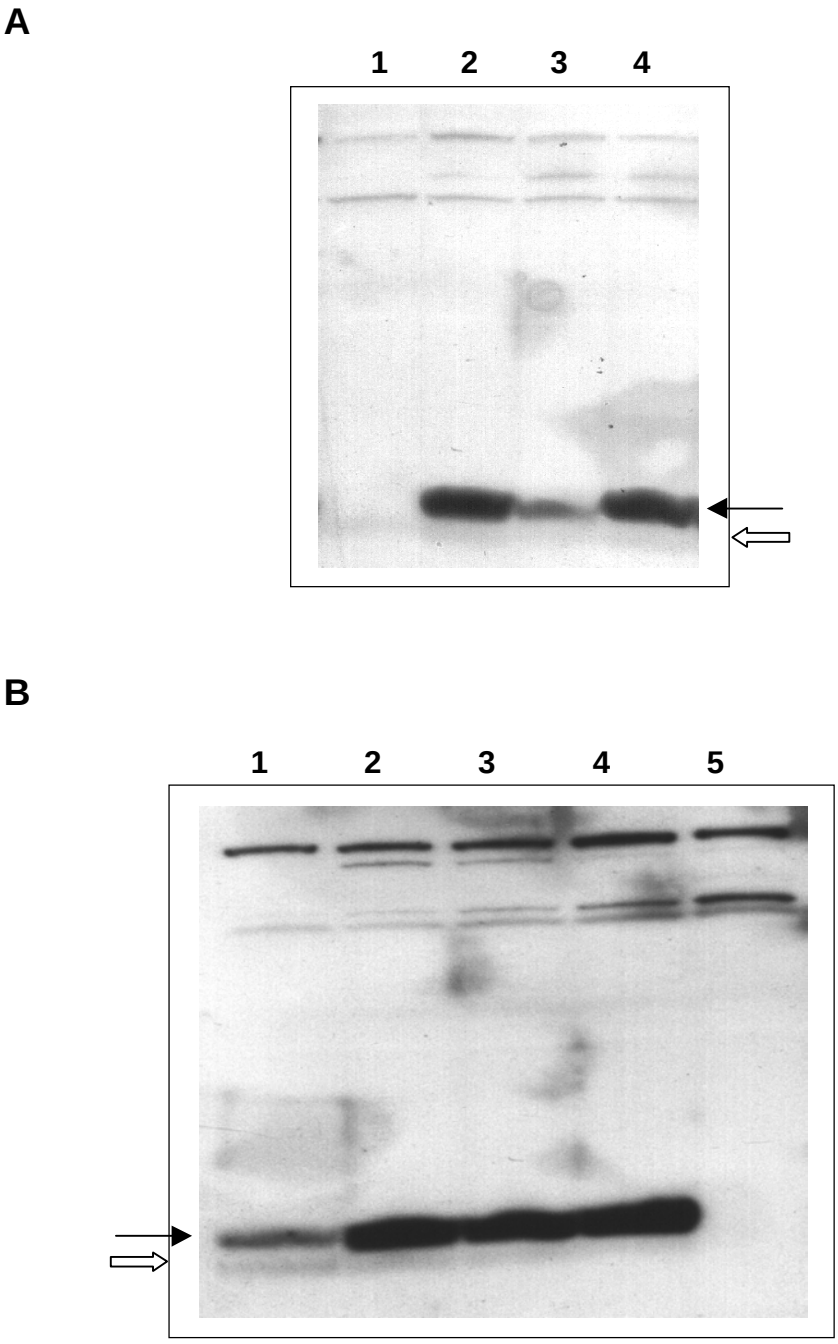


Fig. 3. 10 Holin synthesis in *E. coli* LE392 cells lysogenized with λΔSthf::*hol118*. Panel A. Immunological analysis of inner membrane fraction of *E. coli* cells after induction of temperent λΔSthf carrying different Hol118 variants. Lane 1, λΔSthf; λΔSthf*::hol118 wt,* lane 2, λΔSthf::*hol118 (93);* lane 3, λΔSthf::*hol118 (96);* lane 4. Panel B Time-resolved appearance of Hol118 in the inner membrane of *E. coli* after induction of λΔSthf::*hol118.* The samples were taken at 20 min, (lane 1), 35 min, (lane 2), 45 min, (lane 3) and 55 min, (lane 4) after induction. Lane 5 is a control with λΔSthf taken 60 min after induction.

detected upon production of Hol32-phoA fusion protein. The result indicated that the N-terminal part of the fusion protein (first 32 aa of Hol118) positioned the reporter protein in the cytoplasm of the cell.

3. 6. 4 Hol118 has three possible translational starts

Hol118 starts with two Met codons separated by codons for the amino acids isoleucine (I) and glutamic acid (E) (Fig. 3. 7, Fig. 3. 8). Direct evidence for utilization of AUG(1) and AUG(2) in λΔSthf as start signals for translation was obtained using toeprinting analysis. This method employs primer extention inhibition, and indicates the sites were 30S ribosomal subunits are bound on the mRNA in ternary complexes (Hartz *et al.*, 1988). The polymerase usually stops 15 nucleotides downstream of the AUG codon were the ternary complex is positioned, and a toeprinting signal is formed by accumuation of labelled product on a polyacrylamide gel.

Toeprinting reactions were performed using a run-off transcript from pSP-λΔS*hol118.* The obtained signals suggested the presence of 3 initiation sites (Fig. 3. 11A): The first one is located 15 nucleotides downstream from the AUG(1) (see Fig. 3. 8); the second 15 nucleotides downstream from AUG(2), and a third toeprinting band was located 15 nucleotides apart from a third AUG codon, located 40 nt downstream of the 5´ end of *hol118.* A strong (but unspecific) signal formed inbetweeen AUG(2) and AUG(3), which however, did not correspond to any canonical start codon and was therefore not analysed further.

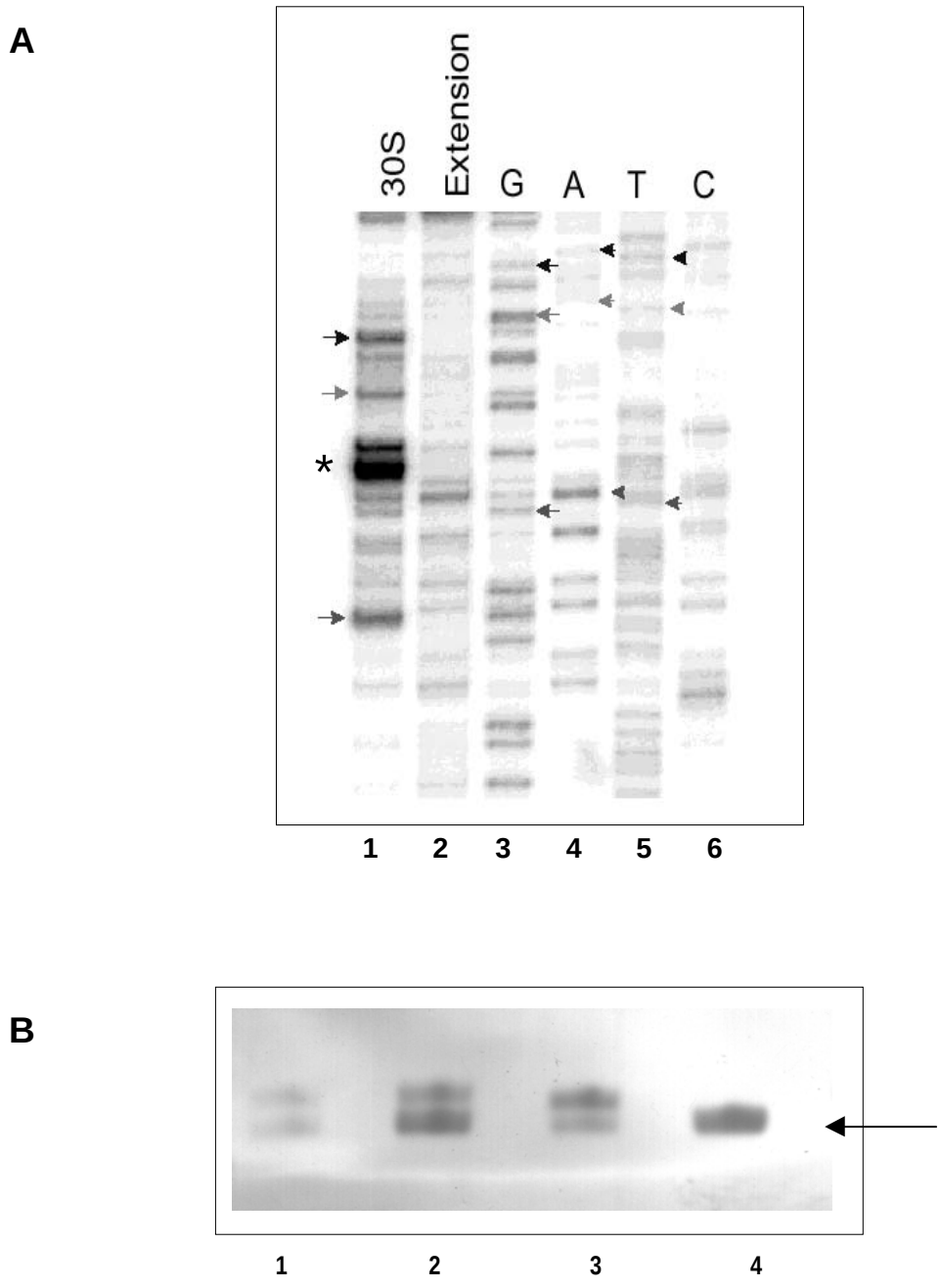


Fig. 3. 11 Ternary complex formation on the translational region of *hol118,* and *in vitro* translation of *hol118* variants. Panel A. The toeprinting signals corresponding to the AUG start codons are marked with arrows, and a signal formed on a noncanonical codon is marked by an asterisk. Lane 1, primer extension in the presence of the 30S ribosomes and $tRNA^{fMet}$. Lane 2, primer extension in the absence of 30S ribosomes and $tRNA^{fMet}$. The corresponding nucleotide sequence of the 5´ flanking and initial coding region of *hol118* is shown on right, (lanes 3-6). Panel B. Detection of *in vitro* holin synthesis from plasmids with different *hol118* variants: lane 1: pSP*hol118* (wt), lane 2: pSP*hol118 (96)*, lane 3 pSP*hol118 (93)* and lane 4 pSP*hol118*Δ*(M1-Y13).* The band corresponding to the Hol118 (83) protein is marked by an arrow.

3. 6. 5 In vitro transcription/translation yielded the expected products

Toeprinting indicated that translation starts from not only the first two "expected" codons, but also from an additional start, AUG(3) (Fig. 3. 8, Fig. 3. 11A). To experimentally demonstrate that this occurs under physiological conditions, an *in vitro* reaction was performed using a coupled transcription-translational system. *Ho118* and variant genes with mutated start codons were transcribed from a T7 promotor. An optimized ribosome-binding site (GAGGAG) with a spacer of 7 nucleotides was positioned upstream of the gene. The products of the *in vitro* reactions were separated by SDS-PAGE electrophoresis and the protein bands visualized by Western analysis using antiHol118 polyclonal antibodies. Four different variants were analysed: *hol118 wt*, *hol118 (96)*, *hol118 (93)* and *hol118 (83).* In all reactions, two bands appeared, were the lower band corresponded to Hol118 (83) protein (Fig. 3. 11B). It should be noticed that proteins starting at AUG(1) and AUG(2) form one band, they cannot be resolved on the electrophoresis system used here.

3. 6. 6 Hol118 is detected in Listeria cells after infection with A118

In order to reveal the lenght of the latent period, lysis profile of A118 in infected *Listeria* cells was analysed by performing a one-step growth curve (Fig. 3. 12). Cell lysis started 60 min after infection, and was complete after about 120 min. To detect the presence of the Hol118 protein in *Listeria* cell membranes, a sample was taken 120 min after infection with A118 and processed as described above. Proteins were separated by SDS-PAGE electrophoresis visualized as described above. Two bands were observed, indistinguishable from the bands detected in *E. coli* cell samples, and by *in vitro* translation of *hol118* (Fig. 3. 10B and Fig. 3.11B).

