DISSERTATION

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Novel antimicrobial agents against Gram-negative and Gram-positive bacteria: A concept study

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Dedication

To my parents, sister and Sona; who have been a constant source of inspiration in my life and career

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2. Zusammenfassung

Am Anfang des zwanzigsten Jahrhunderts erfolgte der Einsatz von Bakteriophagen zur Bekämpfung bakterieller Infektionen, dies wurde jedoch im Zuge der Entdeckung der Antibiotika weitestgehend eingestellt. Ein Neubeginn der Phagentherapie erfolgte erst mit dem Auftreten von Antibiotika Resistenzen in Bakterien. Phagen kodierte Endolysine erwiesen sich in Tiermodell-Studien als effiziente Wirkstoffe gegen Gram-positive Krankheitserreger, die aber aufgrund der geringen Löslichkeit nur bedingt einsetzbar waren. In dieser Arbeit wurde ein chimeres Endolysin mit verbesserter Löslichkeit und hoher Spezifität gegen Staphylococcus aureus (*S. aureus*) entwickelt. Der Bakteriophage P68 gehört zur Familie der Podoviridae und intiziert *S. aureus* Stämme. Versuche das Endolysin P16 des Phagen P68 zu isolieren scheiterten an der geringen Löslichkeit des Proteins. Daher wurde das rekombinante P16-17 Endolysin generiert. Das P16-17 Endolysin besteht aus der enzymatisch aktiven N-terminalen D-alanyl-glycyl-endopeptidase Domäne (DAGE) des P16 Endolysins und der C-terminalen Zellwand bindenden Domäne des P17 Hüllproteins des Phagen P68. Dieses chimere Endolysin zeigte neben guter Löslichkeit eine antimikrobielle Wirkung gegen *S. aureus*. Weiterhin verstärkte das P16-17 Endolysin die Wirkung des Aminoglycosid-Antibiotikums Gentamycin. Dieser synergistische Effekt könnte ausgenutzt werden, um die effektive Dosis von Aminoglycosid-Antibiotika zu reduzieren.

Das bakterielle Ribosom ist ein Hauptangriffspunkt vieler klinisch relevanter Antibiotika und es sind eine Vielzahl von verschiedenen Inhibitionsmechanismen am Ribosom bekannt. Die intensive Nutzung von Antibiotika führte in den letzten Jahren zum Auftreten immer neuer Resistenzen und zu Bakterienstämmen die gegen mehrere Antibiotika (´´Multi-Drug-Resistent Stämme'') resistent sind. Die eingeschränkte Nutzbarkeit von Antibiotika gegen resistente Stämme verlangt nach neuen Therapeutika. In der hier durchgeführten Pilotstudie sollte die Möglichkeit der Inhibition der Assemblierung des Ribosom als neues Ziel eines

Therapeutikums überprüft werden. Das ribosomale Protein S1 ist für die Translation der zellulären messenger RNA (mRNA) in Gram-negativen Bakterien essentiell. Die Bindung dieses Proteins an das Ribosom erfolgt über das ribosomale Proteine S2. Eine Störung der Interaktion der beiden ribosomalen Proteine S1 und S2 könnte somit zu einem Erliegen der Proteinsynthese führen und ist damit ein geeignetes Ziel die Proteinsynthese selektiv in Bakterien zu unterbinden, da in höheren Organismen kein S1 Homolog vorkommt.

In den hier durchgeführten ``Yeast-two-hybrid Interaktionsstudien'' der ribosomalen Proteine S1 und S2 wurden verschiedene verkürzte Varianten des Protein S1 benutzt. Diese Studien zeigten, das die N-terminale Domäne des S1 ($S1_{106}$) ausreichend für die Interaktion mit dem Ribosom ist. In einer Mutationsanalyse konnte weiterhin eine Variante von $S1_{106,}$ gefunden werden, die eine stärkere Interaktion mit dem Protein S2 zeigte.

Weiterhin konnte durch Anwendung der Yeast-two-hybrid Interaktionsstudien der Interaktionsbereich des Proteins S2 mit Protein S1 auf eine Subdomäne (Helix-Turn-Helix zwischen Aminosäure 140 und 150) eingegrenzt werden. Diese Interaktion konnte *in vitro* mit isolierten Mutanten Proteinen bestätigt werden. Die ermittelten Details der Interaktion von Protein S1 und S2 eröffnet eine Basis für das Design von neuen chemischen Verbindungen, die diese Protein-Protein Interaktion verhindern und somit ein selektives Therapeutikum gegen Gram-negative Bakterien bietet könnte.

3. Summary

Bacteriophage therapy has been used at the beginning of the 20th century to combat bacterial infections. With the discovery of antibiotics, phage therapy was largely abandoned in western countries. However, the rise of antibiotic resistant bacteria has created renewed interest in phage as antimicrobials. In addition, bacteriophage endolysins were shown to be effective against Gram-positive pathogens in animal models and resistance to phage endolysins has not been reported. However, most endolysins from phages of Gram-positive bacteria show a poor solubility. In this work, a chimeric endolysin with an improved solubility was created that exerted a high specificity against *Staphylococcus aureus*.

Phage P68 belongs to the family of podoviridae and infects several *S. aureus* strains including nosocomial MRSA (``Methicillin resistant *S. aureus*'') strains. Attempts to purify the P16 endolysin of phage P68 were unsuccessful owing to the insolubility of the protein. This limitation was overcome by the construction of a chimeric P68-derived endolysin, P16-17. P16-17 consists of the inferred N-terminal D-alanyl-glycyl endopeptidase domain (DAGE) of P16 and the C-terminal cell wall binding domain of minor coat protein, P17. The domain swapping approach and the applied purification procedure resulted in soluble P16-17 protein, which exhibited antimicrobial activity towards *S. aureus*. In addition, P16-17 augmented the antimicrobial efficacy of the antibiotic gentamicin. This synergistic effect could be useful to reduce the effective dose of aminoglycoside antibiotics.

The ribosome represents a major target for many clinically relevant antibiotics. However, the extensive use of antibiotics to treat bacterial infections has lead to drug resistant pathogens. Here a pilot study was conducted with the aim to inhibit ribosome assembly. As protein S1 is known to be essential for translation in Gram-negative bacteria and ribosomal protein S2 is required for binding of S1 to the ribosome, the S1/S2 interaction was studied. A yeast two hybrid system was exploited to study the interactions of protein S1 and truncated variants

thereof with protein S2. A significantly better interaction was noticed with the truncated variant of protein S1, $S1_{106,}$ encompassing 106 amino acids from the N-terminal region. A mutant variant of $S1_{106,}$ $S1_{106MUT}$ with changes in two amino acid residues interacted stronger with protein S2 when compared with the wild type $S1_{106}$. Both $S1_{106}$ and $S1_{106MUT}$ were able to bind to ribosomes and caused cell death upon over-production. With the aim to define the protein S2 domain, contacting S1, the helix-turn-helix motif situated between 104 and 150 was scrutinized, and was shown to interact with protein S1 as well as with protein $S1_{106}$. These studies provide a basis for the design of compounds which could possibly disrupt S1/S2 interactions, and thus could act semi-selectively against Gram-negative pathogens.

4. Introduction

4.1. *Staphylococcus aureus*

The genus *Staphylococcus* belongs to the family *Micrococcaceae*, which appear as grape-like clusters when viewed through a microscope. *Staphylococcus aureus* forms large, round, golden-yellow colonies (Fig. 1), often with β-hemolysis when grown on blood agar plates (Lowy, 1998). The golden appearance of the colonies is the etymological root of the bacteria's name: *aureus* means "golden" in latin. *S. aureus* falls in the category of facultative anaerobes and opportunistic pathogens (Wilkinson, 1997).

The genome of *S. aureus* consists of a circular chromosome of approx. 2.8 Mbp. Resistance of *S. aureus* to antibiotics is brought about by genes located either on the chromosome or on plasmids (Novick, 1990). *S. aureus* can adapt to a wide range of growth conditions including growth over a temperature range of 15 to 45 degrees and NaCl concentrations as high as 15 percent. *S. aureus* forms part of the normal flora of humans found on nasal passages, skin and mucous membranes.

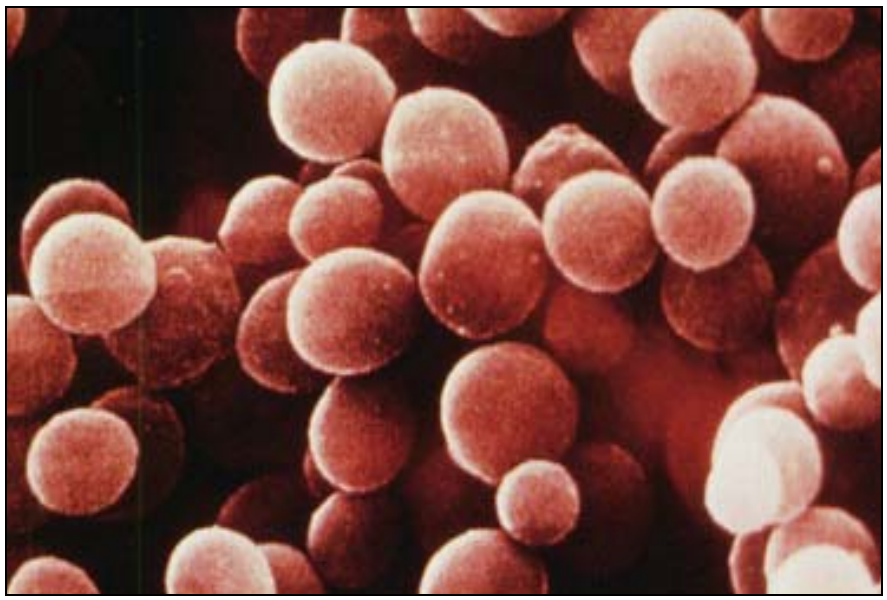

Fig. 1. Electron micrograph of *Staphylococcus aureus*

4.1.1. Cell wall structure and capsule of *S. aureus*

Peptidoglycan constitutes 50% of the weight of *S. aureus*. The peptidoglycan structure of *S. aureus* is comprised of N-acetylglucosamine and N-acetylmuramic acid with β-1, 4 linkages. The tetrapeptide chains bound to N-acetylmuramic acid are cross-linked by penta-glycine linkages (Fig. 2), which is a unique feature of the *S. aureus* cell wall (Lowy 1998). In addition, accessory compounds such as teichoic acids, teichuronic acids, polyphosphates or carbohydrates are attached to the peptidoglycan skeleton. Ribitol teichoic acids are major constituents of the staphylococcal cell wall. The poly-ribitol phosphates, bearing a negative charge, are covalently attached to the peptidoglycan. Lipoteichoic acids are glycerol phosphate polymers linked to a glycolipid terminus anchored in the cytoplasmic membrane (Navarre and Schneewind, 1999).

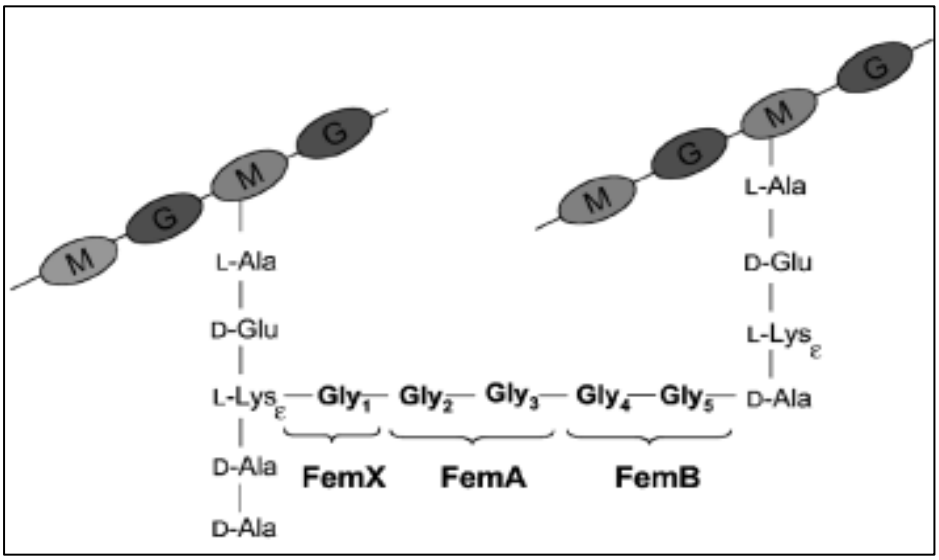

Fig. 2. Cell wall structure of *S. aureus*. The FemX, FemA and FemB are enzymes involved in the formation of the penta-glycine linkages (Schneider *et al*., 2004).

Most of the *staphylococci* produce microcapsules. Microcapsule formation helps the bacteria to evade the immune system of the host and also plays an important role in increasing virulence (Lee and Lee, 2006). Among the 11 types of staphylococcal microcapsular serotypes that have been identified, type 5 and 8 account for 80% of human infections (Arbeit *et al*., 1984). Most methicillin-resistant *S. aureus* isolates belong to type 5. The chemical composition of four of these antiphagocytic polysaccharide microcapsules, including type 5 and 8, has been determined. Type 5 and 8 microcapsules are composed of acidic sugar moieties (Fox *et al*., 1998).

4.1.2. Pathogenicity of *S. aureus*

Staphylococcus aureus causes a variety of infections ranging from minor skin lesions like boils and furunculosis to complicated and life threatening infections like pneumonia, mastitis, phlebitis, meningitis, and urinary tract infections. *S. aureus* also causes deep-seated infections such as osteomyelitis and endocarditis. A significant number of hospital acquired infections are caused by *S. aureus*. *S. aureus* can also cause food poisoning by releasing enterotoxins into food, and toxic shock syndrome by the release of superantigens into the blood stream. Virulence factors (Fig. 3) produced by *S. aureus* include (i) surface proteins that promote colonization of host tissue, (ii) invasins (eg: kinases, hyaluronidase, leukocidin) that promote bacterial spread in tissues, (iii) surface factors (capsule, protein A), which inhibit phagocytic engulfment, (iv) membrane damaging toxins (hemolysins, leukotoxin, leukocidin) that lyse eukaryotic cells and (v) exotoxins (SEA-G, TSST, ET) which can damage host tissue.

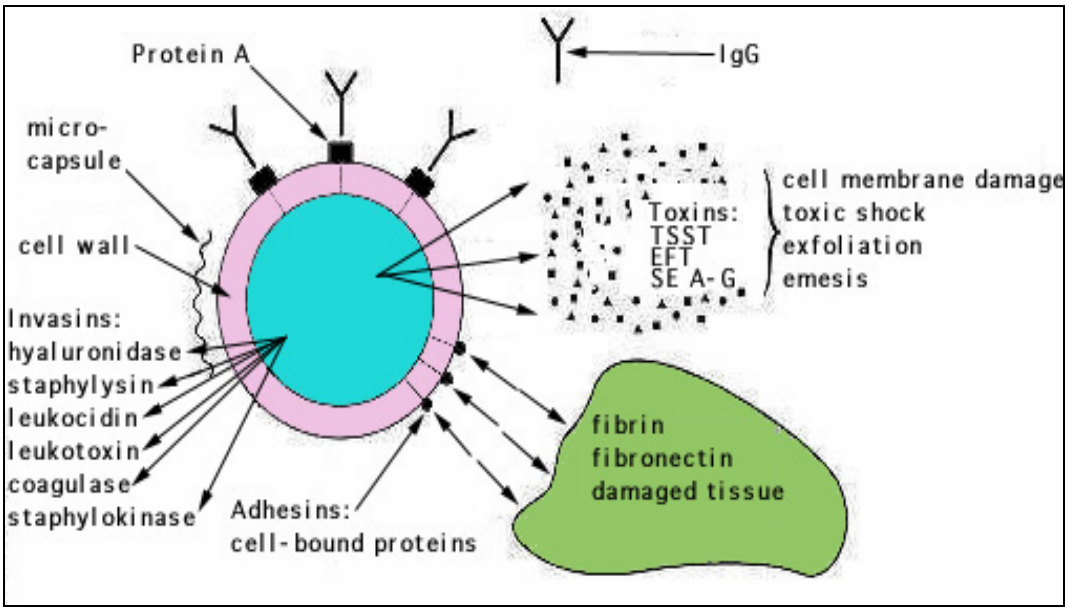

Fig. 3. Virulence factors of *Staphylococcus aureus*

There are several features in common to most of the surface virulence factors produced by *S. aureus*. These include the presence of a secretory signal sequence at the N-terminus, a hydrophobic membrane spanning domain and a C-terminal cell wall anchoring domain. A ligand binding domain at the N-terminal of the surface factors allows these surface proteins to function as adhesins (Foster and McDevitt, 1994). An example of a surface protein functioning as an adhesin is *S. aureus* surface protein G (SasG). SasG promotes adhesion of *S. aureus* to nasal epithelial cells and thereby contributes to colonization (Roche *et al.*, 2003). Moreover, SasG is involved in biofilm formation (Corrigan *et al*., 2007).

S. aureus produces a wide range of toxins. The enterotoxins produced by *S. aureus* are known elements in staphylococci associated food poisoning. *Staphylococcus aureus* strain Fukuoka 5 carries two plasmid encoded enterotoxins, SES and SET. (Ono *et al*., 2008). The extracellular toxins, on the other hand contribute to the pathogenicity of the bacteria (Marrack and Kappler 1990). One of the best studied extracellular toxins are the cytotoxins, which functions in establishing infections by converting the local tissues at the site of infection to a suitable environment that is required for growth of *S. aureus* (Fueyo *et al*., 2005)**.** There are

numerous cytotoxins produced by *S. aureus*. Among these, the most important and widely studied class of cytotoxins are hemolysins and panton-Valentine leukocidin, also known as PV-leukocidin. There are four types of hemolysins, α, β, γ and δ (Dinges *et al*., 2000). The α-hemolysin binds to the membrane of susceptible cells, allowing the toxin to cause small pores in the cell membrane through which monovalent cations are released. In humans, platelets and monocytes are highly sensitive to α-toxin. The cascade of secondary reactions following toxin binding causes the release of cytokines that in turn trigger the production of inflammatory mediators. These events cause various symptoms of septic shock that occur during severe infections caused by *S. aureus* (Bhakdi and Tranum-Jensen, 1991).

The *S. aureus* β-toxin causes damage of membranes rich in sphingomyelin, thus forms the family of sphingomyelinase (Glenny and Stevens, 1989). It is highly haemolytic for sheep erythrocytes but not for rabbit erythrocytes. The differing susceptibility of erythrocytes to β-toxin may be due to the differing sphingomyelin contents of erythrocytes (Doery *et al*, 1965). The sphingomyelinase activity of the β-toxin does not cause lysis of the cells but leaves them vulnerable to a number of other lytic agents (Thelestam, 1983).

The γ-toxins are produced by numerous isolates of *S. aureus* (Lina *et al*, 1999). In contrast to the α and β toxins, γ-toxins are bi-component pore forming toxins (Sugawara *et al.*, 1997; Finck-Barbançon *et al*, 1991; Woodin, 1960). Two genes, *hlgA* and *hlgC,* code for the S component, whereas, *hlgB* encodes the F component of the γ-toxin. The individual components (S or F) of the γ-toxins are non functional (Prévost *et al*., 1995).

There exists a synergistic action between the γ-toxin and the Panton-Valentine leukocidin (PV). The genes *hlgA* and *hlgC,* encoding for the S-component of PV-leukocidin, are carried by a prophage and the F-component is contributed by the γ-toxin that is produced by *S. aureus* (Kamio *et al*, 1993). PV-leukocidin is a leukocytolytic toxin and has been epidemiologically associated with severe cutaneous infections (Dinges *et al*., 2000).

The δ-toxin, produced by many isolates of *S. aureus* is a surface active protein, which gets readily associated with hydrophobic membrane structures, and forms ion channels (Colacicco *et al.*, 1977; Mellor *et al*., 1988). The δ-toxin is able to lyse horse and human erythrocytes. Moreover, it inhibits binding of epidermal growth factor (EGF) to cell surface receptors and also inhibits water absorption in the ileum by activating adenylate cyclase (Kreger *et al*., 1971; Kapral, 1985; Umezawa *et al*, 1980).

A wide range of virulence factors are secreted by *S. aureus.* These include the superantigen toxins which include the enterotoxins (A to M) and the toxic shock syndrome toxin (TSST-1). They are also known as pyrogenic toxin superantigens (Novick *et al*., 2001). The function of TSST-1 include binding to the major histocompatibility complex (MHC II) thereby causing extensive T-cell proliferation and cytokine release, eliciting high fever, rash, diarrhea, vomitting and renal dysfunction (Marrack and Kapler, 1990). Staphylococci also produce various enzymes that are secreted. These include protease, lipase and hyaluronidase. These factors may facilitate spread of the infection to neighbouring tissues.

