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Complete Genome Sequence and Characterization of the Lysis System of the Temperate *Clostridium perfringens* Bacteriophage f3626

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Für meine Frau Josi

"The enemy of my enemy is my friend"

- Arabian Proverb -

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ABBREVIATIONS

× *g*	relative centrifugal force
aa	amino acid(s)
Arg	arginine
bp	base pairs
C-terminal	carboxy-terminal
cfu	colony forming units
Cps	major capsid protein
CsCl	cesium chlorid
DNA	desoxyribonucleic acid
ds	double stranded
EDTA	Ethylenediaminetetraacetic acid
EN	enteritis necroticans
gp	gene product
HMM	Hidden Markov Modell
Ile	Isoleucine
kb	kilobase pairs
MOI	multiplicity of infection
MSD	membrane-spanning domain
N-terminal	amino-terminal
NE	necrotic enteritis
Ni-NTA	nickel-nitrilo-triacetic acid
nt	nucleotides
ORF	open reading frames
pfu	plaque-forming units
SDS	sodium dodecyl sulfate
TM	transmembrane
Tmp	tapemeasure protein
Tsh	major tail protein
Tris	tris(hydroxymethyl)aminomethane

SUMMARY

Two temperate viruses ϕ3626 and ϕ8533 have been isolated from lysogenic *Clostridium perfringens* strains. Phage ϕ3626 was chosen for a detailed analysis, and was inspected by electron microscopy, protein profiling, and host range determination. For the first time, the nucleotide sequence of a bacteriophage infecting a *Clostridium* species was determined. The virus belongs to the Siphoviridae family of the tailed phages, in the order Caudovirales. Its genome consists of a linear dsDNA molecules of 33,507 nucleotides, with invariable 3'-protruding cohesive ends of 9 residues. Fifty open reading frames were identified, which are organized in 3 major life cycle-specific gene clusters. The genes required for lytic development show an opposite orientation and arrangement as compared to the lysogeny control region. A function could be assigned to 19 gene products, based upon bioinformatic analyses, N-terminal amino acid sequencing, or direct experimental evidence. These include DNA packaging proteins, structural components, the dual lysis system, a putative lysogeny switch, and proteins that are involved in replication, recombination, and modification of phage DNA. The presence of a putative sigma factor related to sporulation-dependent sigma factors and a putative sporulation-dependent transcriptional regulator suggest a possible interaction of ϕ3626 with sporulation events in *C. perfringens*. It has been found that the ϕ3626 attachment site *attPP'* has been found to be located in a non-coding region immediately downstream of *int*. Integration occurs into the bacterial attachment site *attBB'*, located within the 3'-end of a *guaA* homologue. This essential housekeeping gene is functionally independent of the integration status, due to the reconstitution of its terminal codon by the phage sequence.

C. perfringens commonly occurs in food and feed, can produce an enterotoxin frequently implicated in foodborne disease, and has a substantial negative impact on the poultry industry. As a step towards new approaches for its control, the lysis system of *C. perfringens* bacteriophage ϕ3626 has been investigated, whose dual lysis gene cassette consists of a holin and an endolysin. Hol3626 features two membrane spanning domains (MSD) and represents a class

II holin. A positively charged beta-turn between the two MSDs suggests that both the amino- and the carboxy-terminus of Hol3626 might be located outside of the cell membrane, a very unusual holin topology. Holin function was experimentally demonstrated by its ability to complement deletion of the heterologous phage λ holin in λΔSthf. The endolysin gene *ply3626* was cloned in *E. coli*. However, protein synthesis only occurred when the bacteria were supplemented with rare $tRNA^{Arg}$ and $tRNA^{Ile}$ genes. Formation of inclusion bodies could be avoided by drastically lowering the expression level. Amino-terminal modification by a hexa-histidine tag did not affect enzyme activity, and enabled purification by metal-chelate affinity chromatography. Ply3626 features a N-terminal amidase domain and an unique C-terminal portion, which might be responsible for the specific lytic range of the enzyme. All forty-eight tested strains of *C. perfringens* were sensitive to the murein hydrolase, whereas other clostridia and bacteria of other genera were generally not affected. The highly specific activity towards *C. perfringens* might be useful for novel biocontrol measures in food, feed, and complex microbial communities.

ZUSAMMENFASSUNG

Aus lysogenen *C. perfringens* Stämmen konnten zwei temperente Phagen, ϕ3626 and ϕ8533, isoliert werden. Eine detaillierte Analyse einschließlich Elektronenmikroskopie, Bestimmung der elektrophoretischen Mobilität der strukturellen Proteine, sowie eine Bestimmung des Wirts-Spektrum wurde mit dem Phagen ϕ3626 durchgeführt. Erstmalig wurde die vollständige Nukleotid Sequenz eines Bakteriophagen bestimmt, welcher Organismen der Gattung *Clostridium* infizieren kann. Der Phage gehört in die Familie der Siphoviridae, innerhalb der Ordnung der Caudovirales (geschwänzte Phagen). Das Genom ist ein lineares dsDNS Molekül mit einer Größe von 33507 bp, welches einzelsträngige, 3'-überstehende, kohäsive Enden von 9 Nukleotiden besitzt. Fünfzig offene Leseraster (ORFs) konnten im ϕ3626 Genom identifiziert werden, deren Anordnung dieses in drei wesentliche Lebenszyklus-spezifische Genbereiche aufteilt. Der Genbereich verantwortlich für die Aufrechterhaltung des lysogenen Lebenszyklus ist im Vergleich zu den übrigen Genbereichen ("frühe" und "späte" Gene des lytischen Zyklus) gegenläufig organisiert. Aufgrund bioinformatischer Analysen, N-terminaler Aminosäure Sequenzierung und funktioneller Charakterisierungen konnten über 19 ORFs funktionelle Aussagen gemacht werden. So konnten folgende Proteine identifiziert werden: Proteine verantwortlich für die DNA Verpackung, strukturelle Proteine, die Proteine des Dualen-Lyse Systems, ein potentieller Lysogenie-Schalter und Proteine, die in der Replikation, Rekombination und Modifikation der Phagen DNA involviert sind. Die Gegenwart eines möglichen Sigma-Faktors, der Ähnlichkeiten aufweist zu Sporulations-abhängigen Sigma-Faktoren, und die eines weiteren Sporulations-abhängigen Transkriptionsfaktors weisen darauf hin, dass ϕ3626 möglicherweise einen Einfluss auf Sporulations-spezifische Prozesse in *C. perfringens* hat. Die Integrationsstelle *attPP'* ist in einem nicht-kodierenden Bereich vom ϕ3626 Genom lokalisiert, direkt in Leserichtung und unterhalb von *int*. Eine Integration findet an der bakteriellen *attBB'* Stelle statt, welche im 3' Ende eines *guaA*-ähnlichen Gens zu finden ist. Dieses essentielle „Haushaltungs"-Gen ist in seiner Funktion unabhängig vom Lysogeniestatus, da durch eine Integration das ursprüngliche Stopp-Codon an gleicher Stelle vom Phagen bereitgestellt wird.

C. perfringens kann häufig aus Futter- und Lebensmitteln isoliert werden und einige Stämme sind in der Lage ein Enterotoxin produzieren, welches ursächlich

ist für die vom Mikroorganismus hervorgerufenen Fälle von Lebensmittelvergiftungen. Das Bakterium wird aber auch mit Erkrankungen in Verbindung gebracht, welche zu beachtlichen wirtschaftlichen Einbussen in der Geflügel-Industrie führen. Daher wurde für die Kontrolle dieses Bakteriums das lytische System des temperenten Bakteriophagen ϕ3626 untersucht. Das zunächst durch Bioinformatik identifizierte Lyse Module codiert ein Holin und ein Endolysin. Hol3626 besitzt zwei transmembrane Bereiche (MSD) und wird folglich den Klasse II Holinen zugeordnet. Jedoch weist der positiv geladene Bereich zwischen den beiden MSD darauf hin, das sowohl das Amino- als auch das Carboxy-Ende des Hol3626 möglicherweise außerhalb der Zellmembran lokalisiert sind, was eine sehr außergewöhnlichen Topologie darstellen würde. Die Funktion des Holins konnte experimentell gezeigt werden durch seine Fähigkeit, das Fehlen des heterologen λ Holins in Testsystem $\lambda\Delta$Sthf zu komplementieren. Das Gen *ply3626,* welches das Endolysin kodiert, wurde zunächst in einen gängigen Stamm von *Escherichia coli* kloniert und exprimiert. Jedoch war eine Protein-Synthese nur möglich, nachdem das Bakterium mit den im Organismus selten vorkommenden $tRNA^{Arg}$ und $tRNA^{Ile}$ Genen sublementiert wurde. Die hierbei nach Induktion zunächst beobachtete Bildung von Einschlusskörpern konnte durch eine starke Absenkung der Expressionsrate vermieden werden. Eine Amino-terminale Veränderung des Enzymes durch das Hinzufügen eines Hexa-Histidine Markers (HPL3626) hatte keinen negativen Effekt auf die enzymatische Aktivität und erlaubte eine Aufreinigung mittels Nickel-Chelat Affinitätschromatographie. Das Endolysin Ply3626 besteht aus einer N-terminalen Amidase Domäne und einem C-terminalen Bereich, welcher möglicherweise für die spezifische Erkennung der *C. perfringens* Zellwand verantwortlich ist. Lebende Zellen von 48 getesteten *C. perfringens* Stämme waren sensitiv gegenüber der Murein Hydrolase, hingegen zeigten andere Clostridien Arten generell keine Sensitivität, und auch andere Bakterien waren nicht sensitiv. Diese hohe Spezifität von Ply3626 gegenüber *C. perfringens* ist von hohem Wert für nun mögliche Anwendungen, zum Beispiel die Entwicklung neuer biologischer Maßnahmen zur Kontrolle der Entwicklung von *C. perfringens* in Lebensmitteln, Futtermitteln oder auch komplexen mikrobiellen Lebensgemeinschaften.

1 INTRODUCTION

1.1 Clostridium perfringens

Clostridium perfringens is an anaerobic, gram-positive, spore-forming, non-motile rod which can be isolated from the environment, and is frequently found in the intestine of humans and domestic and feral animals (SONGER 1996; SONGER 1997; STEVENS 1997; MCCLANE et al. 2000; MCCLANE 2001). Spores can persist in soil, sediments, and areas subject to human or animal faecal pollution (10^4 -10^6 cfu/g are usually found in human feces) (MCCLANE 2001). *C. perfringens* type A can be isolated from virtually all examined soil samples (10^3-10^4 cfu/g) (LABBÉ 1989). Therefore, it is not surprising that *C. perfringens* can be found on many raw and processed foods. About 50 % of raw or frozen meat and poultry contain *C. perfringens* (LABBÉ 1989), making these meat products common vehicles of *C. perfringens* food poisoning.

Tab. 1 Major lethal toxins and types of *C. perfringens*

	Toxins produced			
Type	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

+ : present; - : not produced

The species is divided into five types, A to E, based on the production of four major toxins (Tab. 1) (SMITH 1991). The **α-toxin** is a phospholipase C (PLC) and is produced by all types of *C. perfringens*. α-Toxin is hemolytic, destroys platelets and leukocytes, and increases capillary permeability, effects which are likely to be related to its ability to cleave sphingomyelin and the phosphoglycerides of cholin, ethonolamin and serine present in eukaryotic cell membranes (BRYANT and

STEVENS 1997). The b**-toxin** is produced by *C. perfringens* types B and C. It is recognized as the major virulence determinant in enterotoxaemia and necrotic enteritis in various animals species. The β-toxin is a very potent toxin. The LD_{50} of purified β-toxin in mice has been reported to be between 0.31 and 12.5 µg kg^{-1} by the intravenous route and between 4.5 and 93.5 µg kg^{-1} when administered intraperitoneally. Purified β-toxin has shown to cause hemorrhagic necrosis and severe destruction of intestinal villi and mucosae in guinea-pig intestinal loops (LEARY and TITBALL 1997). The e**-toxin** is the most potent clostridial toxin after botulinum und tetanus neurotoxins. The toxin has an LD_{50} in mice of 78 ng kg^{-1} when administered intravenously. The toxin is produced by *C. perfringens* types B and D. This toxin is the main virulence factor of *C. perfringens* type D, which is responsible for enterotoxemia in sheep, goat, and more rarely in cattle. In experimental animals, epsilon toxin elevates the blood pressure, increases the vascular permeability, and causes edema and congestion in various organs including lungs and kidneys. Necrosis of the kidneys is also observed in lambs that have died from enterotoxemia (PAYNE and OYSTON 1997). The i**-toxin** from *C. perfringens* type E is a binary toxin consisting of two independent proteins, an enzymatic Ia and binding Ib component. Ia catalyses ADP-ribosylation of actin monomers, thus disrupting the actin cytoskeleton (CARMAN et al. 1997).

Besides these major toxins, as many as 17 exotoxins have been described for *C. perfringens*, but their role in pathogeny of the organisms has been shown for only some of them (SONGER 1996).

1.1.1 Genetic organization of virulence factors from *C. perfringens*

Symptoms of a *C. perfringens* infection are caused by extracellular enzymes and toxins produced by the organism (PETIT et al. 1999). The genes for these enzymes can be chromosomal or plasmid-encoded, or, as the enterotoxin *cpe* gene, be located on a transposon (PETIT et al. 1999). It has been reasoned that the similarities of some toxins of *C. perfringens* with toxins found in other organisms is due to horizontal gene transfer based on conjugative plasmids, transposons or bacteriophages (PETIT et al. 1999). In other clostridial species,

toxins are known to be bacteriophage-encoded; prominent examples are the neurotoxins BoNT/C and BoNT/D of *C. botulinum* and the α-toxin from *C. novyi* (HENDERSON et al. 1997; JOHNSON 1997). In contrast to these findings, no toxin gene or other virulence factor has been reported to be encoded by a bacteriophage in *C. perfringens* (ROOD and COLE 1991).

1.1.2 Histotoxic diseases in humans caused by *C. perfringens*

1.1.2.1 Clostridial wound infection

Three types of clostridial wound infections are recognized (STEVENS 1997): **(i)** simple contamination of wounds with the organisms; **(ii)** clostridial (anaerobic) cellulitis, an infection localized in the skin and soft tissue. Some gas is produced by the growth of the clostridia in a wound, but invasion into healthy tissue and bacteremia does not occur; **(iii)** traumatic gas gangrene (clostridial myonecrosis, "Gasbrand"), the most common type of clostridal wound infection, occurs through direct inoculation of a contaminated, ischemic wound. Also, it can occasionally occur after a surgical procedure. Rapid onset of myonecrosis, gas production, and sepsis are the hallmarks of this disease. The toxins produced are responsible for decomposition of muscular tissue. An anaerobic environment is created, which is conducive to further growth of the bacteria. *C. perfringens* is found in 80 % of traumatic gas gangrene cases. The remaining cases are caused by other clostridial species, like *C. septicum, C. novyi, C. histolyticum, C. bifermentas, C. tertium* and *C. fallax* (STEVENS 1997).

Gas gangrene in humans is usually associated with *C. perfringens* type A and is mediated primarily by the α-toxin, and secondarily by the ?-toxin (perfringolysin O, PFO) (ROOD 1998; PETIT et al. 1999).

1.1.2.2 Enteritis Necroticans (EN)

A segmental necrotizing enteritis of the small intestine, also called "Darmbrand", was first described in 1949 in an epidemic in Northern Germany after World War II (KREFT et al. 2000). In the highlands of Papua New Guinea, EN was an endemic disease often called pig-bel, because of the increase of

incidences of EN primarily in children after ritual pork feasts (JOHNSON and GERDING 1997). The disease also occurs sporadically in Africa, South East Asia, and the United States (STEVENS 1997). Predisposing factors are helminthic co-infection (JOHNSON and GERDING 1997) and malnutrition, specifically in patients with diets low in protein and rich in trypsin inhibitor (such as sweet potatoes) (LAWRENCE 1997). This kind of disease is rare in the developed countries, where its occurrence is confined to adults with chronic illnesses like diabetes (PETRILLO et al. 2000; GUI et al. 2002).

The β-toxin produced by *C. perfringens* type C has been considered to be the major virulence factor for the occurrence of this disease in humans (MCCLANE 2001). A formation of holes in lipid bilayers has been shown to be caused by β-toxin and it has been reasoned that the lethal action of the β-toxin is based on the formation of pores in susceptible cells (SHATURSKY et al. 2000).

1.1.3 Food Poisoning

The *C. perfringens* type A-associated diarrhea is the most common disease caused by this organism. The surveillance reports of the Center of Disease Control ranked *C. perfringens* as one of top five common causes of food-borne disease in the US (BEAN et al. 1996; OLSEN et al. 2000).

Highly contaminated food (>10^6-10^7 *C. perfringens* per g food) is the basis of this infection. While sporulating, the ingested clostridia are able to produce an enterotoxin within the intestine (JOHNSON and GERDING 1997; MCCLANE 2001). Most frequently meat products, such as beef, turkey, chicken, or pork are the sources associated with *C. perfringens* food poisoning, but cases connected to seafood, dairy products, fruits and vegetables have also been described (JOHNSON and GERDING 1997). The diarrhea is usually self-limiting, and additional symptoms are abdominal pain, nausea, vomiting and fever.

The enterotoxin (Cpe) is the causative agent responsible for the symptoms of *C. perfringens* food poisoning. Unlike many other enterotoxins, Cpe is not an exotoxin that is produced by the organism and secreted. Its production has been found to be linked to the sporulation of the organism (DUNCAN et al. 1972; LABBÉ

and DUNCAN 1974). It has been shown that only sporulating cells and not vegetative cells are able to produce *cpe* mRNA (CZECZULIN et al. 1996). The enterotoxin is most probably released by the destruction of the mother cell at the final step of sporulation (MCCLANE et al. 2000). In the intestine, it binds to the epithelial cells where it induces morphological damage, which eventually is responsible for intestinal fluid loss, causing the diarrhea (MCCLANE 2001).

The regulation of the expression of the enterotoxin is based on a transcriptional level. Sporulation-dependent promoters upstream of the enterotoxin gene have been identified (ZHAO and MELVILLE 1998). It has been reasoned that, due to similarities of these promoters to the $\sigma^{E/K}$ dependent promoter of *B. subtilis*, the expression of *cpe* might depend on alternative sigma factors that are involved in the late stages of sporulation. This would explain the correlation of sporulation and enterotoxin formation found in the mother cell compartment of *C. perfringens* (ZHAO and MELVILLE 1998).

Sporulation of bacteria has been studied in detail in *Bacillus subtilis* (AGUILAR et al. 2001). The recently completed sequencing of *C. acetobutylicum* allowed the comparison of genes involved in sporulation. Many of the respective genes found in *B. subtilis* are absent in *C. acetobutylicum*, which suggests major differences in the sporulation process between these organisms. However, the repertoire of transcriptional regulators, in particular the sigma factors, are very similar (NÖLLING et al. 2001).