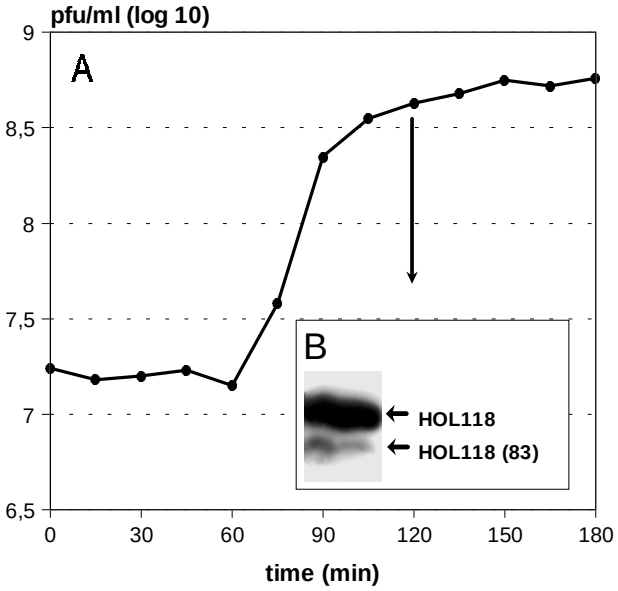


Fig. 3. 12 Detection of Hol118 in the membrane of *Listeria* cells following infection with A118. Panel A. One step growth curve of A118 phage on *Listeria monocytogenes 1001*. Panel B. Immunological analysis of membrane fraction of *Listeria* cells after infection with A118 phage. The sample was taken 120 min after infection with A118. Hol118 specific bands are indicated by arrows.

3. 6. 7 Hol118 (83) inhibits function of Hol118 wt in trans

In order to determine the individual effect of Hol118 (83) on pore formation, the corresponding mutant was inserted into λΔSthf and tested for lytic function. It was found that λΔSthf::*hol118 (83)* did not support R-mediated lysis of *E. coli* upon induction (Fig. 3. 13A). The function of the truncated Hol118 variant was further tested *in trans* to the wildtype Hol118 allele. For this purpose, λ*imm*434*cI::hol118* phage was constructed, which can lysogenize cells carrying λΔSthf phage through the presence of the immunity region from lambdoid phage 434. As shown in Fig. 3. 13B, expression of Hol118 (83) from λΔSthf *in trans* clearly delayed onset of lysis by λ*imm*434*cI::hol118,* compared to the control. The presence of an additional copy of Hol118 in a second control accelerated lysis, likely due to a gene dosage effect (Fig. 3. 13B).

Additionally, the Hol118(83) protein was supplied *in trans* from the medium copy transactivation plasmid pBRT-Hol118(83) (Fig. 3. 13C). Expression of the insert is driven by the $p_{R`}$ promotor, activated exclusively by the Q antitermination factor not present on the plasmid. This enables a co-ordinated expression of holin genes from the phage and plasmid during λ multiplication. The here presented results showed that

Hol118 (83) inhibited cell lysis by the λΔSthf::*hol118* phage (Fig. 4C), which further supports the assumption that the N-terminally truncated protein acts as the inhibitor of lysis.

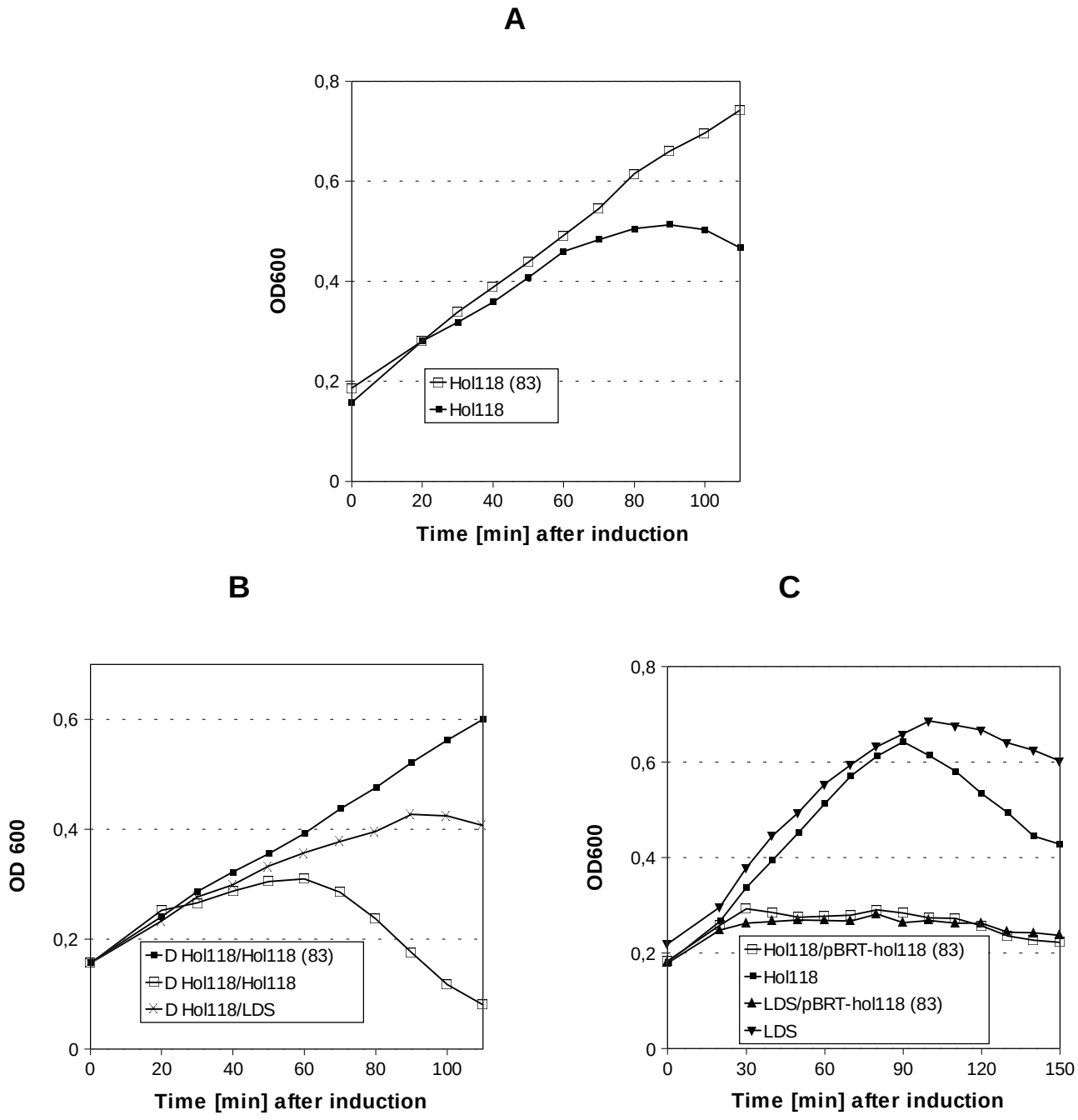


Fig 3. 13 Hol118 (83) has no lytic activity in λΔSthf phage, and causes *in trans* inhibition of Hol118 function. Panel A. Lysis profiles of *E. coli* LE392 cells lysogenized with λΔSthf::*hol118 (83),* λΔSthf::*hol118 wt*. Panel B. Lysis profiles of *E. coli* LE392 double lysogens with λ*imm*434*cI::hol118 wt* and λΔSthf::*hol118 (83),* or λΔSthf::*hol118* and λΔSthf, respectively. Panel C. Lysis profiles of *E. coli* LE392 cells lysogenized with λΔSthf::*hol118* wt, induced in the presence of the transactivation plasmid pBRT-*hol118 (83).*

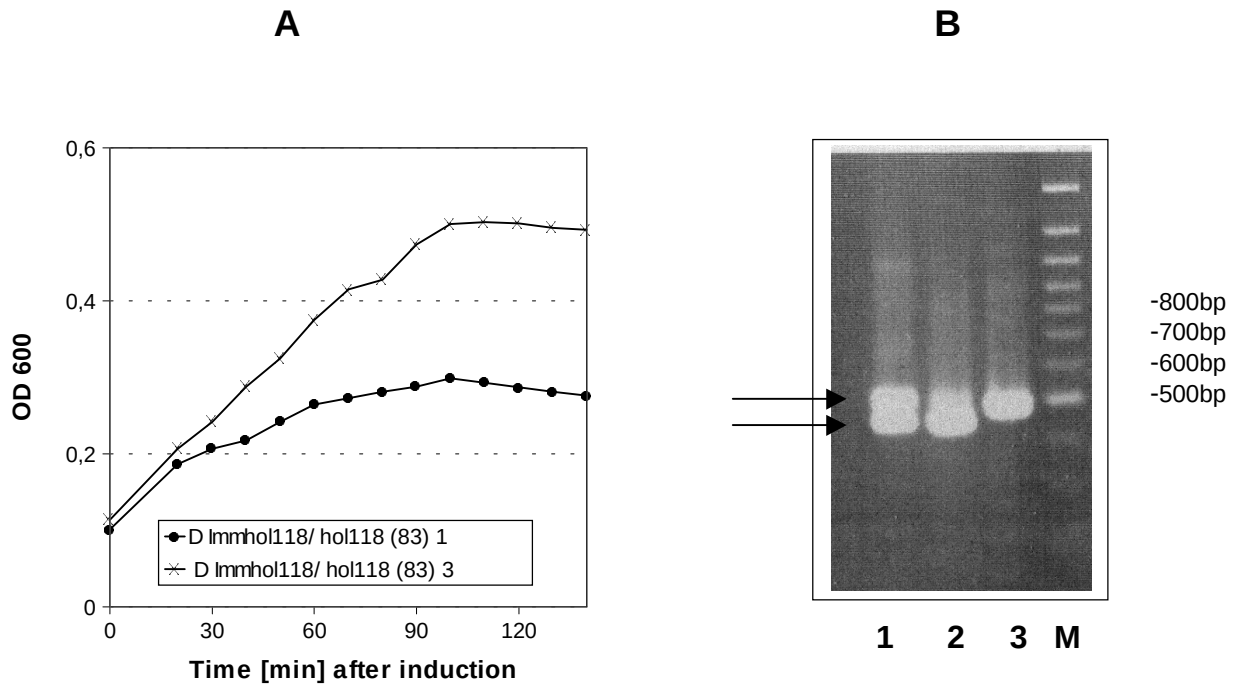


Fig. 3. 14 Cell lysis is influenced by the Hol118:Hol118 (83) ratio. Panel A. Lysis profiles of different *E. coli* double lysogens (1 and 3) carrying: λ*imm*434*cI::hol118* and λΔSthf::*hol118(83),* expressed at different levels after induction. Panel B. PCR performed on liberated phages in the medium after thermal induction. The lower band corresponds to *hol118(83)* in λΔSthf and the upper band to *hol118* in λ*imm*434*cI.* Lane 1. PCR products from clone 1. Lane 2. PCR products from clone 3. Lane 3. PCR on phage λΔSthf::*hol118wt.* Lane 3. 100bp DNA ladder.

This hypothesis is further strengthened by the observation that overexpression of Hol118 (83) to Hol118 in double lysogenes expressing λΔSthf::*hol118 (83)* and λimm434::*hol118,* led also to cell lysis inhibition (Fig. 3. 14A and Fig. 3. 14B).

3. 6. 8 Hol118M14L, Hol118M14I and Hol118M14V are defect in lysis timing

In order to inhibit translation initiation at AUG(3), this start codon was changed to codon CTG or ATT, and the effect of the *hol118M14I and hol118M14L* alleles tested in λΔSthf (Fig. 3. 15). All mutants showed accelerated lysis. λΔSthf::*hol118M14L* had a very short lysis timing, cell density started to decrease 30 min after induction, while λΔSthf*hol118M14I* phage lysed later, 50 min after induction. Obviously, the impaired translation starts for Hol118 (83) protein in *hol118M14I* and *hol118M14L* led to

accelerated cell lysis in both cases, again strengthening the hypothesis that Hol118(83) is the inhibitor of Hol118 function. However, the observed difference in lysis timing of Hol118M14I and Hol118M14L, suggests that the phenotypic change associated with this mutation can not be solely attributed to the lack of the inhibitor function.

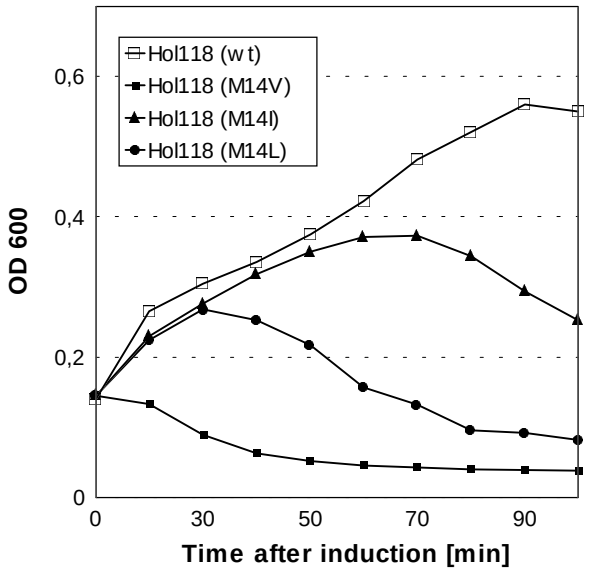


Fig. 3. 15 Hol118M14V, Hol118M15L and, Hol118M14I are early lysis mutants. Lysis profiles of *E.* coli LE392 cells lysogenized with λΔSthf expressing *hol118M14I, hol118M14L,* and *hol118M14V* and induced by heat shock.