4.1.3. *S. aureus* resistance to antimicrobial agents

The acquisition of virulence factors and high level of antibiotic resistance makes *S. aureus* to a leading cause of infections in hospital settings, especially affecting immunocompromised patients (Mandell *et al*., 2001). One of the challenges in the treatment of nosocomial infections worldwide is the appearance of methicillin-resistant *S. aureus* (MRSA). MRSA strains carry an effective drug-resistance mechanism that can protect these pathogens against all members of the β-lactam family of antibiotics including methicillin, dicloxacillin, nafcillin, and oxacillin (Crisóstomo *et al*., 2001). The target of β-lactam antibiotics are penicillin binding proteins (PBP), which are involved in the final step of peptidoglycan synthesis. Bacteria have between four and eight PBPs. In contrast to Gram-negative bacteria, which produce β-lactamases, most Gram-positive bacteria gain resistance towards β-lactam

antibiotics by either changing the specificity of their PBPs or by acquiring new PBPs with lower affinity for antibiotics (Mouz *et al.,* 1998).

There are two types of MRSA strains: community-acquired MRSA (CA-MRSA) and healthcare-associated MRSA (HA-MRSA). CA-MRSA differs from HA-MRSA in clinical, bacteriological and epidemiological aspects (Amador-Miranda *et al*., 2008). The CA-MRSA strains are different from HA-MRSA with regard to antimicrobial susceptibility, which can be attributed to the presence of SCC*mec* (mobile genetic element carrying antimicrobial resistance genes) (Saïd-Salim *et al*., 2003). CA-MRSA produces certain virulence factors and exotoxins like PV-leukocidin. HA-MRSA produces superantigenic exotoxins and staphylococcal enterotoxin A (Tristan *et al.,* 2007). Vancomycin and teicoplanin are the most common glycopeptide antibiotics used to treat both CA-MRSA and HA-MRSA infections (Schentag *et al*., 1998). Vancomycin binds to the peptides of peptidoglycan precursors. This blocks the formation of glycosidic bonds between the sugar moieties by transglycosidase and peptide cross-links by trans-peptidase enzymes, resulting in the formation of a weak peptidoglycan, which is unable to withstand the internal osmotic pressure (Watanakunakorn, 1984). There is also an emergence of vancomycin-resistant *Staphylococcus aureus* strains (VRSA) (Sievert *et al*., 2008; Conly and Johnston, 2002). The resistance of *S. aureus* towards vancomycin can be attributed to the presence of *van* genes, which include *vanA* (codes for D-Ala-D-Lac ligase), *vanH* (encodes a dehydrogenase that produces D-lactate from pyruvate) and *vanX* (encodes a d,d-dipeptidase that destroys D-Ala-D-Ala). In addition, *vanR* and *vanS* encode a signal transduction system that senses vancomycin in the environment and induces expression of resistance genes (Woodford, 2001; Naas *et al*., 2005).

4.2. Bacteriophages

The term bacteriophage accounts for viruses that infect bacteria. Bacteriophages consist of an outer protein shell enclosing the genetic material. The genetic material may be single stranded

RNA, single stranded DNA, double stranded RNA or double stranded DNA. The size of the genetic material can vary from 5 to 500 kilo base-pairs, either in linear or circular orientation. Bacteriophages are ubiquitous and can be found in all reservoirs populated by bacteria, ranging from fresh water to sea water, and psycrophilic to thermophilic environments (Wommack and Colwell, 2000). The circular double stranded DNA phage account for 95% of the bacteriophage population. According to the International Committee on Taxonomy of Viruses, phage can be classified according to morphology and nucleic acid. The broad classification of double stranded tailed phages is shown in Table 1.

Table1. Classification of bacteriophages (Mc Grath and van Sinderen, 2007).

Family	**Morphology**	**Nucleic acid**
Myoviridae	Non-enveloped, contractile tail	Linear dsDNA
Siphoviridae	Non-enveloped, long non- contractile tail	Linear dsDNA
Podoviridae	Non-enveloped, short non- contractile tail	Linear dsDNA
Tectiviridae	Non-enveloped, Isometric	Linear dsDNA
Corticoviridae	Non-enveloped, Isometric	Circular dsDNA
Lipothrixviridae	Enveloped, rod shaped	Linear dsDNA
Plasmaviridae	Enveloped, pleomorphic	Circular dsDNA
Rudiviridae	Non-enveloped, rod shaped	Linear dsDNA
Fuselloviridae	Non-enveloped, lemon shaped	Circular dsDNA
Inoviridae	Non-enveloped, filamentous	Circular ssDNA
Microviridae	Non-enveloped, isometric	Circular ssDNA
Leviviridae	Non-enveloped, isometric	Linear ssRNA
Cystoviridae	Enveloped, spherical	Segmented dsRNA

4.2.1. Bacteriophage mediated lysis of bacteria

For most dsDNA phages, the infection cycle culminates in lysis of the host brought about by at least two phage-encoded proteins, the endolysin and the holin. Endolysins can cleave glycosidic, amide, or peptide bonds of the bacterial peptidoglycan**.** The holins on the other hand are diverse small membrane proteins with two or three trans-membrane domains (Wang *et al*., 2003). In phage λ, the holin and endolysin are encoded by gene S and R, respectively. Both of these genes are located at the beginning of the late transcriptional unit. The endolysin accumulates in the cytoplasm following the activation of the late transcriptional unit (Young *et al*., 2000). At a 'genetically determined time' the holin forms inner membrane lesions through which the endolysin can gain access to the peptidoglycan (Young *et al*., 2000; Young, 1992). The accumulation of the holin protein has negligible effect on the host bacteria until the completion of replication cycle. (Gründling *et al*., 2001; Wang *et al.,* 2000). The sequence of events involved in the formation of membrane lesions by holin is discussed in section 4.2.1.1.

All dsDNA phages utilize the holin-lysin system to achieve host cell lysis (Fig. 4) with the exeption of a few phages of Gram-negative bacteria, which use the *sec* system to target the endolysin to the periplasm (São-José *et al*., 2000). The secreted endolysins remain tethered at the periplasmic site of the inner membrane in an inactive form by the N-terminal SAR (Signal Arrest Release) sequence, that functions as a type II signal anchor (Xu, *et al.,* 2004; Xu, *et al*., 2005). Upon depolarization of the membrane by the holin, the N-terminal SAR sequence of the endolysin is released, which causes the activation of the endolysin, followed by cell lysis (Xu *et al*., 2005). Phages of the Gram-negative bacteria, P1 and 21 use SAR endolysins to evoke host cell lysis. Furthermore, holins are not absolutely essential for cell lysis by SAR-endolysins. However, they are thought to control the timing of the lytic event, by depolarizing the membrane which accelerates the release of the SAR lysozyme from the periplasmic membrane (Xu *et al*., 2004; Park *et al*., 2006).

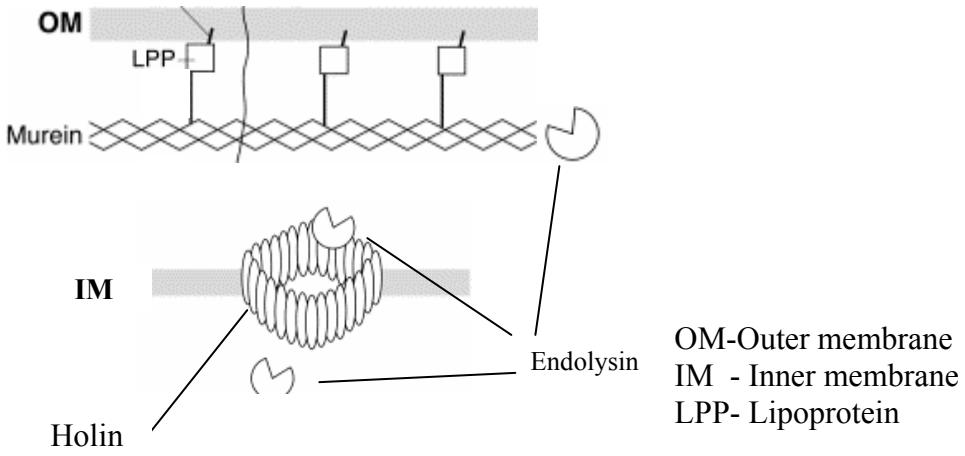

Fig. 4. Lysis of Gram-negative bacteria by a phage-encoded holin-endolysin system (modified from Young *et al*., 2000).

In addition to holins and endolysins, two accessory phage encoded lysis proteins are involved in lysis of Gram-negative bacteria *in vivo* when the outer membrane is stabilized by divalent cations, like Mg^{2+} (Zhang and Young, 1999; Summer *et al*., 2007). In phage λ, these proteins are the gene products of *Rz* and *Rz1*. Equivalents of *Rz* and *Rz1* are found in nearly all phages of Gram-negative bacteria including phages, T7 and P2 (Summer *et al*., 2007). The Rz/*Rz1* equivalent in phage T7 is *gp18.5/gp18.7* and *lysB/lysC* in phage P2. The Rz/*Rz1* and *gp18.5/gp18.7* genes are overlapping in phage λ and T7, whereby the genes *Rz1* and *gp18.7* are embedded within *Rz* and *gp18.5*, respectively. In contrast, *lysB/lysC* from phage P2 exist in 'overlapped' orientation, where about half of the *lysC* gene extends beyong the *lysB* stop codon (Summer *et al*., 2007). The *Rz*, *gp18.5* and *lysB* encode a polypeptide with a predicted N-terminal transmembrane domain (TMD), and the *Rz1*, *gp18.7* and *lysC* genes encode outer membrane lipoprotein (Berry *et al*., 2008; Kedzierska *et al*., 1996). The interactions of the Rz and Rz1 proteins during the lytic cycle of phage λ facilitates the linking of the host inner and

outer membranes, hence lysis in the presence of high concentrations of divalent cations (Summer *et al*., 2007; Berry *et al*., 2008).

Cell lysis by ssDNA and ssRNA phages like ΦX174, MS2 and Qβ is achieved by the interaction of phage encoded proteins with host enzymes. The ΦX174 gene product *E,* required for host cell lysis is localized in the cytoplasmic membrane (Maratea *et al*., 1985). In comparision to other phage ΦX174 proteins, protein E is made in small amounts and was shown to interact with host proteins encoded by genes *slyD* and *MraY.* The SlyD protein is a peptidyl-prolyl *cis-trans* isomerase (PPIase), which is involved in accurate folding and stability of protein E (Young, 1992; Roof *et al*., 1994). The MraY translocase I on the other hand catalyzes the formation of the first lipid intermediate in peptidoglycan synthesis (Ikeda *et al*., 1991). Phage ΦX174 encoded protein E inhibits the MraY catalyzed step in peptidoglycan biosynthesis (Bernhardt *et al*., 2001a). By protein E binding to MraY the incorporation of a specific peptidoglycan precursor, diaminopimelate (DAP), is inhibited (Zheng *et al*., 2008; Bernhardt *et al*., 2001b).

Similar to phage ΦX174 protein E, protein L of RNA phage MS2 is synthesized in very small quantities (Coleman *et al*., 1983; Kastelein *et al*., 1982). Protein L is a membrane protein which seems to be present at membrane adhesion sites of the cell envelope (Walderich *et al*., 1988; Walderich and Höltje, 1989). However, the insertion of protein L alone is not sufficient for lysis. Moreover, the membrane lesion effected by protein L is unable to release the 28 nm MS2 virions. Protein L appears to trigger the lytic transglycosylase and DD-endopeptidase proteins, which exert muralytic activity leading to cell lysis and consequently to phage release (Walderich *et al*., 1988; Goessens *et al*., 1988).

Unlike phage ΦX174 protein E, which specifically inhibits the incorporation of DAP into the murein, protein A_2 of phage Qβ evokes host cell lysis by inhibiting the MurA (Bernhardt *et al*., 2001b). MurA is a UDP-N-acetylglucosamine enol-pyruvyl transferase, which catalyzes the formation of UDP-N-acetylglucosamine enol-pyruvate from UDP-N-

acetylglucosamine (Nanninga, 1998). Protein A_2 inhibits MurA by a tight and direct binding to its 'substrate-binding cleft' (Bernhardt *et al*., 2001b). Interestingly, protein A_2 is a multifunctional capsid protein of phage Qβ. In addition to its role in host cell lysis, protein A_2 also facilitates binding of the phage to the sex pilus of the bacteria during infection (Winter and Gold, 1983). When synthesized, protein A_2 remains inactive until it becomes assembled onto the phage capsid. This spatial activity allows the precise timing of cell lysis (Hatfull, 2001).

4.2.1.1. Holins

As mentioned above, the timing of lysis of host cells during the lytic cycle of many dsDNA phage is controlled by holins (Wang *et al*., 2000; Young *et al*., 2000). Holin proteins induce a non-specific 'hole' in the cytoplasmic membrane through which the endolysins can access the peptidoglycan (Young, 1992). The holin proteins can act in concert with heterologous endolysins to achieve host cell lysis, suggesting that they are not specific for their cognate endolysins (Rietsch and Bläsi, 1993). The holin proteins are synthesized in low amounts throughout the lytic cycle of phage λ, probably with an efficacy of less than one translation per minute per mRNA (Rolfe and Campbell, 1977; Bläsi *et al*., 1989; Chang *et al*., 1995; Nam, 1991). During the lytic cycle, the accumulation of holin has a negligible effect on the host (Gründling *et al*., 2001; Wang *et al*., 2000).

Holins can be classified based on their membrane topologies. Class I holins have three transmembrane domains (TMD) with an 'N-out' 'C-in' topology, i.e. the N-terminus faces the periplasm, whereas the C-terminus is located towards the cytoplasm. Phage λ holin is an example of a class I holin, comprising 105 amino acid residues (Bläsi *et al*., 1999; Gründling *et al*., 2000b). Class II holins, on the other hand are shorter (65-85 amino acid residues) and are comprised of two TMDs in an 'N-in', 'C-in' topology, with both the N-terminal and C-terminal domain facing towards the cytoplasm (Young, 2002). Both, class I

and II holins feature a highly charged C-terminal domain, generally rich in basic residues (Gründling *et al*., 2000b). The C-terminal domain of phage λ holin is non-essential for its function (Bläsi *et al*., 1999). A third type of holin is represented by class III holins. Phage T4 encodes a class III holin, with one TMD and an exeptionally large periplasmic domain (Tran *et al*., 2005).

During the lytic cycle ~1000-5000 molecules of the λS holin protein accumulate in the inner membrane (Zagotta and Wilson, 1990; Smith *et al*., 1998). The precision of host cell lysis by holin protein in phage λ is determined by yet another protein, the anti-holin. In phage λ, the holin and anti-holin are products of the *S* gene. The peculiar feature of the lambda *S* gene is the presence of two initiation codons, Met-1 and Met-3. The initiation of translation at Met-1 yields the anti-holin termed S107, which comprises 107 amino acids. Translation initiation at Met-3 gives rise to the holin protein, designated S105. Despite the fact that the primary gene *S* products differ only by the presence of Met1-Lys2 at the N-terminus of S107, both proteins have opposing functions (Bläsi and Young, 1996; Bläsi *et al*., 1990; Bläsi *et al*., 1989; Raab *et al*., 1988). The opposing function of the S107 has been attributed to the extra positive charge contributed by the Lys2 residue at the N-terminus. This positive charge retards translocation of the N-terminal transmembrane domain (TMD1) of S107 relative to that of S105. As S107 and S105 form heterooligomers, these assemblies prevent formation of a 'hole' in the membrane (Gründling *et al*., 2000a). Dissipation of membrane potential at the end of the lytic cycle or artificially by energy poisons triggers translocation of the N-terminal TMD1 of S107 across the cytoplasmic membrane and allows formation of the lesion in which S105 and S107 proteins are topologically and functionally equivalent (Graschopf and Bläsi, 1999).

Similar to λ *S*, the holin gene of phage 21 has a dual start and encodes a holin ($S^{21}68$) and an anti-holin ($S^{21}71$) (Barenboim *et al*., 1999; Bonovich and Young, 1991). However, the phage 21, $S^{21}68$ holin has only two transmembrane domains with a predicted N-in, C-in

topology (Park *et al*., 2006). The TMD1 of S^{21} is enriched with small nonpolar residues (Gly, Ala) and polar uncharged residues (Ser, Thr). This feature of TMD1 of S^{21} is unusual for canonical transmembrane domains, but found in the SAR domain of phage P1 endolysin (Xu *et al*., 2004). Biochemical and physiological studies have attributed the 'hole forming activity' to TMD2 of S^{21}, thus making the 44 amino acid polypeptide the smallest known protein exhibiting properties of a holin (Park *et al*., 2006). It has been speculated that S^{21} is inserted into the cytoplasmic membrane as a hairpin structure with TMD1/TMD2 interactions. These interactions are thought to either mask the hydrophilic residues of TMD2 or prevent association of TMD2 with another TMD2 and block `hole` formation. Upon termination of the lytic cycle followed by membrane depolarization, the TMD1 exits from the cytoplasmic membrane to the periplasm similar to SAR endolysins (Xu *et al*., 2004; Xu *et al*., 2005). This may allow the interaction of two TMD2 domains, which in turn is facilitated by the interactions of two TMD1 domains in the periplasm, and consequently leads to `hole` formation. The functionality of the phage λ anti-holin equivalent of phage 21, $S^{21}71$, is due to the extra positive charge at the N-terminus, which delays the exit of TMD1 to the periplasm relative to that of S2168 holin (Park *et al*., 2006).

In contrast to the well characterized holins with either three (S105 of phage λ) or two ($S^{21}68$ of phage 21) transmembrane domains, phage T4 has a bitopic topology with one TMD (Tran *et al*., 2005). Phage T4 holin, T, is substantially larger than the other holins with 218 amino acid residues including 163 residues forming the C-terminal periplasmic domain (Wang *et al*., 2000). The anti-holin of phage T4, RI, shares no homology with the holin and is encoded by a different gene. RI inhibits the holin activity by specifically binding to T (Tran *et al*., 2005). Following superinfection of phage T4 infected cells by another T4 phage, there is an observed phenomenon known as `lysis inhibition` (LIN), where the normal lysis timing by the holin is overriden (Doermann, 1948; Hershey, 1946; Tran *et al*., 2005). The LIN state is unstable and requires continues superinfection for its maintenance. The addition of energy

poisons that lead to collapse of the membrane potential completely subverts the LIN state (Abedon, 1994). The holin and anti-holin of phage T4 play a major role in establishing the LIN state in infected cells (Ramanculov and Young, 2001). The anti holin encompass an N-terminal SAR domain, which allows it to be released to the periplasm following the primary infection. However, the anti-holin undergoes spontaneous inactivation and subsequent degradation by DegP in the absence of superinfection. Following superinfection, the 'LIN signal' is recognized by the anti-holin and this interaction stabilizes the active conformation of the anti-holin, which in turn binds to the holin thereby extending the lysis time (Tran *et al*., 2007).

Recently, the holin protein from phage λ, S105, was shown to be cytotoxic against human tumor cells and was able to reduce tumor growth in a mouse model (Agu *et al*., 2006). The λ holin was localized in the endoplasmic reticulum (ER) and mitochondria of eukaryotic cells. It has been speculated that similar to 'channel-forming' proteins encoded by human pathogens, λ-holin directly affects ER calcium homeostasis (Rao *et al*., 2004; Agu *et al*., 2007). In Hela and MCF-7 cells, λ-holin caused mitochondrial membrane depolarization. The microscopic examination of the cell organelles revealed that mitochondria were dilated and rounded. The classical features associated with apoptotic cells including chromatin degradation was absent in λ-holin expressing cells (Kerr *et al*., 1995). However, a substantial number of double membrane-bound vesicles appeared, a characteristic feature of autophagosomes. Autophagosomes are well characterized in eukaryotic cells undergoing a non-apototic mode of cell death. Hence, λ-holin proteins are thought to mediate lysosomal autophagy in contrast to programmed cell death (Agu *et al*., 2007; Gozuacik and Kimchi, 2004; Reggiori and Klionsky, 2005).

4.2.1.2. Endolysins

The classification of phage encoded endolysins is based on the specific linkages they cleave in the peptidoglyacan. Endolysins include, (a) N-acetylmuramidases, (b) endo-β-N-acetylglucosaminidases, (c) transglycosylases, (d) endopeptidases, (e) N-acetylmuramoyl-L-alanine amidase (Fig. 5) (Young *et al*., 2000; Loessner, 2005; Fischetti, 2004; Fischetti, 2005).

N-acetylmuramidases hydrolyze the β-1, 4 O-glycosidic bonds between N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc), thereby, reducing the glycan chains to disaccharide units. Phage T4 endolysin is an example of a N-acetylmuramidases. The T4 endolysin is globular in structure, with a diameter of about 5 nm and exists in solution as a 14,000-M_r monomer (Creighton, 1984; Stojković and Rothman-Denes, 2007).