The enterotoxin gene *cpe* seems to have a variable localization; it can be found chromosomal, plasmid-borne, or is located on a transposon (CORNILLOT et al. 1995; PETIT et al. 1999). Interestingly, there seems to be a correlation of the localization of the genes and the diseases caused by CPE. Most food poisoning cases are caused by *C. perfringens* carrying *cpe* on the chromosome, whereas most isolates from non-food-poisoning disease (such as antibiotic-associated diarrhea), and veterinary disease harbor a plasmid-borne *cpe* gene (CORNILLOT et al. 1995; COLLIE et al. 1998; COLLIE and MCCLANE 1998).

1.1.4 Diseases caused by *C. perfringens* in poultry

C. perfringens is also responsible for several, mostly enteric, diseases in diverse animals (SONGER 1996; SONGER 1997). Enterotoxemic diseases associated to type A strains have been reported for lambs, goats, calves, horses, pigs, and dogs (SONGER 1996). Since necrotic enteritis (NE) was first reported in 1961 (PARISH 1961) it has to be regarded as an important, world wide disease of domestic chicken (NAIRN and BAMFORD 1967; BAINS 1968; KÖHLER ET AL. 1974A; KÖHLER ET AL. 1974B). Its occurrence has also been described also for other birds, such as turkeys or wild geese (WOBESER and RAINNIE 1987; GAZDZINSKI and JULIAN 1992). Predisposing factors for NE are intestinal mucosal damage caused by high-fiber litter (AL-SHEIKHLY and TRUSCOTT 1977), or coccidiosis (co-infection with protozoa like *Eimeria* spec.) (AL-SHEIKHLY and AL-SAIEG 1980).

The cause of NE is *C. perfringens* type A and C (FICKEN and WAGES 1997). Besides NE, *C. perfringens* is also able to cause a mild form of necrotic enteritis (KALDHUSDAL and HOFSHAGEN 1992). Another subclinical condition associated with *C. perfringens* infection has been described: hepatitis or cholangiohepatitis which is associated with liver lesions (LOVLAND and KALDHUSDAL 1999; LOVLAND and KALDHUSDAL 2001). Both conditions are thought be responsible for an impaired production performance (LOVLAND and KALDHUSDAL 2001), associated with an increased feed conversion ratio and retarded growth rate.

Antibiotics, to which in some cases development of resistance of *C. perfringens* strains has been described (DEVRIESE et al. 1993), and are commonly prophylactically used in poultry feed for the control of NE. It has been reasoned that use of antibiotics as growth promoters does increase development of cross-resistance of pathogenic bacteria against therapeutically used antibiotics (WITTE 1998; WEGENER et al. 1999). According to the World Health Organization, the use of any antimicrobial agent for growth promotion in animals should be terminated if it is: used in human therapeutics; or known to select for cross-resistance to antimicrobials used in human medicine (WHO 1997).

Therefore, there is a need for the development of alternatives to antibiotics used in feed, in order to control, for example, the development of NE in poultry. Specifically acting murein hydrolases (see 1.3.2) from bacteriophages infecting *C. perfringens* might be such an alternative.

1.2 Bacteriophages

1.2.1 General Introduction

Bacteriophages (or phages) are viruses that are able to infect bacteria and destroy them by lysis, or hydrolysis of the cell. They can be considered as obligate intracellular parasites, due to the fact that they solely rely on the metabolism of their host to proliferate (KLAUS et al. 1992). It has been assumed that per prokaryotic cell a 10-fold number of bacteriophages can be found, making the phages the most abundant "organism" on earth (BRÜSSOW and HENDRIX 2002).

Bacteriophages have been discovered independently by the microbiologists F. Twort (TWORT 1915) and Félix d'Hérelle (D'HÉRELLE 1917). Phages have frequently been used in the study of bacterial genetics and cellular control mechanisms, largely because their bacterial hosts can be easily grown and infected with phage in the laboratory. Phages were also used in an attempt to destroy bacteria causing diseases, but this approach was largely abandoned in the 1940s when antibacterial drugs became available. The possibility of "phage therapy" has recently attracted new interest among medical researchers, owing to the increasing threat posed by drug-resistant bacteria.

The primary criteria for a classification of bacteriophages into families are (i) the type of their genome (whether it is build up of RNA or DNA, and whether the nucleic acid molecule is double-stranded or single-stranded); and (ii), their morphology (ACKERMANN and DUBOW 1987). Of the bacterial viruses 96 % are tailed phages (Caudovirales) (BRÜSSOW and HENDRIX 2002), which consist of a head with an inner core of DNA - the phage genome - and a tail, whose morphology is a criterion for classification into Myoviridae (long contractile tail),

Siphoviridae (long non contractile flexible tail), and Podoviridiae (short non contractile tail).

The host range of bacteriophages is usually very limited, they frequently infect only one or a few related species of bacteria, sometimes they are even strain-specific (ACKERMANN and DUBOW 1987).

1.2.2 Life cycles

In general there are two major types of bacteriophages, virulent and temperate phages. Virulent phages are only able to propagate in a lytic life cycle, whereas temperate phages are additionally able to switch into a lysogenic life cycle.

1.2.2.1 Lytic life cycle of phages

The life cycle can be divided into four main steps: **(i) Absorption:** Phages adsorb on a specific receptor on the cell surface. **(ii) Infecton:** After a local disruption of the cell wall the phage injects its genome into the bacterium. **(iii) Replication and Maturation:** The phage uses the bacterium's chemical energy and biosynthetic machinery to produce viral proteins, as well as more phage nucleic acid. These produced molecules assemble within the bacterial host into new phage particles. **(iv) Release:** The progeny virions are released after maturation either by lysis, extrusion, or budding (ACKERMANN and DUBOW 1987). Lysis can be readily observed in bacterial cultures growing confluently on solid medium, where groups of lysed cells appear as clear areas, or plaques (ACKERMANN and DUBOW 1987).

1.2.2.2 Lysogenic life cycle of temperate phages

In temperate phages, the infected host is not used for the immediate proliferation of the phage, but the phage genome becomes usually integrated as a prophage into the host chromosome. In this state, known as lysogeny, most of the information contained in the viral nucleic acid is not expressed, only the proteins responsible for the maintenance of the lysogeny are produced. In some rather rare cases, the phage genome can become transiently located extrachromosomally as

an episome (pseudo-lysogeny), a status which seems to be dependent on environmental conditions (RIPP and MILLER 1998).

The establishment of the lysogenic life style and the latent ability of the prophage to change into the lytic life cycle resulting in the production of virus particles and lysis of the host is regulated in a complex way, best understood in phage λ. It depends on a concerted action of the repressors (lysogenic switch) expressed from the phage genome (PTASHNE 1992). In a growing culture the induction of the lytic life cycle is usually strong suppressed. Certain environmental conditions, which have a negative effect on the host, (such as stress, nutrient depletion, inappropriate growth temperatures or antimetabolites) might induce the lytic life cycle. This also occurs spontaneously at low rates (10^{-2} - 10^{-5} per bacterium per generation), but can also be induced with physical or chemical DNA damaging agents (e. g. UV light, mitomycin C) (ACKERMANN and DUBOW 1987).

1.2.3 Bacteriophages of *C. perfringens*

Bacteriophages of the genus *C. perfringens* have been described since the early 1940s, and both, temperate and virulent phages have been described in the following years (MAHONY 1979). However, besides the description of a preliminary typing scheme (YAN 1989) and a preliminary mapping of the integration sites of two temperate phages (CANARD and COLE 1990), no work has been performed on bacteriophages of *C. perfringens* for the last 20 years (H. W. ACKERMANN, personal communication). No sequences of bacteriophages infecting *C. perfringens* are available. Up to now not a single genome of phages infecting any species of the genus *Clostridium* was sequenced and analyzed.

In 1959, Smith screened 152 strains of *C. perfringens* for temperate phages and found that 32 were lysogenic. Additionally, the isolation of virulent phages was described by the author (SMITH 1959). The author also investigated the usefulness of these phages to establish a typing scheme, but found that only 61.2 % of the strains were susceptible to lysis (SMITH 1959). A very narrow host range, which seems to be common in clostridial phages (OGATA and HONGO 1979) was an obvious obstacle in the development of phage typing schemes.

The only example of phage conversion reported so far for *C. perfringens* were experiments performed by Stewart and Johnson. The researches tested a lysogenic strain and its cured and its re-lysogenized derivatives on their ability to sporulate. The results indicated that the lysogens produced spores with a higher efficiency and that the spores were more resistant to heat in comparison to the phage-free derivatives (STEWART and JOHNSON 1977).

1.3 Lysis by Bacteriophages

Generally, there are two different ways how bacteriophages can lyse their host cells to release their progeny at the end of their lytic life cycle. For both, the cell wall is the main target, but the disruption of the cell wall is achieved in different ways. The first way is a blockage of the enzymes involved in peptidoglycan synthesis, exploiting the bacteria's need to continuously synthesize these molecules (HATFULL 2001). This was recently demonstrated for phages Qβ and ϕX174 (BERNHARDT et al. 2001a; BERNHARDT et al. 2001b). The second, more common way, is the use of a cell wall hydrolyzing system, which usually consists of a holin and an endolysin (YOUNG 1992; YOUNG et al. 2000).

1.3.1 Holins

Holins are small hydrophobic proteins that are thought to forms stable and non-specific lesions in the cell membrane and thereby admit the endolysin to pass the membrane and access the cell wall target (YOUNG and BLÄSI 1995). The timing of lysis, which is critical for the viral reproduction, is somehow "programmed" into the structure of the holin and thus controls the length of the vegetative cycle (WANG et al. 2000; YOUNG et al. 2000). Holins are one of the most diverse functional groups, represented by more than 100 known or putative holin sequences, which form more than 30 ortholog groups (WANG et al. 2000). Many holins are organized in a dual gene cluster together with the endolysin (WANG et al. 2000). For the Siphoviridae, it appears as if this cluster is most often located at the 3'-end of the late gene region, next to the lysogeny module (LUCCHINI et al. 1999b).

All holins commonly possess at least one transmembrane domain (TM domains, or membrane spanning domain (MSD)) (YOUNG 1992). The number of TM domains is a criterion for the basic classification of the holins into class I and class II holins. Class I holins do have three TM domains and class II holins do have only two TM domains. Some other holins cannot be grouped into theses classes, since they have only one MSD, and likely function in different ways. (WANG et al. 2000).

A recently developed genetic system can be used to assess the functional properties of various holins of all classes in *E. coli* λΔSthf::*hol* lysogens, where a chromosomally deleted S holin is replaced with a desired holin. Thermal induction of a lysogen allows an observation of a synchronized lytic cycle, and permits reasonably precise assessment of the course and timing of lysis (VUKOV et al. 2000).

1.3.2 Endolysins

Endolysins are mureolytic enzymes. They access the cell wall after the formation of lesions by holins, and hydrolyze the peptidoglycan (YOUNG 1992). With respect to their substrate specificities, they can be grouped into several classes: amidases, glycosidases, muramidases and endopeptidases (YOUNG 1992). A novel activity, L-alanoyl-D-glutamate peptidases, has been described for *Listeria* phages (LOESSNER et al. 1995b).

For endolysins, an astonishing substrate specific lysis actitvity has been described, which is thought to be based upon a modular structure found in many phages lysins from different bacteria (LOESSNER et al. 1995b; LOESSNER et al. 1997; LOEFFLER et al. 2001; NELSON et al. 2001). The enzymatically active polypeptide domain is linked to a corresponding cell wall binding domain, which targets the protein to its site of action (Garcia et al. 1990; Loessner et al. 2002). Consequently, the recognition specificity of the hydrolases is largely conferred by these cell wall binding domains (Baba and Schneewind 1996; Loessner et al. 2002).

Besides the described lysis from within, endolysins are also able to quickly lyse gram-positive phage host cells when applied exogenously (LOESSNER et al. 1995a). Recently, streptococcal phage lysins have shown their potential use in control of pathogenic bacteria *in situ* (LOEFFLER et al. 2001; NELSON et al. 2001). Here, mice were treated nasally and pharyngeally with endolysin obtained from streptococcal phages, which led to a reduction, or even eradication, of previously inoculated streptococci (LOEFFLER et al. 2001; NELSON et al. 2001). In another approach *Lactococcus lactis* has been used to produce and secret a *Listeria* phage amidase (Ply511), which remained fully active and killed *L. monocytogenes* cells in the medium (GAENG et al. 2000).

1.4 Aims of this work

One major aim was to collect essential information on phages infecting *C. perfringens*, with respect to basic morphological characteristics, nucleotide sequence, and potential effect on lysogenized host cells. For this purpose strains of *C. perfringens* were screened for lysogeny and bacteriophages could be obtained from these strains. Further steps were the complete nucleotide sequencing of a bacteriophage genome and its detailed analysis by bioinformatics and experimental studies.

In the prospect of a potential application the lysis system of the bacteriophage was studied in detail. The respective genes were identified and cloned, and the protein tested for activity and its substrate specificity. Here, the goal was to evaluate the potential use of a recombinant endolysin as a novel antimicrobial substance to control *C. perfringens*.

2 MATERIALS AND METHODS

2.1 Organisms, bacteriophages and plasmids

All bacterial strains, phages, and plasmids used are listed in Table 2. Fifty-one *Clostridium perfringens* strains have been obtained from various sources (see Tab. 2). *Escherichia coli* strain DH5αMCR (Invitrogen) in combination with plasmid pBluescript II SK- (Stratagene) was used for cloning. The endolysin gene *ply3626* was cloned into pQE-30 and pSP72, and overexpressed in *E. coli* strains JM109 or JM109 (DE3), respectively. *E. coli* LE392 was used for plating of λ phage and for the generation of lysogens

Tab. 2 Bacterial strains, bacteriophages, and plasmids used

Strain/phage/plasmid	Genotype or relevant properties	Source/reference
Bacteria		
E. coli DH5αMCR	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *supE*44 λ⁻ *thi*-1 *gyrA*96 *relA*1	Invitrogen
E. coli JM109(DE3)	JM109 with λDE3	Promega
E. coli JM109	*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 (r_K- m_K+) *sup*E44 *rel*A1 Δ(*lac-pro*AB) [F' *tra*D36 *pro*AB *lac*I^{q}ZΔM15]	Laboratory stock
E. coli LE392	*hsd*R514(r_K-, m_K+) *sup*E44 *sup*F5*8* *lac*Y1 *gal*K2 *gal*T22 *met*B1 *trp*R55 (phage host; permissive for λgt11)	Promega
C. perfringens WS 2981 to 3002		Twenty-two isolates from various food sources
C. perfringens WS 3006 to 3008		Three isolates from the caecum/ileum of poultry with necrotic enteritis
C. perfringens WS 2955 to 2963		Nine clinical isolates from humans
C. perfringens ATCC 3626, 3628	Toxin type B and C type strains	ATCC, USA
C. perfringens NCTC strains 528, 3110, 6719, 6785, 8081, 8346, 8533, 10240, 10612, 10614, 10719, 11144	Strains with various toxin types: A, B, C, E; NCTC3110: propagation strain for ϕ3626, used as standard in the lysis assay	NCTC, London
C. perfringens DSM strains 756^T, 798, 2943		DSMZ, Heidelberg, Germany

Tab. 2 continued; Bacterial strains, bacteriophages, and plasmids used

Strain/phage/plasmid	Genotype or relevant properties	Source/reference
Phages		
ϕ3626	From *C. perfringens* ATCC3626	This work
ϕ8533	From *C. perfringens* NCTC8533	This work
λΔSthf	λS, single *Eco*RI site, *c*Its857, Amp^r	(Vukov et al. 2000)
λΔSthf::S	native *S* inserted into the *Eco*RI site of λΔSthf	(Vukov et al. 2000)
λΔSthf::*hol3626*	*hol3626* cloned into the *Eco*RI site of λΔSthf	This work
Plasmids		
pBluescript II SK (-)	3.0-kbp cloning vector; Amp^r	Stratagene
pACYC-IRL10	encodes additional copies of ileX, argU and leuW, Cmr	(ZDANOVSKY AND ZDANOVSKAIA 2000)
pQE-30	3.4-kb coning and expression vector; T5 promoter/lac operator element; 5'- 6x His-tag coding sequence; Ampr	Qiagen
pSP72	2.4-kb cloning and expression vector; T7 promoter Ampr	Promega
pHPL3626	Ply3626 cloned into the BamHI and SalI site of pQE-30	This study
pHPL3626ΔC	5' fragment (540 bp) of Ply3626 probably encoding an amidase domain cloned into the BamHI and SalI site of pQE-30	This study
pSPply3626	Ply3626 cloned into the EcoRI site of pSP72	This study

2.2 Media and growth conditions

C. perfringens strains were grown at 37°C in tryptone-yeast extract medium (FACH and GUILLOU 1993) in anaerobic jars (Oxoid) using the Anaerocult A system (Merck). Most of the handling was performed in a flexible vinyl glove-chamber (Coy Laboratories, Michigan), which contained a 95 % N_2 / 5 % H_2 atmosphere. Luria-Bertani (LB) medium was used for the incubation of *E. coli* cells at 37°C. For production of recombinant endolysin, temperature was reduced to 21°C, and to 30°C for propagating lysogens. For the growth of LE392, 0.2 % maltose and 10 mM $MgSO_4$ were added. Plasmid-bearing cells were selected using 100 µg ml^{-1} ampicillin and/or 10 µg ml^{-1} chloramphenicol, when appropriate. Lysogens were selected on media containing 30 µg ml^{-1} ampicillin. Phage stocks were stored at 4°C in SM Buffer.

All media were prepared using demineralized water, all buffers or solutions were prepared using MilliQ water.

TY (per l)

Trypton	30	g
Yeast extract	20	g
HCl cysteine	0.5	g

pH 7.2 ± 0.2

For plates add agarose 1.5 % (w/v) or for top agar 0.4 %.

LB broth (per l)

Trypton	10	g
Yeast extract	5	g
NaCl	8	g

pH 7.4

For plates add agarose 1.5 % (w/v) or for top agar 0.4 %.

SM buffer (per l)

Tris HCl stock solution	50.0	ml
NaCl	5.8	g
$MgSO_4 \times H_2O$	2.0	g

Tris HCl stock solution: 1 M (per l)

Tris	121.1	g

pH 7,5 titrate with HCl

2.3 Isolation of bacteriophages

A total of 51 clostridial strains clostridial strains were screened for lysogeny by UV-irradiation as previously described (LOESSNER and BUSSE 1990). Exponentially growing cells (10 ml) were exposed to UV-light (254 nm, 0.02 J cm^{-2}) for 5 min. After 3 h incubation (37°C) in the dark, cultures were centrifuged (10 min, 8,000 × *g*), and supernatants cleared by filtration (0.2 µm).

Phage activity was tested by the spot-on-the-lawn method, against all available *C. perfringens* strains. 100 µl of exponentially growing *C. perfringens* cultures were spread on a agar plate and allowed to dry for thirty minutes. On the confluently growing bacteria 5-25 µl of phage containing solution were spotted. Lytic activity was observed by the formation of macroplaques after an overnight incubation. To verify whether the macroplaques were formed due to the multiplication of the phages the according solutions were consequently used for the soft agar layer technique (see 2.4).