The character of these mutations was further tested by expressing them *in trans* to Hol118 wt allele in order to observe its dominant or recessive behaviour over the wild type. Lysis timing assays of the tested double lysogenes are summarized in Table 3. 1.

Table 3. 2 Lysis timing of induced double lysogens expressing different hol118 alleles

***E.coli* strain LE392:**	**Hol118 alleles**	**Lysis time [min]**[a]
λ*imm*434*cI::hol118*/λΔSthf	Hol118/---	90
λ*imm*434*cI::hol118*/λΔSthf::*hol118*	Hol118/Hol118	60
λ*imm*434*cI::hol118*/λΔSthf::*hol118M14I*	Hol118/Hol118M14I	70
λ*imm*434*cI::hol118*/λΔSthf::*hol118M14V*	Hol118/Hol118M14V	20
λ*imm*434*cI::hol118*/λΔSthf::*hol118M14L*	Hol118/Hol118M14L	20
λ*imm*434*cI::hol118*/λΔSthf::*hol118 (83)*	Hol118/Hol118-(83)	<110

[a] Lysis time is defined as the time in minutes between the shift to 42°C and onset of lysis.

Hol118M14L, *hol118M14V,* and *hol118M14I* mutants had a dominant effect over the presence of Hol118 wt. A finding which indicated that residue 14 in the first transmembrane domain is important for the oligomerization process of the holin in the membrane. Since Hol118 (83) protein, can not complement the lysis defect of λΔSthf, and from the dominant character of M14 mutations, it may be concluded that the first transmembrane domain is cructial for the permeabilization process leading to pore formation.

Further, Hol118 (83) was expressed *in trans* to Hol118M14I, Hol118M14L and Hol118M14V from pBRT plasmid in cells lysogenized with λΔSthf::*hol118M14V,* λΔSthf::*hol118M14I* or λΔSthf::*hol118M14L* phages. We observed a dominant effect of the mutations at M14 to the presence of the inhibitor expressed *in trans.* It may be speculated that this position within the first transmembrane domain is involved in the interaction between Hol118(96) and Hol118(83) variants.

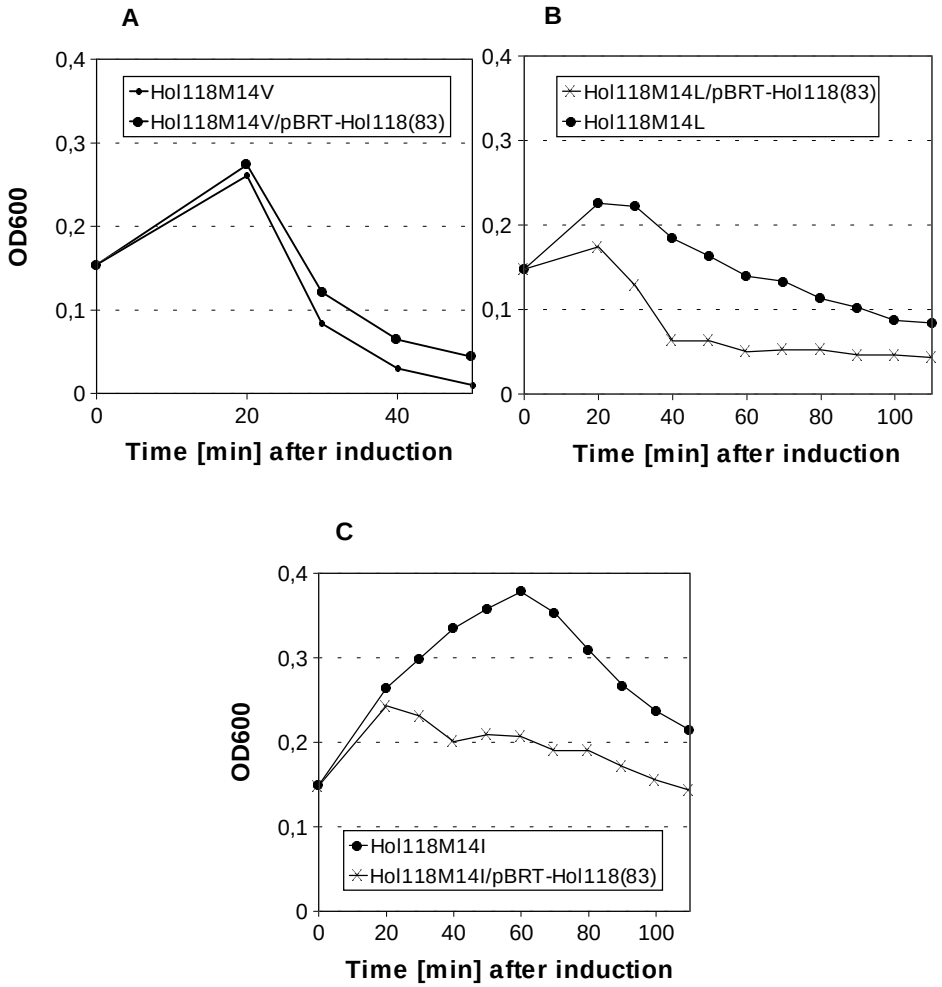


Fig. 3. 16 Mutation at M14 are dominant over the presence of Hol118(83). Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf::*hol118M14V,* panel A; λΔSthf::*hol118M14L,* panel B; λΔSthf::*hol118M14I,* panel C; induced in the presence of pBRT-*hol118(83)*.

3. 6. 9 F84S in Hol118 causes a lysis timing defect

Hol118M1L,F84S mutant was isolated as a spontaneous, plaque forming, mutant of λΔSthf::*hol118M1L* which formed extreamly small plaques. Hol118M1L, F84S mutant, caused rappid cell lysis 20min after induction (Fig. 3. 17) The presence of the single M1L mutation in Hol118 had no significant influence on lysis, so the extreamly early lysis of the double Hol118 mutant could be attributed only to the F84S change in the C-terminal part of the protein.

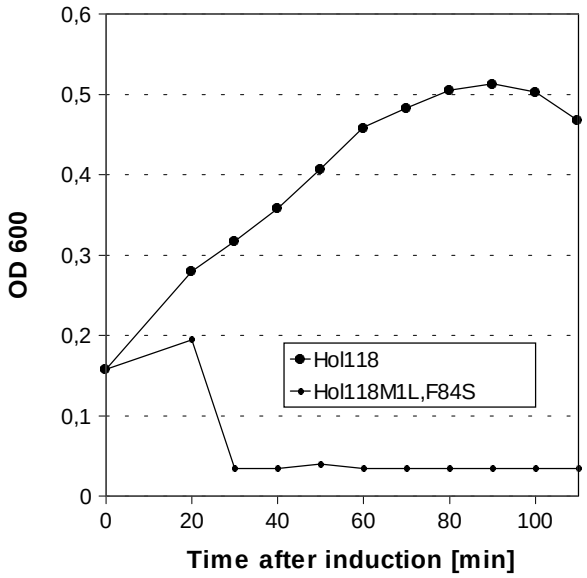


Fig. 3. 17 Hol118F84S is an early lysis mutant. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf::*hol118* phage and λΔSthf::*hol118M1L,F84S.*

3. 7 Analysis of Hol500 from Listeria monocytogenes bacteriophage A500

The lysis gene casette of *Listeria monocytogenes* phage A500 contains the dual start *hol500* gene, preceding the endolysin gene in a different reading frame, with overlapping start/stop codons (Loessner *et al.*, 1995). Inserting *hol500* into λΔSthf complemented the lysis defect of the used phage. λΔSthf::*hol500* phage formed bigger plaque compared to λΔSthf::*hol118*. The significant difference in lysis properties of this two inserted holins was surprising, since they differ in only seven amino acids (Fig. 3. 7). Both holins differ in the organization of the N-terminus. Hol500 has a typical dual start motif, two M codon separated by a codon for a positively charged amino acids. Further changes are in the first ß-turn and the second transmembrane domain of the protein. The C-terminus of Hol500 differs in two amino acid and has an increased positive charge compared to Hol118.

A

λΔSthf::*hol500*

B

Hol118

Hol500

Fig. 3. 18 Translation control region of λΔSthf::*hol500* and amino acid sequences of Hol118 and Hol500. Panel A. mRNA region governing the translation of the *hol500* gene. Single *Eco*RI restriction site in λΔSthf is shown in italics. Ribosom binding site (sdi) region experimentally shown for λ wt (Bläsi *et al* 1989) and a putative RBS in front of the AUG(4) are underlined. The four AUG start codons that gave signals in the toeprinting reaction are indicated. Panel B. Amino acid sequences of Hol118 and Hol500. Amino acids differing in Hol500 and Hol118 are underlined. Translational starts detected in the toeprinting reactions are marked by an asteriskes below the amino acids.

3. 7. 1 Hol500 can be induced prematurely through the dissipation of the membrane potential

We evaluated the lytic properties of both the longer and the shorter possible translation products of *hol500.* The three individual alleles (native *hol500, hol500 (96), hol500(93)*, were tested for lytic competence (Fig. 3. 19A). Cells in which native Hol500 is produced start to lyse 60 min following induction of λΔSthf::*hol500* phage. Synthesis of only the longer Hol500(96) polypeptide (M4L mutation) led to complete lysis, and production of Hol500(93) also enabled membrane disruption and gpR-mediated lysis. These results are clearly different from those obtained with λ S, but also different to the lysis by Hol118 variants (see Fig. 3. 7). Moreover, although the presence of an additional amino-terminal positive charge in the Hol500-96-M2K mutant was found to decrease the lysis-supporting function of this holin, the effect seems somewhat similar to inhibition oberserved with the corresponding mutation in *S107-M3K.*

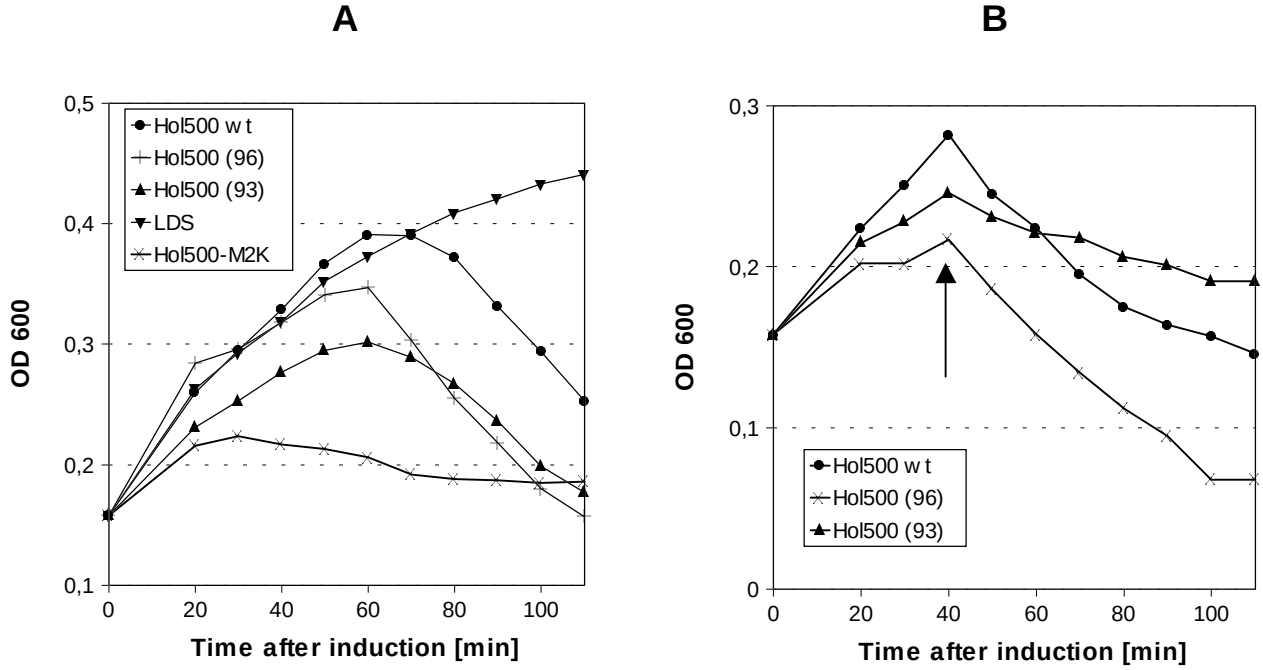


Fig. 3. 19 Hol500 can be induced prematurely by dissipation of the membrane potential. Panel A. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf carrying N-terminally different *hol500* gene variants. Panel B. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf carrying different *hol500* gene variants as specified in the legend, treated with CCCP 40 min after induction (black arrow)

It was further investigated whether Hol500 could be triggered prematurely by destruction of the membrane potential. LE392 cell lysogenized with phages λΔSthf::*hol500,* λΔSthf::*hol500 (96),* λΔSthf::*hol500 (93)* were treated with CCCP 40 min after induction. The lysis curves of induced λΔSthf::*hol500* and λΔSthf::*hol500(96)* responded directly by accelerated cell lysis, while this was not observed for λΔSthf::*hol500(93)* (Fig. 3. 19B). In contrast to Hol118, Hol500 could be triggered by the distruction of the membrane potential. The presence of a typical dual translational start motif, as defined for the S holin, asked for further experiments with respect to the N- terminal portion of Hol500.