Endo-β-N-acetylglucosaminidases hydrolyse the N, N'-diacetyl chitobiose of the asparagine linked sugar moieties of the peptidoglycan (Takegawa *et al*., 1989). Endolysin of the *Streptococcus agalactiae* bacteriophage B30 is an example of a N-acetylglucosaminidase (Huard *et al.*, 2003; Donovan *et al*., 2006).

Another class of endolysins, the transglycosylases act similar to N-acetylmuramidases, cleaving β-1, 4 linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine residues (Höltje *et al*., 1975). Unlike muramidases, transglycosylases are not hydrolases and the cleavage of the β-glysocidic linkages yields 1, 6-anhydromuramyl residues (Blackburn and Clarke, 2001). Transglycosylases are the only class of endolysins which do not possess hydrolase activity (Loessner, 2005; Fischetti, 2004). The endolysin of phage ΦKZ, gp144 is an example of phage encoded transglyosylases (Fokine *et al*., 2008).

Endopeptidases cleave the peptide moiety of the peptidoglycan. The compact structure of the peptidoglycan is provided by the cross-linking of the backbone of the peptidoglycan sugar moieties by tetrapeptide linkages (Höltje, 1998). The endolysin lysH5 of the *S. aureus*

bacteriophage ΦH5 comprises endopeptidase activity, which specifically cleaves the D-alanyl-glycyl bond specific for *S. aureus* peptidoglycan (Obeso *et al*., 2008).

The most widely studied class of endolysins are the N-acetylmuramoyl-L-alanine amidases (NAM), which cleave the linkages between N-acetylmuramoyl and L-amino acid residues in the peptidoglycan (Hermoso *et al*., 2007). Phage T7 and *S. aureus* phage Φ11 endolysins are examples for NAM activity (Loessner, 2005; Hermoso *et al*., 2007).

The majority of the phage encoded endolysins display only one type of muralytic activity. However, a few phage encoded endolysins are bifunctional. The endolysin of *S. aureus* phage Φ11 harbours both, an amidase and an endopeptidase activity (Navarre *et al*., 1999). The *Streptococcus agalactiae* phage B30 endolysin possesses a glucosaminidase and an endopeptidase activity (Pritchard *et al*., 2004). Similarly, the *S. agalactiae* phage NCTC 11261 and *Staphylococcus warneri* M phage ΦWMY endolysins display endopeptidase/muramidase and endopeptidase/amidase activities, respectively (Cheng *et al*., 2005; Yokoi *et al*., 2005).

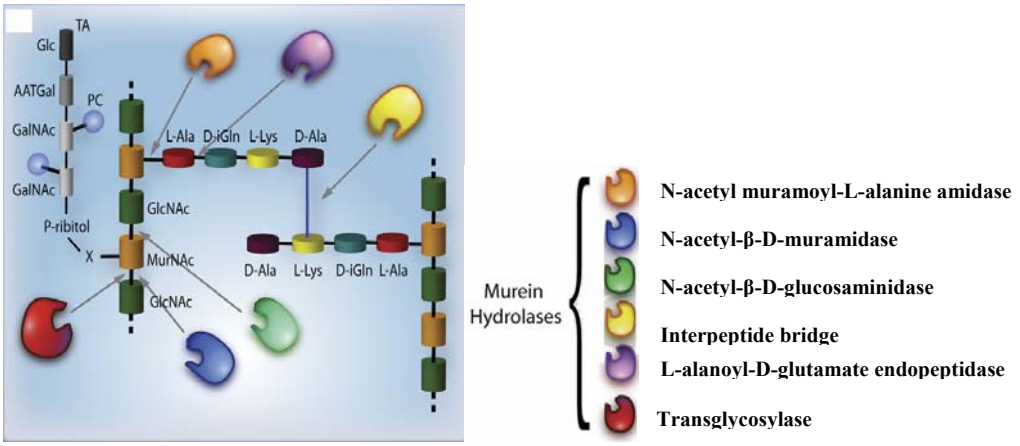

Fig. 5. Mode of action of different murein hydrolases (modified from Hermoso *et al*., 2007).

Most endolysins encoded by phages infecting the Gram-positive bacteria are characterized by a two-domain structure, comprising a muralytic active domain and a cell wall binding domain (Diaz *et al*., 1990; Garcia *et al*., 1990). The N-termini of these endolysins usually harbour the

catalytic domain, whereas the C-termini encompass the cell wall binding domain (López *et al*., 1997; Fischetti, 2005).

A few endolysins encoded by phages of Gram-positive bacteria have a multi-domain structure. Staphylococcal phage Φ11 endolysin has two catalytic domains linked to a single cell wall binding domain (Navarre *et al*., 1999). Streptococcus phage C_1 endolysin, PlyC, is a multimeric protein made up of several copies of PlyCA (Catalytic domain) and PlyCB (Cell wall binding domain) (Nelson *et al*., 2006).

The first phage endolysin with a choline binding domain (ChBP) to be crystallized was the Cpl-1 endolysin of pneumococcal phage Cp-1. The Cpl-1 endolysin (Fig. 6) is a flexible protein, where the catalytic domain and the cell wall binding domain are separated by a 10 amino acid hydrophilic linker sequence (Hermoso *et al*., 2003). The catalytic module of Cpl-1 has an irregular $(\beta/\alpha)_5\beta_3$ barrel, and the cell wall anchoring module is formed by six similar choline-binding repeats. The choline binding domains target the endolysin to the choline containing teichoic acid of the pneumococcal cell wall (Hermoso *et al*., 2003).

Similarly, the crystal structure of the cell wall binding domain of endolysin PlyB, encoded by phage BcpI reveals an irregular $(\beta/\alpha)_5\beta_3$ barrel. The PlyB endolysin displays muralytic activity against *B. anthracis* (Schuch *et al*., 2002). Similar to other phage endolysins, PlyB encompass a bi-domain structure with a catalytic domain and a cell wall binding domain. The cell wall binding domain is homologous to the bacterial SH3b domain (Porter *et al*., 2007). The specific target of the SH3b domain in PlyB endolysin is not known. However, it has been shown that the bacterial SH3b domain from the glycyl-glycyl endopeptidase, ALE-1 targets penta-glycine bridges within the *S. aureus* peptidoglycan (Lu *et al*., 2006). However, *Bacillus* spp. do not possess penta-glycine crossbridges in the peptidoglycan, and therefore an alternative role of the SH3b domain in saccharide binding cannot be excluded.

The activation of SAR endolysins is initiated following the membrane depolarization by holins. The periplasmic membrane-bound SAR endolysins exit the bilayer in an active form. Although the process of the activation of SAR endolysins is not fully understood, it has been speculated that an intramolecular disulfide isomerization causes its activation (Xu *et al*., 2005). The SAR endolysins from phage Mu, T1, P1 and phage 21 appear to make use of the same strategy of intramolecular disulfide isomerization for activation (Hermoso *et al*., 2003).

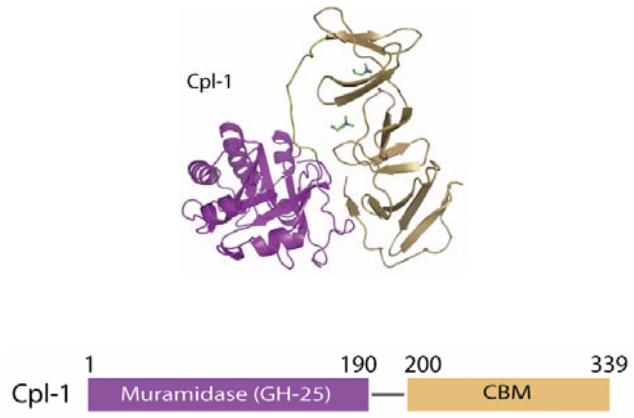

Fig. 6. Three-dimensional structure of the Cp1-1 endolysin showing both the catalytic and the cell wall binding domain (Hermoso *et al*., 2007).

4.2.2. *S. aureus* bacteriophages

4.2.2.1. Phage Twort

S. aureus bacteriophage Twort belongs to the family of myoviridae. There are two genes in phage Twort required for the host cell lysis, *holTW* and *plyTW*. HolTW shares homology with ORF3, a putative holin of prophage Φ11 (Weerakoon and Jayaswal, 1995). Several features of the holin protein families are shared by HolTw. These include, (a) two membrane spanning domains, (b) a β-turn linker separating the antiparallel transmembrane domains, (c) a charged C-terminal domain (Young and Bläsi, 1995). The *holTw* encodes a 185 aa protein and

overlaps with *plyTW* (encodes endolysin) by 35 bp but in a different reading frame, suggesting translational coupling of the two genes. PlyTW shares significant homology to the LytA amidase of Φ11 (42% identity). The C-terminal domain of PlyTW shares high degree of homology with the C-terminus of lysostaphin. HolTw complements a lambda *S* gene mutation (Loessner *et al*., 1998).

4.2.2.2. Phages Φ11, Φ12 and Φ13

Phages Φ11, Φ12 are dsDNA phages which utilize a unique holin-endolysin system to achieve host cell lysis. The endolysins encoded by both Φ11 and Φ12 are comprised of three domains. The N-terminal region comprises the endopeptidase domain (CHAP), cleaving the D-alanyl-glycyl linkage. The central region of the endolysin harbours the amidase domain which cleaves the N-acetyl-muramoyl-L-alanine linkage. The C-terminal region contains the cell wall binding motif which comprises a SH3b domain (Baba and Schneewind, 1996; Sass and Bierbaum, 2007).

Phage Φ13 is a dsRNA temperate phage with a genome of 42.7 kb and belongs to serogroup F (Carroll *et al*., 1995). Phage Φ13 mediates a negative effect on β-hemolysin gene expression and a postive effect on staphylokinase production. The negative effective on β-hemolysin expression is achieved by phage integration into the host attB site, which is located within the β-hemolysin gene, *hlb* (Carroll *et al*., 1993). Phage Φ13 carries a gene for staphylokinase. The expression of this gene contributes to the formation of a phenotype with increased staphylokinase production and thereby to an increased virulence of the host (Zou *et al*., 2000). The staphylokinase activity leads to the formation of plasmin from plasminogen, which in turn digests fibrin clots, thereby facilitating a spreading of the infection. Furthermore, staphylokinase cleaves immunoglobulin G (IgG) and the complement component, C3b (Bokarewa *et al*., 2006).

4.2.2.3. Phage 187

Phage 187 differs from all other *S. aureus* phages in its host range, distinctive virions and its DNA restriction pattern (Lee and Stewart, 1985; Loessner *et al*., 1999). Phage 187 recognizes a distinctive teichoic acid (galactosamine ribitol teichoic acid) (Karakawa and Kane, 1971). In contrast to the lysis gene organization of other *S. aureus* phages, where holin genes overlap with endolysin genes, in phage 187 the holin gene is fully embedded in the 5' region of the endolysin gene *ply187*. The N-terminal domain of phage 187 endolysin, Ply187, harbours the muralytic active domain and is thought to act as an N-acetylmuramoyl-L-alanine amidase due to its significant homology to LytA of phage Φ11 (Wang *et al*., 1991).

4.2.2.4. Phage 68

Phage P68 (Fig. 7) belongs to the family of podoviridae, which consists of the smallest known dsDNA phages (Tao *et al*., 1998). Phage P68 comprises an isometric head with a short non-contractile tail of ~ 40 nm in length. The pre-neck appendage of phage 68 is a hallmark of 'Φ29-like phages'. The total G+C content of the genome of phage 68 is 29.3%. Identical inverted terminal sequence repeats with a length of 210 bp were found at the end of the DNA. There are 22 open reading frames (ORF) within the genome of phage 68, out of which 12 are organized in an opposite direction. The lysis gene cassette of phage 68 comprises ORF15, which encodes the holin and ORF16, encoding the endolysin. Interestingly, the holin gene is almost completely embedded (overlap of 253 bp) within the endolysin gene (Vybiral *et al.,* 2003).

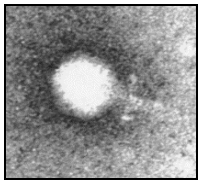

Fig. 7. Electron micrograph of the phage P68 negatively stained with 2% uranyl acetate (Vybiral *et al.,* 2003).

4.2.2.4.1. Phage 68 endolysin (Lys16)

The endolysin gene (ORF16) of phage P68 shows significant similarity to the endopeptidase domain of the *S. aureus* phage Twort endolysin (Loessner, *et al*., 1998) and to the endolysin of phage Φ11 (Wang *et al*., 1991). The endolysins of phage Twort and Φ11 encompass three domains composed of an N-terminal D-alanyl-glycyl endopeptidase domain, a central *N*-acteylmuramoyl-L-alanine amidase domain and a C-terminal cell-wall-targeting domain. However, the endolysin of phage P68, Lys16 lacks the central *N*-acteylmuramoyl-L-alanine amidase domain found in Twort and Φ11 endolysins (Fig. 8) (Navarre *et al*., 1999). A significant homology of 43% and 57% respectively, was found between the N-terminal 112 amino acids of P68 Lys16 with the inferred D-alanyl-glycyl endopeptidase domains of the Twort and the Φ11 endolysins. The C-terminal domain of P68 Lys16 is homologous to the cell-wall-targeting domains of both the Twort and Φ11 endolysins (Takáč *et al*., 2005).

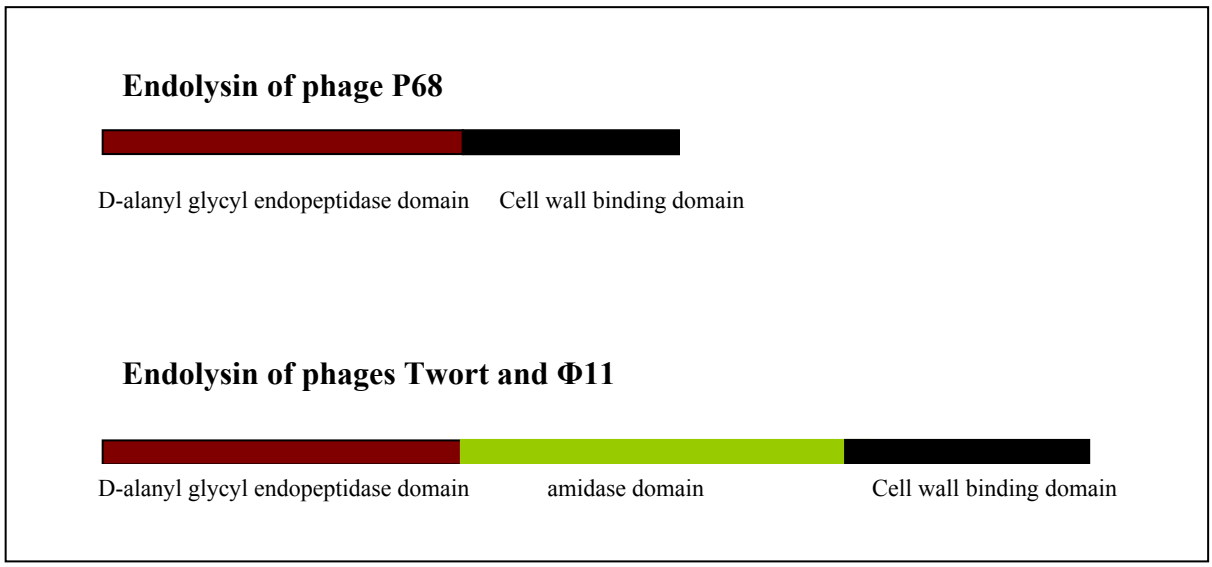

Fig. 8. Proposed domain organization of phage P68, Twort and Φ11 endolysins (Navarre *et al*., 1999; Takáč *et al*., 2005)

4.2.2.4.2. Phage 68 protein 17

The cell-puncturing device of lytic phages functions to penetrate the outer membrane of the host bacteria by locally dissolving the peptidoglycan to allow injection of the phage genome (Arisaka *et al*., 2003). In phage T4, protein 5 is located on the phage tail and forms a member of the cell-puncturing complex (Kao and McClain, 1980; Nakagawa *et al*., 1985). In phage P68, protein 17 is part of the phage virion (Takáč and Bläsi, 2005). Protein 17 shows 45% similarity within a 487 amino acid overlap to the product of ORF56 of the temperate *S. aureus* phage φETA (Yamaguchi *et al*., 2000) as well as to ORF636 of the *S. aureus* temperate phage φSLT (Narita *et al*., 2001) within a 558 amino acid overlap. These two proteins share similarity with the minor structural protein gp89 of *L. delbrueckii* bacteriophage LL-H (Mikkonen *et al*., 1996). Similar to phage T4 protein 5, protein 17 of phage 68 displayed muralytic activity against *S. aureus* peptidoglycan (Labedan and Goldberg, 1979; Takáč and Bläsi, 2005). It has been suggested that the lysozyme activity of protein 5 digests the peptidoglycan locally, allowing the T4 tail tube to penetrate the periplasmic space and henceforth allowing the infection process to be completed (Kao and McClain, 1980; Mosig *et al*, 1989; Nakagawa *et al*, 1985). The substrate specificity of protein 5 is similar to that of T4 gpE lysozyme, which is a N-acetylmuramidase (Kanamaru *et al*., 1999). It has also been demonstrated that the *L. lactis* phage Tuc2009 tail tip protein Tal_{2009} has muralytic activity. Tal_{2009} appears to undergo autocatalytic processing, which was attributed to its endopeptidase activity (Kenny *et al*., 2004). In contrast to the Tal_{2009} protein, there is no evidence for a putative protease motif in protein 17 (Takáč and Bläsi, 2005). Protein 17 recognizes a different receptor with respect to phage P68 on *S. aureus*. It has been speculated that protein 17 targets a carbohydrate moiety found in the *S. aureus* cell wall (Takáč and Bläsi, 2005).

4.3. Enzybiotics

The term enzybiotic has been coined for phage encoded endolysins that are capable to lyse bacteria when added exogenously (Veiga-Crespo *et al*., 2007; Nelson *et al*., 2001; Zimmer *et al*., 2002). The local degradation of the exposed peptidoglycan of Gram-positive bacteria results in lysis since the cytoplasmic membrane can not withstand the osmotic pressure of the cytoplasm (Fischetti, 2006).

4.3.1. Enzybiotics against pathogenic bacteria

Several recent studies suggest that enzybiotics can be used to treat bacterial infections. These include, the therapeutic application of purified phage endolysin against Streptococci in mice (Nelson *et al.*, 2001; Loeffler *et al.*, 2001) and against several other Gram-positive pathogens including *Enterococcus faecalis* and *E. faecium* (Yoong *et al.*, 2004), *Clostridium perfringens* (Zimmer *et al.*, 2002), group B Streptococci (Cheng *et al.*, 2005) and *B. anthracis* (Schuch *et al.*, 2002). Bacteriophage Cp-1 encoded endolysin, Cpl-1, was active against *Streptococcus pneumoniae* in a murine sepsis model (Jado *et al*., 2003). A single dose of CpI-1 (1100U) endolysin administrated intraperitoneally rescued the mice challenged with *S. pneumoniae* (Jado *et al*., 2003). Similarly, the endolysin from a phage infecting group C streptococci was efficient in the prevention and elimination of group A Streptococci from the upper respiratory tract of mice (Nelson *et al*., 2001).

Endolysin PlyC from phage γ was effective against *B. anthracis in vitro* and *in vivo*. The capsule of *B. anthracis* did not prevent the access of PlyC to the cell wall. The intraperitonial injection of *B. anthracis* in mice was fatal and caused the death of mice within 5 h or less. However, treatment of the mice with PlyC (50U) intraperitoneally 15 min following infection had a significant theraupeutic effect, rescuing 68.4 % of the infected mice (Schuch *et al.*, 2002).

4.4. The prokaryotic ribosome

The protein biosynthesis is an important and complex process which is performed by a large ribonucleoprotein machinery, the ribosome. The prokaryotic ribosome (70S) has a mass of approximately 2.6-2.8 MDa and encompasses two distinct subunits, the small 30S subunit and the large 50S subunit (Fig. 9), each composed of rRNA and proteins. In the recent years, the structure of the ribosome has been determined at atomic resolution (Selmer *et al*., 2006). These data support the biochemical studies, which indicate that the catalytic activities of the ribosomes are mainly performed by the rRNA. It therefore is termed a protein-stabilized ribozyme (Culver, 2003). The 30S subunit has a molecular weight of approx. 0.85 MDa and is involved in decoding of the mRNA based genetic code into the amino acid sequence of a protein, using adaptor molecules known as transfer RNAs (tRNAs) (Laursen *et al*., 2005). It is composed of 16S rRNA (ribosomal RNA, Fig. 10) and 21 proteins designated S1-S21. It contains the messenger decoding-site and mediates the interaction between messenger RNA (mRNA) and transfer RNA (tRNA). The 50S subunit consists of two RNA molecules, the 5S and 23S rRNAs, and 34 associated proteins. During protein synthesis it catalyzes peptide bond formation at the peptidyl transferase center, which is formed exclusively by the 23S rRNA. The 50S subunit harbors the sites of interaction with several GTP binding proteins, which assist in various translational processes such as initiation, elongation and termination. The 5S rRNA with a size of 120 nt acts as a seventh domain of the 50S subunit and confers stability to its structure (Sergiev *et al*., 2000).