2.4 Propagation and purification of phages

The soft agar layer technique (ADAMS 1959) was used for the purification and propagation of the phages. Dilutions of the supernatants displaying lytic activity were added to 3.5 ml of molten soft agar inoculated with 0.1 ml log-phase culture of the propagation strain. The mixture was poured on TY plates and incubated overnight. Single plaques were picked and placed into 0.45 ml of TY medium. The phages were allowed to diffuse from the agar into the medium by incubation of 4 h at 4°C. After centrifugation at 8000 $\times g$ for 15 min the supernatant was cleared by sterile filtration (0.22 µm) and used for a several rounds of purification.

Bacteriophage ϕ8533 was propagated by this technique to high titers (10^9 pfu ml^{-1}). This was achieved by using a soft agar layers of confluently lysed plates for the propagation. The complete soft agar layers were transferred into disposable 50 ml centrifuge tubes containing SM buffer. After incubation (4°C, 4 h) the solution was centrifuged (8000 $\times g$; 15 min) and cleared by sterile filtration.

For high titer stocks of ϕ3626 (>10^9 pfu ml^{-1}), liquid cultures were used. Cultures were infected at an OD_{600} of 0.1 using an MOI of 1. Afterwards, growth was monitored photometrically, and following lysis, phages were harvested by centrifugation (10,000 × *g*, 10 min) and sterile-filtration of the culture supernatant.

Purification of viruses from high titer stocks has been described earlier (ZINK and LOESSNER 1992). The phages of the high titer stock solutions were precipitated using the standard protocol for the isolation of lambda bacteriophages

(SAMBROOK et al. 1989) by adding 1 M NaCl and 10 % (w/v) polyethylene glycol 8000. After an incubation for overnight at 4°C, with a slight agitation for the first 10 hours of incubation, the phages were collected by centrifugation (10000 × *g*, 15 min, 4°C) and the pellet was resuspended carefully in 5 ml of SM buffer. These preparations could be used for the electron microscopy (see 2.6).

For further molecular work ϕ3626 was purified using a pre-formed stepped CsCl gradient (densities: 1.7/1.5/1.45/1.4/1.3/1.2 g cm^{-3}) at 85000 × g_{max} for 16 h at 10°C. A blue-greyish band at a buoyant density of approximately 1.45 g cm^{-3} was carefully withdrawn by a sterile syringe (~ 5 ml). The CsCl was removed from the purified bacteriophages by dialysis against SM buffer (2 l) for 2 h and a second dialysis for overnight (4°C) in autoclaved cellulose ester tubings.

2.5 Determination of the lytic range of the isolated bacteriophages

The ability of the two phages to lyse *C. perfringens* strains was tested by the drop-on-the-lawn-technique. Ten µl of the purified phage stocks (10^7 pfu ml^{-1}, see 2.4) were placed on the plates inoculated with *C. perfringens* strains. The lytic activity was observed after overnight incubation.

2.6 Electron microscopy

Phage particles were examined by electron microscopy (EM) as reported before (ZINK and LOESSNER 1992). Briefly, a small drop of the CsCl solution (5 µl) was placed on top of a carbon film fixed on a copper grid (400 mesh) for 1.5 min, to allow phage to attach to the carbon film. Excess solution was removed. The surface of the grid was repeatedly washed with water, and finally negatively stained using 2 % uranyl acetate. Pictures of the virus particles were taken with a transmission electron microscope (Zeiss EM-10A) at an acceleration voltage of 60 kV, with a magnification of 100,000 fold.

2.7 DNA manipulation techniques

2.7.1 DNA isolation from bacteriophages

The DNA of ϕ3626 was obtained using the standard method for the extraction of bacteriophage λ DNA (SAMBROOK et al. 1989) with the exception of doing the extraction with one time phenol, twice with phenol/chloroform/isoamylalcohol (50:48:2, v/v/v) and once with chloroform. No dialysis was performed and the DNA was precipitated with EtOH. Due to a contamination with RNA an additional treatment with RNAse A was necessary and the DNA was finally isopropanol precipitated.

2.7.2 DNA isolation from *C. perfringens*

For the identification of the attachment site it was necessary to isolate and purify the genomic DNA of its lysogenic host *C. perfringens* ATCC3626. This was done according to an alternative protocol for the isolation of DNA from gram positives (FLISS et al. 1991). A 25 ml culture grown overnight at 37°C was harvested by centrifugation at 10000 g_{max} for 10 min. The pellet was washed once with 0.1 × SSC (for 20 × SCC: 3 M NaCl; 0.3 M Na-citrate; pH 7.0) recentrifuged and resuspended in 500 µl TES (30 mM Tris/HCl [pH8]; 50 mM NaCl; 5 mM EDTA [pH8]; 20 % saccharose [w/v]) containing 8.4 µg mutanolysin and 0.6 mg lysozyme to ensure the destruction of the cell wall. After an incubation of 30 min at 37°C 5 µl of RNAse A (~ 80 U) was added. After another incubation for 30 min 4.5 ml of lysis buffer (10 mM Tris/HCl [pH8]; 1 mM EDTA [pH8]; 0.8 % SDS [w/v]) and 300 µg proteinase K was added. The cells lysed within the following incubation at 50°C for 2 h (gently agitated). The DNA was extracted using a phenol, (2 ×) phenol/chloroform/isoamylalcohol (50/48/2, [v/v/v]), chloroform extraction. Finally the DNA was ethanol precipitated.

2.7.3 DNA precipitation by Ethanol / Isopropanol

DNA in a sample was precipitated by the addition of 0.3 M sodium acetate (pH 5.2, 0.1 volume of 3 M) along with 2 to 2.5 volumes of ethanol (-20°C) and incubation for 30 min at 21°C. The sample was then centrifuged at 4°C (maximum

speed) in a microcentrifuge (Eppendorf) and washed once with 70 % ethanol. After a second centrifugation the pellet was left to dry and finally resuspended in MilliQ water or ½ TE. Instead of using 2/2.5 volumes of ethanol 1 volume of isopropanol can also be used for precipitation of DNA.

TE buffer

10 mM	TRIS/HCl
1 mM	EDTA
pH 8.0	

2.7.4 Ligation

Ligations were performed using 1 U T4 DNA ligase (Promega, Madison, USA) and 10x reaction buffer (Promega) for 4 h at 21°C or for 18 h at 4°C.

2.7.5 Electrotransformation

Transformation of *E. coli* was carried out by electroporation according to the protocols by (DOWER ET AL. 1988) using a BioRad Gene Pulser and sterile, individually wrapped 2-mm electroporation cuvettes (EquiBio Ltd., Kent, UK). Selection for resistance to antibiotics in *E. coli* was performed with ampicillin (100 µg ml^{-1}). Colonies harboring recombinant plasmids were selected by blue-white screening according to standard methods (SAMBROOK et al. 1989). Recombinant plasmids were isolated using the Qiaprep Miniprep Kit (Qiagen, Hilden, Germany).

2.7.5.1 Preparation of competent cells

500 ml of LB (*E. coli*) broth were inoculated with 1 ml of an overnight liquid culture of the *E. coli* and incubated at 37°C shaking at 150 rpm. After reaching an optical density of OD_{600} = 0.5-0.6, cells were harvested by centrifugation (8000 × g, 4°C, 10 min) and stored on ice. Cell Pellet was resuspended and washed with: 500 ml 5 % glycerol (4°C), 250 ml 5 % glycerol (4°C), 10 ml 10 % glycerol (4°C). Finally the pellet was resuspended in 1 ml 10% glycerol (4°C) and stored in 40 µl aliquots at -70°C

2.7.5.2 Electroporation

20 µl of the ligation mixture or plasmid was dialyzed for 30 min using dialysis membrane filter (0.025 µm pore size, Millipore) and electoporated into 40 µl of *E. coli* competent cells (see 2.7.5.1) on ice using a Gene-Pulser (BioRad) under the following conditions:

Resistance:	200 Ω
Capacitance:	25 µFD
Volt:	2.5 kV (12.5 kV/cm)
Time constant:	~4.7

Following electroporation, 500 µl LB-medium was added directly to the electroporation cuvettes. For regeneration of the host cells, the mixture was incubated at 37°C for 1 h. 100 µl aliquots (and ten-fold dilutions) were plated on LB plates supplemented with the corresponding antibiotic. Plates were incubated for 18 h at 37°C.

2.7.6 Separation of DNA fragments by agarose gel electrophoresis (AGE)

To prepare a 1.2 % gel, 0.5 g agarose (SeaKem LE, FMC BioProducts) were dissolved in 40 ml 1 x TAE buffer using a microwave. The hot agarose solution was poured into a gel tray with slot-formers. 5 µl of DNA containing samples were mixed with 1 µl 6x gel loading buffer (GLB) Blue/orange 6x loading dye (Promega, Madison, USA) and pipetted into the gel slots. Additionally an appropriate molecular weight marker was loaded (see Tab. 3) After electrophoresis, the gel was stained with ethidiumbromide EtBr (0.5 µg/ml) for 30-45 min and visualized by UV-translumination using the ImageMaster® VDS (Pharmacia, Biotech). Electrophoresis was performed using the following equipment: GNA-100 submarine electrophoresis unit (Pharmacia Biotech); Easy-Cast™ Electrophoresis system, Model #B1A (Owl Scientific, Inc.); Electrophoresis Power Supply EPS 600 (Pharmacia Biotech).

TAE (Tris/acetate/EDTA) electrophoresis buffer 50 x stock solution (1 l):

2 M	Tris-base
1 M	NaAc
50 mM	EDTA
(pH 8.0)	

Gel loading buffer: (GLB 6x)

0.10 %	Xylen-Cyanol FF (XCFF)
0.25 %	Bromophenolblue
0.20 %	SDS
5 mM	EDTA
50% (v/v)	Glycerol
(pH 8.0)	

Tab. 3 Size of the fragments of the used molecular markers

Marker		Fragment size (bp)
DNA Ladder (MBI Fermentas, Vilnius)	100 bp	100, 200, 300, 400, 500, 600, 700, 800, 900, 1031, 1200, 1500, 2000, 3000 bp
DNA Ladder (MBI Fermentas, Vilnius)	1 kb	250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10.000 bp

2.7.7 Preparation of DNA fragments from agarose gels

Gel extraction was performed by using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer:

2.7.8 Polymerase Chain Reaction (PCR)

Oligonucleotides used for PCR and sequencing reactions were obtained from MWG Biotech (Ebersberg, Germany). PCR amplification was performed with a Techne Progene automated thermocycler with 0.2-ml thin-walled PCR tubes (Advanced Biotechnologies). Reactions were carried out in 50-µl volumes containing 5 µl 10x PCR reaction buffer (containing 2.0 mM $MgCl_2$), 100 pmol of each oligonuleotide primer, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP, Eurogentech) and 1 U of *Taq* DNA polymerase (Qiagen). The standard PCR protocol: After an initial denaturation step (94°C, 5 min) amplification was carried out with 25-35 cycles (denaturation at 94°C for 450s, annealing at 50°C for 60s and extension at 72°C for 30s) followed by a final extension step at 72°C for 10 min. The annealing temperature was adjusted to the melting temperature of the primers and the extention time to the size of the expected fragment.

2.7.9 Purification of PCR products

PCR products were purified using spin columns with the QIAquick-spin PCR Purification Kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer.

2.8 Analysis and amino-terminal sequencing of f3626 structural proteins

The isolation and purification of the structural proteins was performed essentially as described earlier (ZINK and LOESSNER 1992; LOESSNER et al. 1994). Virion proteins were separated by Sodium dodecyl sulphate polyacrylamid gel electrophoresis (SDS-PAGE) according to (LAEMMLI 1970) on a discontinous 8-18% gradient gel (Excel Gel, Pharmacia LKB). Equal volumes of the peptide

containing samples and gel loading buffer (2x GLB) were mixed and boiled at 100°C for 10 min. Electrophoresis was performed at 12°C on a horizontal system (2217 Ultrophor Electrofocusing Unit, LKB Broma) at constant voltage (200 V for the first 20 min, 600 V for the remaining 60 min, 50 mA, 35 W) using the 2303 Multidrive XL 3.5 kV power supply (LKB, Broma). As molecular-weight standard SDS-*7L* (Sigma) was used.

Electrophoresis buffer: (10x Tris-Glycine-buffer) (per l)

144.2 g	Glycine
30.3 g	Tris
10.0 g	SDS

SDS-GLB (Gel-loading-buffer) (50 ml)

2,5 ml	Tris-HCl (1.25 M, pH 6,8)
1,0 g	SDS
2,5 ml	2-Mercaptoethanol
5,8 ml	glycerole (87%)
5 mg	bromphenolblue

Gels that were not used for semidry-blotting were dyed for 25-30 min using prewarmed (60°C) coomassie R-350 dye solution. The background dye was removed by washing (2 × 30 min) the gel with prewarmed wash solution and finally the gel was placed for 30 min in a preserve solution. The percentage of the major structural bands were determined by densitometrical scanning.

Coomassie R350 dye solution

coomassie R-350 tablets	4 ×
methanol	25 %
acetic acid	10 %

Wash solution

methanol	25 %
acetic acid	10 %

Preserve solution

methanol	25 %
acetic acid	10 %
glycerol	10 %

For microsequencing proteins were electroblotted on a polyvinylidene difluoride (PVDF) membrane (GelmanSciences) using the 2117-250 Novablot electrophoretic transfer kit (LKB) in a Multiphor II device (Pharmacia) according to (KYHSE-ANDERSEN 1984) at 20°C for 1 h at 0.8 mA cm^{-2}. The blot was build up of the immobilizing membrane and the gel placed between filters soaked with transferbuffers that are in direct contact to the graphite-electrodes, according to the manufacturers manual (Pharmacia).

Anodic buffer I

0.3 M	Tris
20%	methanol

Anodic buffer II

25 mM	Tris
20 %	methanol

Cathodic buffer

40 mM	6-Aminocapronic acid
0.01 %	SDS
20 %	methanol

The membrane was rinsed with methanol once and dyed using 1/2 Coomassie R-350 dye solution Three major bands were excised from the

membrane and the dye was removed by washing the membrane in 45 % methanol and 10 % acetic acid. The first 10 amino acids (aa) each of the individual proteins were determined using an automated sequencer (Applied Biosystems Procise 492-01) by the service department of the Arbeitsgruppe Proteinanalytik (Universität Münster)

2.9 Bioinformatics

The program DNASIS and the Husar Analysis Package (version 4.0; http://genome.dkfz-heidelberg.de) were used for analysis of the nucleotide and amino acid sequences. The BLAST algorithms (ALTSCHUL et al. 1997) were used for similarity searches in the databases available through the NCBI (http://www.ncbi.nlm.nih.gov), or the Husar Analysis Package. COILSCAN (LUPAS et al. 1991) was used to predict probabilities to form a coiled-coil structure. The HMMscan program (Pfam database; release 5; http://pfam.wustl.edu) identifies protein families using the Hidden Markov Model (DURBIN et al. 1998), and TmHMM (v. 2.0) protein analysis uses this model for prediction of transmembrane domains (SONNHAMMER et al. 1998).

2.10 Cloning, nucleotide sequencing, and identification of the *cos*-site

The DNA of ϕ3626 was extracted and purified using standard techniques (see 2.7), and the construction of genomic libraries of ϕ3626 was performed essentially as described previously (LOESSNER et al. 2000). Here, limited digests with *Tsp*509I (New England Biolabs), and complete digests using *Hin*dIII (MBI Fermentas) or *Taq*I (Roche) were performed, fragments of 1-2 kb length ligated into pBluescript, and transformed into *E. coli*. Blue-white screening on Xgal-containing agar plates was used for the identification of insert-bearing clones. Plasmids from small-scale cultures were digested with *Pau*I (MBI), and fifty-eight clones carrying inserts varying in size were identified by agarose gelelectrophoresis. These plasmids were used for sequencing using IRD-800-

labelled primers complementary to sequences flanking the multiple cloning site. Sequencing was performed using a heat-stable polymerase (SequiTherm EXCEL II; Epicentre Technologies) on an automated DNA sequencer (4200 IR^2; LI-OR).

The obtained sequences were edited and aligned using the software DNASIS (version 2.1; Hitachi). Gaps were closed by direct sequencing of ϕ3626 chromosomal DNA, with the aid of specific primers derived from the contigs. Distinct chain termination signals were generated at the ends of the molecule, i.e., the putative single stranded ends (*cos*-sites). The genome sequence was finalized by the determination of the sequence of the *cos*-site overlaps, by PCR amplification of DNA from lysogenic host bacteria (see 2.11), using primers complementary to sequences upstream and downstream of the *cos*-site.

2.11 Identification of *attPP'* and *attBB'*

Purified genomic DNA of ϕ3626 was used as template for the identification of the attachment site using inverse PCR (OCHMAN et al. 1988). *AttPP'* was expected to be located in a non-coding region immediately downstream of *int*. A *Sau*3AI restriction site is present within the *int* gene, and this enzyme (Roche) was used for complete digestion of the bacterial DNA. Fragments were treated with T4 DNA ligase to obtain self-ligated circular molecules. Divergent primers were designed, complementary to an area located within *int*: Att3up (5'-CTCAAATGATAGCAA-CAACAGG-3') and Att3dw (5'-CTTTTACTTTTAGGAGTTTGGG-3'), and used for PCR amplification of ligated fragments. The product were purified and sequenced using the same primers. The obtained sequence contained the *attBP'* site, and the non-prophage part of the sequence displayed 100 % identity over 663 nucleotides to a sequence of the unfinished *C. perfringens* genome available from the Institute for Genome Research (TIGR, http://www.tigr.org). Additional sequence was obtained, in order to design a primer attB1 (5'-GACAATCATATTAAAATGACT-GCC-3') that, in combination with primers complementary to the prophage DNA, att5dw (5'-CTCAAATGATAGCAACAACAGG-3'), produced a fragment containing the *attPB'* site, which was also purified and sequenced.

2.12 Cloning and functional analysis of the holin gene *hol3626*

To study the function of the holin, a specialized vector system (λΔSthf) was used (VUKOV et al. 2000). *Hol3626* was amplified by PCR using primers hol3626-up (5'-ATCAGAATTC**TTA**ATTTTCTTTTTTATTAAATCCTTCTTT-3'), and hol3626-down (5'-ATCAGAATTC**ATG**TTTAAATTTATACCAGAAGTAATAAGT-3') (*Eco*RI sites are underlined, start and stop codons are in boldface [the native TAG stop codon was changed to TAA]). The product was cloned into the *Eco*RI site of λΔSthf, and packaged using a λ DNA packaging extract (Stratagene). A preliminary test for holin function was the ability to form plaques on *E. coli* LE392. Single plaques were isolated, and identity of *hol3626* verified by sequencing using primers as described earlier (VUKOV et al. 2000). Purified λΔSthf*::hol3626* was then used to lysogenize LE392. Growth and lysis kinetics of the lysogenic cells in liquid cultures was monitored by measuring optical density (OD_{600}). Lytic development was induced by a temperature shift from 30°C to 42°C for 20 min, followed by incubation at 37°C for 120 min. Lysogens carrying the wildtype *S* holin gene from λ (λΔSthf::*S*) served as positive control, and lysogens carrying λΔSthf without holin as negative control.