3. 7. 2 Mutation in the N-terminus of Hol500

The effect of two different changes in the N-terminus of Hol500 were tested: deletion the first Met, and mutation of the first AUG codon into a CTG. Recombinant phages were selected for plaque forming ability. Both, λΔSthf::*hol500ΔM1* and λΔSthf::*hol500M1L* were difficult to select with the correct sequence of the inserted holin. In both phages the same protein is translated, starting at the second AUG codon. The only difference between them is the reduced spacer in λΔSthf::*hol500ΔM1*for three nucleotides. Both mutants had impaired permeabilization properties as shown on Fig. 3. 20. Among the sequenced clones, two interesting mutants were characterized: *Hol500M1L2* (Hol500 M1L, M4I), which caused earlier cell lysis compared Hol500 wt, and Hol500ΔM1F81L. Deleting M1 almost destroyed lytic activity, so it was possible to conclude that the protein starting from the second AUG(2) has impaired lytic activity compared to wild type.

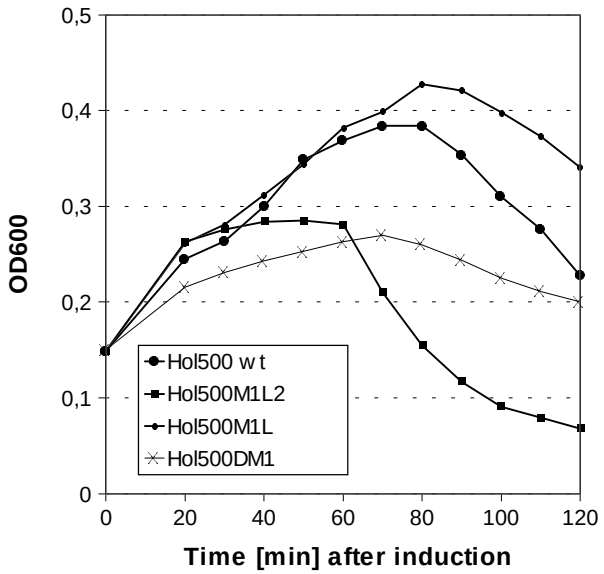


Fig. 3. 20 Lysis profiles of N-terminal variants of Hol500. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf carrying N-terminally different *hol500* gene variants as specified in the legend of the figure.

3. 7. 3 Membrane insertion of different Hol500 N- terminal variants

In order to correlate the lytic properties of Hol500 variants in λΔSthf with the presence of the proteins in the membrane, inner membrane fraction of *E. coli* cells lysogenized with phages λΔSthf*hol500wt,* λΔSthf*hol500(93),* λΔSthf*hol500(96),* λΔSthf*hol500M1L,* λΔSthf*hol500M1L2,* λΔSthfhol500ΔM1 *and* λΔSthf were isolated 55 min after induction (Fig. 3. 21). Membrane proteins were resolved by SDS-PAGE electrophoresis, and immunoblotted using Hol118 antiserum. A correlation between the band intensity observed in the Western blot and lytic efficiency of holins was observed (Fig. 3. 20, Fig. 3. 21). Variants with accelerated cell lysis all had increased band intensities compared to Hol500 wt. The changes intoduced into the N-terminus of Hol500 influenced membrane insertion of the protein, but did not reveal functional differences for proteins starting at the double translational start of Hol500 as observed for λ S.

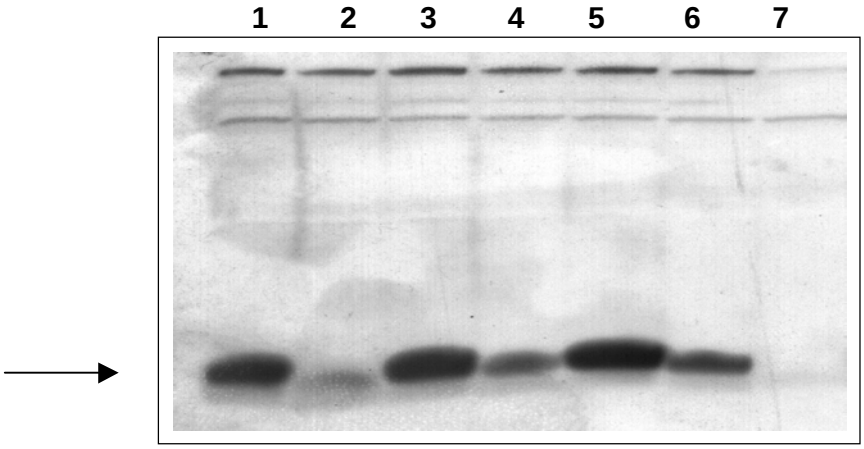


Fig. 3. 21 Membrane insertion of N-terminal variants of Hol500. Western blot analysis of inner membrane fractions of *E. coli* cells after induction of prophages: Lane 1. λΔSthf::*hol500*; Lane 2. λΔSthf::*hol500 (93);* Lane 3. λΔSthf::*hol500(96);* Lane 4. λΔSthf::*hol500M1L;* Lane 5. λΔSthf::*hol500M1L2;* Lane 6. λΔSthf::hol500ΔM1*;* Lane *7.* λΔSthf. Arrow indicate the Hol500 specific band.

Table 3. 3 Hol500 alleles and mutants tested in this study

Holin	N-terminal amino acid sequence[a]	Lysis time [min][b]
Hol500 wt	+ - + - **MM**KMEFGKE	65-70
Hol500 (93)	- + - - - - **M**EFGKE	65-70
Hol500 (96)	+ - +- **MM**K***L***EFGKE	65-70
Hol500-M2K	+ + - + - **M*K***K*L*EFGKE	no lysis
Hol500ΔM1	+ - + - - **M**K**M**EFGKE	80
Hol500M1L	+ - + - - **M**K**M**EFGKE	80
Hol500M1L2	+ - + - - **M**K***I***EFGKE	60

[a] Translation start codons for *hol500* are indicated by bold letters, charged amino-acids by + and - above the sequence, and mutated amino acids are shown in italics

[b] Lysis time is defined as the time in minutes between the shift to 42°C and onset of lysis.

3. 7. 4 M14 is used as a translational start in hol500

In order to see which start codons in *hol500* are actually used as translational starts, primer extention inhibition (toeprinting) analysis was performed. The upstream nucleotide sequence which comprises the sdi structure was amplified together with the *hol500* sequence from λΔSthf::*hol500 wt* and the fragment inserted into pSP72 under the control of T7 promotor. The toeprinting reaction was carried out using a run-off transcript from pSP-λΔS*hol500* (Fig. 3. 22, Fig. 3. 11). The obtained signals suggested the presence of 3 initiation sites immediately at the 5´end of the gene. A fourth toeprinting band was located 15 nucleotides apart from AUG(4), located 40 nt downstream of the 5´ end of *hol500.* A strong (but unspecific) signal formed inbetween AUG(3) and AUG(4), which however, did not correspond to any canonical start codon and was therefore not analysed further.

In order to inhibit translation initiation at AUG(4), this start codon was changed into ATT. Hol500M14I was tested in the λΔSthf phage and compared to the permeabilization properties of the Hol500 wt (Fig. 3. 23). Toeprinting analysis revealed that M14 codon in *hol500,* is used as a translation start for a truncated protein starting within the gene. Changing this start codon into ATT led to accelerated cell lysis, as observed in *hol118.* Due to the overall homology between *hol118* and *hol500* it can be concluded that the same type of inhibitor is translated starting from M14 position in the gene.

3. 7. 5 Hol118 (83) inhibits Hol500 lysis in trans

In order to detect the effect of Hol118 (83) protein on Hol500, the protein was expressed *in trans* to λimm434::*hol500* phage. Clear inhibition of lysis could be observed when Hol118(83) is expressed to Hol500 (Fig. 3. 24). Hol118 (83) and the postulated Hol500 (83) protein would differ in only four amino acid positions, so the *in trans* effect of Hol500(83) should not be very different compared to the effect of Hol118(83). From these results it was concluded that the same type of inhibitor is involved in the regulation of lysis timing in A500 as observed for bacteriophage A118.

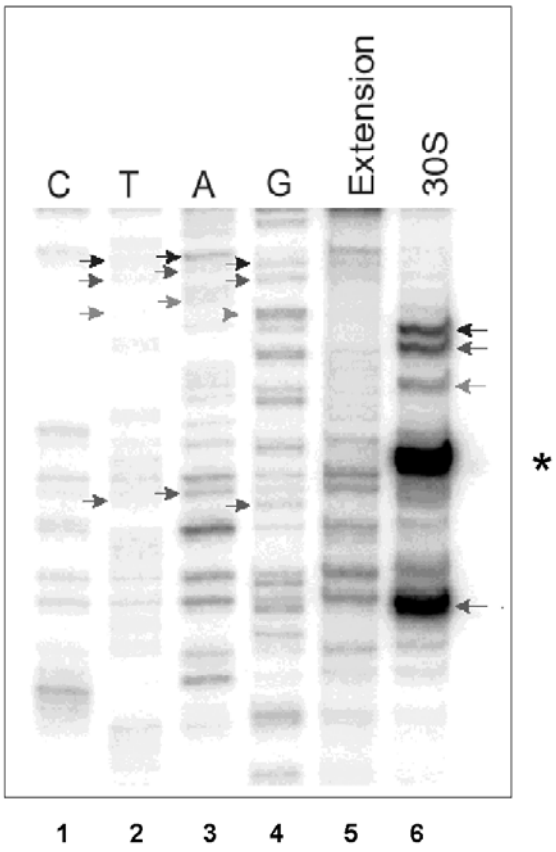


Fig. 3. 22 Ternary complex formation on the translational region of *hol500*. The toeprinting signals corresponding to the AUG start codons are marked with arrows, and a signal formed on a noncanonical codon is marked by an asterisk. Lane 1-4. mRNA sequence of the 5´ flanking and initial coding region of *hol500* is shown at the left. Lane 5. Primer extension in the absence of 30S ribosomes and $tRNA^{fMet}$. Lane 6. Primer extension in the presence of 30S ribosomes and $tRNA^{fMet}$.

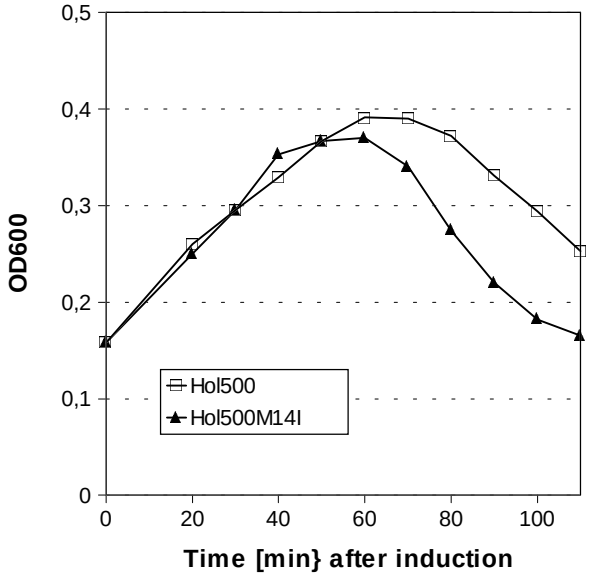


Fig. 3. 23 M14I change in Hol500 causes accelerated cell lysis. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf::*hol500* wt and λΔSthf*::hol500M14I.*

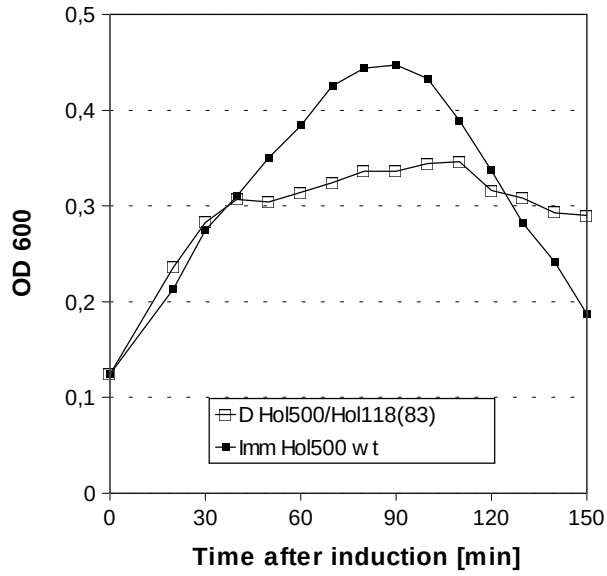


Fig. 3. 24 Hol118(83) inhibits Hol500 *in trans*. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λimm434::*hol500,* and of an *E. coli* double lysogen carrying λimm434::*hol500* and λΔSthf::*hol118(83)* phage.