There are three functional sites on the ribosome where tRNAs bind: (i) The A-site is where the decoding takes place. Here, the correct aminoacyl-tRNA is selected based on its codon-anticodon interaction, respective to the codon of the mRNA currently displayed (ii) The P-site, where the peptidyl tRNA is located, carrying the elongating polypeptide chain (iii) The E-site, or the exit site, harboring the deacylated tRNA, ready to exit the ribosome (Fig. 11).

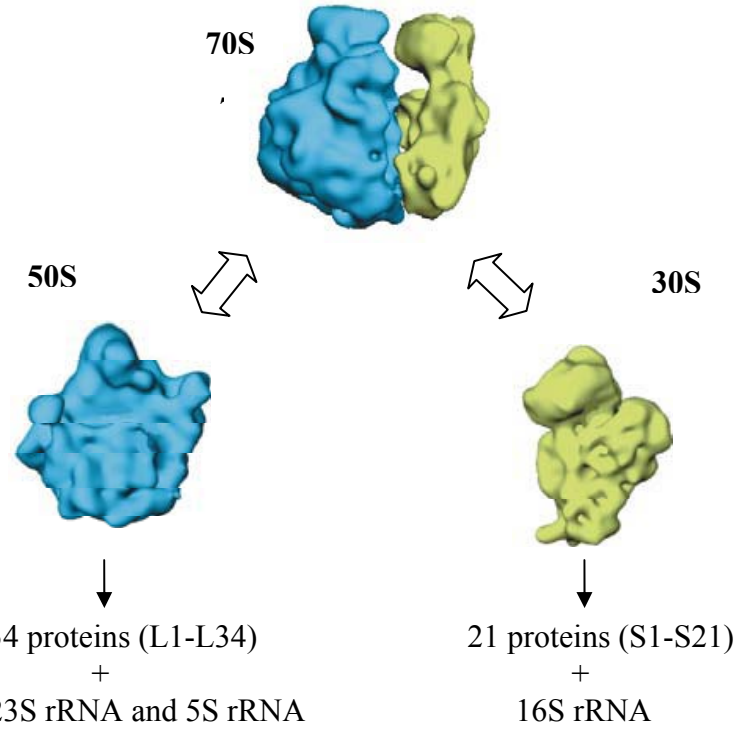

Fig. 9. Composition of the *E. coli* ribosome (modified from Iskakova, 2005).

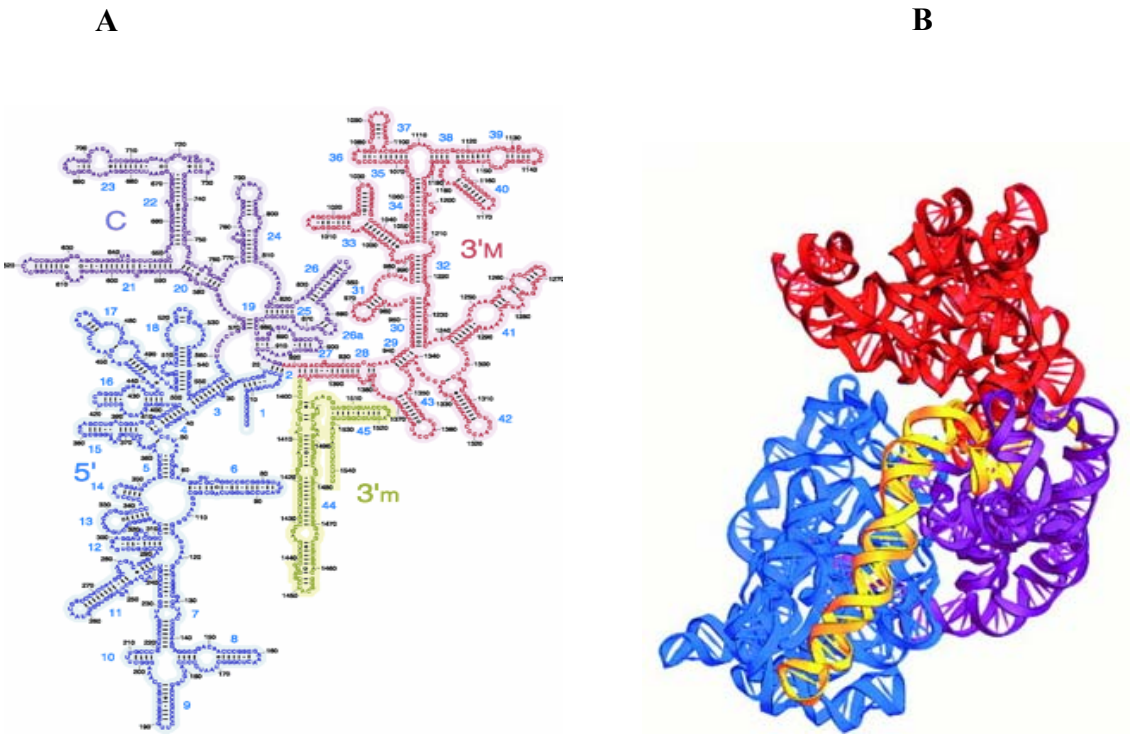

Fig. 10. Structure of the16S rRNA. (A) The 3D structure of the 16S rRNA. (B) Three-dimensional fold of 16S rRNA in 70S ribosomes (Yusupov *et al*., 2001). The colors are identical to (A). Blue: domain I; violet: domain II; Red: domain III; yellow: domain IV.

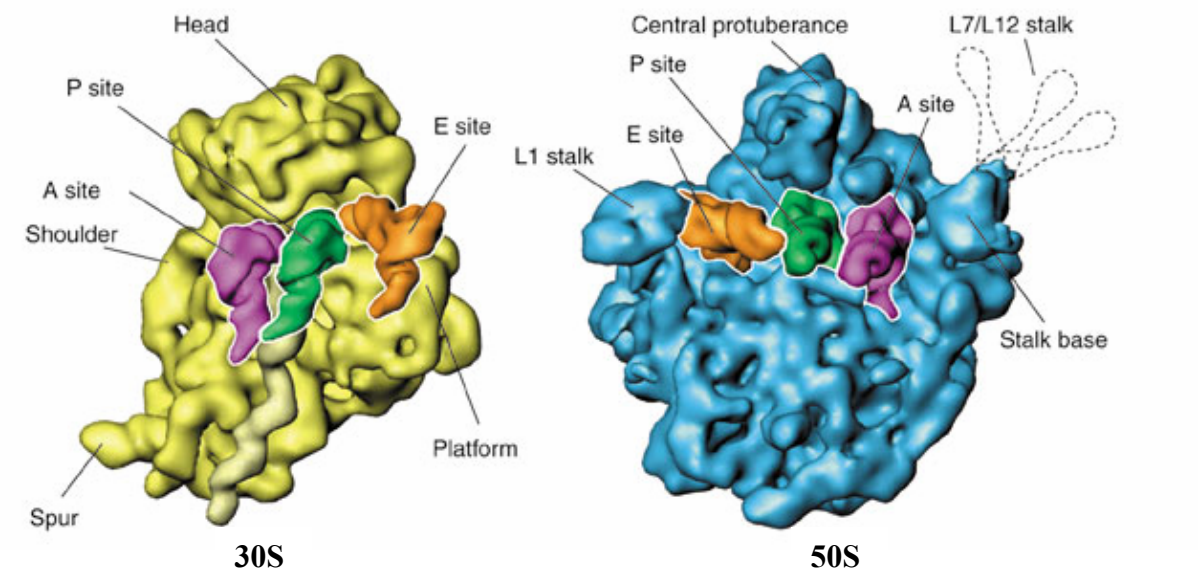

Fig. 11. The location of A (violet), P (green) and E (yellow) site tRNAs in the 50S and 30S ribosomal subunit (Frank, 2003).

4.5. Protein synthesis

During protein synthesis a single ribosome can incorporate 10-20 amino acids (aa) per second (Bremer and Dennis, 1996). The accuracy of aa incorporation is very high having only one misincorporation per 3000 aa (Bouadloun *et al*., 1983). The process of protein synthesis can be divided into four consecutive phases: initiation, elongation, termination and ribosome recycling (Fig. 12).

4.5.1. Initiation

Translation initiation is the rate limiting step in protein synthesis. During initiation, the ribosome, the aminoacylated and formylated initiator tRNA (fMet-tRNA$_f^{Met}$), the mRNA, and intiation factors IF1, IF2 and IF3 are involved (Laursen *et al*., 2005). Initially, the anti-Shine Dalgarno (anti-SD) sequence on the 3'-end of 16S rRNA interacts with the Shine Dalgarno-sequence (SD) of the mRNA, thereby facilitating the positioning of the 30S subunit on the

ribosome binding site on the mRNA. Following the SD-anti-SD interaction, the initiator tRNA is bound to the P-site of the 30S subunit in three steps, codon-independent binding, codon-dependent binding and fMet-$tRNA_f^{Met}$ adjustment (Tomsic *et al*., 2000; Laursen *et al*., 2005). These steps are promoted by IF2, which directly interacts with fMet-$tRNA_f^{Met}$ and the ribosome. In addition, IF2 also prevents the binding of the aminoacylated elongator tRNAs during ternary complex formation (Gualerzi and Pon, 1990; Hartz *et al*., 1989). Furthermore, the binding of the fMet-$tRNA_f^{Met}$ to the P-site is stabilized by IF3 (Gualerzi *et al*., 1977). IF3 acts as fidelity factor in translation initiation. It selectively destabilizes non cognate start codon-anti-codon interaction by directly associating with the ribosome, anti-codon and fMet-$tRNA_f^{Met}$ (Subramanian and Davis, 1970; Gualerzi *et al*., 1971; Sussman *et al*., 1996; Hartz *et al*., 1990; Gold, 1988; Yusupov *et al*., 2001; La Teana *et al*., 1995). In addition, IF1 participates in initiation complex formation by specifically binding to the A-site of the 30S subunit. It is suggested to block the A-site and thereby it positions the initiator tRNA towards the P-site (Carter *et al*., 2001; Dahlquist and Puglisi, 2000). The unstable pre-initiation complex comprising the 30S subunit, IF1, IF2, IF3, mRNA and fMet-$tRNA_f^{Met}$, which is bound to the P site by a codon-independent manner undergoes a confirmational change which facilitates the codon-anticodon interactions leading to a stable 30S initiation complex (Pon and Gualerzi, 1984). Following the formation of the stable 30S initiation complex, IF1 and IF3 dissociate and, upon GTP-hydrolysis catalysed by IF2, the association of the the large 50S subunit to the 30S initiation complex is stimulated. This leads to the formation of the 70S initiation complex the release of IF2 (Marzi *et al*., 2003). However, GTP hydrolysis is neither essential for the interaction with the initiator tRNA and IF2 nor required for the formation of the initiation complex (Petersen *et al*., 1979). However, it is speculated that the GTP-hydrolysis is required for the correct adjustment of the fMet-$tRNA_f^{Met}$ in the P site (La Teana *et al*., 1996). The 70S initiation complex with the initiator tRNA located in the P site

consequently enters the elongation cycle (Boelens and Gualerzi, 2002; Gualerzi *et al*., 2000; Gualerzi and Pon, 1990; Sonenberg *et al*., 2000; Sørensen *et al*., 2001).

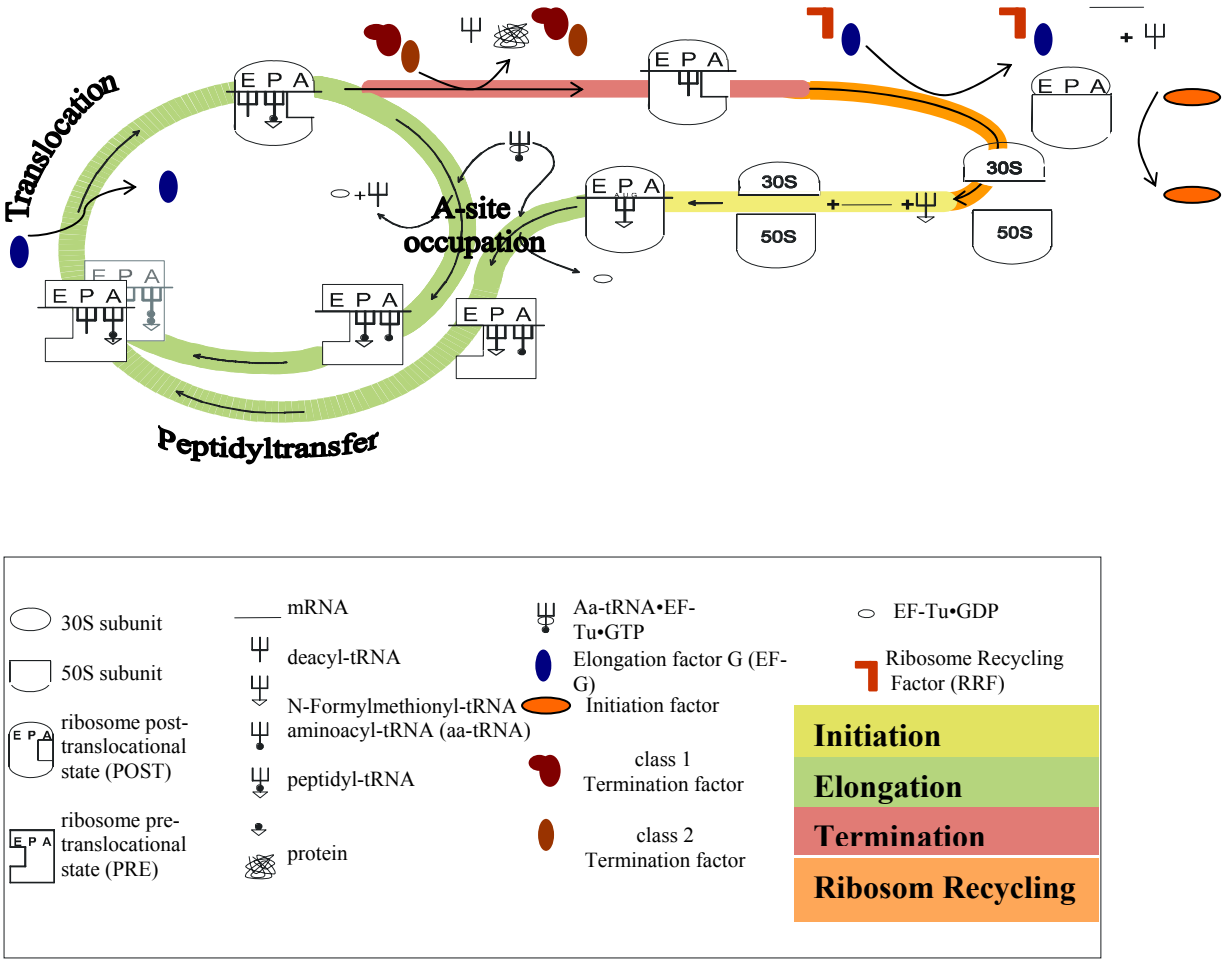

Fig. 12. Protein synthesis in bacteria (modified from Iskakova, 2005)

4.5.2. Elongation

The elongation of the nascent peptide by one aa according to the corresponding codon on the mRNA is performed by the ribosome in a cyclic manner (Vesper and Nierhaus, 2004). Two main elongation factors, EF-Tu and EF-G are involved in the elongation cycle, which can be divided into three important steps: (i) A-site occupation, (ii) peptide bond formation, and (iii) translocation. The A-site occupation can be subsequently divided into two reactions. In the

first reaction, the EF-Tu associated with GTP and the aminoacyl-tRNA (ternary complex) (aa-tRNA**.**EF-Tu**.**GTP) is recruited to the A-site based on the codon-anti-codon interaction. Following the delivery of the aa-tRNA to the A-site, the GTPase center of EF-Tu is activated leading to the GTP hydrolysis and release of the EF-Tu**.**GDP complex from the ribosome. The second reaction is known as the accommodation step, wherein the release of the EF-Tu**.**GDP from the ribosome allows the aa-tRNA to be bound to the A-site by docking the aminoacyl residue of the aa-tRNA to the peptidyl transfer center (PTC) of the 50S subunit. The A and P site occupied aminoacyl tRNAs are now ready for the formation of the peptide bond. This state of the ribosome is called the 'pre-translocational' state (PRE) (Vesper and Nierhaus, 2004). The second step of the elongation cycle is the peptide bond formation between the peptidyl residue of the donor (P-site) and the aminoacyl residue of the acceptor (A-site) (Vesper and Nierhaus, 2004). This leads subsequent formation of a peptidyl-tRNA at the A-site and a deacylated tRNA at the P-site. The third and final step of the elongation cycle is the translocation. During translocation, the tRNAs located in the A-site and P-site change their position. Precisely, the peptidyl-tRNA at the A-site moves to the P-site and the deacylated tRNA of the P site moves to the E site. This state of the ribosome is called the 'post-translocational state' (Vesper and Nierhaus, 2004). The P- and E-sites of the ribosome are occupied and the A-site is vacant and waits to receive the new aa-tRNA.EF-Tu.GTP ternary complex based on the codon on the mRNA, thus the elongation cycle is repeated. However, during the next A-site occupation the E site tRNA has to be released (Vesper and Nierhaus, 2004). This movement of the tRNA**.**mRNA complex within the ribosome by one codon is catalyzed by elongation factor G (EF-G) (Spahn and Nierhaus, 1998; Wilson and Noller, 1998).

4.5.3. Termination

The termination of protein synthesis occurs when a stop codon (UAA, UAG or UGA) is located in the A-site of the ribosome. This process is followed by the subsequent hydrolytic release of the completed polypeptide from the peptidyl-tRNA catalyzed by release factors RF1 and RF2. Subsequently, RF3 stimulates the termination process in a GTP-dependent manner, thereby releasing RF1 and RF2 from the post-termination complex (Freistroffer *et al*., 1997; Zavialov *et al*., 2001). However, RF3 has been shown not to be essential in *E. coli*.

4.5.4. Ribosome recycling

Ribosome recycling is regarded as the fourth step in protein synthesis. Upon termination of protein synthesis the ribosome recycling factor (RRF) -in conjunction with EF-G and IF3- promotes the dissociation of the ribosome into its subunits. Since an RRF deficient *E. coli* is not viable, dissociation of the ribosomes is suggested to be essential for cell growth (Janosi *et al*., 1998). EF-G performs distinct functions in protein synthesis including translocation and ribosome dissociation. During both processes, EF-G hydrolyses GTP to GDP and Pi. The translocation step is less dependent on GTP hydrolysis by EF-G. However, the ribosome disassembly by the RRF and EF-G is blocked when GTP is replaced by a non-hydrolyzable analog of GTP (Rodnina *et al*., 1997; Peske *et al*., 2005; Zavialov *et al*., 2005). Recently, it was shown that the Pi released during GTP hydrolysis is important for ribosome dissociation (Savelsbergh *et al*., 2009). The binding of EF-G to the RRF bound ribosome triggers an inter-domain rotation of RRF, which destabilizes two strong intersubunit bridges of the ribosome (B2a and B3), consequently separating the two subunits (Gao *et al*., 2007). Upon dissociation of the ribosomal subunits, IF3 binds to the 30S subunit, thereby preventing reassociation (Hirashima and Kaji, 1973; Peske *et al*., 2005; Zavialov *et al*., 2005; Vesper and Wilson, 2006).

4.6. Ribosomal proteins S1 and S2

Protein S1 is the largest protein in the *Escherichia coli* ribosome and has a molecular mass of 61 kDa. It associates to the 30S subunit during late stage of ribosome biogenesis (Subramanian, 1983; Sharma *et al*, 2005). In Gram-negative bacteria, protein S1 plays an essential role in the translation initiation. It interacts with a pyrimidine-rich region within the mRNA upstream of the SD-sequence (Boni *et al*., 1991, Sengupta *et al*., 2001), thereby increasing the concentration of the translational start site in the vicinity of the decoding site on the ribosome. In addition, protein S1 has been suggested to assist in positioning of the 30S subunit in close proximity to the translational start site by destabilizing secondary structures (de Smit and van Duin, 1994). Therefore, S1 is indispensable for the translation of canonical mRNAs and is therefore essential for *E. coli* and likely of all Gram-negative bacteria possessing a S1 homologue (Sørensen *et al*., 1998). However, for the translation of leaderless mRNAs lacking a 5' UTR protein S1 is dispensable (Moll *et al*., 2002a and b). Furthermore, it has been suggested to play a role in translation elongation (Potapov and Subramanian, 1992). In addition to its function in the initiation of protein synthesis it has been reported to be involved in the rescue of stalled ribosomes *via* the tmRNA mediated trans-translation (Saguy *et al*., 2007; Qi *et al*., 2007). In this process, where a degradation tag is added to the incomplete peptide bound to the stalled ribosome (Muto *et al*., 2000; Withey and Friedman, 2003), the tmRNA functions as both, tRNA and mRNA (Qi *et al*., 2007).