2.13 Cloning of endolysin gene *ply3626*

For cloning of *ply3626* into pSP72, primers PlyEcoStart (5'- ATCAGAATT-C*GAGGAG*AAATTACT**ATG**AAGATAGCAGAAAGAGGCGGTCATAAT-3'), and PlyEcoStop (5'-ATCAGAATTC**TTA**TATTCTTTCTAAATATTTAGCTGTAAA-3') (*Eco*RI sites are underlined, start/stop codons are in boldface, ribosome binding site is in italics) were used for amplification of the endolysin gene, from purified phage DNA. The PCR product was digested with *Eco*RI (Roche), and ligated into the *Eco*RI site of pSP72, which has been prepared by treatment with shrimp alkaline phosphate (U.S. Biochemicals). Ligation reactions were transformed into *E. coli* JM109(DE3). For cloning into pQE-30, primers PlyBamStart (5'-TCTAGGATCC**ATG**AAGATAGCAGAAAGAGGCGGTCATAAT-3'), PlySalStop (5'-

ATAAGTGTCGAC**TTA**TATTCTTTCTAAATATTTAGCTGTAAA-3') were used. A truncated Ply 5'-fragment encoding only the first 180 aa of the endolysin was amplified using PlyBamStart and PlySalMid (5'-AAGTGTCGAC**TTA**TTGTCCCCC-AGCAACTTTACAAACTCC-3'), the latter of which is complementary to bp 513-540 of *ply3626* and equipped with a stop codon. The PCR products were purified, digested with *Bam*HI and *Sal*I (MBI Fermentas), and ligated into pQE-30. Ligation reactions were transformed into *E. coli* JM109(pACYC-IRL10). Correct insertion and sequence of the genes was checked by nucleotide sequencing of the plasmid inserts.

2.14 Overexpression and purification of Ply3626

JM109(DE3)(pPly3626) failed to produce enzymatically active protein upon induction of protein synthesis by addition of IPTG (1 mM). With respect to JM109(pACYC-IRL10)(pHPL3626), IPTG induction at 37°C incubation temperature resulted in the rapid formation of inclusion bodies. Incubation temperature was then reduced to room temperature (21°C), and growth was monitored photometrically (OD_{600}) over a period of 20 hours. At regular intervals, samples of the growing cultures were taken, and protein synthesis documented by SDS-PAGE. At a final OD of approximately 1.0, cells were harvested by centrifugation (8,000 × *g*, 15 min at 4°C), and pellets frozen at -20°C overnight. After thawing, cells were resuspended in 1/50 volume in PBS (pH 8.0) or in Ni-NTA-buffer (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, pH 8.0). Crude extracts were prepared by single passage through a French-Press cell (SLM Aminco) at 100 MPa. Debris was removed by centrifugation (30,000 × *g*, 10°C, 25 min), and supernatants containing the active enzyme were cleared by filtration (0.22 µm pore size), and stored at -20°C.

Purification of the hexa-His-tagged endolysin was carried out using immobilized metal-chelate affinity chromatography (IMAC), as previously described (LOESSNER et al. 1996). Individual fractions were checked by SDS-PAGE and assayed for lytic activity. Protein concentration was determined using

with a modified Bradford Assay (Nanoquant; Roth), and buffer exchange and concentration of the proteins were performed using centrifugal concentrators (Fugisep Maxi, 10 kDa cut-off, Intersep Systems).

2.15 Lytic activity and substrate specificity

A total of 48 *C. perfringens* strains, 30 strains of other *Clostridium* species. (*C. absonum, C. barati, C. beijerinckii, C. bifermentans, C. botulinum, C. butyricum, C. difficile, C. fallax, C. novyi, C. pasteurianum, C. sporogenes, C. tertium, C. tetani, C. tetanomorphum* and *C. tyrobutyricum*), and 34 bacterial strains of other genera (*Bacillus cereus, B. megaterium, B. polymyxa, B. subtilis, B. thuringiensis, B. weihenstephanensis, Bifidobacterium gallicum, Bf. gallinarum, Bf. ruminantium, Brochothrix thermosphacta, Campylobacter jejuni, Enterobacter cloacae, E. gallinarum, Enterococcus faecalis, Ec. faecium, Escherichia coli, Lactobacillus aviarius, Lb. plantarum, Lb. ruminis, Lactococcus lactis, Leuconostoc carnosum, Leu. citreum, Leu. mesenteroides, Listeria innocua, L. ivanovii, L. monocytogenes, Pediococcus pentosaceus, Salmonella enterica, Staphylococcus aureus, S. epidermidis, S. gallinarum, Streptococcus pyogenes* and *Strep. thermophilus*) were selected and tested for sensitivity against the recombinant phage lysin. Cells were resuspended in PBS to an optical density of approximately 1, and directly used as substrate, that were carried out in microplates at 37°C. Cell suspension (180 µl) were mixed with 20 µl (1:10) of crude extract of JM109(pACYC-IRL10)(pHPL3626), or 5 µl purified HPL3626. The decrease in optical density was monitored using a Victor^2 1420 platereader (Perkin-Elmer). As a negative control, cell extract of JM109(pACYC-IRL10) was used. The activity of the C-terminally truncated endolysin was determined with cell extracts from JM109(pACYC-IRL)(pHPL3626ΔC).

2.16 Nucleotide sequence accession numbers

The DNA sequences reported here can be accessed in the EMBL, Genbank, and DDBJ databases under accession numbers AY082069 and AY082070 (Appendix).

3 RESULTS

3.1 Isolation of *C. perfringens* phages

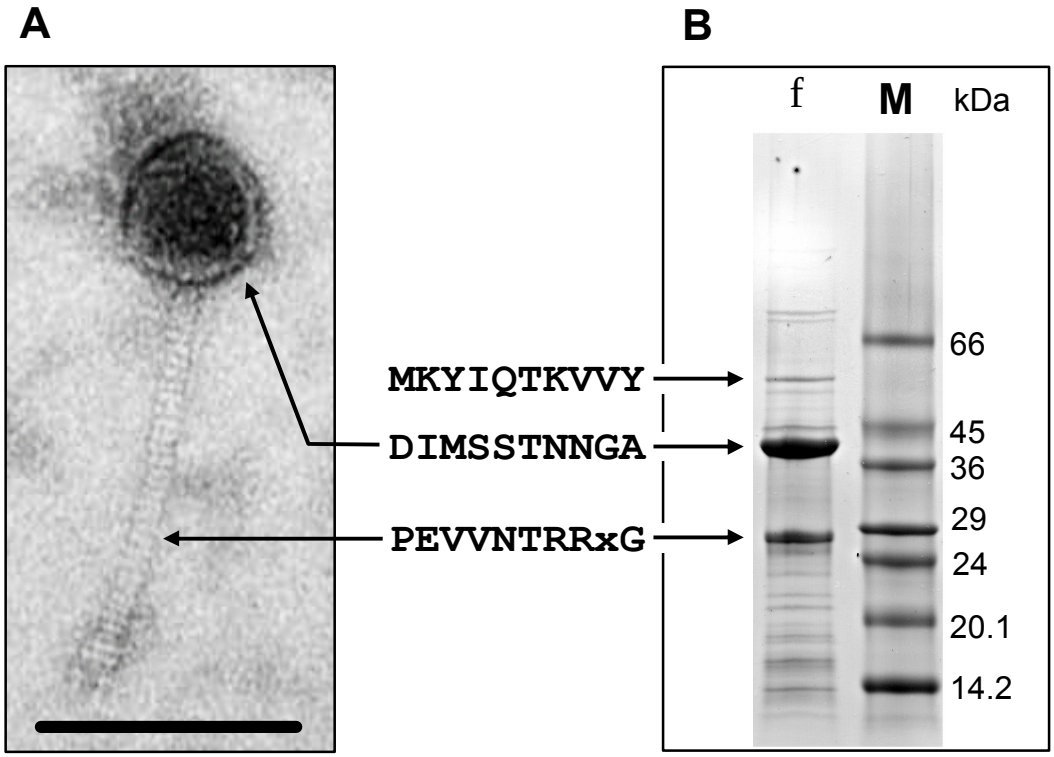
Lytic activity was observed in UV-induced culture supernatant of two of the tested strains (ATCC 3626 and NCTC 8533), which was shown to be due to the presence of bacteriophages. Their lytic ranges on 51 *C. perfringens* strains were different: Phage ϕ3626 was lytic against 11 strains (21.6 %) while phage ϕ8533 was able to lyse only 4 strains (7.8 %), which were also all sensitive to ϕ3626. The optimal propagation hosts were determined to be *C. perfringens* NCTC 3110 for ϕ3626, and ATCC 3628 for ϕ8533.

Since phage ϕ3626 displayed a broader host range and, at least in the framework of this project, was easier to propagate, it was chosen for further studies. Electron microscopy (Fig. 1) revealed that the ϕ3626 virion has an isometric capsid (55 nm diameter ± 2 nm; a total 7 virion particles were measured) and a long, flexible, non-contractile tail (170 nm ± 5 nm length). Therefore, ϕ3626 belongs to the Siphoviridae family of doublestranded DNA bacterial viruses in the order Caudovirales (ACKERMANN 1998).

3.2 Nucleotide sequencing and determination of the *cos*-sites

The genome of ϕ3626 was sequenced by a shotgun approach. Sequencing the various plasmid inserts yielded contigs which allowed the design of primers to close the gaps in-between the contigs. The sequence was finalized by determination of the *cos*-site core sequence from amplified bacterial chromosomal DNA (Fig. 2). Predicted restriction maps of the ϕ3626 DNA were in perfect agreement with the experimental achieved pattern, indicating that the sequences were assembled correctly (results not shown). The complete genome has a size of 33,507 nucleotides with 3'-protruding, singlestranded cohesive ends of 9

nucleotides (Fig. 2). Its average molar G+C content of 28.4 mol% is slightly higher than the 24-27 mol% reported for its host (HIPPE et al. 1991), or for the clostridial plasmid pIP404 (25 mol%).



C

In silico Translated Sequence	ORF	Functional Assignment
1-MKYIQTKVVY	16	Minor Structural Protein
115-DIMSSTNNGA	6	Major Head Protein [Cps]
2-PEVVNTRRCG	11	Major Tail Protein [Tsh]

Fig. 1 Electron micrograph and protein profile of f3626

(A) Electron micrograph of ϕ3626, showing its isometric capsid and flexible tail. Scale marker is 100 nm. **(B)** SDS-PAGE showing the protein profile of ϕ3626 (left lane) and molecular mass marker proteins (right lane). N-terminal amino acid sequences from selected structural proteins are shown between the images. The arrows point to the respective SDS-PAGE bands and individual, corresponding viral components, as deduced by bioinformatic analysis. **(C)** Genetic identity and functional assignments for the three proteins are given. The mature major head protein begins with amino acid D115 of the deduced sequence, indicating proteolytic processing.

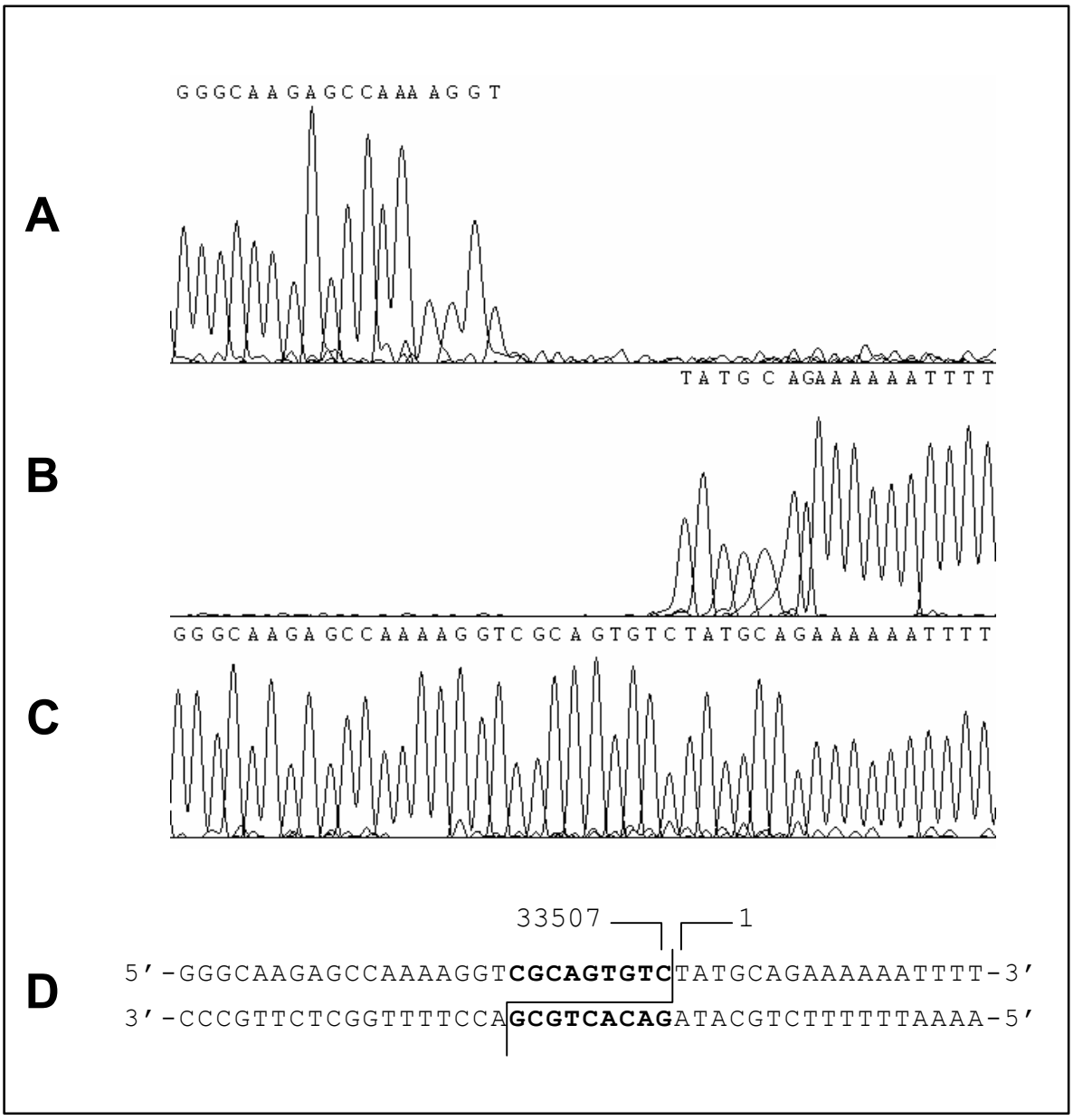


Fig. 2 Identification of single stranded cohesive ends in ϕ3626 DNA.

(A) Chromatogram showing the sequence from the right end of the genome towards the cos-site (coordinates 33481-33498). **(B)** Chromatogram of the sequence from the left end of the non-circular genome towards the cos-site (shown inversed; coordinates 1-17). **(C)** Sequence of a PCR product from ϕ3626 prophage, spanning the entire cos-site. **(D)** Corresponding sequence of the ligated cohesive ends, joining the left and right arm of the DNA molecule. The single stranded 3'-protruding ends are printed in bold letters.

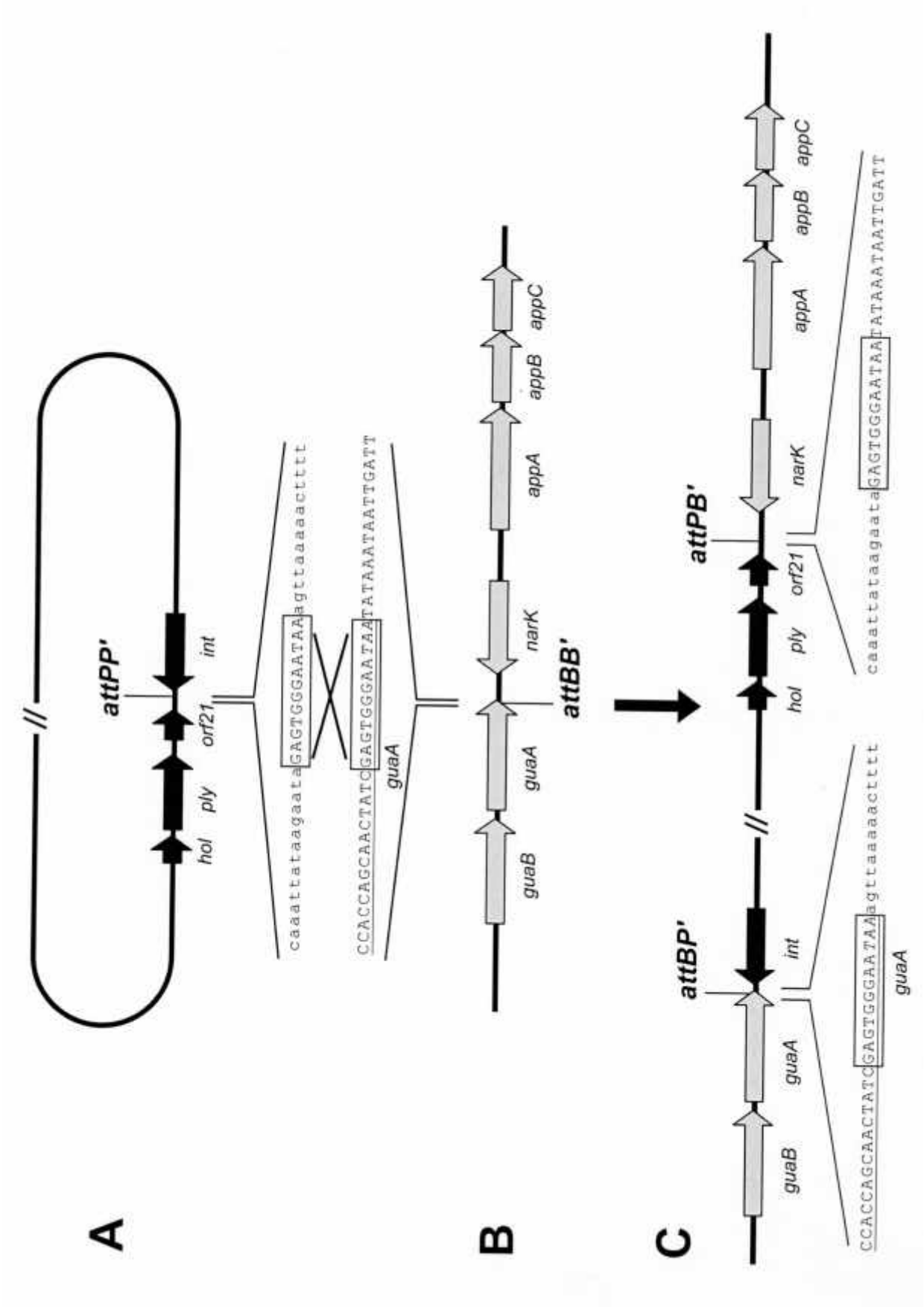
3.3 Identification and nucleotide sequence of the attachment sites *attPP'* and *attBB'*

The integration site of the bacteriophage ϕ3626 was identified using an inverse PCR approach. Sequencing of the first PCR product yielded 663 nt bacterial sequence. This ("left-arm") prophage-host junction was designated *attBP'* (Fig. 3C). The bacterial sequence was found to be 100 % identical to a portion of a contig (10.804 bp) of the unfinished genome of *C. perfringens* (TIGR, http://www.tigr.org). A second PCR product yielded additional sequence information of the host (183 nt), which was also identical over its full length to the *C. perfringens* sequence, and encompassed the *attPB'* site, at the junction of the "right arm" of ϕ3626 and the bacterial chromosome.