3. 7. 6 Lysis timing mutant in the third transmembrane domain of Hol500

A λΔSthf::*hol500ΔM1F81L* was isolated as a spontaneous mutant during the construction of λΔSthf::*hol500ΔM1* phage (Fig. 3. 19. and Fig. 3. 20). This mutation in the third transmembrane domain revelead a very early lysis timing phenotype compared Hol500 wt.

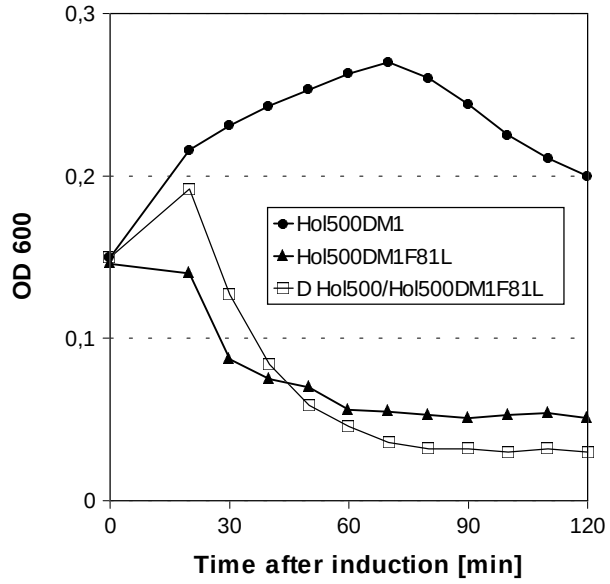


Fig. 3. 25 F81L possibly influences oligomerization. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf::*hol500ΔM1,* λΔSthf::*hol500ΔM1F81L* and double lysogen carrying λimm434::*hol500* and λΔSthf::*hol500ΔM1F81L.*

The phenotype of the mutant was further tested by expressing it in double lysogens to Hol500 wt in λimm434::*hol500.* This change had a clear dominant effect over the Hol500 wt (Fig. 3. 25) indicating its possible influence on oligomerization. The F81L change created a LL motif of Leucins at the end of the third transmembrane protein. This motif has been found in all holins, except Gp 17,5, tested in this work and can therfore be of functional and structural importance for this protein (Fig. 3. 5, Fig. 3. 7) group.

3. 8 Complementation of the λΔSthf lysis defect with Hol2438 from a Listeria innocua cryptic prophages

The *Hol2438* gene was discovered in a cloned fragment of DNA from *Listeria innocua,* which showed lytic activity against *Listeria* cells (Zink *et al.*, 1995). The gene is located upstream of *cpl*2438 endolysin. The only difference between Hol2438 and Hol500 is the lack of the last amino acid residue, a positively charged lysine (K) (Fig. 3. 7), thereby reducing the positive charge of the C-terminus. Here, *hol2438* was amplified by PCR using *Listeria* chromosomal DNA as a template, and inserted into λΔSthf. Recombinant phages were selected for plaque forming ability, and phage-carrying cells were further tested for lysis timing.

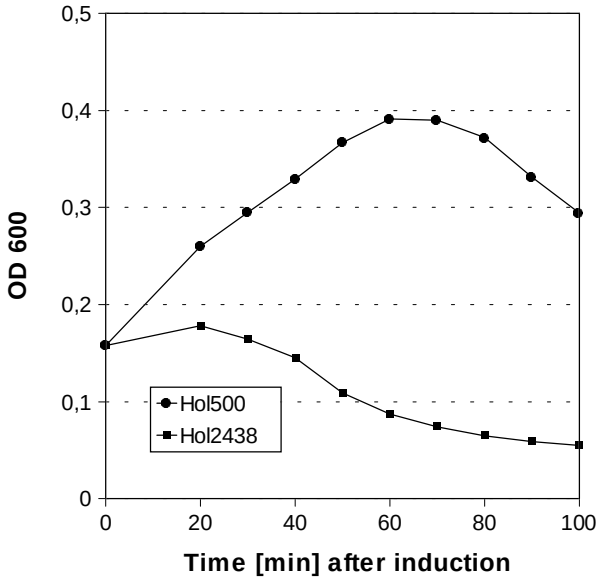


Fig. 3. 26 Reduction of the positive charge at the C-terminus influences lysis timing of Hol500. Lysis profiles observed follwing thermal induction of *E. coli* LE392 lysogenized with λΔSthf::*hol500* and λΔSthf::*hol2438* phages.

It was surprising to find that only the lack of one K residue had such a profound effect on the lysis timing of the holin (Fig. 3. 26). These results are in accordance with results obtained from extensive analysis of the C-terminal region of λ S holin which revealed that the reduction of positive charges on the C-terminus accelerated cell lysis.

3. 9 MscL can not complement the lysis defect of λΔSthf

MscL, the large mechanosensitive channel belonges to the group of high-conductance ion channels gated by mechanical forces on cell membranes, and functions in osmoregulation. These proteins protect bacteria from lysis upon osmotic shock. The *mscL* gene codes for a 15 kd protein present in the cytoplasmic membrane. Homologous genes have been identified both in gram-negative and gram-positive bacteria (Blount *et al.*, 1999). MscL has two transmembrane domains, and the N-terminus and C-teminus positioned in the cytoplasm of the cell (Blount *et al.*, 1996; Chang *et al.*, 1998). The exact conformation of MscL homologous channel from *Mycobacterium tuberculosis* has been determined by x-ray crystallography. It has been shown that the protein forms a homopentamer channel in the membrane (Chang *et al.*, 1998). Further, hypotonic shock causes MscL channel to pass solutes, including small proteins as thioredoxin but also elongation factor Tu and DnaK proteins through the cytoplasmic membrane (Ajouz *et al.*, 1998; Berrier *et al.*, 2000). The topology of MscL protein is similar to the topology of the class II holin proteins: N-terminus and C-terminus are positioned in the cytoplasm, with two transmembrane domains traversing the cytoplasmic membrane. The fact that, through the MscL channel, proteins can be transferred from the cytoplasm to the periplasm of the bacterial cell promted to test whether MscL can complement the lysis defect of λΔSthf.

For this purpose, a gene fragment encoding MscL was inserted into λΔSthf, phages packaged, and plated to test for plaque forming ability. However, no plaque forming ability for MscL could be detected. The recombinant λΔSthf::*mscL* phage was then selected by direct lysogenization of LE392 cells. No lysis could be observed following induction of λΔSthf::*mscl* lysogens.

It was shown here that a very similar protein to holins, in the organization of basic structural domains, was unable to complement the lysis defect of λΔSthf (Fig. 3. 7). Therefore, holin function seems to be specific, in spite of the great diversity of holin protein sequences yet discovered

4. Discussion

The last compilation of holins and putative holin sequences revealed more than 100 protein sequences (Wang *et al.*, 2000). Most genes coding for putative holins are found upstream of adjacent endolysin genes. They specify small membrane proteins having at least one transmembrane domain flanked by charged and hydrophilic N- and C- terminal domains. Not very many holins and putative holin protein were experimentally tested for their function. Holins start a permeabilization process, "hole" formation, in the membrane which enables the endolysin to pass through the membrane and reach the periplasm to degrade the peptidoglycan. Holins form pores in the membrane in a time dependent manner and the whole process assumes a crucial timing function. This function can be fulfilled by very different proteins, which are currently classified (according to sequence similarities into at least 34 holin families (Young *et al.*, 2000). A novel genetic system is described in this work, which allows to assess at least qualitatively the functional properties of various holins of all classes, in *E. coli* λΔSthf::*hol* lysogens. Induction of a lysogen permits observation of a synchronized lytic cycle, and allows reasonably precise assessment of the course and timing of lysis.

In this study, holins from phages infecting both gram-negative and gram-positive bacteria where analysed, with a special focus on holins from *Listeria monocytogenes* bacteriophages A118 and A500. Two proteins were also tested which do not belong to holins *per se:* Gp orf 2 from *Listeria monocytogenes* bacteriophage A511, and MscL, the mechanosensitive channel from *E. coli.* Both proteins were unable to complement the lysis defect of λΔSthf. It was demonstrated that λΔSthf can be used to specifically select a protein with holin function.

4. 1 Holins from Escherichia coli phages

Three different holins from *E. coli* phages were tested here: λ *S*, and two holins from non-lambdoid phages T7 and T4. *S* belongs to the class I, while Gp 17.5 was classified as class II, and T4, T as a completely different holin type (Wang *et al.*, 2000).

Function of *S* is post-transcriptionally controlled by a dual start motif, which permits synthesis of two N-terminally different polypeptides, S107 and S105 with different functions (Bläsi and Young, 1996; Blasi *et al.*, 1989). The timing and rate of cell lysis established here for wild-type S and S105 were similar to the values reported

earlier (Raab *et al.*, 1988). However, it was reported that lysis in an induced λ lysogen expressing only *S107* was almost absent. This is in contrast to here reported results, where induction of λΔSthf::*S107* resulted in lysis starting at approximately 60 min. This may be explained by the different translation initiation sites present in the two constructs. In native λ, a relatively inefficient Shine-Dalgarno sequence with a very short spacer directs the ribosome to the first start codon, whereas the layout of λΔSthf enables identical translation conditions for all the cloned holin genes, since they all exhibit a start codon immediately downstream of the *Eco*RI site. In terms of comparative holin analysis, this can be regarded as an advantage.

The difference in lytic capacity of *S105* and *S107* is determined by the distribution of amino-terminal charged residues, and the total amino-terminal net charge (Steiner and Bläsi, 1993). The here presented finding that the S107-M3K protein resulted in a complete non-lytic phenotype confirms results obtained using a similar mutant (Graschopf and Bläsi, 1999). It has been observed that presence of additional positive charge in the N-terminus in S hindered it from translocating through the membrane, and therefore prevented formation of the active N_{out}-C_{in} topology (Graschopf and Bläsi, 1999). Our results are in excellent agreement with these data, and justify the conclusion that λΔSthf represents a quick and reliable system for evaluation of holin function in an easy-to-use λ background.

Three lysis genes have been identified in bateriophage T7 encoding: Gp 3,5 a murein hydrolase which can also bind and inhibit the fuction of T7 RNA polymerase, Gp 17,5 and Gp 18,5 which is homologous to the λ Rz (Young, 1992). It has been suggested that Gp 17,5 and Gp 3,5 have the same function in T7 as S and R in λ phage. Tested in λΔSthf, Gp 17,5 caused a sudden and saltatory onset of lysis 30 min after induction, which confirmed its holin function. Gp 17,5 was initially classified as a class II holin (Wang *et al.*, 2000; Young, 1992). However, analysis for potential transmembrane domains using the TMHMM 2.0 software predicts only one transmembrane domain to be formed and a high probability for a N_{out}-C_{in} topology for the holin (Fig. 3. 5).

It has been shown that T from phage T4 can complement the *S* gene defect of λ and it was postulated that T is a functional equivalent of S (Lu and Henning, 1992). In a λ hybrid were T was inserted in the place of the S, T causes an abrupt cell lysis starting about 20 min after the induction (Ramanculov and Young, 2001a). Lysis timing of λΔSthf::*t* is about 30 min and this difference reflects different expression conditions in

these two phages. The most interesting observation was the finding that Gp 17,5 and Gp T have almost identical lysis timing tested in λΔSthf. T has a single transmembrane domain near the N-terminus of the protein with a large C-terminal part positioned in the periplasm of the cell (Ramanculov and Young, 2001b). This topology was established using gene fusions with *pho*A (alkaline phosphatase) and *lac*Z (ß-galactosidase) genes and is in perfect accordance with the prediction for transmembrane domains made by TMHMM 2.0. Experimental data for gp 17,5 toplogy of are not available. However, according to the prediction for transmembrane domains T and Gp 17, 5 would group into the same class of holins which posess only one transmembrane domain. This similar behaviour of these very different holins (S, T, Gp 17, 5) of *E. coli* phages tested in an identical *E. coli*-dependent λΔSthf system is not based on similarity of protein sequences. (Fig. 3. 5). It may be hypothesized that holin function (pore formation and timing) relies on some underlying structural principles in the sequences of the transmembrane domains of holin proteins.