S1 consists of six consecutive motifs (Fig. 13; Bycroft *et al*, 1997). The two N-terminal motifs of S1 are required for binding to the small ribosomal subunit. The four C-terminal motifs of approximately 70aa in length are facing to the solvent side of the ribosome. They are involved in interaction with mRNAs and tmRNAs at single stranded RNA and RNA pseudoknots (Giorginis and Subramanian, 1980; Ringquist *et al*., 1995). In addition, it has been shown that the extreme C-terminal domain of ribosomal protein S1 is involved in the autogenous regulation of the S1 expression (Boni *et al*., 2000).

Some of the peculiar features of ribosomal protein S1 include the acidity of the protein. All other ribosomal proteins except the two large subunit proteins, L7/L12 are basic. In addition, S1 is the only protein which binds to the ribosome by protein-protein interaction (Boni *et al*, 1982). Supporting this observation, cross-linking experiments and X-ray crystallographic studies demonstrated that protein S2 is located in close proximity to S1 on the ribosome (Sengupta *et al*, 2001; Laughrea and Moore, 1978; Bollen *et al*, 1979). In addition, studies by Moll *et al* (2002b) revealed that ribosomes devoid of protein S2 also lack S1. These results indicate that protein S2 is essential for binding of protein S1 to the 30S ribosomal subunit.

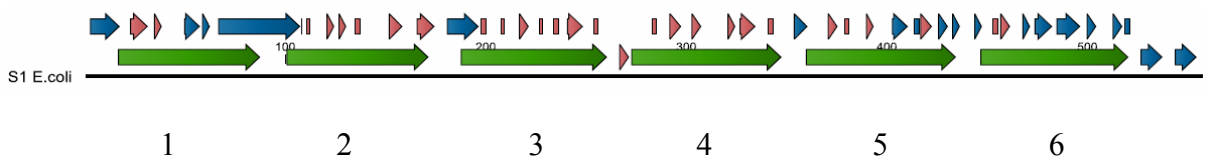

Fig. 13. Domain organization of protein S1 of *E. coli*

Ribosomal protein S2 is the second largest protein associated with the 30S subunit (Fig. 14). It is a highly conserved protein throughout all domains of life and is indispenble for translation in prokaryotes, eukaryotes, chloroplasts and mitochondria (Ardini *et al*., 1998; Wilson and Nierhaus, 2005). In prokaryotes, protein S2 is suggested to protect and stabilize the SD- anti-SD duplex located between the head and platform of the 30S subunit (Kaminishi *et al*., 2007). Structural analyses have shown that S2 is a slightly elongated protein with a two-domain organization that has been shown to be more sensitive to proteolytic cleavage compared to other ribosomal proteins under the same conditions (Georgalis *et al*., 1981; Wittmann *et al*., 1980; Wimberly *et al*., 2000; Brodersen *et al*., 2002). During ribosome assembly protein S2 binds to the 30S subunit at a very late step (Culver, 2003). However,

protein S2 binds to the 16S rRNA (Mizushima and Nomura, 1970; Culver, 2003) and can be cross linked to ribosomal proteins S1, S3, S5 and S8 (Littlechild *et al*., 1977; Traut *et al*., 1980).

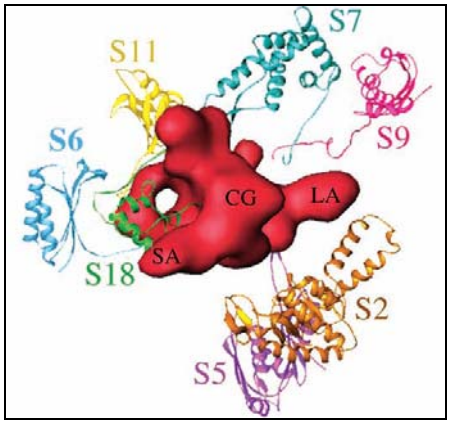

Fig. 14. Proposed position of protein S1 (Red) and S2 along with other ribosomal proteins in the 30S subunit (Sengupta *et al.,* 2001).

4.7. Antibiotics targeting the ribosomes

Due to the high complexity of the ribosome it represents a major target for clinically relevant antibiotics (Gale *et al*., 1981). As the rRNA, which is responsible for most of the pivitol steps in protein synthesis, accounts for most of the contacts with antibiotics (Yonath, 2005), they target functionally important sites within the ribosome, like (i) the decoding site within the 30S subunit (paromomycin and streptomycin, Brodersen *et al*., 2000), (ii) the mRNA tunnel (edeine and spectinomycin, Carter *et al*., 2000; Pioletti *et al*., 2001; Brodersen *et al*., 2000), (iii) the A-site of the 30S subunit (tetracycline; Pioletti *et al*., 2001), (iv) the peptidyl transfer center (PTC) in the 50S subunit (chloramphenicol and sparsomycin, Pioletti *et al*., 2001; Schlünzen *et al*., 2001, Hansen *et al*., 2003), and (v) the protein exiting tunnel (troleandomycin, Berisio *et al*., 2003).

The 30S subunit is primarly targeted by pactamycin, tetracyclines and aminoglycoside antibiotics. Pactamycin is a cyclopentane derivative that interacts with helix H23b and the helix H24a of the 16S rRNA, close to the tRNA binding cleft (Brodersen *et al*., 2000). It mimics the di-nucletotide of the mRNA in the E-site, which suggests that it blocks the path of movement of mRNA through the ribosome. Pactamycin does not interfere with initiation, subunit association or tRNA binding to the A-site. However, the translocation of A- and P-site tRNA to the P- and E-site seems to be affected (Dinos *et al*., 2004).

4.7.1. Aminoglycoside antibiotics

Aminoglycoside antibiotics are produced by *Streptomycetes* and generally composed of a sugar moiety and an amino group. They are the most commonly used antibiotics as clinical therapeutics, like gentamicin, streptomycin, neomycin, and kanamycin. Aminoglycosides mainly target the 30S ribosomal subunit, where they usually bind to the 16S rRNA (Shakil *et al*., 2008). They specifically bind to the A-site internal loop within the deep groove of helix 44 of the 16S rRNA in the highly conserved region of the decoding center (Carter *et al*., 2000), where they interfere with two flexible adenine residues (A1492, A1493; Ogle *et al*., 2001). These residues are involved in the selection of the cognate aminoacyl tRNA during elongation. Consequently, these antibiotics affect the fidelity of protein translation (Moazed and Noller, 1987; Fourmy *et al*., 1996; Carter *et al*., 2000; Pape *et al*., 2000). Taken together, aminoglycoside antibiotics seem to act as rigid molecular braces that prevent conformational changes important for ribosome function.

Kasugamycin (Ksg), was shown to prevent the formation of the pre-initiation complex by interfering with fMet-tRNA$_f$, which binds to the ribosomal P-site in *E. coli* ribosomes (Poldermans *et al*., 1979; Woodcock *et al*., 1991). In addition, recent studies have shown that treatment of the *E. coli* cells with Ksg *in vivo* leads to the formation and accumulation of stable 61S ribosomal particles. These particles comprise an intact 50S subunit. However,

many proteins of the small subunit were absent (Kaberdina *et al*., 2009). In addition, it has been shown that erythromycin affects the assembly of 50S subunit and the paromycin and neomycin inhibit 30S assembly in *S. aureus* (Chittum and Champney, 1995; Mehta and Champney, 2003).

4.7.2. Polyketide antibiotics

Polyketides are usually secondary metabolites of bacteria, fungi, animals or plants formed by the polymerization of propionyl or acetyl subunits by claisen condensation (Robinson, 1991). Tetracycline is one among the most commonly used polyketide antibiotics. It belongs to the octahydronaphtacene derivatives with an overall L-shape structure. Several binding sites of tetracycline on the 30S subunit have been identified by crystallographic studies (Pioletti *et al*., 2001; Brodersen *et al*., 2000). The drug interacts with the shallow groove of the helix H34, including contacts to helix H31. This interaction prevents binding of the aminoacyl-tRNA to the A-site, consequently leading to the inhibition of the first step in elongation (Pioletti *et al*., 2001; Brodersen *et al*., 2000).

4.8. Bacterial resistance towards antibiotics

Bacterial resistance to antibiotics is a common problem in modern therapeutics. The mode of resistance is created by random mutations in a gene of bacteria and in turn evolved by natural selection. Resistance of bacteria against antibiotics could also be a result of horizontal gene transfer (Berglund and Söderquist, 2008). The most common nature of resistance towards β-lactam antibiotics is by enzyme catalyzed chemical modification (Wright *et al*., 1998; Mingeot-Leclercq *et al*., 1998; Kotra *et al*., 2000). The resistance against tetracycline which belongs to polyketide antibiotic group occurs by efflux pump, ribosomal protection and enzymatic degradation (Chopra and Roberts, 2001). The resistance towards antibiotics targeting the ribosomes is usually achieved through point mutations within the rRNA. For

instance, the resistance to streptomycin is achieved by point mutations in the 16S rRNA gene (505U→526A, 506A→525U and 507U→524A), which confer resistance to the drug but do not affect the functionality of the ribosome (Springer *et al*., 2001). Likewise, the resistance to other aminoglycoside antibiotics such as neomycin, kanamycin and gentamicin is conferred by point mutations in the 16S rRNA gene (De Stasio *et al*., 1989; Beauclerk and Cundliffe, 1987). Resistance against kasugamycin is caused by the lack of di-methylation of two adjacent adenosines positioned at nucleotides 1518/1519 in helix 45 at the 3'-end of the 16S rRNA (Helser *et al*., 1971). This post-transcriptional modification has been shown to alter the conformation and flexibility of the ultimate stem-loop in 16S rRNA (Rife and Moore, 1998).

4.9. The S1-S2 interaction: A new target for antimicrobials

As mentioned above, several chemically diverse antibiotics target the ribosome at a few locations, which results in an overlap of their binding sites. As a consequence, a single mutation in the 16S rRNA gene can potentially confer resistance to several antibiotics (Skinner *et al*., 1983). Moreover, these binding sites are predominantly localized in regions of conserved function(s) in eukaryotic, bacterial as well as in mitochondrial ribosomes. Therefore, these drugs can have adverse side effects when used as clinical therapeutics. Thus, there is need for alternative antimicrobials, which do not interfere with ribosome function, but inhibit assembly of functionally important proteins. In contrast, any mutation interfering with such a protein-protein interaction would be detrimental to cell growth of bacteria. It is therefore conceivable, that resistance mechanisms towards drugs, which are based on an alteration of the drug target site (i.e. protein-protein interaction sites), are more difficult to develop as point mutations in 16S rRNA. Taken together with the semi-selective action against Gram-negative pathogens, such antimicrobials could be advantageous over conventional antibiotics.

In the past decades, major progress in drug development has been made by using a binding-pocket model of drug targets. Small molecules were designed to fit into substrate binding sites of proteins. Since it was easier to screen for low molecular weight inhibitors that block their active sites, in principal enzymes have been used as therapeutic drug targets (Arkin and Wells, 2004). However, given that specific protein-protein interactions are essential in most biological processes, they represent likewise promising opportunities for therapeutic intervention based on selective inhibition of these interactions. For example, highly specific protein-protein interactions in bacteria that are not found in human hold promise for treatment of infectious diseases (Arkin and Wells, 2004). As S1 is essential for translation initiation in *E. coli* and probably in most Gram-negatives, and requires S2 for binding to the ribosome, the studies aiming at identifying novel antimicrobials, which specifically inhibit the S1-S2 interaction, would prove to be fruitful. In contrast, there is no functional S1 homologue in the low "GC" group of Gram-positive bacteria, which include beneficial commensals such as probiotic lactic acid bacteria (Vellanoweth and Rabinowitz, 1992). In addition, mitochondria lack functional homologues of ribosomal protein S1 (Vellanoweth and Rabinowitz, 1992; Cavdar-Koc *et al.,* 2001) and eukaryotic ribosomes employ different mechanisms for translation initiation (Marintchev and Wagner, 2004). Therefore, a drug, which interferes with the S1-S2 interaction, would act selectively against a number of pathogenic *Escherichia coli* strains. Given that protein S1 is highly conserved in the gamma subdivision of proteobacteria, this drug has the potential to act as well as an antimicrobial against other Gram-negative bacterial pathogens, including *Pseudomonas aeruginosa, Salmonella enterica* and *Klebsiella pneumoniae*.

5. Aim of the study

The first aim of the study was to develop a specific enzybiotic against *S. aureus*. The strategy for developing a novel antimicrobial agent against Gram positive *S. aureus* included the genetic engineering of bacteriophage P68 endolysin with the aim to increase the solubility of the enzybiotic.

The second aim targets towards the design of an antimicrobial compound affecting ribosome assembly in Gram-negative bacteria. As mentioned above, the interaction between the two ribosomal proteins S1 and S2 has the potential to represent a target site for antimicrobial activity. A drug, which would disrupt the S1/S2 interaction, could thus act semi-selectively against Gram-negative pathogens. In order to verify the interaction of proteins S1 and S2, a yeast-two-hybrid analysis has been performed. In addition, a random mutagenesis study has been performed to elucidate the amino acid residues involved in the S1/S2 interaction.

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7. Publications and manuscripts

7.1. Antimicrobial activity of a chimeric enzybiotic towards *Staphylococcus aureus*.

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Antimicrobial activity of a chimeric enzybiotic towards *Staphylococcus aureus*

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ABSTRACT

Phage lytic enzymes (enzybiotics) have gained attention as prospective tools to eradicate Gram-positive pathogens resistant to antibiotics. Attempts to purify the P16 endolysin of *Staphylococcus aureus* phage P68 were unsuccessful owing to the poor solubility of the protein. To overcome this limitation, we constructed a chimeric endolysin (P16-17) comprised of the inferred N-terminal D-alanyl-glycyl endopeptidase domain and the C-terminal cell wall targeting domain of the *S. aureus* phage P16 endolysin and the P17 minor coat protein, respectively. The domain swapping approach and the applied purification procedure resulted in soluble P16-17 protein, which exhibited antimicrobial activity towards *S. aureus*. In addition, P16-17 augmented the antimicrobial efficacy of the antibiotic gentamicin. This synergistic effect could be useful to reduce the effective dose of aminoglycoside antibiotics.

1. Introduction

Host cell lysis by double-stranded DNA phage is generally achieved by a phage encoded holin in conjunction with a muralytic enzyme (endolysin). In general, the holin induces a non-specific lesion in the cytoplasmic membrane, which allows the endolysin to degrade the peptidoglycan at a genetically programmed time after infection (Young, 1992). Ancillary proteins can either serve as negative regulators of the holin, or act by destabilizing the outer membrane (Young and Bläsi, 1995; Wang et al., 2000). The endolysins are enzymes with broad activities cleaving either glycosidic, amide, or peptide bonds of the peptidoglycan (Young, 1992). The phage encoded endolysins are principally synthesized without a signal sequence and accumulate fully folded and active in the cytosol. However, a few endolysins exploit the host *sec* system to reach the peptidoglycan. These endolysins bear a N-terminal *SAR* (signal *a*rrest *r*elease) sequence by which they are tethered to the outer site of the inner membrane until the membrane potential is dissipated by the holin (Xu et al., 2005). In contrast to Gram-negative bacteria, endolysins can act as exolysins on Gram-positive bacteria, of which the peptidoglycan is externally accessible (Garcia et al., 1987; Fischetti, 2003; Loessner, 2005). Purified phage endolysins have been used as therapeutics against Streptococci in mice (Nelson et al., 2001; Loeffler et al., 2001), and have been proven effective against other Gram-positive pathogens including *Enterococcus faecalis* and *E. faecium* (Yoong et al., 2004), *Clostridium perfringens* (Zimmer et al., 2002), group B Streptococci (Cheng et al., 2005) and *Bacillus anthracis* (Schuch et al., 2002). The term enzybiotics has been coined to classify these antimicrobials (Nelson et al., 2001; Hermoso et al., 2007).

In addition to classical endolysins, which are synthesized during phage infection, phage virion proteins can as well exert muralytic activity. These virion associated murein hydrolases are believed to facilitate the entry of phage DNA during infection. The presence of such activities has been previously reported for the *Staphylococcus aureus* phage ϕ11 and ϕ85 (Moak and Molineux, 2004), for the *Lactococcus lactis* phage Tuc2009 (Kenny et al., 2004), and for the T4 phage tail protein 5 (Nakagawa et al., 1985). Moreover, we have recently demonstrated a muralytic activity of *S. aureus* phage P68 virion protein 17, which has a bipartite structure comprising the muralytic activity in the N-terminal part, whereas the C-terminal part encompasses the cell wall binding domain (Takáč and Bläsi, 2005).

The human pathogen *S. aureus* is a cause of severe infections in immuno-compromised individuals and a source of nosocomial infections. The widespread appearance of drug resistant strains of *S. aureus* especially to methicillin and vancomycin is a major issue in combating this pathogen (Assadullah et al., 2003). The *S. aureus* phage P68 endolysin (Lys16) was shown to exhibit muralytic activity against clinical isolates of *S. aureus* (Takáč et al., 2005). However, the protein proved to be rather insoluble. To improve the solubility and to create a specific enzybiotic against *S. aureus,* here we combined the inferred N-terminal D-alanyl-glycyl endopeptidase domain of P68 Lys16 with the C-terminal binding domain of P68 protein 17. The chimeric P16-17 enzybiotic displayed antimicrobial activity towards *S. aureus*, and a combination treatment with P16-17 and sub-inhibitory concentrations of the antibiotics gentamicin showed that the P16-17 can be used to reduce the effective dose.

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Table 1
Strains and plasmids used in the study.

	Genotype/relevant features	Source/reference
Bacterial strains		
E. coli XL-1	Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene, USA
E. coli B	F$^-$ *ompT gal hsdS*$_B$	New England Biolabs, USA
S. aureus 68	Host strain for phage P68	Vybiral et al. (2003)
S. aureus AS145	*mec ΔfemAB::tetK*	Stranden et al. (1997)
S. aureus RN4420	*ΔtagO*	Gründling and Schneewind (2006)
S. aureus strains P1, P2, P12, P16 and P17	Clinical strains	Lab. collection
B. subtilis		Lab. collection
Plasmids		
pQE30	*E. coli* expression vector, *lac*UV5 promoter Amp^r	Qiagen, Germany
pUCLYS16	pUC18::793 bp XbaI–XhoI fragment from pDLYS16	Takáč et al. (2005)
pMUH17	pET16b::gene *17* of P68	Takáč and Bläsi (2005)
pDAGE1	pQE30::423 bp PCR product containing the 5′-end of P16 gene under control of the *lac*UV5 promoter	This work
pHYBR1	pQE30::423 bp of the 5′-end of *lys16* fused to 75 bp of the 3′-end of *gene 17* under control of the *lac*UV5 promoter	This work

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. The *S. aureus* strains were cultivated in BHI broth (Merck) at 37 °C. *E. coli* and *B. subtilis* strains were cultivated in Luria-Bertani medium (LB) at 37 °C. When appropriate, ampicillin (100 μg ml^{-1}), tetracycline (10 μg ml^{-1}), gentamicin (40 μg ml^{-1}) or streptomycin (40 μg ml^{-1}) was added. Where indicated, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM.

2.2. Construction of plasmids

To construct a plasmid encoding the chimeric protein P16-17, a 445 bp fragment comprising 423 bp of the 5′-end of phage P68 *gene 16* (pUCLYS16, Table 1) was amplified using primers FP1a and RP2a (Table 3; Supplementary material). The PCR fragment was inserted into the *Bam*HI/*Kpn*I site of the expression vector pQE30 (Table 1), creating plasmid pDAGE1. The PCR amplification of *gene 17* using plasmid pMUH17 (Table 1) with the primers FP12b and RP2b (Table 3; Supplementary material) generated a DNA fragment of 95 bp comprising the 3′-terminal 75 bp of gene *17*. This fragment was inserted into plasmid pDAGE1 cleaved with *Kpn*I and *Hind*III, resulting in plasmid pHYBR1 (Table 1). Transcription of the P16-17 protein gene in pHYBR1 is driven by the *lac*UV5 promoter.