Alignment of the two *att* site flanking sequences revealed a core sequence of 12 nt (Fig. 3). On the circularized phage genome, *attPP'* is located in a non-coding region of 273 bp between ORF 21 and *int*. Sequence analysis showed that the *attBP'* is encompassed within the 3'-end of a *C. acetobutylicum guaA* homologue, encoding a guanosine monophosphate (GMP) synthetase (89 % similarity over 220 aa). The core sequence of 12 bp represents the terminal 4 tripletts of *guaA*, including a TAA stop codon (Fig. 3). The region immediately downstream of *attPB'* (189 nt) does not contain coding capacity.

Fig. 3 Organization of phage and bacterial attachment sites (next page)

(A) Schematic representation of the circularized ϕ3626 genome with its *attPP'* site (see Table 2 for identity of ORFs). **(B)** Partial sequence of the *C. perfringens* genome, encompassing attBB' and surrounding genes (see text). **(C)** Integrated prophage status within *attBP'* and *attPB'*. The ORFs flanking *attPP'* and *attBB'* are indicated by arrows (black arrows, phage/prophage ORFs; grey-shaded arrows, *C. perfringens* ORFs). Partial sequences of junction fragments from the phage/prophage are denoted in small letters, host sequence in capital letters, the homologous att site core sequence (12 bp) is boxed. Underlined sequence corresponds to the 3'-end of *guaA*, the stop codon is shown in italic letters. Figure is not to scale.


A
attPP'
hol
ply
orf21
int
caaattataagaaataGAGTGGGAATAAagttaaaaactttt
CCACCAGCAACTATCGAGTGGGAATAATATAAATAATTGATT
guaA
B
guaB
guaA
narK
attBB'
appA
appB
appC
C
attBP'
guaB
guaA
int
attPB'
hol
ply
orf21
narK
appA
appB
appC
caaattataagaaataGAGTGGGAATAATATAAATAATTGATT
CCACCAGCAACTATCGAGTGGGAATAAagttaaaaactttt
guaA

In order to obtain a more detailed picture of the chromosomal localization of *attBB'* and *guaA*, a 10.8 kb sequence contig of the unfinished genome of *C. perfringens* (The Institute for Genomic Research, TIGR) was annotated. Upstream of *guaA*, *guaB* could be identified, encoding an inosin-5'-monophosphate (IMP) dehydrogenase (82 % similarity over 478 aa, with *C. actetobutylicum* GuaB (NÖLLING et al. 2001)). Downstream of *attBB'*, four coding regions could be identified, the products of which are similar to NarK of *Pseudomonas aeruginosa* (Acc.-No. Y15252; 40 % over 315 aa), and AppA, AppB, and AppC, which are part of an oligopeptide transporter operon present for example in *C. actetobutylicum* (NÖLLING et al. 2001) (similarities of 53 % over 580 aa, 63 % over 288 aa, and 71 % over 323 aa, respectively).

3.4 Identification and organization of ϕ3626 ORFs

Bioinformatic analysis revealed the existence of 50 putative protein coding regions (Tab. 4) on the ϕ3626 genome, covering 94.1 % of the sequence. The criteria for the characterization of a potential ORF were the existence of an ATG, GTG (5 present), or TTG (3 present) start codon, and a minimum coding capacity of 40 amino acids. Except for ORF41, all ORFs where proceeded by a recognizable potential ribosome binding site complementary to the 3'-end of *C. perfringens* 16S rRNA (GARNIER et al. 1991).

On the whole, the transcriptional units of ϕ3626 (as deduced from bioinformatic analysis) can be organized into three major functional clusters, which is also reflected by the transcriptional direction of the ORFs (Fig. 4). The first cluster from the *cos*-site at coordinate 1 up to position 19804 is transcribed rightward in the genomic map (Fig. 4), and represents genes encoding structural proteins and the lysis system. These genes can be summarized as 'late genes'. The second cluster (nt 19805 to 23645) likely encodes products responsible for the control of lysogeny including the *att*-site, an integrase, the repressor and a putative *cro*-like protein. The third cluster (nt 23680 to 33507) includes rightward-

facing ORFs, whose putative products represent the early genes, involved in replication, recombination and modification of phage DNA.

3.5 Structural proteins of f3626

The proteins building up the virion particle were separated by SDS polyacrylamide electrophoresis (Fig. 1). Microsequencing yielded N-terminal sequences of three structural proteins, which enabled identification of the corresponding genes.

The major capsid component Cps (NH_2-DIMSSTNNGA, encoded by ORF6) resembles 43.3 % of total phage protein (Fig. 1). The N-terminal sequence indicated that the gene product must be post-translationally processed between R_{114} and D_{115}, which results in a decrease in size from 47.7 kDa to 34.3 kDa. Cps displays a high probability to form a coiled-coil structure in the N-terminal portion removed by processing, a finding similar to what has been reported for phages Sfi21 and HK97 (CONWAY et al. 1995; DESIERE et al. 1999).

The major tail protein Tsh (NH_2-PEVVNTRRXG, encoded by ORF11) corresponds to 12.7 % of the total protein, with an apparent size of 27 kDa (Fig. 1). The predicted size was 22.5 kDa. This observation was also made for other phages, such as *Listeria* phage A118 (LOESSNER et al. 2000). The amino acid sequence differs from the predicted sequence only by the absence of the initiator methionine, as is often observed in prokaryotes with proline as the penultimate amino acid (HIREL et al. 1989)

A minor structural protein (Gp16, 2.1 % total protein content) was also isolated, and its N-terminal sequence (NH_2-MKYIQTKVVY) was in perfect agreement with the putative product of ORF16. The predicted size of 55.1 kDa is identical to the experimentally determined size.

3.6 Bioinformatic analysis of f3626 gene products

Deduced amino acid sequences of the fifty ORFs were compared with known sequences from the databases to uncover similarities to genes with known function. Functional assignments and significant homologies to other proteins are listed in Tab. 4. The findings are described below in more detail.

Tab. 4 Open reading frames, gene products, functional assignments, and amino acid sequence homologies of ϕ3626

ORF	Start	Stop	Gp (kDa)	Gp (pI)[a]	Functional assignments	Homologies (gp/orfs are given in brackets)[b]
1	54	557	19.2	7.2	Terminase, small subunit	ϕ105 (gp21) Sfi19 (gp161) bIL309 (gp38)
2	550	2295	67.7	5.9	Terminase, large subunit	ϕ105 (gp22 & 23) ϕSLT (gp563) D3 (gp2)
3	2296	3564	48.7	8.3	Portal protein	ϕ105 (gp25) ϕSLT (gp412) D3 (gp4)
4	3554	3715	6.4	9.7		
5	3720	4325	23.1	4.7	Putative prohead protease	ϕPV83 (gp41) ϕC31 (gp35) ϕPVL (gp5a)
6[c]	4366	5631	47.7	4.9	Major capsid protein [Cps]	ϕ105 (gp27) bIL285 (gp44) ϕadh (gp395)
			(34.3)	4.7	Processed form, cleaved between R_{114} and D_{115}	
7	5641	5919	10.8	4.9		ϕ105 (gp30)
8	5912	6235	13.2	8.8		
9	6236	6685	17.0	8.7		ϕ105 (gp32)
10	6687	7037	14.0	4.4		
11[c]	7054	7650	22.5	4.9	Major tail protein [Tsh]	ϕ105 (gp34)
12	7665	7979	11.9	9.7		
12.1[d]	7665	8485	32.2	4.3		
13	8515	11400	103.8	10.0	Tail tape-measure protein [Tmp]	ϕSLT (gp1374) TP901-1 (TMP) ϕPVL (gp15)
14	11401	12102	27.4	4.8		PSA (gp13) bIL285 (gp53)
15	12102	15053	110.0	6.2		PSA (gp14) bIL285 (gp54) ϕg1e (gp1608)
16[c]	15106	16584	55.1	5.5	Minor structural protein	ϕ105 gp42
17	16597	16905	12.1	4.8		
18	16914	17669	28.2	4.8		bIL170 (gpI12, accession number AF009630)
19	17684	18061	14.3	9.6	Holin	ϕ105 (gp45)
20	18149	19192	38.8	5.9	Endolysin	pIP404 (ORF10) *C. perfringens* (ORF 10C) PSA (Ply) 12826 (Ply12)
21	19382	19804	15.9	4.8		
22	21119	20070	41.3	9.9	Integrase	T12 (Int) PSA (gp24) T270 (Int)
23	21587	21135	17.6	5.2		PSA (gp26) ϕSLT (gp153) A118 (gp35)

continued

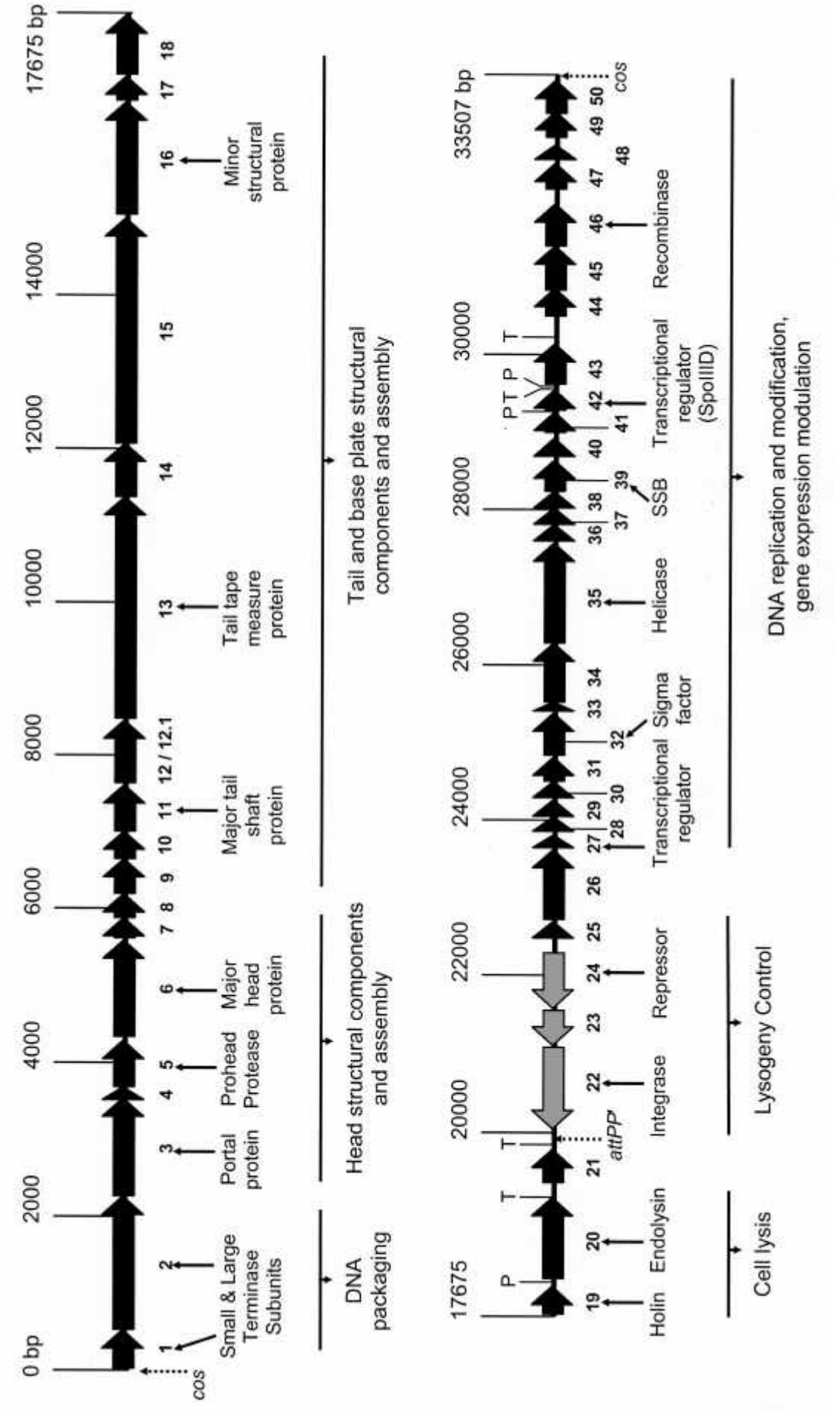
Tab. 4 continued

ORF	Start	Stop	Gp (kDa)	Gp (pI)[a]	Functional assignments	Homologies (gp/orfs are given in brackets)[b]
24	22330	21608	27.4	5.1	Repressor	ϕSLT (gp101a) ϕg1e (gp132), PBSX (Xre)
25	22504	22722	8.3	9.2	Cro	A2 (Cro)
26	22749	23645	34.5	8.7		ϕSLT (gp81a) ϕadh (Tec)
27	23680	23856	6.8	9.6	Transcriptional regulator	ϕETA (gp13,) ϕPV83 (gp12)
28	23903	24082	6.7	9.8		
29	24093	24299	7.9	9.2		
30	24319	24537	8.5	10.3		
31	24527	24853	13.1	4.5		
32	24867	25412	21.3	9.4	Sigma factor	*C. acetobutylicum*, *B. subtilis* and of *C. perfringens*
33	25428	25547	4.6	8.9		
34	25563	26315	29.3	9.4		ϕETA (gp22) ϕSLT (gp256) r1t (gp11)
35	26325	27608	48.3	8.3	Helicase	P1 (Ban) SPP1 (gp40) D3 (gp74)
36	27625	27825	7.9	4.4		
37	27825	28061	9.3	4.9		
38	28058	28270	7.9	6.4		
39	28263	28679	15.4	4.9	SSB	Ul36 (gp141) bIL286 (SSB) A118 (SSB)
40	28706	28942	9.1	4.1		
41	29020	29295	10.9	10.0		
42	29304	29576	10.5	10.9	SpoIIID	*C acetobutylicum*, *B. subtilis* and other Bacilli
43	29638	30168	21.1	8.7		
44	30515	30865	13.5	9.5		
45	30858	31424	21.4	4.7		
46	31428	31973	21.4	10.2	Recombinase	L2 (gp5) Wϕ (Int) 186 (Int,)
47	32153	32500	13.4	9.5		
48	32533	32718	7.4	4.6		
49	32735	33058	12.6	9.9		
50	33039	33467	16.6	9.8		ϕSLT (gp104b) bIL286 (gp39) ϕadh (gp170)

Tab. 4 continued [a] Predicted by computer analysis; [b] For reference or accession numbers, see text; [c] Experimentally shown; [d] Translational frameshift might result in two C-terminally different products (see 3.5.4).

Fig. 4 Schematic representation of the f3626 genome. (next page)

Assumed ORFs, some functional assignments and overall genetic organization. The ORFs are numbered consecutively (see Table 3) and are indicated by arrows or arrowheads that point in the direction of transcription. Black arrows indicate rightward transcription, grey-shaded ORFs are oriented leftwards. Their relative position on the genome (33,507 nt) is indicated by coordinates. The *attPP'* and the *cos*-sites are shown by a dashed arrows. P indicates some specific putative sporulation dependent promoters, and T stands for putative rho-independent transcription terminators (see text for explanation)


0 bp
2000
4000
6000
8000
10000
12000
14000
17675 bp
cos
1
2
3
4
5
6
7
8
9
10
11
12 / 12.1
13
14
15
16
17
18
Small & Large Terminase Subunits
Portal protein
Prohead Protease
Major head protein
Major tail shaft protein
Tail tape measure protein
Minor structural protein
DNA packaging
Head structural components and assembly
Tail and base plate structural components and assembly
17675
20000
22000
24000
26000
28000
30000
33507 bp
P
T
T
19
20
21
attPP'
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
39
38
40
41
42
43
P
T
T
44
45
46
48
47
49
50
cos
Holin
Endolysin
Integrase
Repressor
Transcriptional regulator
Sigma factor
Helicase
SSB
Transcriptional regulator (SpoIIID)
Recombinase
Cell lysis
Lysogeny Control
DNA replication and modification, gene expression modulation

3.6.1 ORF 1 and 2

Fifty-four nucleotides downstream of the *cos*-site, two ORFs have been allocated that likely represent the small and large subunit of the terminase, which introduces specific cuts into the concatemeric DNA at the *cos*-site to initiate genome packaging. Gp1 shares similarities with gp21 of *Bacillus subtilis* phage ϕ105, gp161 of phage Sfi19 of *Streptococcus thermophilus* (DESIERE et al. 1998) and gp38 of prophage bIL309 of *Lactococcus lactis* (CHOPIN et al. 2001), with similarities between 43-47 % over stretches of 132-169 aa. The large subunit (gp2) displayed significant similarities to the gp22 and gp23 ϕ105 (55 % over 416 aa and 48 % over 88 aa, respectively), and to terminases from various other phages.

3.6.2 ORF 3-5

Gp3 encodes a putative portal protein, based on similarities to gp25 of ϕ105, and the portal proteins of ϕSLT (NARITA et al. 2001) and D3 (KROPINSKI 2000) (39-45 % identity over 313-397 aa). The localization of ORF4 disrupts the pattern found in ϕC31, D3 and HK97, which consists of a consecutive order of portal protein, prohead protease and a N-terminally processed major capsid protein (DUDA et al. 1995; SMITH et al. 1999; KROPINSKI 2000). No similarities to any potential protein could be found for Gp4. Gp5 is similar to putative prohead proteases from various phages, including *S. aureus* phages ϕPV83 gp41 (KANEKO et al. 1997) and ϕPVL gp5a (KANEKO et al. 1998), *Streptomyces* phage ϕC31 gp35 and bIL309 gp36. Homologies range from 58-62 % similarity over stretches from 147-172 aa.

3.6.3 ORF 7 and 9

For these ORFs, no functional assignments could be made. Only a congruent gene order is obvious by the similarities to putative gene products 30 and 32 of ϕ105 with similarities of 50 % over 87 aa and 45 % over 121 aa, respectively. Also, the deduced sequence of the major tail protein (encoded by ORF11) displays shares similarities to gp34 of ϕ105 (42 % over 146 aa).