4. 2 HolTw from Stapylococcus aureus bacteriophage Twort

HolTw from *S. aureus* bacteriophage Twort belongs to the class II holins (see Fig. 3. 7), has a positively charged N-terminus, and an extensive, negatively charged C-terminus (Loessner *et al.*, 1998). λΔSthf::*holTW* showed an "early lysis" phenotype and, as a consequence, conferred a plaque forming defect. Surprisingly, the mutant polypeptide HolTW-M in λΔSthf::*holTW-M* delayed the lysis event as compared to the native protein. This difference is due to the presence of 5 additional C-terminal residues in the mutant protein, and a V52L substitution. It is not entirely clear which of the two changes is responsible for the observed effect. The V52 residue is located within the predicted ß-turn that separates the two possible membrane spanning domains, and substitution by leucine was not predicted to alter folding properties and protein structure. However, the addition of two charged residues at the C-terminus increases hydrophilicity of this domain. Important findings in λ S were that the hydrophilic C-terminal domain is not essential for oligomerization (Rietsch *et al.*, 1997), but represents a regulatory domain that interacts with the membrane in order to schedule lysis timing (Bläsi *et al.*, 1999). These results obtained with the mutated HolTW-M protein suggest that (i) the extensively charged C-teminus somehow schedules lysis timing, and (ii) show that mutations which add charged residues without affecting net charge can influence holin function. Although it seems obvious that the primary amino-acid

structure of this class II holin determines a N_{in} - C_{in} topology (TMHMM 2.0) of the protein in the membrane, the exact function of the large C terminus needs to be further elucidated.

4. 3 Hol118 from Listeria monocytogenes bacteriophage A118

Holins of λ and A118 are structurally similar but functionally different. Hol118 belongs to the same holin class as the λ S holin. Analysis for potential transmembrane domains (TMHMM 2.0) (Sonnhammer *et al.*, 1998) identified three transmembrane domains and indicated a high probability for a N_{out}- C_{in} topology (Fig. 4. 1). The activity of a Hol32-*lac*Z fusion protein over the lack of Hol32-*pho*A fusion protein activity provided experimental evidence for the N_{out} position of the N-terminal domain of Hol118. This domain has a negative charge of -2, and two potential translational starts (Fig. 3. 7A). However, these do not seem to have an important role in regulating its function, since no significant difference in lysis could be seen among the different N-terminal variants. Changing AUG(2) codon into CTG had no major effect, and changing of AUG(1) into CTG did also not influence lysis. The Hol118(93) protein, (truncated for the first three amino acids) can complement the lysis defect of λΔSthf but inserts into the membrane of *E. coli* less efficiently compared to Hol118(96). It was surprising to find that disruption of the membrane potential had no premature activating effect on Hol118. Due to the overall negative charge of the N-terminal portion of Hol118, it is reasonable to conclude that the energized membrane plays an active role in positioning the N-terminus in the periplasm of the cell (Cao *et al.*, 1995). Negatively charged amino acids in the N- terminal domains together with hydrophobic forces are important for the proper orientation of proteins in the membrane depending on the intact membrane potential (Kiefer and Kuhn, 1999; Kiefer *et al.*, 1997).

Hol118 appeared in the cytoplasmic membrane of *E. coli* 20 min after induction of λΔSthf::*hol118,* so that the lysis process must be delayed to the point determined by the "lysis clock" of Hol118. Here a model is proposed in which Hol118(83), translated from the AUG(3) start codon, acts as the intragenic inhibitor of Hol118 permeabilization.

A translational start at M14 was detected in *hol118*, and *in vitro* translation of *hol118* yieled two bands were the smaller corresponds to Hol118(83). Western blotting detected two identical Hol118 polypeptides in membrane fractions of induced *E. coli* (λΔSthf::*hol118*) lysogens, and in *Listeria monocytogenes* infected with A118.

Expressed in λΔSthf, Hol118(83) had no lytic activity by itself, and recombinant λΔSthf::*hol118(83)* was unable to form plaques. Hol118 (83) was expressed *in trans* from λΔSthf and from a transactivation plasmid. In double lysogenes carrying λΔSthf::*hol118(83)* and λimm434::*hol118*, Hol118(83) caused delayed lysis. Cell lysis interference by Hol118(83) was also observed when Hol118(83) was made *in trans* from pBRT-Hol118(83). Taken together, all these data strongly suggest that the lysis process is inhibited by Hol118(83). In spite of the lack of a permeabilization function which leads to pore formation, Hol118(83) had toxic effect on cells, when expressed from a plasmid. In fact, it was not possible to clone *hol118(83)* on any high copy plasmid (data not shown).

The start codon for the Hol118(83) holin is positioned within the first transmembrane domain of the protein. In order to prevent translation initiation, the M14 codon, was changed into CTG or ATT. Both mutations resulted in accelerated cell lysis, but to a different degree. Changes in the start codon for Hol118(83) also influenced the amino acid sequence of the protein starting upstream. It is important to emphasize that three mutations changing the AUG(3) codon into GTG, ATT or CTG had a dominant effect on lysis timing when expressed *in trans* to wild type Hol118. It was reported earlier that dominant mutations in holins point to a possible influence on the oligomerization process, leading to "hole" formation (Raab *et al.*, 1988). From the character of changes at this position, it is obvious that M14 position forms part of the intrinsic lysis clock of Hol118, besides being a start codon for Hol118(83) protein. For Hol118(83), TMHMM 2.0 software predicts only two transmembrane domains to be formed, which correspond to the second and third transmembrane domain of Hol118 (Fig. 4. 1). The protein could not complement the lysis defect of λΔSthf, but it was able to delay lysis timing of Hol118 *in trans*. Further, the mutation in M14 influenced the interaction with Hol118(83). We have observed that lysis timing could not be delayed in cells expressing Hol118(83) from pBRT to induced λΔSthf::*hol118M14I,* λΔSthf::*hol118M14V,* or λΔSthf::*hol118M14L* phages. Hence, Hol118(83) had a recessive effect on lysis timing of Hol118M14I, Hol118M14L and Hol118M14V variants, but was able to delay the lysis timing of the wildtype holin. From the present results, it can be postulated that Hol118(83) constitutes part of the lysis clock of Hol118 and is superimposed on the intrinsic lysis clock. Results also clearly demonstrated that the first transmembrane domain is crucial for permeabilization function and influences the interaction with Hol118(83). The actual effector/inhibitor ratio and the molecular basis of

lysis inhibition and its regulation has to be further elucidated in the A118 genetic background.

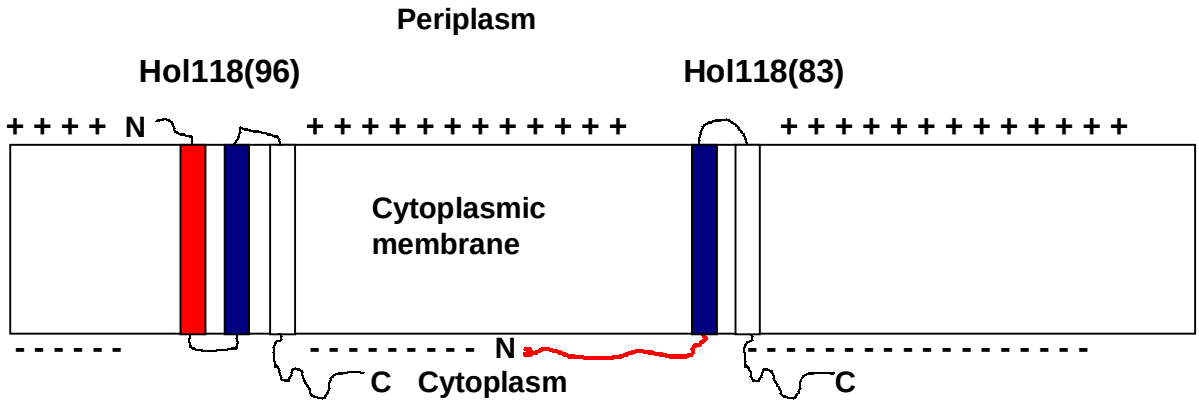


Fig. 4. 1 Membrane topology for Hol118(86), and Hol118(83). Membrane topology was depicted according to a bioinformatic analysis by TMHMM 2.0 software (http://www.cbs.dtu.dk/krogh/TMHMM/).

4. 4 Hol500 from Listeria monocytogenes bacteriophage A500 and differences between Hol118, Hol500, and Hol2438

Hol500, Hol118 and Hol2438 have almost identical amino acid sequences (Fig. 3. 7). The N- terminus of Hol500 has an overall neutral charge; in the first ß-turn a L34I change is present; the second transmembrane domain differs in two amino acids: I46V and F56S, and the C- terminus of Hol500 is different in the overall charge, caused by the E93K substitution and through a S88A change. The only difference of Hol2438 to Hol500 is the lack of the last lysine in the C-terminal tail of the protein. In spite of these small changes in the amino acid sequences, their detected lytic properties in λΔSthf were significantly different.

Hol500 has a possible dual translational motif, two start codon separated by a codon for lysine. The *hol500* dual start alleles that were tested in this work were defined by their similarity to λS and to the corresponding translational initiation sites in *hol118* gene of *Listeria monocytogenes* bacteriophage A118. However, the gpR-dependent cell lysis mediated by the individual *hol500* alleles is different. The longer Hol500(96) polypeptide enabled reasonably quick cell lysis. The protein starting at M4,

Hol500(93), could not be defined as the effector of lysis, and changing the AUG(4) codon into a CTG, Hol500(96) led to an abrupt cell lysis not observed for other Hol500 alleles Cell lysis mediated by Hol500 holin could be triggered prematurely through the destruction of the intact membrane potential of the cell, so that the lysis process is inhibited by the energized membrane. The presence of aditional positive charge in Hol500-M2K mutant inhibits cell lysis which is in accordance with the properties of S mutants were the excess of positive charge on the N- terminus completely blocks the cell lysis event (Graschopf and Bläsi, 1999). Western blot analysis showed a correlation between protein concentration in the membrane of the *E. coli* cells and the observed lysis pattern. Mutants in the N-terminus, which accelerated lysis had, in the same time point, more holin proteins present in the membrane. These results confirm the postulated critical-concentation model, in which lysis time depends on the concentration level of the protein in the membrane of the bacterial cell (Gründling *et al.,* 2001). All three M codons at the beginning of *hol500* were used as transational starts in λΔSthf, and M14 within the first transmembrane domain. As observed for Hol118, mutations in the first M codons did not significantly influence the lytic property of *hol500.* However, changing AUG(4) codon into AUU led to accelerated cell lysis of Hol500; the same mutation had a similar effect on lysis in Hol118. Hol118(83) was able to inhibit *in trans* cell lysis mediated by Hol500. The effect of the cognate Hol500(83) has to be tested separately in order to see the *in trans* effect of this protein. Due to few differences in the amino acid composition of Hol118(83) and Hol500(83), the inhibitory function for Hol500(83) could be extrapolated from the function of Hol118(83). At any rate, it would be interesting to test the inhibitory effect of Hol500(83) in an experiment because of the I46V and F56S changes in the second transmembrane domains between the two *Listeria* holins. Both changes could have an effect on the interaction between transmembrane domains; valine through its branched aliphatic chain, and serine through the hydroxyl group which was found to stabilize interactions between membrane spanning domains (Lemmon *et al.*, 1997; Zhou *et al.*, 2000).

It was surprising to find that the influence of a reduced net charge of +1 (Hol2438 compared to Hol500) led to such a pronounced effect, causing lysis to start 30 min before Hol500. The C-terminal domain of Hol118 is also reduced in its positive charge in comparision to Hol500, but lysis timing is not influenced by this feature, based on the observation that the lysis timing is much delayed for Hol118 compared to Hol500. Therefore it may be possible to conclude that other amino acids in the C-terminal domain or in other domains compensate this charge difference in the C-terminal

domains of Hol118 and Hol500. It was found that the single effect of charged amino acids in the C-terminal domain, as observed in Hol2438 has the same influence on timing regulation as observed in λ. Excess positive charge in the C-terminus delayed lysis timing.