2.3. Purification of P16-17

E. coli XL-1 Blue bearing plasmid pHYBR1 was grown to an OD_{600} of 0.3. Then IPTG was added and incubation was continued at 37 °C for 4 h. The cells were collected by centrifugation at 5000 × *g* for 10 min. As the synthesized P16-17 protein was insoluble (Fig. 1A) and accumulated in inclusion bodies (not shown), the cell pellet was resuspended in 20 ml denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M Urea, pH 8.0), and incubated at room temperature for 1 h followed by sonication. After sonication, the cell lysate was centrifuged at 28,000 × *g* for 30 min, the supernatant was mixed with Ni-NTA agarose (Qiagen,), and incubated at room temperature for 1 h. The Ni-NTA bound P16-17 was eluted using 1.0 ml of buffer E (100 mM NaH_2PO_4, 10 mM Tris–Cl, 8 M Urea, pH 4.5). The eluate containing P16-17 was inactive as judged by zymogram assays (not shown). Therefore, the P16-17 fraction eluted from the Ni-NTA column was subjected to size exclusion chromatography (Biogel A-15m, Bio-Rad) with the reasoning that denatured protein may refold after removal of urea by passage through this gel filtration column (Batas and Chaudhuri, 1996). Prior to loading, the column (bed volume: 10 ml) was equilibrated with 25 mM Tris–Cl, pH 7.5. The proteins were eluted with 1.0 ml of equilibration buffer.

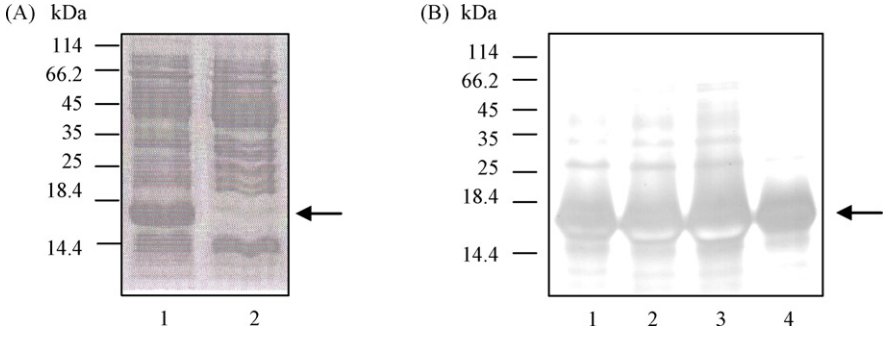

Fig. 1. (A) Synthesis of the recombinant P16-17 in *E. coli* XL-1 Blue (pHYBR1). Following IPTG induction of gene *16-17* in *E. coli* XL-1 Blue (pHYBR1) the cells were harvested and subjected to sonication. After centrifugation at 28,000 × *g*, equal amounts of protein from the pellet (lane 1) and supernatant (lane 2) were resolved on a 12% SDS polyacrylamide gel. The gel was stained with Coomassie blue G250. (B) Purification of P16-17 by size exclusion chromatography. Lane 1-4, equal amounts of fractions containing P16-17 were resolved on a 12% SDS polyacrylamide gel, which was then subjected to silver staining. The position of marker proteins is shown on the left and that of P16-17 is indicated by an arrow.

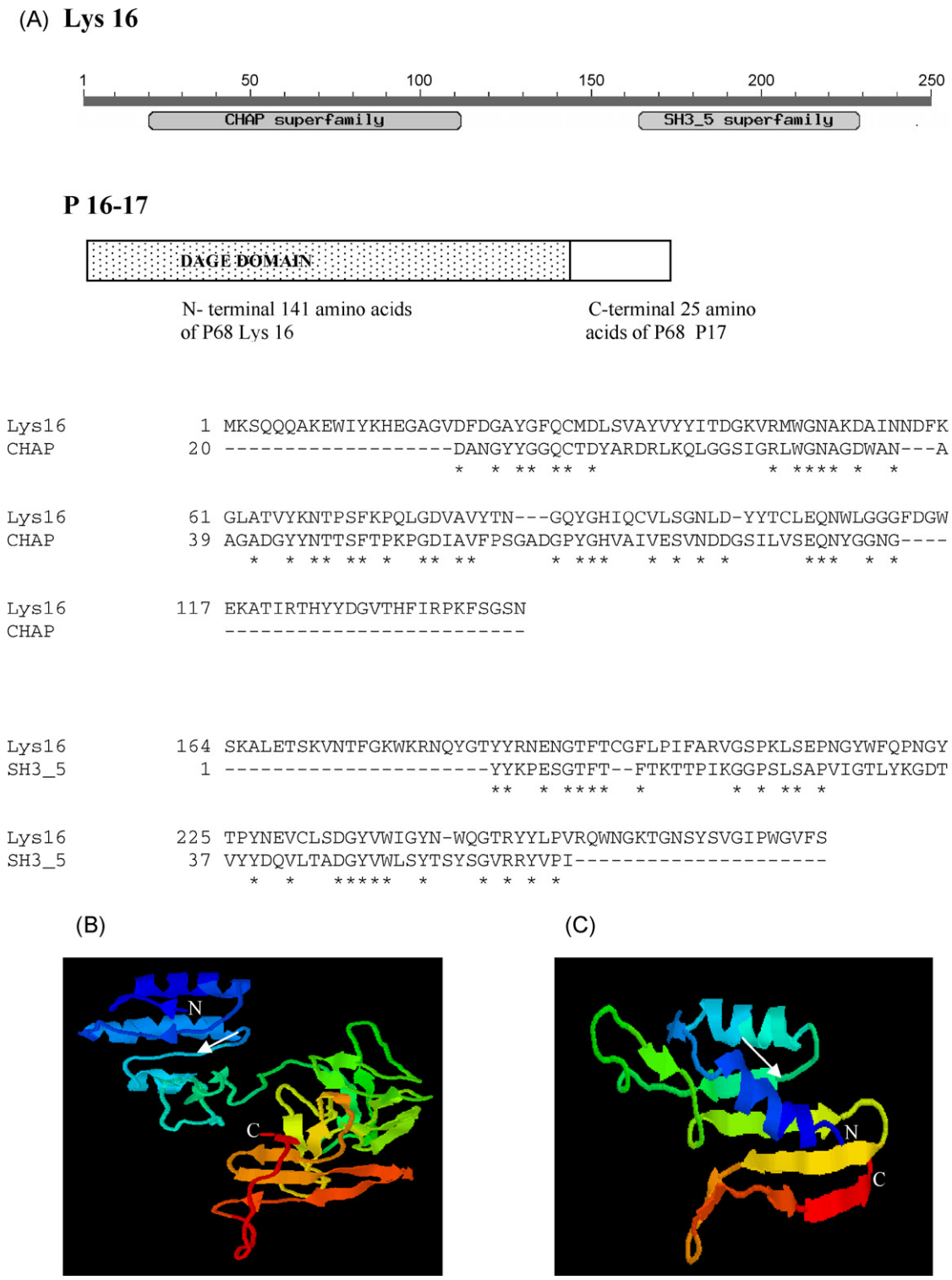

Fig. 2. BLAST analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi?RID=4WN 45HN3015&mode=all) of the Lys16 domains and prediction of the 3-dimensional structure of Lys16 and P16-17. (A) The location of the predicted CHAP and SH3_5 domains (Lu et al., 2006) within the Lys16 sequence is shown on top. A schematic depiction of the P16-17 chimeric endolysin, comprising the N-terminal 141 aa of Lys16 and the C-terminal most 25 aa of P17 and the alignments of Lys16 with the consensus CHAP and SH3_5 domains are shown below. Conserved residues are marked by stars. (B) The 3-D structure of Lys16 was predicted using the LOOPP program available at http://cbsuapps.tc.cornell.edu/index.aspx. The C-terminal end of the 141 aa fragment containing the predicted DAGE domain of Lys16 is indicated by an arrow. (C) Predicted 3-D structure of P16-17 (http://cbsuapps.tc.cornell.edu/index.aspx). The arrow denotes the junction between the Lys16 141 N-terminal aa and the C-terminal 25 aa of P17 in the hybrid protein.

As judged by SDS-PAGE and silver staining P16-17 was 95% pure. The protein was soluble and no precipitation was observed (not shown). The P16-17 fraction shown in Fig. 1B, lane 4 was used for further experiments. The activity, yield and purity of the P16-17preparation(s) in *E. coli* lysates and after the different purification steps is documented in Table 4 (Supplementary material).

2.4. SDS-polyacrylamide gel electrophoresis and zymogram assays

SDS-polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). The gels were either stained with Coomassie brilliant blue G250 or by using the silver staining kit PAGESilver™ (Fermentas). The zymogram assays were carried out as described by Lepeuple et al. (1998). Briefly, 0.2% autoclaved *S. aureus* 68 cells were embedded in SDS-gels. After electrophoresis of the protein samples, the gel was incubated for 30 min in distilled water at room temperature, and then transferred into buffer containing 25 mM Tris (pH 7.5) and 0.1% Triton X-100, followed by further incubation for 16 h at 37 °C. The gel was rinsed with distilled water, stained with 0.1% methylene blue in 0.001% KOH for 2 h at room temperature, followed by de-staining with distilled water. Peptidoglycan hydrolase activity was visualized as a clear zone on dark blue background.

2.5. Cell wall binding assay

Five hundred nanograms of the P16-17 fraction was added to 500 μl of an overnight culture of *S. aureus*. After incubation at 37 °C for 30 min the cells were spun down at 15,000 × *g*. The pellet was washed with BHI media followed by centrifugation at 15,000 × *g*. Unbound protein present in the supernatant was precipitated with 5% TCA at 4 °C for 1 h. *S. aureus* bound P16-17 present in the cell pellet and unbound P16-17 present in the TCA precipitate derived from the supernatant were detected by Western-blotting using polyclonal antibodies raised in rabbits against whole phage 68 particles, i.e. against virion proteins like P17.

3. Results and discussion

3.1. Construction of the chimeric P16-17 protein

Initially, we attempted to purify phage 68 Lys16 with the aim to test its antimicrobial efficacy towards clinical isolates of *S. aureus*. However, these experiments were unsuccessful owing to the poor solubility of the protein (not shown). The N-terminal 112 amino acids of phage 68 Lys16 are 50% homologous to the D-alanyl-glycyl endopeptidase (DAGE) domain of the phage Twort endolysin (Loessner et al., 1998), and a BLAST analysis confirmed the presence of a CHAP domain (Fig. 2A), a typical signature found in enzymes with N-muramoyl-L-alanine amidase and D-alanyl-glycyl endopeptidase activity (Bateman and Rawlings, 2003). The BLAST analysis

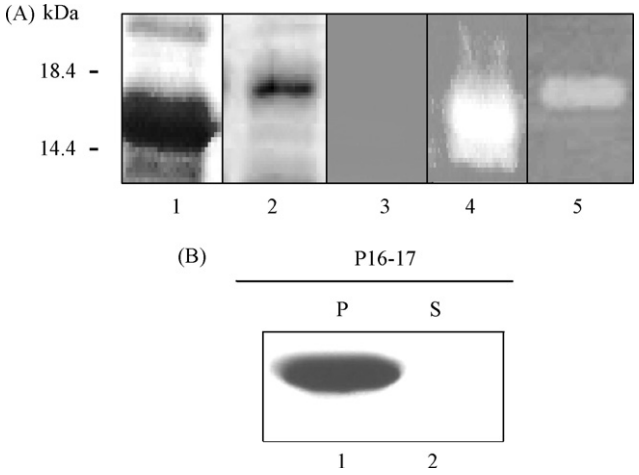

Fig. 3. P16-17 displays muralytic and cell wall binding activity. (A) The protein fractions were resolved on a 12% SDS polyacrylamide gel in the absence (lane 1 and 2) or in the presence of autoclaved *S. aureus* B68 (lanes 3, 4 and 5) murein, respectively. Lanes 1 and 2, electrophoretic pattern of protein fractions containing the enriched $Lys16_{141}$ fragment and the enriched P16-17 protein, respectively. Lanes 3–5, zymogram assays with protein fractions, wherein P16-17 was absent (lane 3), containing $Lys16_{141}$ (lane 4) and enriched P16-17 (lane 5), respectively. The position of marker proteins is indicated on the left. (B) Binding of P16-17 to the cell surface of *S. aureus* strain 68 was revealed by means of immunodetection using antibodies raised against P68 phage particles (see Section 2). Only the relevant part of the gels/Western-blot is shown. Lane 1, P16-17 was found in the cell pellet (P), i.e. the protein associated with the cell surface; Lane 2, supernatant fraction (S).

further suggested the presence of a cell wall binding domain at the C-terminus of Lys16 that is homologues to the bacterial SH3_5 domain (Fig. 2A). The Learning, Observing and Outputting Protein Patterns (LOOPPs) program of the computational biology service unit, Cornell University (http://cbsuapps.tc.cornell.edu/index.aspx) was used to assess the structure of the Lys16 protein. The predicted structure of Lys16 revealed similarity with the crystal structure of the modular pneumococcal phage CPl-1 lysozyme (Hermoso et al., 2003) and with the endolysin of *Listeria monocytogenes* phage PSA (Korndörfer et al., 2006). The putative N-terminal catalytic domain and C-terminal cell wall binding domain in Lys16 were predicted to be separated by a linker sequence, which is likewise present in the endolysins of the phages CPl-1 and PSA (Fig. 2B).

First, we constructed several C-terminally truncated Lys16 proteins to narrow down the catalytic domain (not shown). In agreement with the sequence conservation of the N-terminus of Lys16 and with the bioinformatic predictions, the shortest Lys16 variant, encompassing the N-terminal 141 aa (P16-141) exerted muralytic activity towards *S. aureus* 68 murein in zymograms (Fig. 3A, lane 4). Similar to the entire Lys16 protein, P16-141 showed a poor solubility (not shown). With the rationale to construct a soluble enzybiotic specific for *S. aureus*, next we determined the minimal sequence of the cell wall targeting domain of phage 68 protein 17. The functional

Table 2
Antimicrobial activity of P16-17 in combination with gentamicin towards clinical *S. aureus* strains.

S. aureus	Control (% reduction in viability)	P16-17-f (% reduction in viability)	Gentam. (% reduction in viability)	P16-17-f+Gentam. (% reduction in viability)	Cell wall binding of protein 17
P1	0	25 ± 5	13 ± 2	60 ± 6	+
P2	0	33 ± 2	17 ± 5	62 ± 3	+
P12	0	0	9 ± 6	8 ± 3	–
P16	0	32 ± 3	17 ± 4	59 ± 3	+
P17	0	36 ± 1	13 ± 7	52 ± 8	+

The antimicrobial activity of 10 μg ml^{-1} of the protein fraction enriched for P16-17 (P16-17-f), of 40 ng ml^{-1} gentamicin (Gentam.), and of a combination of 10 μg ml^{-1} of P16-17-f and 40 ng ml^{-1} gentamicin towards clinical *S. aureus* strains was tested by exposing 1×10^7 cfu of the respective strains to the antimicrobial agents or to 10 μg ml^{-1} control protein fraction, wherein P16-17 was absent. The CFU and % survival (average of three experiments) was determined as described in the legend to Fig. 4. Cell wall binding (+) or lack of binding (–) of protein 17 to the respective *S. aureus* strains was determined by Takáč and Bläsi (2005).

dissection of Lys16 prompted us to construct a series of chimeric P16-17 proteins comprising the 141 N-terminal aa of Lys16 abutted to different numbers of C-terminal aa of P17. The cell wall binding capacity of the corresponding protein 16-17 variants were tested as outlined in Section 2 for P16-17. In summary, these studies revealed that the C-terminal most 25 aa of P17 were sufficient to target P16-17 to the cell wall of *S. aureus* strain 68 (Fig. 3B, lane 1). This was rather surprising as the cell wall targeting domains of endolysins are generally larger in size (references in Loessner, 2005).

Recent attempts to identify the ligand required for binding of P17 to the cell surface made proteins, lipids and lipoteichoic acids less likely in serving this function. In a further search for a receptor of the P17 cell wall binding domain, we used the *S. aureus* mutant strains RN4420Δ*tagO* (Gründling and Schneewind, 2006) and AS145 (Stranden et al., 1997), which are defective in the formation of polyribitol wall teichoic acids and in the pentaglycine linkage of the murein, respectively. The pentaglycine linkage is known to be required for binding of lysostaphin (Gründling and Schneewind, 2006). However, the P16-17 protein was shown to bind to the cell wall of either *S. aureus* mutant further excluding both structures as receptors (not shown).

3.2. Antimicrobial activity of P16-17 towards S. aureus

The enriched P16-17 (purity ~95%) was soluble and displayed muralytic activity towards *S. aureus* peptidoglycan in a zymogram assay (Fig. 3A, lane 5). The antimicrobial activity of P16-17 was tested by exposing 1×10^7 cells of exponentially growing *S. aureus* strain 68 to different concentrations of the P16-17 containing sample. A decline in colony *f*orming *u*nits (cfu) was observed after 240 min following addition of the P16-17 containing fraction at a protein concentration of 500 ng ml^{-1}, 2 and 10 μg ml^{-1}, resulting in the reduction of viable cells of *S. aureus* strain 68 to 32 ± 3, 52 ± 6 and 95 ± 3%, respectively (Fig. 4A). As anticipated, the addition of 10 μg P16-17 containing fraction did neither affect the viability of *E. coli* nor that of *B. subtilis*, underlining the specificity of P16-17 for *S. aureus*. Next, we monitored the loss of viability as a function of time upon addition of 10 μg ml^{-1} of the P16-17 containing fraction to 1×10^7 cells of exponentially growing *S. aureus* 68. Following addition, a reduction in the cfu to 48 ± 4 and 6 ± 2% was observed after 30 and 60 min, respectively (Fig. 4B). As the addition of 10 μg ml^{-1} control extract (Fig. 4A and B) and heat inactivated P16-17 (Fig. 4B) did not affect the cell viability, we attributed the observed antimicrobial activity to P16-17.

3.3. P16-17 reduces the effective dose of gentamicin

We have recently shown that infection of the Gram-negative *P. aeruginosa* with a filamentous phage reduced the effective dose of several antibiotics and that a combination treatment with phage and sub-inhibitory doses of the aminoglycoside antibiotic gentamicin, rescued mice from lethal *P. aeruginosa K* infections (Hagens et al., 2006). The augmentation of the antimicrobial efficacy of antibiotics by the filamentous phage can be most probably ascribed to the formation of an aqueous channel in the cell envelope by a phage specific protein (Russel, 1995). Similarly, cell wall-active antibiotics such as penicillin and vancomycin have been shown to act synergistically with membrane impermeable gentamicin, by increasing the

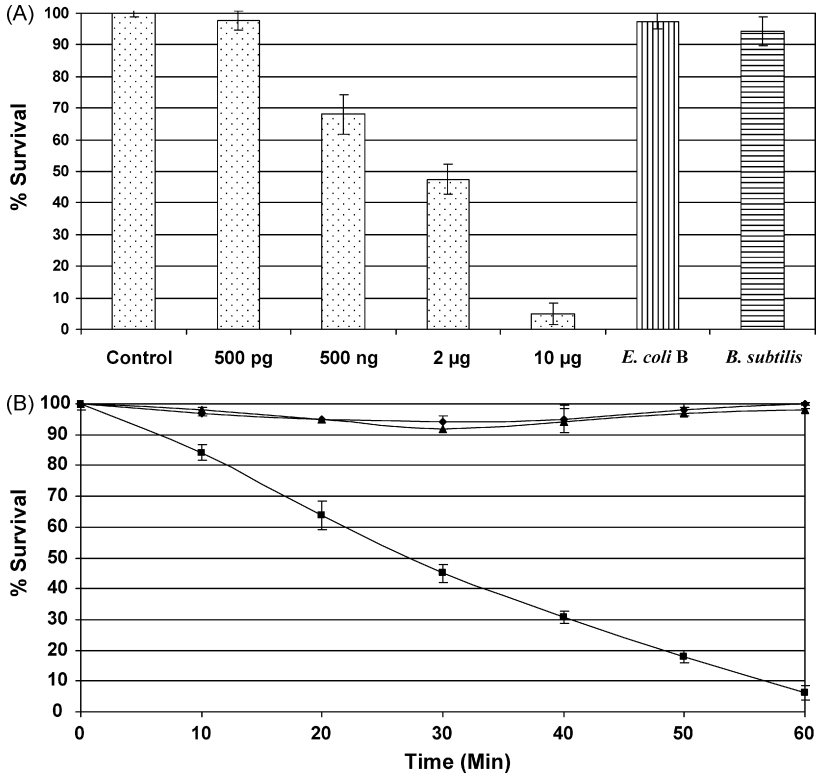

Fig. 4. Antimicrobial activity of protein P16-17. (A) The antimicrobial activity of P16-17 was tested by exposing 1×10^7 cells of exponentially growing *S. aureus* strain 68 for 240 min to 10 μg ml^{-1} of cell lysate obtained from *E. coli* XL-1 Blue (pHYBR1) without induction of P16-17 synthesis (control) and 500 pg ml^{-1}, 500 ng ml^{-1}, 2 and 10 μg ml^{-1} of the protein fraction containing P16-17 (⬚). The antimicrobial activity of P16-17 towards *E. coli* B (▥) and *B. subtilis* (▤) was tested by exposing 1 × 107 cfu of exponentially growing cells to 10 μg ml^{-1} of the protein fraction containing enriched P16-17. (B) 1 × 107 cfu of exponentially growing *S. aureus* strain 68 was exposed to 10 μg ml^{-1} of the protein fraction containing P16-17 (■) and (♦), *S. aureus* strain 68 was incubated with 10 μg ml^{-1} of cell lysate obtained from *E. coli* XL-1 Blue(pHYBR1) without induction of P16-17 synthesis and (▲), with 10 μg ml^{-1} of the P16-17 fraction, which was boiled for 5 min. The cfu of the respective cultures were determined by serial plating after 240 min (A) or at different times (B) after exposure to the protein fractions. Percent survival denotes the reduction in plating efficiency when compared to the control. The experiments were performed in triplicate. The error bars represent standard deviations.

penetration of the latter into the cell (Cottagnoud et al., 2003). These studies prompted us to test whether perturbation of the cell wall by P16-17 would likewise increase the sensitivity of the Gram-positive *S. aureus* towards genetamicin. The minimal inhibitory concentrations of gentamicin for the clinical *S. aureus* isolates P1, P2, P12, P16 and P17 were determined with 1 $\mu g\,ml^{-1}$ (not shown). For instance, a reduction in viability of 32 ± 3 and $17 \pm 4\%$ was observed when *S. aureus* strain P16 (1×10^7 cfu) was treated with 10 $\mu g\,ml^{-1}$ of the P16-17 containing fraction or the sub-inhibitory concentration of gentamicin of 40 $ng\,ml^{-1}$ (Table 2). However, the concomitant addition of 10 $\mu g\,ml^{-1}$ of the P16-17 containing fraction and 40 $ng\,ml^{-1}$ of gentamicin resulted in a further reduction in viability of $59 \pm 3\%$ (Table 2). Comparable results were obtained with the clinical *S. aureus* isolates P1, P2 and P17. The clinical *S. aureus* strain P12 to which phage 68 protein 17 failed to bind (Takáč and Bläsi, 2005) served as a control. As anticipated, P16-17 did not affect the viability and the synergistic effect of a combination treatment was not observed with this strain (Table 2).