3.6.4 ORF 12/12.1

Tailed bacteriophages frequently have a pair of overlapping ORFs between the major tail protein gene and the tail length tape measure gene that are expressed by a translational frameshift (JUHALA et al. 2000). This seems to be the case for ORF12/12.1, resembling the situation in λ phage (LEVIN et al. 1993). ORF12 starts at nt 7665, and the obvious stop codon is located at 7979. However, just 27 nucleotides upstream of this stop codon, a putative heptanucleotide 'slippery sequence' (GGGUUUU) can be allocated, encoding the dipeptide Gly-Phe in both, the zero and -1 reading frames (Fig. 5). At this site ribosomes might shift frames and continue in the -1 frame until termination at nt 8485, resulting in a larger gene product as described for the gpG-T of λ phage (LEVIN et al. 1993).

```
    7921  ATGATAAAGCTTTTAAAGCGAGTGC***GGGTTTT***ATGAAAGGCAAGAAATTCAAGAGA**TAG**G

 0 FRAME  N  D  K  A  F  K  A  S  A  G  F  M  K  G  K  K  F  K  R  *
-1 FRAME                           G  F  Y  E  R  Q  E  I  Q  E  I  G
```

Fig. 5 Putative translational frameshift.
A fragment of the ORF12/12.1 is shown and the deduced amino acid sequence. The "slippery sequence" is in bold and italic letters. The stop codon is in bold letters and the amino acids encoded by the "slippery sequence" are in italic and underlined

3.6.5 ORF 13

The gene product of ORF13 encodes a large protein (103.8 kDa) sharing similarities with the tape measure proteins (Tmp) of lactococcal bacteriophage TP901-1 (PEDERSEN et al. 2000), and gp1374 of ϕSLT, gp15 of ϕPVL, and many other phages.

3.6.6 ORF 15

The deduced gp15 seems to consist of a mosaic sequence. A HMMscan indicated two areas with similarities to different protein families. The area between aa 892-975 displayed a high probability being a peptidase M37, and the area from aa 681-801 displayed a similarity to a phage lysozyme domain. The latter domain showed similarities to the *Salmonella* phage P22 endolysin (VANDER BYL and

KROPINSKI 2000), and to the lactococcal phage C2 endolysin (WARD et al. 1993), (41-42% over 157-169 aa).

3.6.7 ORF 19 and 20

These two ORFs located at the distal end of the "late gene" region encode a dual lysis system consisting of a holin (Hol) and an endolysin (Ply). This genetic organization is common to many viruses of the Siphoviridae family (LUCCHINI et al. 1999b). Together, they are responsible for the release of phage progeny from infected host cells.

The deduced amino acid sequence from ORF19 displayed similarity to a putative holin of *B. subtilis* phage ϕ105 (KOBAYASHI et al. 1998) (50 % over 105 aa). A dual start motif (YOUNG and BLÄSI 1995) is not present in hol3626. Bioinformatics revealed that hol3626 encodes a protein with two highly hydrophobic amino acid sequence regions (Fig 6 B) which, by TmHMM analysis, were strongly suggested to function as membrane spanning domains (MSD). Applying the "positive-inside" rule (VON HEIJNE 1992), the positively charged amino acids located in the putative beta-turn region between the two MSDs (coordinates 33-46; Fig. 6A) suggest a location of this domain on the "inside" of the cytoplasmic membrane, when the polypeptide is inserted into the membrane (Fig. 6 C). This suggests that both the N- and the C-terminus are most likely located on the "outside" of the membrane (Fig. 6 C)

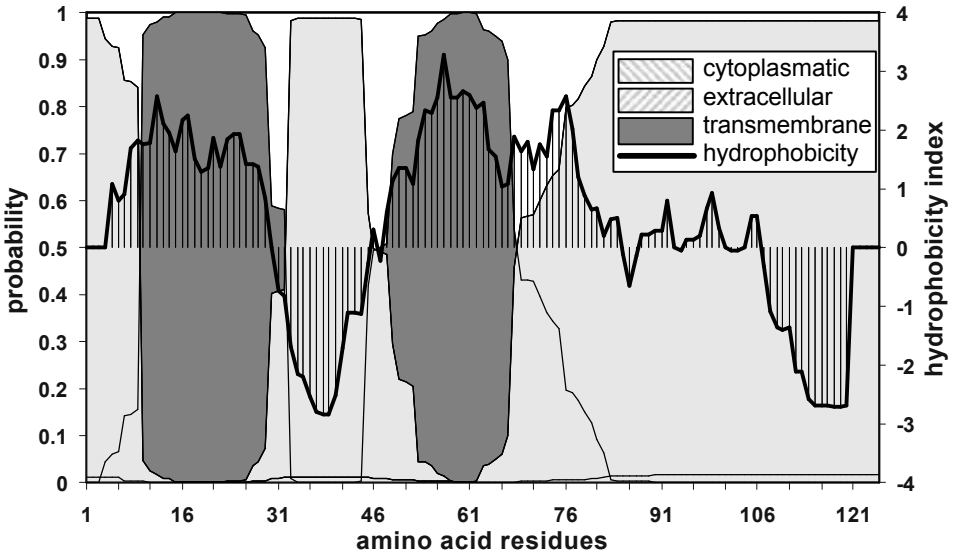
Fig. 6 Bioinformatic analysis of Hol3626. (next page)
(A) Amino acid sequence of Hol3626. Charged amino acid residues are indicated by + and - signs, respectively, and sequences with a high probability to form MSD (by TmHMM, see text) are boxed. **(B)** The probability to form MSD (left y-axis, dark grey areas) was predicted by TmHMM, and probable cytoplasmic and extracellular regions of the polypeptide are indicated by grey diagonal stripes. An hydrophobicity plot (right y-axis) (KYTE and DOOLITTLE 1982) was overlaid (bold black line). Hydrophobic region are in the positive area, whereas a negative hydrophobicity indicates a hydrophilic region. **(C)** The putative model of Hol3626 topology when inserted in the cell membrane (see text). Grey bars indicated the membrane spanning domains. Charged amino acid residues in the hydrophilic area between the two putative MSD are indicated.

A

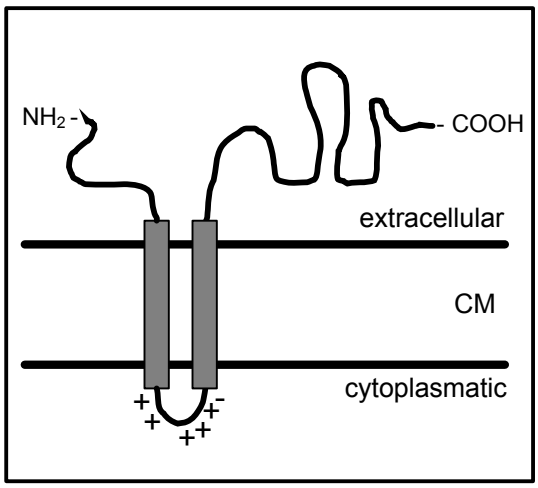
```
          10        20        30        40        50        60
MFKFIPEVISWLLVLYIGFKIIDMILGVLKTIKNKNYRSRKMRDGIIRWVAELMAIAFVL
  +   -           +  -      +  + +  + ++ +-   +   -

          70        80        90       100       110       120
ILDMFLGLKFTVIGVTLALFAYKEAGSIVENLGECGVELPEIVSEKLEVLNKNNKNKEGFNKKEN
  -     +            +-     -   -   -  -   -+ -   +  + +-   ++-
```

B



C



ORF20 probably encodes an N-acetylmuramoyl-L-alanine amidase. A HMMscan analysis revealed a N-acetylmuramoyl-L-alanine amidase domain in a 116 amino acid N-terminal portion of Ply3626 (Fig. 7). This assignment was further supported by sequence alignments, which also indicated relatedness of the Ply3626 N-terminal region to amidases, in particular the PlyPSA enyzme from *L. monocytogenes* phage PSA (SATTELBERGER et al. 2002), the *B. subtilis* autolysin CwlB, and the *B. cereus* phage 12862 endolysin (KURODA and SEKIGUCHI 1991; LOESSNER et al. 1997; SATTELBERGER et al. 2002). Interestingly, extended similarities (72-75% over 265-346 amino acids) exist to hypothetical proteins of unknown function of *C. perfringens* or the *C. perfringens* plasmid pIP404 (GARNIER and COLE 1988; LYRISTIS et al. 1994; SHIMIZU et al. 1994).

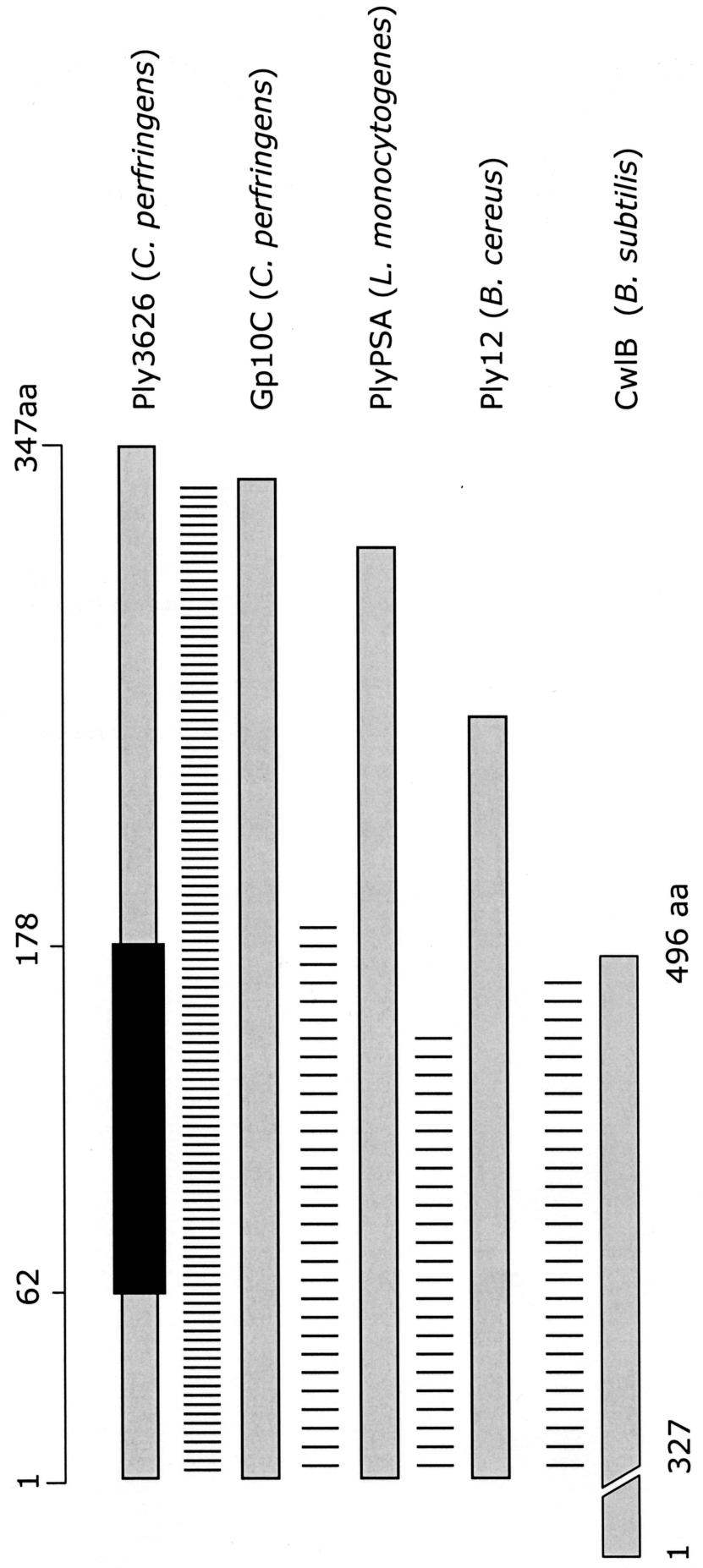
The two lysis genes have been cloned and their function was demonstrated experimentally (see 3.8 and 3.9)

3.6.8 ORF 22

This ORF is located immediately upstream of the attachment site (see 3.3). A HMMscan indicated that it encodes a phage integrase belonging to the λ integrase family, responsible for the site-specific recombination of ϕ3626 into the *C. perfringens* chromosome. Furthermore a relationship to Int459 of the transposon like element CW459*tet*(M) from *C. perfringens* was found (ROBERTS et al. 2001) (similarity of 47 % over 363 aa). Many other integrases of phage origins also displayed homology

Fig. 7 Alignment of Ply3626 with other amidases (next page)

Alignment of the amino acid sequence comparisons of Ply3626, with the gene product of ORF 10C from *C. perfringens*, and three amidases of other origin. Size of the proteins (in aa) are indicated by the ruler. The black boxed area indicates the N-acetylmuramoyl-L-alanine amidase domain identified by HMMscan. The vertical bars indicate the extend and degree of similarities among the different polypeptides.


Ply3626 (C. perfringens)
Gp10C (C. perfringens)
PlyPSA (L. monocytogenes)
Ply12 (B. cereus)
CwlB (B. subtilis)
1
62
178
347aa
1
327
496 aa

3.6.9 ORF 23

No functional assignment was possible with gp23, but similar putative gene products are found in the same loci between the integrase and the repressor in other bacteriophages, such as PSA (gp26) (SATTELBERGER et al. 2002), ϕSLT (gp153) and A118 (gp35) with similarities of 45-49% over 90-123 aa

3.6.10 ORF 24-25

Gp24 displays in the N-terminus homologies to several repressors of phages. Strongest hits are the repressor of ϕSLT, the repressor Xre of the *Bacillus* prophage PBSX (TAKAMI et al. 2000) and the repressor of ϕg1e (KODAIRA et al. 1997)) (similarities from 53-64 % over 62-70 aa). In the opposite direction, ORF25 encodes a product with similarity to Cro of *Lactobacillus casei* phage A2 (LADERO et al. 1999) (47 % similarity over 70 aa). Both proteins contain putative H-T-H motifs similar to CI and Cro of λ (results not shown), indicating their potential to bind to DNA.

3.6.11 ORF 26

The product of this gene displays similarity to a putative anti-repressor of phage ϕSLT (56 % similarity over 71 aa). The amino terminus also displayed similarity (67 % over 60 aa) to the Tec protein (topological equivalent of *cro*) found in ϕadh. This implicates that gp26 might also be involved in lysogeny control.

3.6.12 ORF27

Gp27 displays similarities (64 % over 54 aa) to a transcriptional regulator of *B. subtilis* (KUNST et al. 1997), but also to phage proteins with unknown function, such as the gp13 form *S. aureus* phage ϕETA (YAMAGUCHI et al. 2000), and gp12 of ϕPV83, both with 66% similarity over a length of 45 aa.

3.6.13 ORF 32

Both HMMscan and BLAST searches suggest that gp32 is a sigma factor, similar to sporulation-specific sigma factors of *C. acetobutylicum* (σ^F) (NÖLLING et al. 2001), *B. subtilis* (σ^E) (KUNST et al. 1997) and *C. perfringens* (σ^K) (STIREWALT and MELVILLE 2000) (45 % to 53 % over 174-219 aa).

3.6.14 ORF 34

ORFs with similar gene products can be found in ϕETA (gp22), in ϕSLT (gp256) and in the phage r1t from *Lactococcus lactis* (VAN SINDEREN et al. 1996), all with no known function.

3.6.15 ORF 35

Gp35 seems to be a helicase, responsible for the unwinding of DNA before replication. The strongest similarities are with DnaC of *C. acetobutylicum* (NÖLLING et al. 2001) and *B. subtilis* (KUNST et al. 1997) (51-55 % over 424 aa), but also to phage helicases from SPP1 (ALONSO et al. 1997) and others.

3.6.16 ORF 39

HMMscan and BLAST analysis suggest that this gene product most probably is a single stranded binding protein (SSB). Many phage-encoded SSB show a similarity to gp39; some strongest hits are the *L. lactis* phage ul36 (BOUCHARD and MOINEAU 2000), bIL286 (CHOPIN et al. 2001) and A118 (LOESSNER et al. 2000), with similarities of 62-65% over 125-144 amino acids.

3.6.17 ORF 42

Gp42 shows significant similarities (66-71 % over 74 aa) to σ^E/σ^K-dependent transcriptional regulators, also known as a stage III sporulation protein D (SpoIIID), found in *C. acetobutylicum* (NÖLLING et al. 2001), *B. subtilis* (KUNST et al. 1997), and other Bacilli (YOSHISUE et al. 1995; LINDBACK and KOLSTO 1999; TAKAMI et al. 2000).

3.6.18 ORF 46

As gp22 the gp46 probably belongs to the family of λ integrases. Similarities are found to several bacterial recombinases; C. actetobutylicum, B. haolurans or S. aureus (TAKAMI et al. 2000; KURODA et al. 2001; NÖLLING et al. 2001) (53-61 % similarity over 174-179 aa). The mycoplasma virus L2 integrase (MANILOFF et al. 1994), the coliphage Wϕ integrase (LIU and HAGGARD-LJUNGQUIST 1999), and the integrase of the coliphage 168 (KALIONIS et al. 1986) share sequence similarities ranging from 42-47 % over 147-152 aa.

3.6.19 ORF 50

Although no function could be assigned to gp50, similar proteins are encoded by many phages, such as ϕSLT (gp104b), bIL286 (gp39) and ϕadh (gp170), with similarities from 45-59 % over 91-133 aa.

3.7 Other features of the f3626 genome

Several potential stem-loop forming sequences were identified at the ends of possible transcriptional units, likely representing rho-independent transcription terminators: (i) between the lysis cassette and ORF 21, at position 19195-19230 (ΔG = -32.3 kcal mol^{-1}); (ii) between ORF 21 and the lysogeny control region (19856 - 19888; ΔG = -18.0 kcal mol^{-1}); (iii) downstream of ORF 42 (SpoIIID homologue) (29587-29620; ΔG = -17.1 kcal mol^{-1}); and (iv) in a non-coding region downstream of ORF43 (30462-30488; ΔG = -15.4 kcal mol^{-1}). Sequences similar to the P1 promoter upstream of the *C. perfringens* enterotoxin gene *cpe* and to the *B. subtilis* σ^K dependent promoter (ZHAO and MELVILLE 1998) have been allocated upstream of ORF42, possibly encoding a σ^K/σ^E regulated SpoIIID homologue (coordinates 29260-29287), but could also be found upstream of *ply* (18007-18033) and upstream of ORF43 (29688-29713). Interestingly, putative stem-loop structures are present downstream of all these coding regions, suggesting a tightly regulated, spatial expression of specific genes.

3.8 Functional characterization of the f3626 holin

For functional analysis of the holin in an established background, *hol3626* was cloned into λΔSthf background (VUKOV et al. 2000). Recombinant λΔSthf::hol3626 was able to form normal sized plaques on *E. coli* LE392, apparently due to the ability of *hol3626* to support R-mediated lysis in *E. coli*. Thermal induction of the lytic cycle of λΔSthf::*hol3626* in lysogenized LE392 resulted in lysis kinetics relatively similar to λΔSthf::S with a slight delay in lysis onset of approximately 15 min (Fig. 8). Taken together, these results clearly demonstrated that the *hol3626* gene product function as a holin.