5. General conclusions

Holins are major regulatory proteins of the lysis process mediated by bacteriophages which use the dual protein strategy in order to lyse bacterial cells at the end of the lytic growth cycle (Young, 1992). These small membrane proteins permeabilize the membrane in a time dependent manner, allow endolysin access to the peptidoglycan of the cell wall which leads to its degradation and subsequent cell lysis. Holin constitute the largest group of functional homologous protein with almost no similarity on the level of primary protein structure. In order to investigate the relationship between the structure and function within holins, λΔSthf phage vector has been constructed which was used to asses qualitatively functional properties of holins from very different bacteriophages and with various structural features. Holin function has been defined as the possibility of a membrane protein to complement the lysis defect of λΔSthf phage, which has a deletion of it own holin gene. From the here presented results, it is possible to conclude that holin function is specific; membrane proteins such as MscL with a similar domain structure to holins can not complement the lysis defect of the phage. This finding indicates that the postulated common domain structure (hydrophylic N-terminal tail, two or more transmembrane domains, highly charged and hydrophylic C-terminal tail) is not enough to fulfill holin function. Lytic properties of holins from bacteriophage T7, (Gp 17.5), T4, (T) and A118, (Hol118) revealed that at least one transmembrane domain is necessary and sufficient for the permeabilization function. Gp 17.5 and T holin have a different organization of structural domains but very similar lytic properties. The structural basis for this similar function has to be sought in the features of the transmembrane domains of T and Gp 17, 5. Holins oligomerize in the membrane of the cell in the process of membrane permeabilization (Zagotta and Wilson, 1990). Oligomerization depends on specific and unspecific interactions between membrane spanning domains of membrane proteins (Lemmon *et al*. 1997). Therefore, the key structural determinants for holins must reside within this segments.

Hol118 and Hol500 from *Listeria* bacteriophages A118 and A500 have the same overall organization of the basic structural domains as λ S holin: charged N-terminal tail, three potential transmembrane domains and hydrophylic, charged C-terminal tail. However, functional analysis of Hol118 revealed difference in lytic features to S holin. It has here been demonstarted that the potential double translational starts present at the 5´ end of *hol118* and *hol500* is fuctionally non-homologous to the double start of *S*. Differences in the lenght and amino acid composition of the N-terminal portion of Hol118 and Hol500 influenced insertion of proteins in the cytoplasmic membrane. Lysis timing of Hol118/Hol500 was influenced by mutations in the region of the first and third transmembrane domain and the C-terminal tail. A model has been proposed in which a protein starting at the fourteenth codon (M14), within the gene, Hol118(83) or Hol500(83), is acting as the inhibitor of lysis and regulates the lysis timing superimposed on the intrinsic lysis timing which is determined by the features of the transmembrane domains. Experimental evidences have been presented for this proposed model. Further experiments which are designed to elucidate the interaction between the truncated inhibitor Hol118(83) and the effector in the membrane of *Listeria* cells are desirable, in order to unambiquously prove the novel mode of holin inhibition function proposed here.

6. References

Ackermann, H.W., and Dubow, M.S. (1987) *General properties of bacteriophages..In: Viruses of procaryotes:.* Boca Raton, Florida: CRC Press.

Ackermann, H.W., and DuBow, M.S. (1987) *Viruses of procaryotes.* Boca Raton, Florida, USA: CRC Press.

Ajouz, B., Berrier, C., Garrigues, A., Besnard, M., and Ghazi, A. (1998) Release of Thioredoxin via the Mechanosensitive channel Mscl during osmotic downshock of *Escherichia coli* cells. *J Biol Chem* **273**: 26670-26674.

Arendt, E.K., Daly, C., Fitzgerald, G.F., and van de Guchte, M. (1994) Molecular characterization of Lactococcal bacteriophage Tuc2009 and identification and analysis of genes encoding lysin, a putative holin, and two structural proteins. *Appl Environ Microbiol* **60**: 1875-1883.

Barenboim, M., Chang, C.Y., dib Hajj, F., and Young, R. (1999) Characterization of the dual start motif of a class II holin gene. *Mol Microbiol* **32**: 715-727.

Bernhardt, G., T., Wang, I-N., Struck, K., D., and Young, R. (2001) A protein antibiotic in the phage Qb virion: Diversity in the lysis targets. *Science* 292: 2326-2329.

Berrier, C., Garrigues, A., Richarme, G., and Ghazi, A. (2000) Elongation factor Tu and Dnak are transferred from the cytoplasm to the periplasm of *Escherichia coli* during osmotic downshock presumably via the Mechanosensitive channel Mscl. *J Bacteriol* **182**: 248-251.

Birge, A.E. (1994) *Bacterial and bacteriophage genetics*: Springer-Verlag.

Bläsi, U., and Young, R. (1996) Two beginnings for a single purpose: The dual-start holins in the regulation of phage lysis. *Mol Microbiol* **21**: 675-682.

Bläsi, U., Chang, C.Y., Zagotta, M.T., Nam, K.B., and Young, R. (1990) The lethal λ S gene encodes its own inhibitor. *EMBO J* **9**: 981-989.

Bläsi, U., Fraisl, P., Chang, C.Y., Zhang, N., and Young, R. (1999) The C-terminal sequence of the λ holin constitutes a cytoplasmic regulatory domain. *J Bacteriol* **181**: 2922-2929.

Bläsi, U., Nam, K., Hartz, D., Gold, L., and Young, R. (1989) Dual translational initiation sites control function of the λ *S* gene. *EMBO J* **8**: 3501-3510.

Blount, P., Sukharev, S.I., Moe, P.C., Martinac, B., and Kung, C. (1999) Mechanosensitive channels of bacteria. *Methods Enzymol* **294**: 458-482.

Blount, P., Sukharev, S.I., Moe, P.C., Schroeder, M.J., Guy, H.R., and Kung, C. (1996) Membrane topology and multimeric structure of a Mechanosensitive channel protein of *Escherichia coli*. *EMBO J* **15**: 4798-4805.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heyneker, H.L., and Boyer, H.W. (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**: 95-113.

Bon, J., Mani, N., and Jayaswal, R.K. (1997) Molecular analysis of lytic genes of bacteriophage 80 α of *Staphylococcus aureus*. *Can J Microbiol* **43**: 612-616.

Campbell, J.H., and Rolfe, B.G. (1975) Evidence for a dual control of the initiation of host-cell lysis caused by phage Lambda. *Mol Gen Genet* **139**: 1-8.

Cao, G., Kuhn, A., and Dalbey, R.E. (1995) The translocation of negatively charged residues across the membrane is driven by the electrochemical potential: Evidence for an electrophoresis-like membrane transfer mechanism. *EMBO J* **14**: 866-875.

Chang, C.Y., Nam, K., and Young, R. (1995) S gene expression and the timing of lysis by bacteriophage Lambda. *J Bacteriol* **177**: 3283-3294.

Chang, G., Spencer, R.H., Lee, A.T., Barclay, M.T., and Rees, D.C. (1998) Structure of the MscI homolog from *Mycobacterium tuberculosis*: A gated mechanosensitive ion channel. *Science* **282**: 2220-2226.

Chauthaiwale, V.M., Therwath, A., and Deshpande, V.V. (1992) Bacteriophage Lambda as a cloning vector. *Microbiol Rev* **56**: 577-591.

Damman, C.J., Eggers, C.H., Samuels, D.S., and Oliver, D.B. (2000) Characterization of *Borrelia burgdorferi* Blya and Blyb proteins: A prophage-encoded holin-like system. *J Bacteriol* **182**: 6791-6797.

Doermann, A.H. (1952) The intracellular growth of bacteriophages. I. Liberation of intracellular bacteriophage T4 by premature lysis with another phage or with cyanide. *J. Gen. Physiol.* **35**: 645-656.

Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**: 6127-6145.

Ellis, E.L., and Delbrück, M. (1939) The growth of bacteriophage. *J. Gen. Phisiology* **23**: 365-384.

Gasson, M.J., and Anderson, P.H. (1985) High copy number plasmid vectors for use in *Lactic streptococci*. *FEMS Microbiol Lett* **30**: 193-196.

Graschopf, A., and Bläsi, U. (1999) Molecular function of the dual-start motif in the λ S holin. *Mol Microbiol* **33**: 569-582.

Gründling, A., Bläsi, U., and Young, R. (2000a) Genetic and biochemical analysis of dimer and oligomer interactions of the λ S holin. *J Bacteriol* **182**: 6082-6090.

Gründling, A., Bläsi, U., and Young, R. (2000b) Biochemical and genetic evidence for three transmembrane domains in the class I holin, λ S. *J Biol Chem* **275**: 769-776.

Gründling, A., Manson, M.D., and Young, R. (2001) Holins kill without warning. *Proc Natl Acad Sci U S A* **98**: 9348-9352.

Gründling, A., Smith, D.L., Bläsi, U., and Young, R.Y. (2000c) Dimerization between the holin and holin inhibitor of phage λ. *J Bacteriol* **182**: 6075-6081.

Gutierrez, C., and Devedjian, J.C. (1989) A plasmid facilitating *in vitro* construction of *pho*A gene fusions in *Escherichia coli*. *Nucleic Acids Res* **17**: 3999.

Hartz, D., McPheeters, D.S., Traut, R., and Gold, L. (1988) Extension inhibition analysis of translation initiation complexes. *Methods Enzymol* **164**: 419-425.

Henrich, B., Binishofer, B., and Bläsi, U. (1995) Primary structure and functional analysis of the lysis genes of *Lactobacillus gasseri* bacteriophage ϕ adh. *J Bacteriol* **177**: 723-732.

Johnson-Boaz, R., Chang, C.Y., and Young, R. (1994) A dominant mutation in the bacteriophage lambda *S* gene causes premature lysis and an absolute defective plating phenotype. *Mol Microbiol* **13**: 495-504.

Kashige, N., Nakashima, Y., Miake, F., and Watanabe, K. (2000) Cloning, sequence analysis, and expression of *Lactobacillus casei* phage Pl-1 lysis genes. *Arch Virol* **145**: 1521-1534.

Kiefer, D., and Kuhn, A. (1999) Hydrophobic forces drive spontaneous membrane insertion of the bacteriophage Pf3 coat protein without topological control. *EMBO J* **18**: 6299-6306.

Kiefer, D., Hu, X., Dalbey, R., and Kuhn, A. (1997) Negatively charged amino acid residues play an active role in orienting the Sec-independent Pf3 coat protein in the *Escherichia coli* inner membrane. *EMBO J* **16**: 2197-2204.

Krogh, S., Jorgensen, S.T., and Devine, K.M. (1998) Lysis genes of the *Bacillus subtilis* defective prophage PBSX. *J Bacteriol* **180**: 2110-2117.

Kyhse-Andersen, J. (1984) Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem Biophs Methods* **10:** 203-209.

Lehninger, L. A. (1982) Principles of Biochemistry. New York: Worth Publishers, Inc

Lemmon, A., Mark, MacKenzie, R., Kevin, Arkin, T., Isiah, and Engelman M., D. (1997) Transmembrane α- helix interactions in folding and oligomerization of integral membrane proteins. In *Membrane protein assembly*. Heijne, G.v. (ed): R. G. Landes Company.

Lewin, B. (1997) *Gene VI.* New York: Oxford University Press.

Loessner, M.J. (1991) Improved procedure for bacteriophage typing of *Listeria* strains and evaluation of new phages. *Appl Environ Microbiol* **57**: 882-884.

Loessner, M.J., and Busse, M. (1990) Bacteriophage typing of *Listeria* species. *Appl Environ Microbiol* **56**: 1912-1918.

Loessner, M.J., Wendlinger, G., and Scherer, S. (1995) Heterogeneous endolysins in *Listeria monocytogenes* bacteriophages: A new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. *Mol Microbiol* **16**: 1231-1241.

Loessner, M.J., Schneider, A., and Scherer, S. (1996) Modified *Listeria* bacteriophage lysin genes (ply) allow efficient overexpression and one-step purification of biochemically active fusion proteins. *Appl Environ Microbiol* **62**: 3057-3060.

Loessner, M.J., Gaeng, S., and Scherer, S. (1999) Evidence for a holin-like protein gene fully embedded out of frame in the endolysin gene of *Staphylococcus aureus* bacteriophage 187. *J Bacteriol* **181**: 4452-4460.

Loessner, M.J., Maier, S.K., Daubek-Puza, H., Wendlinger, G., and Scherer, S. (1997) Three *Bacillus cereus* bacteriophage endolysins are unrelated but reveal high homology to cell wall hydrolases from different bacilli. *J Bacteriol* **179**: 2845-2851.

Loessner, M.J., Gaeng, S., Wendlinger, G., Maier, S.K., and Scherer, S. (1998) The two-component lysis system of *Staphylococcus aureus* bacteriophage Twort: A

large TTG-start holin and an associated amidase endolysin. *FEMS Microbiol Lett* **162**: 265-274.

Lu, M.J., and Henning, U. (1992) Lysis protein T of bacteriophage T4. *Mol Gen Genet* **235**: 253-258.

Manoil, C., and Beckwith, J. (1985) Tn*pho*A: A transposon probe for protein export signals. *Proc Natl Acad Sci U S A* **82**: 8129-8133.

Martín, C. A., López, R., and García, P. (1998) Functional analysis of two-gene lysis system of the Pneumococcal phage Cp-1 in homologous and heterologous host cells. *J Bacteriol* **180:** 210-217.

Muniesa, M., Recktenwald, J., Bielaszewska, M., Karch, H., and Schmidt, H. (2000) Characterization of a shiga toxin 2e-converting bacteriophage from an *Escherichia coli* strain of human origin. *Infect Immun* **68**: 4850-4855.