We interpret this as showing that P16-17 causes membrane damage, which increases the intracellular penetration of gentamicin. In this way, P16-17 seems be useful to increase the uptake of aminoglycosides with a low membrane permeability, as well as to counteract resistance mechanisms affecting the cellular import of these antibiotics. P16-17 was less effective on the clinical *S. aureus* strains when compared to strain 68 (Table 2 and Fig. 4). Among other possibilities, one variable accounting for this could be the amino-acid composition of the peptidoglycan cross-bridges that can vary in different *S. aureus* strains (De Jonge et al., 1993). Nonetheless, these initial experiments hold promise that the concomitant use of an enzybiotic and an antibiotic can reduce the effective dose of the latter, which seems to be especially desirable for those antibiotics which can exert severe side effects during high dose treatment, such as gentamicin (Wersäll et al., 1969; Forge and Schacht, 2000).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2008.09.003.

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7.2. Direct interaction between ribosomal proteins S1 and S2

Keywords: ribosomal protein S1, ribosomal protein S2, protein-protein interaction

Abstract

In recent years, the structure of the prokaryotic ribosome has been solved at atomic resolution. However, the binding site of the largest and essential ribosomal protein S1 of the 30S subunit is still questionable. There is biochemical evidence for interaction between proteins S1 and S2 and recent studies revealed the requirement of protein S2 for assembly of S1 to the ribosome. Nevertheless, a direct interaction between these proteins has never been shown. Here, we scrutinized this protein-protein interaction employing a yeast-two hybrid system. The results presented indicate a direct S1-S2 interaction. Precisely, the first N-terminal domain of protein S1 ($S1_{106}$), encompassing 106 amino acids was shown to interact with the α2 domain of protein S2, which was suggested to localize in close proximity to protein S1 on the ribosome. In addition, a random mutagenesis study of protein $S1_{106}$ revealed specific amino acids involved in binding to protein S2 pointing towards the interaction surface on S1. Furthermore, we were also able to show that over-production of $S1_{106}$ affects bacterial growth by binding to the 70S ribosomes and thereby blocking the assembly of endogenous protein S1. Taken together, these results indicate the direct S1-S2 interaction, which is essential for translation in Gram-negative bacteria. Moreover, these results support the notion that this interaction surface could serve as a potential target for the design of novel antimicrobial compounds acting semi-selectively against Gram-negative pathogens.

Introduction

Ribosomal protein S1 is the largest ribosomal protein in *E. coli* with a molecular weight of 61 kDa. It is one of the last proteins which associate with the 30S subunit during ribosome biogenesis (Culver, 2003) and binding of protein S1 to the 30S subunit is weak and reversible (Subramanian, 1983). Sequence analysis of protein S1 reveals the presence of six repeating motifs, the 'so-called' S1 domains (Fig. 1; Bycroft *et al.*, 1997). The two N-terminal domains are involved in binding to the ribosome (N1-N2; McGinness and Sauer, 2004; Subramanian *et al*., 1981). Domains R1-R3 bind ssRNA and RNA pseudoknots (Fig. 1; Boni *et al*., 1991; Subramanian, 1983; Aliprandi *et al*., 2008). The distal domain R4 has been suggested to be important for autogenous regulation of *rpsA* (Boni *et al*., 2001). Protein S1 is essential for translation initiation of canonical mRNAs and especially for the translation of highly structured mRNAs (Szer *et al*., 1975; van Dieijen *et al*., 1976). It interacts with a pyrimidine-rich region upstream of the SD-sequence and in addition, it has been shown to unwind RNA secondary structures (Bear *et al*., 1976; Thomas *et al*., 1978; Rajkowitsch and Schroeder, 2007), thereby facilitating the positioning of the 30S subunit in close proximity to the translational start site (de Smit and van Duin, 1994). However, S1 is dispensible for translation of leaderless mRNAs (Tedin *et al*., 1997; Moll *et al*., 2002a). In addition, a recent study suggests the formation of 61S ribosomes in the presence of the antibiotic kasugamycin *in vivo* (Kaberdina *et al*., 2009). These 61S particles are devoid of several ribosomal proteins of the small subunit including the functionally important proteins S1 and S2. However, these ribosomal particles are proficient in translation of leaderless mRNAs (Kaberdina *et al*., 2009).

Protein S1 is the only 30S subunit protein which binds to the ribosome by means of protein-protein interactions (Boni *et al*., 1982). Cross linking experiments suggest a direct interaction between proteins S1 and S2 (Laughrea and Moore, 1978; Bollen *et al*., 1979). In addition, cryo-electron microscopy indicates that the N-terminal domain of S1 penetrates into the head of the 30S subunit, whereas the C-terminal part is exposed to the solvent side, facing

protein S2 (Sengupta *et al*., 2001). Studies performed by Moll *et al.* (2002) revealed that protein S2 is required for the binding of protein S1 to the ribosome. These results support the possible interaction between proteins S1 and S2.

In this study, we verified the direct interaction between ribosomal proteins S1 and S2. Our data revealed that the N-terminal domain of protein S1 (referred here as $S1_{106}$) and the α2 domain of protein S2 is essential for this interaction. In addition, mutational analysis of $S1_{106}$ indicates the amino acid residues important for the interaction with the α2 domain of protein S2.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultivated in Luria-Bertani medium (Miller, 1972) at 37^{o}C. When appropriate, ampicillin (100 μg ml^{-1}), was added. Where indicated, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 50 μm or 1 mM. Yeast strain (HF7c) was grown in YPD medium or in synthetic dropout medium lacking Leu, Trp or both (Clontech, US). In order to prevent false positive results during the restoration of His auxotrophy, 3-amino-1,2,4-triazole (Sigma, US) was used to a final concentration of 5 mM when mentioned.

Construction of plasmids

To purify the truncated variant of protein S1, genes encoding the ribosomal protein S1 and truncated variant of protein S1 ($S1_{106}$), were amplified by primers FPS1 and RPS1, FPS1 and RPS1106 (Table 2) thereby yielding a 1671 and 318 bp fragment, respectively from the *E. coli* (MC4100) bacterial chromosome. The amplified fragments were cloned as *Nde*I/*Xho*I into pET22b (+) vector (Novagen) creating the plasmids pS1 and pS1106 (Table 1), respectively. The transcription of the S1 and $S1_{106}$ protein gene in pS1 and pS1106 is

controlled by a T7-promoter. Similarly, the gene encoding the mutant $S1_{106}$ protein was amplified by primers FPS1 and RPS1106 using pGBTS1106MUT (Table 1) as template. The PCR amplified 318 bp fragment was cloned into pET22b plasmid as *Nde*I/*Xho*I, creating the plasmid pPETMUTS1106.

For elucidating protein-protein interactions *in vivo*, the yeast two hybrid system was employed. Two plasmids, pGBT9 (harboring the Gal4 DNA-binding domain, DBD) and pGAD424 (harboring the Gal4 activation domain, AD) were used for the studies. Genes encoding the ribosomal proteins S1 and S2 were amplified using the primers FPY2HS1/RPY2HS1 and FPY2HS2/RPY2HS2 (Table 2), respectively. To obtain the Gal4 DBD- and AD-protein fusion genes, the PCR amplified products (1671 bp and 750 bp, respectively) were cloned into the *EcoR*I/*BamH*I site of the plasmid pGBT9 and pGAD424, thereby creating the plasmids pGBTS1 and pGADS2, respectively (Table 1). Similarly, the gene encoding $S1_{106}$ and the mutant $S1_{106}$ were amplified by the primers FPY2HS1 and RPY2HS1106. The amplified product of size 318 bp was cloned into pGBT9 plasmid as *EcoR*I/*BamH*I creating the plasmid pGBTS1106 and pGBTS1106MUT (Table 1). The gene encoding a truncated variant of protein S1 harboring both N-terminal domains ($S1_{194}$) was amplified by primers FPY2HS1 and RPY2HS1194 yielding a 582 bp fragment. This amplified PCR product was cloned as *EcoR*I/*BamH*I into the plasmid pGBT9, thus creating the plasmid pGBTS1194 (Table 1). The gene encoding the α2 domain of protein S2 was cloned into pGAD424 vector following amplification using primers FPY2HS2 and RPY2HS2helix (Table 2). The 150 bp PCR amplified fragment was cloned as *EcoR*I/*BamH*I, creating the plasmid pGADS2helix (Table 1).

Random mutagenesis using error prone PCR

In order to introduce mutations into gene encoding for protein $S1_{106}$, plasmid pS1106 was amplified with primers FPS1 and RPS1106. The error prone PCR has been performed under

the following conditions: 7 mM $MgCl_2$, 50 mM KCl, 20 mM Tris (pH 8.4), 0.5 mM $MnCl_2$, 1 mM dTTP, 1 mM dCTP, 0.2 mM dGTP, 0.2 mM dATP. The amplified 318 bp fragment was cloned into corresponding plasmid as described above.

Analysis of protein interactions using a yeast two hybrid system

The yeast two hybrid system experiments were performed according to manufacturers description (Clontech, US). The β-galactosidase assays from yeast cells were performed as described (Guarente, 1983; Miller, 1972). Three independent assays were performed for each mutant.

Purification of proteins S1106 and mutant S1106.

E. coli TUNER bearing plasmid pS1106 and pPETMUTS1106 were grown to an OD_{600} of 0.8. Then 1.0 mM IPTG was added and incubation was continued at 37 °C for 2 h. The cells were harvested by centrifugation at 6000 × *g* for 10 min. The pellet was resuspended in 20 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 100 mg ml^{-1} lysozyme, 24 mg ml^{-1} RNase A, pH 8.0), followed by freezing and thawing in liquid nitrogen and sonication. Upon centrifugation at 30,000 × *g* for 15 min, the supernatant was mixed with Ni-NTA agarose (Qiagen, US), and incubated at 4°C for 1 h. The Ni-NTA bound $S1_{106}$ and $MUTS1_{106}$ were washed with buffer C (1.5 M NaCl, 50 mM NaH_2PO_4, 0.1 % Triton-X 100, 20 mM Imidazole, pH 8.0) and eluted using 2.0 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8.0). The eluates containing the purified proteins were dialyzed against buffer 1x VD buffer (10 mM Tris-Cl, 10 mM MgOAc, 60 mM NH_4Cl, 3 mM β-Mercapto-EtOH, 100 mM PMSF, 10 % glycerol, pH 7.5).

Purification of crude ribosomes upon overexpression of the truncated rpsA gene

The *E. coli* strain TUNER harboring plasmid pS1106 was grown in LB to an OD_{600} of 0.2. At time points 0, 20, 40, 60, and 90 min upon addition of 0.5 mM IPTG, the cells were harvested by centrifugation at 6000 x g for 10 min followed by the resuspension of the pellet in VD buffer (10 mM Tris-Cl, 10 mM MgOAc, 60 mM NH_4Cl, 3 mM β-Mercapto-EtOH, 100 mM PMSF, 100 mM Benzamidine, Dnase I, 100 mM DTT, pH 7.5). The cells were disrupted by sonocation and upon centrifugation at 12000 x g for 10 min the supernatant was withdrawn and re-centrifuged at 15000 x g for 30 min. The supernatant was layered onto 1.1 M sucrose cushion made up in VD buffer. Upon centrifugation at 30000 x g for 60 min the pellet containing crude ribosomes was resuspended in VD buffer and stored at -80^{o}C.

Purification of 30S(-S1)

The 30S ribosomal subunits were prepared essentially as described by Spedding (Spedding, 1990). Protein S1-depleted 30S ribosomes were prepared by affinity chromatography using poly(U)-Sepharose 4B (Pharmacia) (Suryanarayana and Subramanian, 1983).

In vitro binding of S1106 and S1106Mut to 30S(-S1) ribosomes

The purified $S1_{106}$ and $S1_{106Mut}$ (100 pm) were incubated with 30S(-S1) (50 pm) ribosomes at 37^{o}C for 20 min followed by loading onto a sucrose gradient (10-30%) made up in VD buffer. Upon centrifugation for 5 h at 45000 x g, the gradients were fractionated using an ISCO UV/VIS detector (ISCO, US). The fractions containing 30S ribosomes were treated with 5% TCA at 4^{o}C and precipitated proteins were separated on a 12% SDS-PAGE as described by Laemmli (1970). The S1 variants bound to the 30S ribosomes were detected by western blot analysis.

Western blot analysis

Upon separation of the ribosomal proteins by SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Whatman, Germany) by employing a semi-dry blotting apparatus. The nitrocellulose bound proteins were detected by either monoclonal anti-bodies specific for His-tag raised in rabbits (Qiagen) or with polyclonal antibodies specific against ribosomal proteins derived from goat.

Results

Overexpression of $S1_{106}$ affects growth of E. coli

The over production of the N-terminal domain of protein S1 comprising the first two domains (194 aa) is toxic for *E. coli* (McGinness and Sauer, 2004). It is suggested that the fragment binds to the ribosome and displaces endogenous protein S1, which leads to inhibition of protein synthesis and cessation of cell growth. Here, we aimed to determine whether the very N-terminal domain of S1 consisting of 106 amino acids from the N-terminus inhibit cell growth. Therefore, *E. coli* strain Tuner harbouring the plasmid pS106, which codes for the truncated variant $S1_{106}$, was over produced by addition of IPTG. As shown in Fig. 2A, the over production of $S1_{106}$ affects the growth of *E. coli* comparable to overexpression of the truncated *rpsA194* gene encoding protein $S1_{194}$. The growth of both, $S1_{106}$ and $S1_{194}$ overproducing strains, ceased 80 min upon induction. As control, overexpression of the *rpsA* gene encoding the full length protein S1 was included, which resulted in a slight reduction of cell growth. In order to confirm the binding of $S1_{106}$ to the ribosome, concomitantly samples were withdrawn from the respective culture at specific time points following induction of $S1_{106}$ synthesis and crude ribosomes were purified. To determine whether $S1_{106}$ binds to the 70S ribosomes the ribosomal proteins were precipitated using TCA and subjected to SDS-PAGE analysis as described in Materials and Methods. Protein $S1_{106}$ was identified by Western blot analysis using antibodies specific for the histidine tag (Fig 2B). As loading

control protein S2 was identified using S2 specific antibodies (Fig. 2B, upper panel). As shown in Fig. 2B, we were able to verify the binding of protein $S1_{106}$ to the ribosome already 20 min upon induction (Fig. 2B, lane 3). Furthermore, the amount of protein $S1_{106}$ increased during prolongued induction compared to the amount of protein S2 (lanes 4-6).

Interaction of $S1_{106}$ with S2

Biochemical studies suggested the close proximity of interaction between proteins S1 and S2 on the ribosome (Sengupta *et al*., 2001). Furthermore, the association of protein S1 to the 30S subunit is dependent on protein S2 (Moll *et al*., 2002; Laughrea and Moore, 1978; Bollen *et al*., 1979). As $S1_{106}$ has been shown to bind to the 70S ribosome, the next aim was to elucidate, whether the first domain of protein S1 is responsible for the interaction with protein S2. To monitor the protein-protein interaction, we employed a yeast two hybrid approach as described in materials and methods, where interaction of the proteins resulted in the synthesis of β-galactosidase. As shown in Fig. 3 the β-galactosidase activity given in Miller units (MU) increased when the plasmids coding for the Gal4DBD-$S1_{106}$ fusion protein and the Gal4AD-S2 fusion protein were used, compared to the assay when the whole length S1 and $S1_{194}$ (two N-terminal domain of S1: 194 aa) was used. These data indicate the direct interaction between $S1_{106}$ and S2. These results are in line with the previous observation that ribosomes lacking S2 in turn lacks S1 (Moll *et al*., 2002). It is therefore likely that the binding of S1 to the 30S subunit requires direct interaction of its N-terminal domain with protein S2.

Identification of amino acids involved in S1/S2 interaction

To determine which amino acids of $S1_{106}$ are involved in interaction with protein S2, next a random mutagenesis study was performed. Employing error prone PCR random mutations were introduced into the gene encoding protein $S1_{106}$ and the mutant proteins were tested for their ability to interact with protein S2 using the yeast two hybrid approach. Upon

transformation of the plasmid library harboring the genes encoding mutant $S1_{106}$ into the yeast strain harboring plasmid pGAD424S2, we selected transformants yielding reduced or increased β-galactoside activities compared to the wild type $S1_{106}$, indicating an affected affinity between the $S1_{106mut}$ and S2. The subsequent sequencing of the genes coding for the mutant $S1_{106}$ proteins were performed (Agowa, Germany). As shown in Fig. 4A, three mutants of protein $S1_{106,}$ $S1_{106Mut}$#13, $S1_{106Mut}$#18, and $S1_{106Mut}$ #19 yielded no significant MU read out, suggesting that they are not able to interact with protein S2. However, sequence analysis revealed the presence of an internal stop codon in #13 and #18, which explains the lack of interaction with protein S2 (Fig. 4A). However, $S1_{106Mut}$ #19 harbours a Val→Ile mutation at position 35 (Fig. 4A). In addition, increased β-galactoside activity yielded by the mutant $S1_{106Mut}$#2, which harbours two point mutations at position 40 (Ala→Ser) and position 43 (Lys→Ile), indicated a stronger interaction with protein S2 as compared to the wild type $S1_{106}$. As shown in Fig. 4B these residues are conserved throughout Gram-negative bacteria. Taken together, these results indicate the importance of the region covering aa 34-45 for interaction with protein S2. *In silico* analysis of the N-terminal domain of protein S1 suggests that this motif represents a hinge between two a-helices, as depicted in Fig. 4B. Therefore, changes in the amino acid sequence might lead to a structural rearrangement which might affect protein-protein interaction.

To further validate the binding of proteins $S1_{106}$ and $S1_{106Mut}$#2 to 30S(-S1) ribosomes *in vitro*, both proteins were purified using affinity chromatography as described in Materials and Methods. Upon incubation of the S1-depleted 30S subunits with the truncated S1-variants, the mixture was layered onto a sucrose gradient and ribosomal subunits were separated from free $S1_{106}$ proteins by centrifugation. The fractions containing the 30S subunits were tested for the presence of the respective S1 variants by Western blot analysis. As shown in Fig. 4C, although the 30S subunits were not completely depleted for S1, both variants of S1, $S1_{106}$ and $S1_{106Mut}$#2, are present in the 30S fraction, indicating their interaction with the ribosome.

$S1_{106}$ interacts with the α2 domain of S2

The α-helix domain of protein S2 was suggested to be located in the close proximity to S1 (Sengupta *et al*., 2001; Fig. 5A). This helix-loop-helix motif spans the region from amino acid 104 to amino acid 150 of protein S2 (Fig. 5B). We therefore anticipated that this protruding domain of protein S2 might be involved in interaction with S1. To validate this possiblity, the interaction with protein $S1_{106}$ was analyzed using the yeast two hybrid system. Compared to the β-galactosidase activity determined for the interaction between $S1_{106}$ and full length protein S2 (Fig. 5C, lane 3), the results show a comparable interaction between the α2 domain of protein S2 and $S1_{106}$ (Fig. 5C, lane 6).