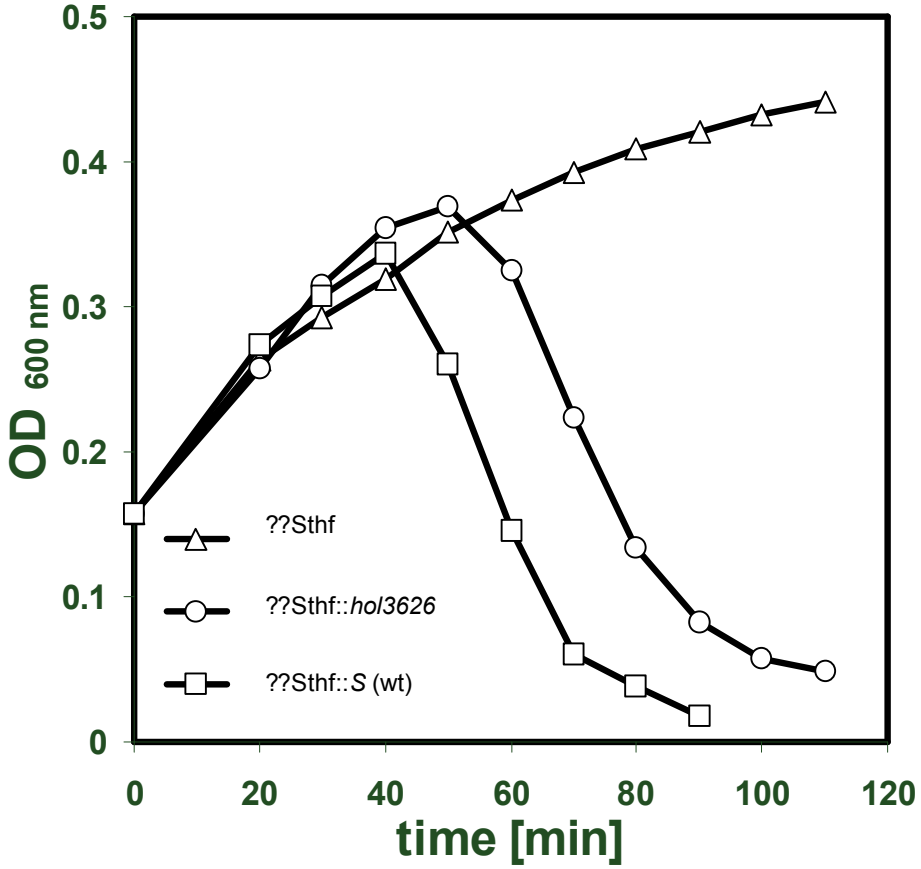


Fig. 8 Lysis profile of Hol3626

Lysis profiles observed following thermal induction of *E. coli* LE392 lysogenized with λΔSthf::hol3626 (squares), λΔSthf::S (circles) and λΔSthf (control without holin) (triangles).

3.9 Functional characterization of ply3626

3.9.1 Recombinant Ply3626 can only be synthesized in *E. coli* supplemented with rare tRNAs

First attempts to produce functional, recombinant Ply3626 were based upon a combination of JM109(DE3) and pSP72, which had repeatedly shown to be useful for this purpose (LOESSNER et al. 1995b; LOESSNER et al. 1997; LOESSNER et al. 1998). However, cloning of *ply3626* did not yield the desired enzymatically active product. In fact, SDS-Page revealed that no appreciable amount of recombinant protein was synthesized (results not shown). Next, a pQE-30 backbone was used, and the N-terminus of Ply3626 was modified by adding a hexa-histidine tag (yielding HPL3626), in order to allow concentration of enzyme from dilute cell extracts. However, still no lytic activity could be found. One reasonable explanation was that the failure to express a low G+C gene of *C. perfringens* origin in an *E. coli* background might be due to different codon usage. In fact, ply3626 contains 12 copies of the isoleucine codon ATA (4.8 % in *C. perfringens*), and 16 copies of the arginine codon AGA (2.7 % in *C. perfringens*). In contrast, these codons are rarely used in *E. coli* (0.7 % and 0.4 %, respectively) (see Codon Usage Database at http://www.kazusa.or.jp/codon). Thus, the third approach was designed such that proper tRNA genes were substituted in order to efficiently express ply3626 in E. coli. These were provided on the compatible plasmid pACYC-IRL10, encoding tRNAs for isoleucine, arginine, and leucine (ZDANOVSKY and ZDANOVSKAIA 2000). For this purpose, pACYC-IRL10 and pHPL3626 were sequentially introduced into *E. coli* JM109. However, induction with IPTG resulted in rapid formation of insoluble inclusion bodies. These contained inactive enzyme, and attempts to resolubilize the polypeptides into an active form were unsuccessful (results not shown). This phenomenon could be avoided by culturing the bacteria at room temperature (21°C) without addition of IPTG. Synthesis of the desired protein was monitored over time (Fig. 9), and revealed that approximately 20 h incubation are required for sufficient protein production. Surprisingly, under these conditions

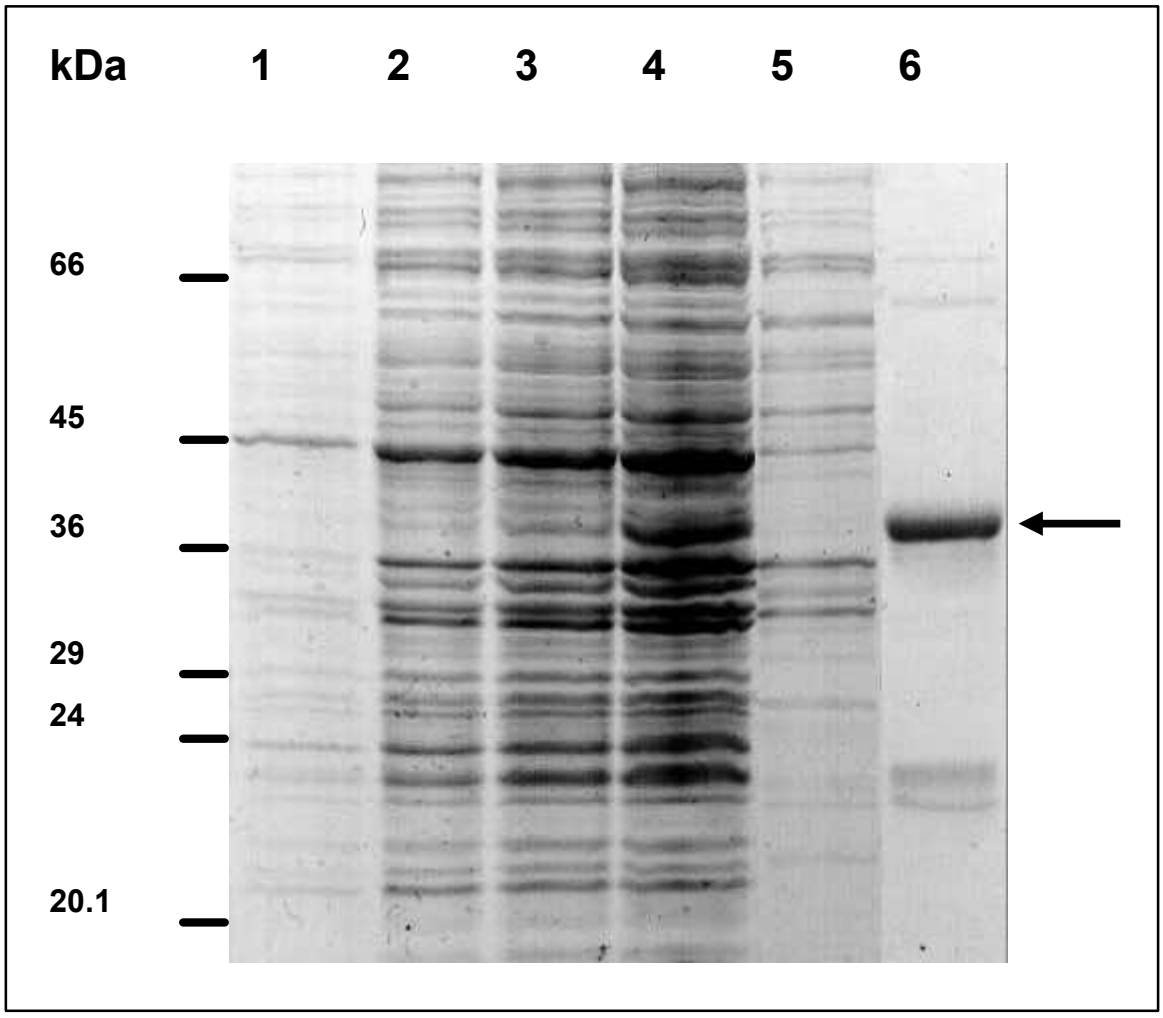


Fig. 9 Synthesis of recombinant endolysin in *E. coli* (pACYC-IRL10) (pHPL-3626), and purification of the protein (indicated by black arrow). Lanes 1-4, samples of the culture at different time point after induction (0, 10, 15, 20 hours). Lane 5, wash fraction from Ni-NTA affinity chromatography. Lane 6, HPL3626 after elution and buffer exchange

background expression from the strong T5 promoter on pQE-30 was sufficient for appreciable gene expression. Crude cell extracts were then prepared, and HPL3626 purified by Ni-NTA affinity chromatography (Fig. 9). Using this method, a total yield of approximately 1.3 mg purified enzyme per liter of culture could be obtained.

3.9.2 The endolysin lyses *C. perfringens* cell walls

For the sensitivity testing towards enzymatic lysis, bacterial cell suspensions were exposed to extracts of *E. coli* (pACYC-IRL10)(pHPL3626). The rapid

decrease in optical density of a cell suspension of *C. perfringens* NCTC 3110 (standard propagation strain for ϕ3626) is shown in Fig. 10. Further tests showed that HPL3626 was able to lyse all 48 tested strains of *C. perfringens* although some variation in the sensitivity of the different strains was observed (see Fig. 10). None of the other bacteria were affected by the enzyme. A single exception was a strain of *Clostridium fallax,* which also displayed sensitivity. Nevertheless, the lytic activity of Ply3626 seems to be generally restricted to *C. perfringens.* Identical results were obtained using purified HPL3626, again demonstrating the nature of the lytic activity. In contrast, the C-terminally truncated form HPL3626ΔC was found to be inactive.

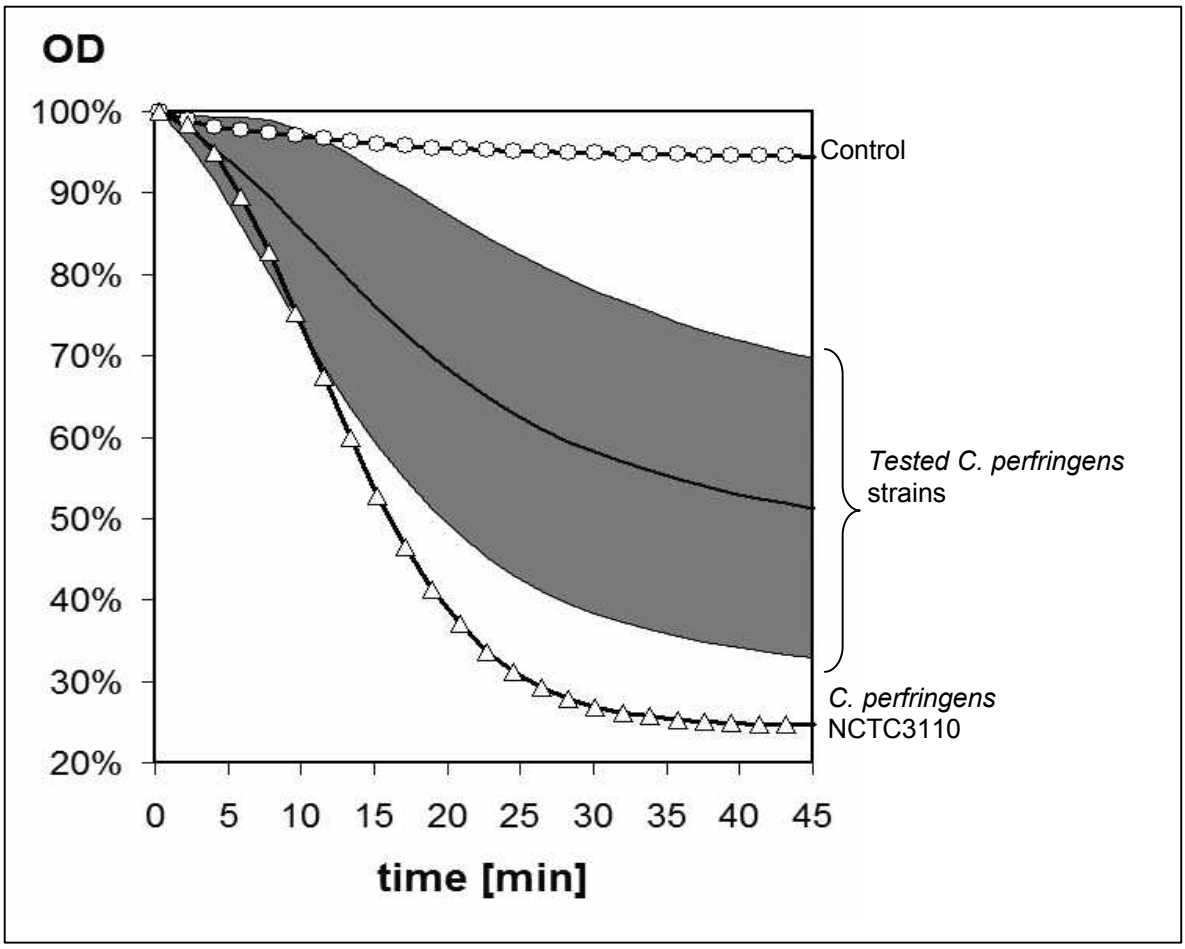


Fig. 10 Lysis Assays with HPL3626

Summary of lysis assays using the enzyme produced by *E. coli* (JM109 pACYC-IRL10) (pHPL3626). The decrease in percent optical density (OD, y-axis) over time is shown here, following addition of enzyme to standardized cell suspensions of C. perfringens. Open triangles indicate the lysis obtained with *C. perfringens* NCTC 3110 cells, the propagation host of ϕ3626. The control assay (extract from JM109 (pACYC-IRL10) (pQE-30)) is indicated by open circles. The average cell lysis response of 48 additional, different *C. perfringens* strains is represented by a black line, and the standard deviation as a grey shaded area

4 DISCUSSION

4.1 Genome organization of f3626 and relationships to other phages

In the framework of this thesis, two novel bacteriophages were isolated from lysogenic strains of the pathogen *C. perfringens*. ϕ3626, a member of Siphoviridae, is the first *C. perfringens* phage characterized on a molecular level, and it is the first bacteriophage of the genus *Clostridium* whose complete genome sequence is available. While 19 of the fifty potential polypeptides allowed functional assignments, twenty-two gene products found no match in the current databases and represent new entries. Most similarities were found with proteins of other phages infecting low G+C gram-positive bacteria, from the genera *Bacillus, Streptococcus, Staphylococcus, Lactococcus, Listeria* and *Lactobacillus*, but also, to a more limited extend, to phages from *E. coli* or *Pseudomonas*. Intergeneric relationships of ϕ3626 genomic modules are particularly pronounced in the "left-end" module, encompassing the DNA packaging and capsid building machinery. The consecutive order of the genes encoding small and large terminase, portal, prohead protease and major head protein is common to phages from low G+C gram-positives (DESIERE et al. 2001), and to lambdoid viruses in general (JUHALA et al. 2000). Here, it is disrupted by ORF 4 with unknown function.

From an evolutionary point of view, it is interesting to note that most amino acid sequence similarities were found to proteins from other Sfi21-like *cos*-site phages (BRÜSSOW and DESIERE 2001) from low G+C bacterial hosts, in particular *B. subtilis* phage ϕ105 and *S. aureus* phages ϕSLT, ϕPVL and ϕPV83. The convincing relatedness of DNA packaging and head proteins points to a vertical passage and evolution of at least this module (BRÜSSOW and DESIERE 2001). However, clostridia occupy different ecological niches and, due to their anaerobic nature, require different growth conditions than the other hosts. Nevertheless, the conserved gene order among their viruses implies a rather tight genetic

conservation. However, it should be noted that the similarities of ϕ3626 are solely based on amino acid sequences, whereas alignment of nucleotide sequences did generally not yield significant homologies. This finding suggests a lack of recent horizontal genetic exchange and therefore implicates that at least the line of viruses represented by ϕ3626 may have diverged at an earlier point. The foreseeable availability of more clostridial phage sequences in the nearer future may help to clarify this situation. Generally, however, these findings are in agreement with the hypothesis (HENDRIX et al. 1999) that most tailed phage genomes are genetic mosaics which have been build from a large common pool by genetic exchange, but were access was, and is, not uniform among the different host-dependent taxonomical groups.

4.2 Cohesive ends of the f3626 genome

The ϕ3626 chromosome features nine nucleotides 3'-protruding, single-stranded, cohesive end. Thirty-five bp upstream of this *cosN* site a nucleotide sequence G_4AG_4 can be allocated, and 37 bp downstream a run of 5 consecutive G nucleotides occur (Fig. 11). A situation reminiscent to bacteriophage BK5-T, where a C_3TC_5 sequence and a run of 7 Gs was found (MAHANIVONG et al. 2001). Also in the bacteriophages Sfi19 and Sfi21 similar findings have been described, where the sequence C_5GC_5 is located in close proximity to the *cosN*-site (LUCCHINI et al. 1999a). MAHANIVONG et al. speculated that in the background of low-G+C content organisms theses findings could be interpreted that these G+C rich

```
 -87 ATACTCAAGA TGAATTGTTT AAGCTAATTC CTAAT***GGGGA*** ***GGGG***TAGGGC AAAAAGTTTT
                                    +1
 -27 GGGCAAGAGC CAAAAGGT**CG CAGTGTC**TAT GCAGAAAAAA TTTTTTTCGG TTTATAAAGT

  34 GAAA***GGGGG***T ATATTATAGTTTGGCTAGAAA GATGATTTCA GTTAATCAAA TTATTGCAAA
```

Fig. 11 The putative *cosB* sequence. The *cosN* sequence is shown in bold. Underlined residues indicate runs of at least four identical bases. The G_4AG_4 and G_5 sequences are indicated in bold italics.

sequences might constitute the recognition site for terminase binding (*cosB*). Additionally, in BK5-T and also in λ the *cosN*-site is directly surrounded by a number of runs of four to seven identical bases of adenines and thymines that are important to DNA bending which occurs upon integration host factor binding (YEO et al. 1990; MAHANIVONG et al. 2001).

4.3 Integration site

Phage ϕ3626 was isolated from a temperate *C. perfringens* strain, where, in the lysogenic stage, the prophage is integrated into the host chromosome within the 3' terminus of *guaA.* The *attPP'* site complements the disrupted coding sequence, which enables termination of *guaA* translation at the expected site (see Fig. 3), independent of the integration status. *GuaA* encodes a GMP synthetase, a housekeeping protein responsible for *de-novo* biosynthesis of the purine nucleotide GMP. *GuaA* null mutants become guanine auxotrophs (NYGAARD 1993), and it is obvious that integration of phage must not destroy its function. Phage ϕ3626 exhibits a particularly elegant way to retain genetic function by duplication of terminal codons including a translational stop signal. This strategy is reminiscent of phages such as PSA of *L. monocytogenes* which integrate into the 3'-ends of a tRNA gene without negative effects (LAUER et al. 2002).