Nam, K., Bläsi, U., Zagotta, M.T., and Young, R. (1990) Conservation of a dual-start motif in P22 lysis gene regulation. *J Bacteriol* **172**: 204-211.

Neely, M.N., and Friedman, D.I. (1998) Functional and genetic analysis of regulatory regions of coliphage H-19B: Location of shiga-like toxin and lysis genes suggest a role for phage functions in toxin release. *Mol Microbiol* **28**: 1255-1267.

Oki, M., Kakikawa, M., Yamada, K., Taketo, A., and Kodaira, K.I. (1996) Cloning, sequence analysis, and expression of the genes encoding lytic functions of bacteriophage ϕ g1e. *Gene* **176**: 215-223.

Parreira, R., Sao-Jose, C., Isidro, A., Domingues, S., Vieira, G., and Santos, M.A. (1999) Gene organization in a central DNA fragment of *Oenococcus oeni* bacteriophage fOg44 encoding lytic, integrative and non-essential functions. *Gene* **226**: 83-93.

Pontarollo, R.A., Rioux, C.R., and Potter, A.A. (1997) Cloning and characterization of bacteriophage-like DNA from *Haemophilus somnus* homologous to phages P2 and Hp1. *J Bacteriol* **179**: 1872-1879.

Raab, R., Neal, G., Sohaskey, C., Smith, J., and Young, R. (1988) Dominance in lambda *S* mutations and evidence for translational control. *J Mol Biol* **199**: 95-105.

Ramanculov, E., and Young, R. (2001a) Functional analysis of the phage T4 holin in a λ context. *Mol Genet Genomics* **265**: 345-353.

Ramanculov, E., and Young, R. (2001b) Genetic analysis of the T4 holin: Timing and topology. *Gene* **265**: 25-36.

Ramanculov, E., and Young, R. (2001c) An ancient player unmasked: T4 *rI* encodes a T-specific antiholin. *Mol Microbiol* **41**: 575-583.

Regamey, A., and Karamata, D. (1998) The N-acetylmuramoyl-l-alanine amidase encoded by the *Bacillus subtilis* 168 prophage SPß. *Microbiology* **144**: 885-893.

Rietsch, A., Fraisl, P., Graschopf, A., and Bläsi, U. (1997) The hydrophilic C-terminal part of the lambda S holin is non-essential for intermolecular interactions. *FEMS Microbiol Lett* **153**: 393-398.

Sambrook, J., Maniatis, T., and Frisch, E.F. (1989) *Molecular cloning: A laboratory manual.* Cold Spring Harbour, NY: Cold Spring Harbor Laboratory Press.

Sao-Jose, C., Parreira, R., Vieira, G., and Santos, M.A. (2000) The N-terminal region of the *Oenococcus oeni* bacteriophage fOg44 lysin behaves as a bona fide signal peptide in *Escherichia coli* and as a cis-inhibitory element, preventing lytic activity on Oenococcal cells. *J Bacteriol* **182**: 5823-5831.

Schägger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range from 1 to 100 kda. *Analytical Biochemistry* **166**: 368-379.

Schmidt, C., Velleman, M., and Arber, W. (1996) Three functions of bacteriophage P1 involved in cell lysis. *J Bacteriol* **178**: 1099-1104.

Sonnhammer, E.L., von Heijne, G., and Krogh, A. (1998) A Hidden markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol* **6**: 175-182.

Steiner, M., and Bläsi, U. (1993) Charged amino-terminal amino acids affect the lethal capacity of Lambda lysis proteins S107 and S105. *Mol Microbiol* **8**: 525-533.

Tedin, K., Resch, A., Steiner, M., and Bläsi, U. (1995) Dual translational start motif evolutionarily conserved in the holin gene of *Bacillus subtilis* phage ϕ 29. *Virology* **206**: 479-484.

van Sinderen, D., Karsens, H., Kok, J., Terpstra, P., Ruiters, M.H., Venema, G., and Nauta, A. (1996) Sequence analysis and molecular characterization of the temperate Lactococcal bacteriophage r1t. *Mol Microbiol* **19**: 1343-1355.

Vukov, N., Scherer, S., Hibbert, E., and Loessner, M.J. (2000) Functional analysis of heterologous holin proteins in a $\lambda\Delta$S genetic background. *FEMS Microbiol Lett* **184**: 179-186.

Wang, I., Dykhuizen, E.D., and Slobodkin, B.L. (1996) The evolution of phage lysis timing. *Evolutionary ecology* **10**: 545-558.

Wang, I.N., Smith, D.L., and Young, R. (2000) Holins: The protein clocks of bacteriophage infections. *Annu Rev Microbiol* **54**: 799-825.

Young, I., Wang, I., and Roof, W.D. (2000) Phages will out: Strategies of host cell lysis. *Trends Microbiol* **8**: 120-128.

Young, R. (1992) Bacteriophage lysis: Mechanism and regulation. *Microbiol Rev* **56**: 430-481.

Zagotta, M.T., and Wilson, D.B. (1990) Oligomerization of the bacteriophage lambda S protein in the inner membrane of *Escherichia coli*. *J Bacteriol* **172**: 912-921.

Zhang, N., and Young, R. (1999) Complementation and characterization of the nested *Rz* and *Rz1* reading frames in the genome of bacteriophage lambda. *Mol Gen Genet* **262**: 659-667.

Zhou, F.X., Cocco, M.J., Russ, W.P., Brunger, A.T., and Engelman, D.M. (2000) Interhelical hydrogen bonding drives strong interactions in membrane proteins. *Nat Struct Biol* **7**: 154-160.

Ziermann, R., Bartlett, B., Calendar, R., and Christie, G.E. (1994) Functions involved in bacteriophage P2-induced host cell lysis and identification of a new tail gene. *J Bacteriol* **176**: 4974-4984.

Zimmer, M., Scherer, S., and Loessner, J. M. (2002) Genome Analysis of *Clostridium perfringens* bacteriophage ϕ3626, which integrates into *guaA* and possibly affects sporulation. *J. Bacteriol* 184: 4359-4368.

Zink, R., Loessner, M.J., and Scherer, S. (1995) Characterization of cryptic prophages (monocins) in *Listeria* and sequence analysis of a holin/endolysin gene. *Microbiology* **141**: 2577-2584.

Appendices

Appendix A *Table of the degenerated genetic code* (Lewin, 1997)

First base ↓ **Second base** →

	U		C		A		G	
U	UUU		UCU	**Ser**	UAU		UGU	
	UUC	**Phe**	UCC		UAC	**Tyr**	UGC	**Cys**
	UUA		UCA		UAA		UGA	*Stop*
	UUG	**Leu**	UCG		UAG	*Stop*	UGG	**Trp**
C	CUU		CCU		CAU		CGU	
	CUC	**Leu**	CCC	**Pro**	CAC	**His**	CGC	**Arg**
	CUA		CCA		CAA		CGA	
	CUG		CCG		CAG	**Gln**	CGG	
A	AUU		ACU		AAU		AGU	
	AUC	**Ile**	ACC	**Thr**	AAC	**Asn**	AGC	**Ser**
	AUA		ACA		AAA		AGA	
	AUG	*Met (Start)*	ACG		AAG	**Lys**	AGG	**Arg**
G	GUU		GCU		GAU		GGU	
	GUC	**Val**	GCC	**Ala**	GAC	**Asp**	GGC	**Gly**
	GUA		GCA		GAA		GGA	
	GUG		GCG		GAG	**Glu**	GGG	

Appendix B Amino Acid Shorthand (Lehninger, 1982)

Amino acid	Three - letter abbreviation	One - letter symbol
Alanine	Ala	**A**
Arginine	Arg	**R**
Asparagine	Asn	**N**
Aspartic acid	Asp	**D**
Cysteine	Cys	**C**
Glutamine	Gln	**Q**
Glutamic acid	Glu	**E**
Glycine	Gly	**G**
Histidine	His	**H**
Isoleucine	Ile	**I**
Leucine	Leu	**L**
Lysine	Lys	**K**
Methionine	Met	**M**
Phenylalanine	Phe	**F**
Proline	Pro	**P**
Serine	Ser	**S**
Threonine	Thr	**T**
Tryptophan	Trp	**W**
Tyrosine	Tyr	**Y**
Valine	Val	**V**

Danksagung/Acknowledgements

Die vorliegende Arbeit wurde unter der Leitung von Prof. Dr. Siegfried Scherer am Institut für Mikrobiologie, FML Weihenstephan der Technischen Universität München in Freising durchgeführt.

Ich möchte mich bei Prof. Dr. Siegfried Scherer für die Möglichkeit am Institut für Mikrobiologie zu promovieren, für dieses wunderschöne Thema, das ich in meiner Dissertation bearbeiten konnte, seine jahrelange Unterstützung und Vertrauen herzlich bedanken.

PD Dr. Martin Loessner möchte ich danken das er mich in seine Arbeitsgruppe aufgenommen hat, für die Betreuung der Arbeit in allen Phasen, und Unterstützung. Ebenso bedanke ich mich für die Korrektur dieser Arbeit und der wissenschaftlichen Veröffentlichungen.

Prof. Dr. Udo Bläsi, PD Dr. Martin Loessner und Prof. Dr. Siegfried Scherer für die Möglichkeit einige Zeit im Institut für Mikrobiologie und Genetik des Biozentrum´s der Universität Wien verbrinngen zu können. Prof. Dr. Udo Bläsi danke ich für die Anregung zu Analysen der Translationsinitiationspostionen (Toeprints) an den *Listeria* Holin Genen und hilfreiche Disskusionen und Dr. Isabella Moll für die experimentelle Durchführung der Toeprints die in dieser Arbeit vorgestellt sind und für ihr Interesse und Zusammenarbeit an diesem Thema.

Prof. Dr. Erhard Bremer und Dr. Claude Gutierrez bedanke ich mich für die Plasmide und Stämme für die Protein-Fusion Analysen mit der Alkalischen Phosphatase (PhoA).

Prof. Dr. Dieter Langosch möchte ich für die Übernahme des Korreferates danken.

Allen Mitarbeitern des Instituts für Mikrobiologie am FML, Weihenstephan danke ich für eine freundliche und herzliche Atmosphäre und große Hilfsbereitschaft während meiner Arbeit. Besonders möchte ich mich bei Markus Zimmer, Dr. Susanne Gäng und Patrick Schiwek bedanken für die Zusammenarbeit im Labor und ihre Hilfe.

Bibliography

1. **Nataša Vukov and Branka Vasiljevic**. (1998) Analysis of plasmid pMZ1 from *Micromonospora zionenzis. FEMS Microbiol Lett* 162: 317-23.

2. **Nataša Vukov, Siegfried Scherer, Edward Hibbert and Martin J. Loessner**. (2000) Functional analysis of heterologous holin proteins in a λΔS genetic background. *FEMS Microbiol Lett* 184: 179-186.

3. **Markus Zimmer, Nataša Vukov, Siegfried Scherer and Martin J. Loessner**. (2002) The murein hydrolase of the bacteriophage ϕ3626 dual lysis system is active against all *Clostridium perfringens* cells (submitted).

4. **Nataša Vukov, Isabella Moll, Udo Bläsi, Siegfried Scherer, and Martin J. Loessner.** (2002) Functional regulation of the *Listeria monocytogenes* bacteriophage A118 holin by an intragenic inhibitor lacking the first transmembrane domain (submitted).

5. **Nataša Vukov, Isabella Moll, Siegfried Scherer, and Martin J. Loessner**. (2002) Comparision of three ralated *Listeria* bacteriophage holins: minor changes cause major differences (in preparation).

Curriculum vitae

Name	Nataša Vukov
Date of birth	25. 03. 1967 in Senta, Serbia, Yugoslavia

Education:

1985	Final exam in Chemistry, Secondary School with Nature-Mathematical education, Senta, Serbia, Yugoslavia
1985-1992	Graduate studies in Molecular Biology and Physiology, Faculty of Biology, University of Beograd, Yugoslavia
1992	Diploma Thesis in Molecular Biology and Physiology: *Analysis of plasmid pMZ1 from Micromonospoa zionenzis*
1992-1997	Postgraduate studies in Molecular Genetics and Genetic engineering
1997	Master Thesis in Biology: *Analysis of plasmid pMZ1 from Micromonospoa zionenzis*
1997-2002	Ph. D. student at the Institute for Microbiology, FML, Weihenstephan at the Technical University, München
2002	Ph. D. Thesis: *Functional analysis of heterologous holins in a λΔS genetic background*

Working experiences:

1993-1997	Research assistant at the Institute of Molecular Genetics and Genetic Engineering, Beograd, Serbia, Yugoslavia
1997-2002	Research at the Institute for Microbiology, FML, Weihenstephan at Technische Universität, München, financially supported by a fellowship from the "Vereinigung der Freunde und Förderer des FML Weihenstephan"