The putative integrase present in ϕ3626 displays similarities to *int459* of the transposon-like element CW459*tet*(M) of *C. perfringens* CW459 (ROBERTS et al. 2001). This element has been partially sequenced, and downstream of the integrase gene, part of *guaA* (*gmp*) can be allocated. These two genes are transcribed in opposite directions, just like the situation found in the ϕ3626 prophage. The coding region of *guaA* (from CW459*tet*(M)) displays high nucleotide homology (98% identical over 151 nucleotides) to the sequence upstream of *attBP'*. Interestingly, for CW459*tet*(M), the nucleotide sequence in the area between *int* and *guaA* differs from the one found in the lysogenic host (rendering the stop codon of *guaA* functional). This might be the reason why CW459*tet*(M) seems to be unable of transposition of its *tet*(M) gene (ROBERTS et al. 2001).

In the phage genome, *attPP'* is located immediately downstream of *int*. This is a common organization and forms an ideal basis for building a site-specific integration vector. No such system is available for *C. perfringens*, and its construction could be of interest for molecular and genetic research on this pathogen.

4.4 Gene products of f3626

The ORF upstream of *int* might encode the repressor and a Cro homologue, being important for the control of lysogeny. Both share helix-turn-helix (HTH) motifs known to be responsible for the necessary protein-DNA interaction. Additional evidence that supports this functional assignments is derived from using non-sequence alignment based homology parameters, such as size and charge of the proteins (CHANDRY et al. 1997). Gp24 and gp25 have biochemical properties that are quite similar to the λ proteins, i.e., λ CI is acidic (predicted pI 4.9) similar to gp24 (pI 5.1), and λ Cro is very basic (pI 10.2) as is gp25 (pI 9.2). Relative sizes of these two proteins are also analogous. These data taken together, it is tempting to speculate that gp24 and gp25 govern the genetic switch of A118.

Post-translational processing of the major head protein is frequently found in bacteriophages. During capsid maturation, ϕ3626 Cps is processed by removal of the first 114 residues. A similar processing can be observed in many other phages which lack a scaffold protein. As described for Sfi21, ϕPVL (DESIERE et al. 1999) and HK97 (CONWAY et al. 1995), ϕ3626 Cps revealed a possible coiled-coil structure in the amino terminal part of the protein which is removed. It has been assumed by Duda and co-workers (DUDA et al. 1995) that this domain might be a functional equivalent of a scaffold protein, fused to the capsid protein.

The length of the phage tail seems to be determined by a ruler-mechanism, dependent on the size of the so-called tape measure protein (KATSURA 1987). Findings in the analysis of the phage support this theory: the designated Tmp (gp13, 962 aa) is about 13 % larger than the size of the λ protein (gpH, 853 aa)

and the ϕ3626 tail is approximately 13 % longer than the λ tail (170 nm and 150 nm, respectively).

The finding of a putative translational frameshift in the overlapping pair of ORF 12/12.1 which are located between the *tsh* and the *tmp* allows the speculation that this resembles the situation found in λ.(LEVIN et al. 1993) and other bacteria (JUHALA et al. 2000). It was postulated that a programmed frameshift allows a regulation of protein expression on a post-transcriptional level and leads to the production of two proteins which share a identical sequence similarity in the N-terminus. In the λ system, the frameshift occurs with a low frequency (4 %). The recently sequenced *Listeria* phage PSA uses this mechanism for the production of its major structural proteins of the head and the tail (SATTELBERGER et al. 2002). In ϕ3626, ORF12 is followed by an overlapping ORF12.1. Just nine codons upstream of the stop codon of ORF12, a 'slippery' sequence could be identified which encodes the dipeptide -G-F- in both the zero and the -1 reading frame (Fig. 5).

Bioinformatics suggest that the product of ORF15 is a "mosaic" sequence which may have at least two functional domains. A phage lysozyme domain to which several phage endolysins showed similarities was found and additionally an endopeptidase domain was located. Both domains were located in the C-terminus of the gene product. The localization of ORF15 between *tsh* and the lysis genes suggest that gp15 might have a function comparable to the T4 tail lysozyme (NAKAGAWA et al. 1985).

4.5 A Link Between Sporulation and Lysogeny ?

The finding of an ORF possibly encoding a putative sporulation-associated sigma factor within the early genes (ORF32) suggest a possible function in 'programming' the RNA polymerase, as shown for sigma gp28 of *B. subtilis* phage SPO1 (COSTANZO and PERO 1983). ORF42 encodes a protein with convincing similarity to a *B. subtilis* sporulation-dependent transcriptional regulator (SpoIIID), which is part of the sigma factor cascade resulting in sporulation. Moreover, sequence motifs possibly reflecting sporulation- or σ^K-dependent promoters (ZHAO

and MELVILLE 1998) are present in the ϕ3626 genome. Interestingly, SpoIIID and some of the bacterial sigma factors showing homology are mother-cell specific, and are made only in the late stages of sporulation, after which the mother cell lyses. Albeit it is not clear what the precise function of homologous factors in ϕ3626 could be, an earlier study on the effect of lysogeny on sporulation of *C. perfringens* offers a possible explanation: Curing of a lysogenic strain resulted in less efficient sporulation and decreased heat resistance of spores. Re-introducing the prophage reversed the effect (STEWART and JOHNSON 1977). Unfortunately, no further details on the nature of the phage or its interaction with the host are known. However, similar findings were reported for some Bacilli, were spore-converting bacteriophages were isolated, enhancing the sporulation of infected cells (SILVER-MYSLIWIEC and BRAMUCCI 1990).

There are additional findings that seem to support this theory. Lewis et al. considered an evolutionary link between prophage induction and sporulation (LEWIS et al. 1998), based on the findings that in *Bacillus subtilis* the repressor proteins of phage 434 and SinR responsible for the sporulation inhibition, displays a high structural similarity within their DNA binding domains and share functional similarities. Loessner et al. described that the endolysins of several *Bacillus* phages displayed similarities to autolysins of *Bacillus* species (LOESSNER et al. 1997). Some autolysin are known to be involved in the process of differentiation leading to sporulation and finally lysis of the mother cell. It is important to mention that Ply3626 also displayed similarities to autolysins, such as CwlB (LytC) from *Bacillus*. Another interesting finding is that the *skin* element in *Bacillus subtilis* is a cryptic prophage. This prophage is integrated into the gene encoding the mother cell specific RNA polymerase sigma factor σ^K and must be excised by a site-specific recombinase, encoded in the *skin* element itself. This maturation results in a functional σ^K, which is needed for the transcription of late sporulation genes (STRAGIER et al. 1989; KROGH et al. 1996).

Altogether, these findings point towards a potential effect of lysogeny on sporulation. In fact, correlation of prophage carrier state and sporulation ability is

presently being investigated, and preliminary results (not included in this thesis) support the above hypothesis.

No direct evidence for an influence of ϕ3626 on the pathogenicity or virulence of *C. perfringens* is yet available. However, it is noteworthy that the expression of the Cpe enterotoxin, which is responsible for the food poisoning effect of *C. perfringens*, is also dependent on sporulation events, and transcription of *cpe* relies on the activition of sporulation-associated promoters (ZHAO and MELVILLE 1998). Further studies are needed to identify the precise role of these sequences in ϕ3626. It is intriguing to note that they are homologous to the recognition sites of sporulation-dependent sigma factors from *B. subtilis*, which are also similar to the proposed ϕ3626 sigma factor Gp32.

4.6 Lysis system of ϕ3626

New approaches are needed for the control of disease-causing organisms such as *Clostridium perfringens*, which produces an enterotoxin frequently implicated in foodborne disease and has a strong impact on the poultry industry (SONGER 1996; STEVENS 1997; MCCLANE et al. 2000; MCCLANE 2001). As a first step towards this approach, the cell wall lytic system of *C. perfringens* phage ϕ3626 has been investigated. This is the first description of lysis functions from a clostridial bacteriophage. The genes are localized at the 3'-end of the "late" genes region next to the lysogeny module, in a dual gene cluster. These findings are consistent with the situation in several other bacteriophages of the Caudovirales family (LUCCHINI et al. 1999b; WANG et al. 2000).

The function of the proposed holin and endolysin gene products could been demonstrated experimentally. Holins are proteins which are thought to first insert themselves into the cytoplasmic membrane, and subsequently oligomerize to form unspecific lesions to permit the cognate endolysin to access the murein (YOUNG and BLÄSI 1995). The ϕ3626 *hol* gene does not contain a dual start motif, which plays an important role in S-mediated lysis timing and was also found in many other holin genes (YOUNG and BLÄSI 1995; BLÄSI and YOUNG 1996). However,

expression in a λ background showed that Hol3636 can substitute for S in R-mediated cell lysis, indicating not only that the clostridial phage holin is fully functional in an *E. coli,* but also suggesting that it contains its own intrinsic timing function. Moreover, one can speculate that its mode of action might be substantially different from S. The primary structure of Hol3626 features two MSDs, which classifies it as a novel entry among the group II holins (YOUNG and BLÄSI 1995). As reasoned in 3.6.7, the "positive-inside" rule (VON HEIJNE 1992) suggests that the charged domain in between the MSDs might be located in the cytoplasm, which would mean an "outside" location of the termini (see Fig. 6 C). According to WANG et al. the most conserved feature of holins, regardless of their class, is that they possess a highly charged C-terminal domain, which with its multiple basic residues, seems likely to be disposed in the cytoplasm (WANG et al. 2000). As shown by protease accessibility in spheroblasts and inverted membrane vesicles this is at least true for the S holin from bacteriophage λ, the class I prototype holin (BLÄSI et al. 1999). Additionally, for λS, it has been shown that the C-terminal sequences define a cytoplasmic regulatory domain which affects the timing of lysis. Also Phage 21 S, the prototype class II holin seems to have the C-terminus located in the cytoplasm (RAMANCULOV and YOUNG 2001). Interestingly, the T4 holin, which seems to have only one MSD, is thought to be orientated in a way that the C-terminus is also not located in the cytoplasm (RAMANCULOV and YOUNG 2001). All these findings indicate that Hol3626, with a "C-outside, N-outside" orientation, represents an unusual and novel topology among the extremely diverse family of holin proteins which certainly warrants further investigations.

Located immediately downstream of the holin gene, *ply* encodes the ϕ3626 endolysin, which probably functions as an amidase. In contrast to the N-terminus, the Ply3626 C-terminal portion is unrelated to other lytic enzymes. It is known that bacterial peptidoglycan hydrolases, in particular those encoded by phages, display a modular domain structure. They usually harbor at least one enzymatically active domain, linked to a corresponding cell wall binding domain, which targets the enzyme to its site of action (GARCIA et al. 1990; LOESSNER et al. 2002). It has been

demonstrated that such binding domains confer recognition specificity on the hydrolases (BABA and SCHNEEWIND 1996; LOESSNER et al. 2002). Since Ply3626 exhibits a very highly evolved specificity towards *C. perfringens* cell walls, it is reasonable to assume that its unique C-terminus harbors a cell wall binding domain, which targets the enzyme to a specific receptor found exclusively on these cells. Loss of lytic activity in the truncated HPL3626ΔC enzyme (containing the isolated amidase module) also indicated the crucial role of the C-terminus in lysis. This hypothesis is also supported by the similar structure and function of two *Listeria* phage endolysins (Loessner et al. 2002). However, it is in contrast to the findings for the multidomain murein hydrolases of *Staphylococcus aureus* phages, where up to 75% of the enzymes could be deleted, sometimes with even increased lytic activity (BABA and SCHNEEWIND 1996; LOESSNER et al. 1998; LOESSNER et al. 1999). This difference of the staphylococcal endolysins to the ϕ3626 endolysin might be explained by the multiple domain architecture of these relative large enzymes, which allows the removal of some portions of the native proteins without losing the actual lytic action (NAVARRE et al. 1999). It has been shown that this is not the case for the listerial endolysins (LOESSNER et al. 2002) and it also seems to be the case for *ply3626.*

The gene encoding for Ply3626 was cloned and expressed in *E. coli.* Efficient production of native and modified (6×His-tag) endolysin was only possible under conditions compensating for the unusual codon usage by *ply3626*, due to a very low G+C content. An *E. coli* vector containing additional copies of the rarely used t-RNA genes *ileX* and *argU* under control of a constitutive promoter became recently available (ZDANOVSKY and ZDANOVSKAIA 2000). Although these observations were similar to the difficulties encountered by other researchers attempting expression of clostridial proteins in *E. coli* (GARNIER and COLE 1986; GARNIER and COLE 1988; ZDANOVSKY and ZDANOVSKAIA 2000), the next hurdle in production of these enzymes, i.e., formation of insoluble inclusion bodies after induction, was unexpected. This seems to be rare with respect to highly soluble and charged proteins such as cytoplasmic endolysin. Lowering the expression level by omitting IPTG and reducing growth temperature apparently promoted

correct protein folding and solubility, so that inclusion bodies did not form. Still, more research is needed to better understand the molecular and biochemical properties of the Ply3626 enzyme, in particular its stability and aggregation behavior.

HPL3626 exhibited stringent substrate specificity towards *C. perfringens* cells, although some variation in lysis sensitivity was observed among the different strains. Besides *C. perfringens*, only *Clostridium fallax* was found to be sensitive to the murein hydrolase. The latter organism is also regarded as a pathogen associated with cases of gas gangrene (STEVENS 1997), albeit with a lower frequency. Most of the clostridial species tested in this study belong to a phylogenetically closely related 16S rDNA cluster I (STACKEBRANDT and RAINEY 1997), encompassing more than half of the pathogenic species (STACKEBRANDT et al. 1999). Here it is interesting to note that *C. perfringens* and some strains of *C. fallax* share the same type of peptidoglycan (A3?), which features LL-diaminopimelic acid-glycine bridge to the terminal alanine of the opposite peptide chain, instead of meso-diaminopimelic acid as found in most other clostridia (SCHLEIFER and KANDLER 1972). These peptide subunits are in close proximity to the bond that is cleaved by the amidase, and, beside the putative cell wall binding domain, might also have an effect on substrate specificity.

The enzyme is very active on clostridial cells when added exogenously, because of the direct accessibility of cell wall peptidoglycan from "without". The high sensitivity to lytic enzymes under otherwise non-disruptive conditions is a particular feature of most gram-positive bacteria, and is particularly "useful" when applying native or recombinant phage endolysins for cell lysis (LOESSNER et al. 1995b; LOESSNER et al. 1997; LOESSNER et al. 1998; LOESSNER et al. 1999; LOEFFLER et al. 2001; NELSON et al. 2001). The highly specific action of Ply3626 on *C. perfringens* cells forms the basis for several potential applications of this enzyme, preferably as a recombinant product. One of the foremost goals for future developments will be the evaluation of its usefulness as a novel antimicrobial additive, with respect to the control or growth prevention of *C. perfringens* in poultry intestine. In such applications, it is of utmost importance not to affect

organisms other than the target bacteria, in order to leave the natural microbial communities undisturbed. Based on the present data, Ply3626 may fulfill this requirement. The application as a novel biopreservative in food items such as raw chicken or turkey may also be considered. The enzyme could be directly added to food or feed (LOEFFLER et al. 2001; NELSON et al. 2001), or, alternatively, be produced by recombinant bacteria. *Lactococcus lactis* has been used to produce and secret a *Listeria* phage amidase endolysin, which remained fully active and was able to kill *L. monocytogenes* cells in the medium (GAENG et al. 2000).

The use of the bacteriophage itself, in a "therapeutic" approach to control *C. perfringens,* may be also be considered as a possible way to harness the specificity of bacterial viruses (SUMMERS 2001). However, due to its narrow lytic host range, ϕ3626 would reach only approximately 22 % of the tested *C. perfringens* strains, and the lytic range of the other *C. perfringens* phage ϕ8533 is also only 8 %. These relatively narrow host ranges would require pre-therapeutic testing, or pooling of a larger number of different phages. Moreover, the likely appearance of resistance and lysogenization would limit the killing potential of native, unmodified phage. On the other hand, development of a resistance against the activity of bacteriophage endolysins has not been reported. This finding has been linked to the necessity of the phage not to be trapped inside the host cell and thereby targeting unique and highly conserved bonds in the cell wall (LOEFFLER et al. 2001).

The results of the study of the lytic system of ϕ3626 are certainly very promising. However, the potential use of Ply3626 for the prevention or biological control of the pathogen *C. perfringens* requires further studies, in particular the function of the enzyme in a food environment, and its potential effects in the intestine of infected poultry.

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APPENDIX

Following Genbank entries can also be retrieved from the National Center for Biotechnology Information (NCBI) under:

AY082069

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=nucleotide&list_uids=19908292&dopt=GenBank

AY082070

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=nucleotide&list_uids=19908294&dopt=GenBank

Genbank entry AY082069

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LOCUS       AY082069                834 bp    DNA     linear   BCT 02-APR-2002
DEFINITION  Clostridium perfringens GMP synthetase (guaA) gene, partial cds.
ACCESSION   AY082069
VERSION     AY082069.1  GI:19908292
KEYWORDS    .
SOURCE      Clostridium perfringens.
  ORGANISM  Clostridium perfringens
            Bacteria; Firmicutes; Bacillus/Clostridium group; Clostridia;
            Clostridiales; Clostridiaceae; Clostridium.
REFERENCE   1  (bases 1 to 834)
  AUTHORS   Zimmer,M., Scherer,S. and Loessner,M.J.
  TITLE     Complete genome sequence, molecular analysis and integration
            properties of Clostridium perfringens bacteriophage phi3626
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 834)
  AUTHORS   Zimmer,M. and Loessner,M.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAR-2002) Institute of Microbiology, FML
            Weihenstephan, Technical University of Munich, Weihenstephaner Berg
            3, Freising 85350, Germany
FEATURES             Location/Qualifiers
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      121 gaaagtggaa cagatacttc agcagtaatt aagagtcacc ataacgttgg aggacttcca
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DEFINITION  Bacteriophage phi3626, complete genome.
ACCESSION   AY082070
VERSION     AY082070.1  GI:19908294
KEYWORDS    .
SOURCE      bacteriophage phi3626.
  ORGANISM  bacteriophage phi3626
            Viruses; dsDNA viruses, no RNA stage; Caudovirales; Siphoviridae.
REFERENCE   1  (bases 1 to 33507)
  AUTHORS   Zimmer,M., Scherer,S. and Loessner,M.J.
  TITLE     Bacteriophage phi3626 complete genome
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 33507)
  AUTHORS   Zimmer,M., Scherer,S. and Loessner,M.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (05-MAR-2002) Institute of Microbiology, FML
            Weihenstephan, Technical University of Munich, Weihenstephaner Berg
            3, Freising 85350, Germany
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CURRICULUM VITAE

MARKUS ZIMMER

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