Discussion

Direct interaction between protein S1 and S2

Protein S1 is one of the most important proteins of the 30S subunit and is involved in translation initiation, elongation and trans-translation of canonical mRNAs in Gram-negative bacteria (Tzareva *et al*, 1994; Potapov and Subramanian, 1992). It has been shown that over production of the two N-terminal domains of S1 ($S1_{194}$; McGinness and Sauer, 2004) was toxic for the cells. Therefore, these domains were termed the "ribosome binding domain" of S1. Here, we were able to show that expression of the *$rpsA_{106}$* gene coding for the first N-terminal domain of S1 ($S1_{106}$) is sufficient to inhibit cell growth (Fig. 2A). Moreover, we were able to verify binding of $S1_{106}$ to crude ribosomes upon overexpression (Fig. 2B). Therefore, it could be assumed that the toxicity of the synthesis of $S1_{106}$ could be attributed to the accommodation of the truncated $S1_{106}$ within the binding pocket of whole length S1 on the 30S subunit. Consequently, this binding would inhibit assembly of endogenous protein S1 to the ribosome. Taken together, our data suggest that the N-terminal domain of S1 ($S1_{106}$) is sufficient for the interaction with the ribosome. Several studies indicate the close proximity of

S1 to the ribosomal protein S2 (Sengupta *et al*., 2001), and recent work by Moll *et al.* (2002) support the hypothesis, that protein S2 is required for assembly of S1 on the ribosome. Here, we were able to support this notion by employing a yeast two hybrid analysis, which reveals the interaction between ribosomal proteins S1 and S2. Furthermore, these results demonstrate that the N-terminal domain of S1 ($S1_{106}$) is sufficient for efficient binding to S2.

In addition, employing a random mutation analysis we identified amino acids and domains of $S1_{106,}$ which might contribute to the interaction with protein S2. Significantly, two point mutations in $S1_{106}$ located at amino acid position 40 and 43, which are conserved in Proteo-bacteria, increased the affinity to protein S2. Precisely, the non-polar alanine residue at position 40 was replaced to a polar serine residue. The second mutation comprised the change of the polar lysine residue at position 43 to the non-polar isoleucine (Fig. 4B). Interestingly, in the low "GC" group of Gram-positive bacteria, which do not require binding of S1 to the ribosome for translation, these amino acids are not conserved. Furthermore, since these mutations are located between two predicted α-helices in protein $S1_{106,}$ these amino acids could be important for the positioning of these helices, which might provide the interaction surface for protein S2.

In addition to the mutational analysis on protein $S1_{106}$, another aim of the study was the identification of a possible interaction domain on protein S2. The structural analysis of protein S2 reveals that it is a slightly elongated protein with a two-domain organization (Georgalis *et al*., 1981; Wittmann *et al*., 1980) The elongated α2 domain of protein S2 is located in close proximity to protein S1 on the ribosome (Sengupta *et al*., 2001), suggesting that it might serve as site of interaction for S1. Therefore, we tested whether the α2 domain, a helix-turn-helix motif situated between amino acids 104 and 150 of S2, is able to bind to protein S1. Using the yeast two hybrid system, we observed a significant interaction between the α2 domain and protein S1 as well as $S1_{106}$ (Fig. 5C). This observation leads to the

conclusion that interaction between proteins S1 and S2 occurs between the N-terminal S1-domain and the S2-α2 domain.

The interaction surface between proteins S1 and S2: A possible target for novel antimicrobials

Antibiotics are low molecular weight compounds produced by microorganisms that kill or inhibit the growth of other microorganisms at concentrations in the μM range. The majority of clinically relevant antibiotics target the ribosome. Precisely, numerous antibiotics bind to functionally important regions within the 16S rRNA in order to establish contact with the ribosome and block its function (Yonath, 2005). As a consequence, point mutations in the 16S rRNA gene exploited by '*natural selection*' often confer bacterial resistance towards several antibiotics targeting the ribosome (Skinner *et al*., 1983). In addition, since these functionally imortant sites of the protein synthesis machinery are conserved within eukaryotes, bacteria and mitochondria, these drugs can have adverse side effects when used as clinical therapeutics. Thus, there is a need for alternative antimicrobials, which exhibit a higher specificity.

Since specific protein-protein interactions are essential in most biological processes, disruption of highly bacterial specific protein-protein interactions by competitive molecules could represent potential tool to specifically inhibit growth of bacteria (Arkin and Wells, 2004; Toogood, 2002). As mentioned above, S1 is an important protein bound to the ribosome by the help of protein S2. Since our results suggest that interaction between protein S1 and S2 plays a crucial role in the translation initiation in *E. coli* and as a S1 homologue is absent in the low GC-group of Gram-positive bacteria, it is conceivable that a compound with the potential to inhibit the assembly of S1 on the ribosome by interfering with S1/S2 interaction could be a potential antimicrobial that specifically inhibits growth of Gram-negative bacteria. Moreover, resistance towards drugs, which are based on an alteration of a

protein-protein interaction site, are more difficult to develop as point mutations in 16S rRNA, since most alterations would be detrimental for the cell.

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Fig. 1.

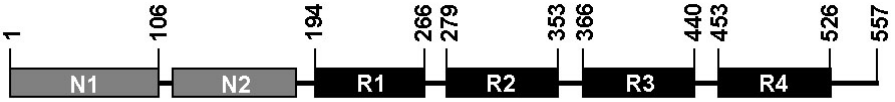
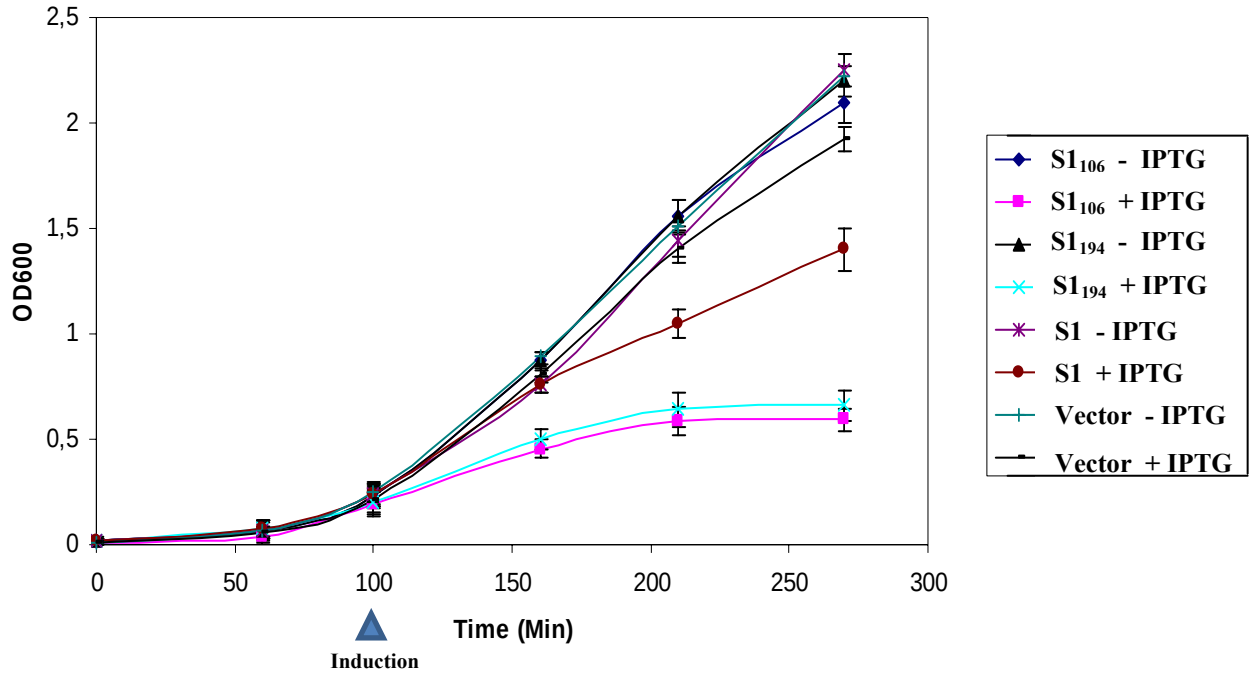


Fig. 2A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2
1,5
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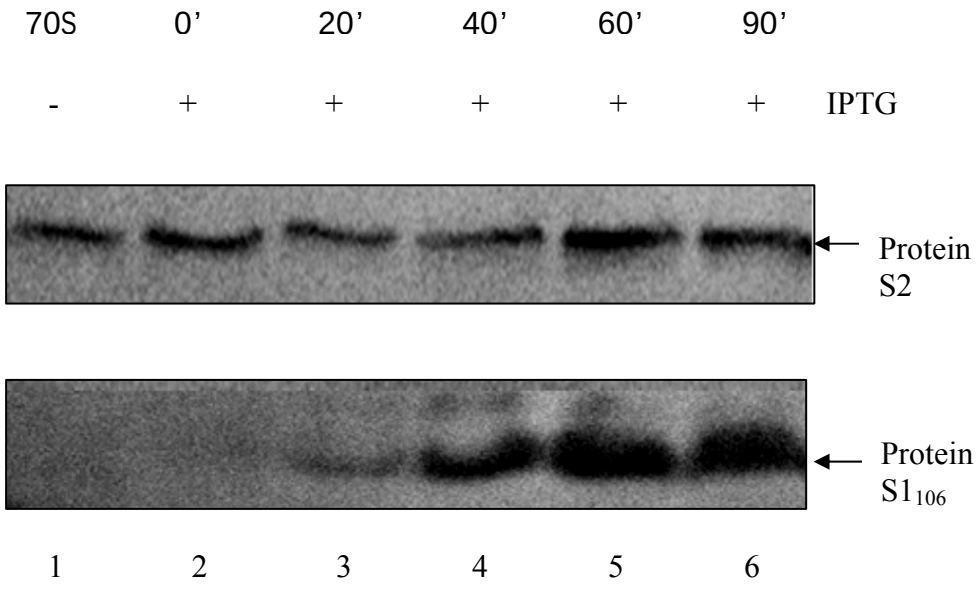
0
OD600
0
50
100
150
200
250
300
Time (Min)
Induction
S1106 - IPTG
S1106 + IPTG
S1194 - IPTG
S1194 + IPTG
S1 - IPTG
S1 + IPTG
Vector - IPTG
Vector + IPTG

Fig. 2B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IPTG
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Protein S1106
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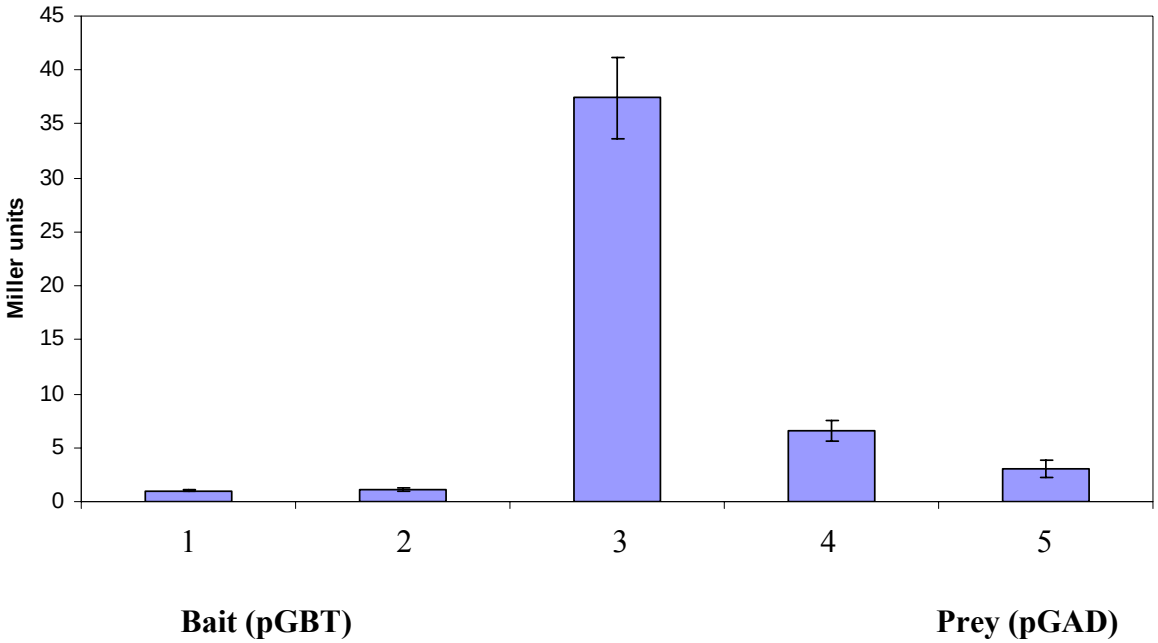
Fig. 3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Miller units
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Bait (pGBT)
Prey (pGAD)

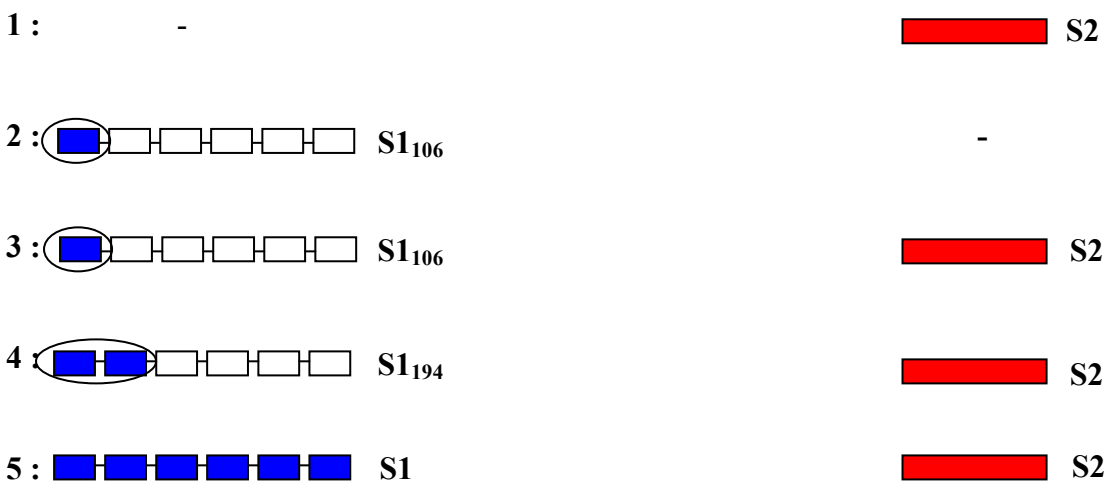
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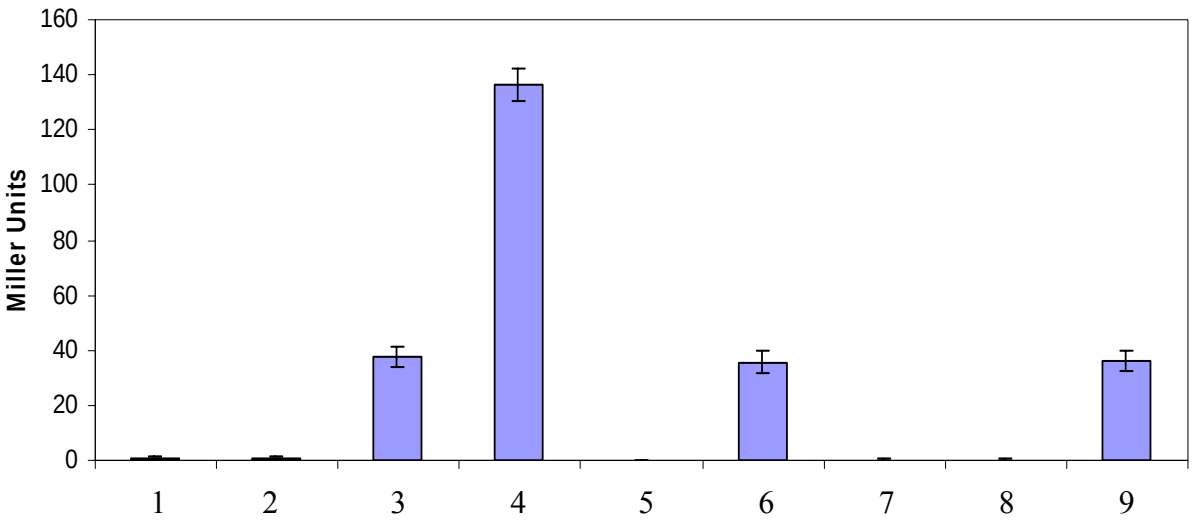
Fig. 4A



	Bait (pGBT)	Prey (pGAD)
1.	–	S2
2.	$S1_{106}$	–
3.	$S1_{106}$	S2
4.	$S1_{106Mut}$ #2	S2
5.	$S1_{106Mut}$ #13	S2
6.	$S1_{106Mut}$ #15	S2
7.	$S1_{106Mut}$ #18	S2
8.	$S1_{106Mut}$ #19	S2
9.	$S1_{106Mut}$ #20	S2

$S1_{106}$	MTESFAQLFEESLKEIETRPGSIVRGVVVAIDKDVVLVDAGLKSESAIPAEQFKN AQGELEIQVGDEVDVALDAVEDGFGETLLSREKAKRHEAWITLEKAYEDAET
$S1_{106Mut}$ #2	MTESFAQLFEESLKEIETRPGSIVRGVVVAIDKDVVLVD**S**GL**I**SESAIPAEQFKN AQGELEIQVGDEVDVALDAVEDGFGETLLSREKAKRHEAWITLEKAYEDAE**L**
$S1_{106Mut}$ #13	MTESFAQLFEESLKEIETRPGSIVRGVVVAIDKDVVLVDAGLKSESAIPAEQF#N AQGELEIQVGVEVDVALDAVEDGFGETLLSREKAKRHEAWITLEKAYEDAEL
$S1_{106Mut}$ #15	MTESFAQLFEESLKEIETRPGSIVRGVVVAIDKDVVLVDAGLKSESAIPAEQFKN AQGELEIQVGDEVDVALDAVEDGFGETLLSREKAKRHEAWITLEKAYEDAE**L**
$S1_{106Mut}$ #18	MTESFAQLFEESLKEIETRPGSIVRGVVVAIDKDVVLVDAGLKSESAIPAEQFKN AQGELEIQV**S**DE**L**TLLWTQ@KTASVKLCCPVRELNVTEPGSRWIKLTKMLN
$S1_{106Mut}$ #19	MTESFAQLFEESLKEIETRPGSIVRGVVVAIDKD**I**VLVDAGLKSESAIPAEQFKN AQGELEIQVGDEVDVALDAVEDGFGETLLSREKAKRHEAWITLEKAYEDA**DT**
$S1_{106Mut}$ #20	MTESFAQLFEESLKEIETRPGSIVRGVVVAIDKDVVLVDAGLKSESAIPAEQFKN AQGELEIQVGDEVDVALDAVEDGFGETLLSREKAKRHEAWITLEKAYEDAE**L**

Fig. 4B

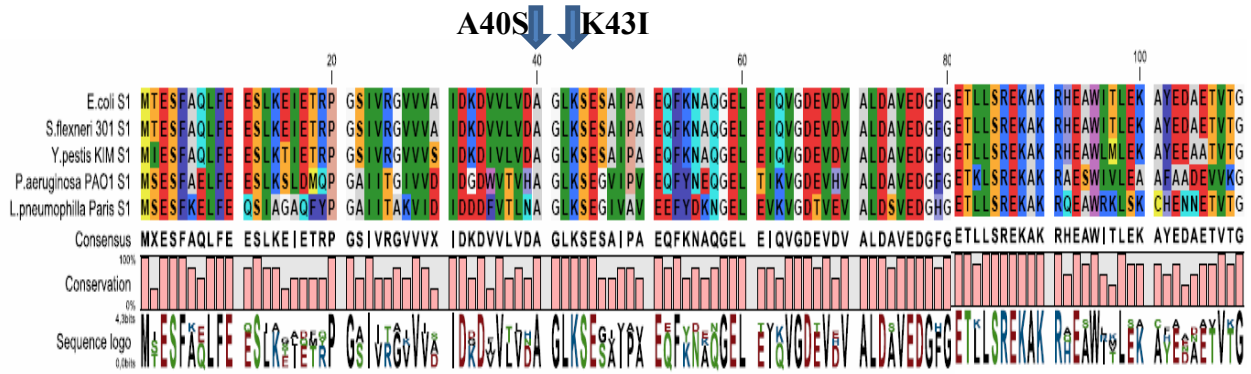
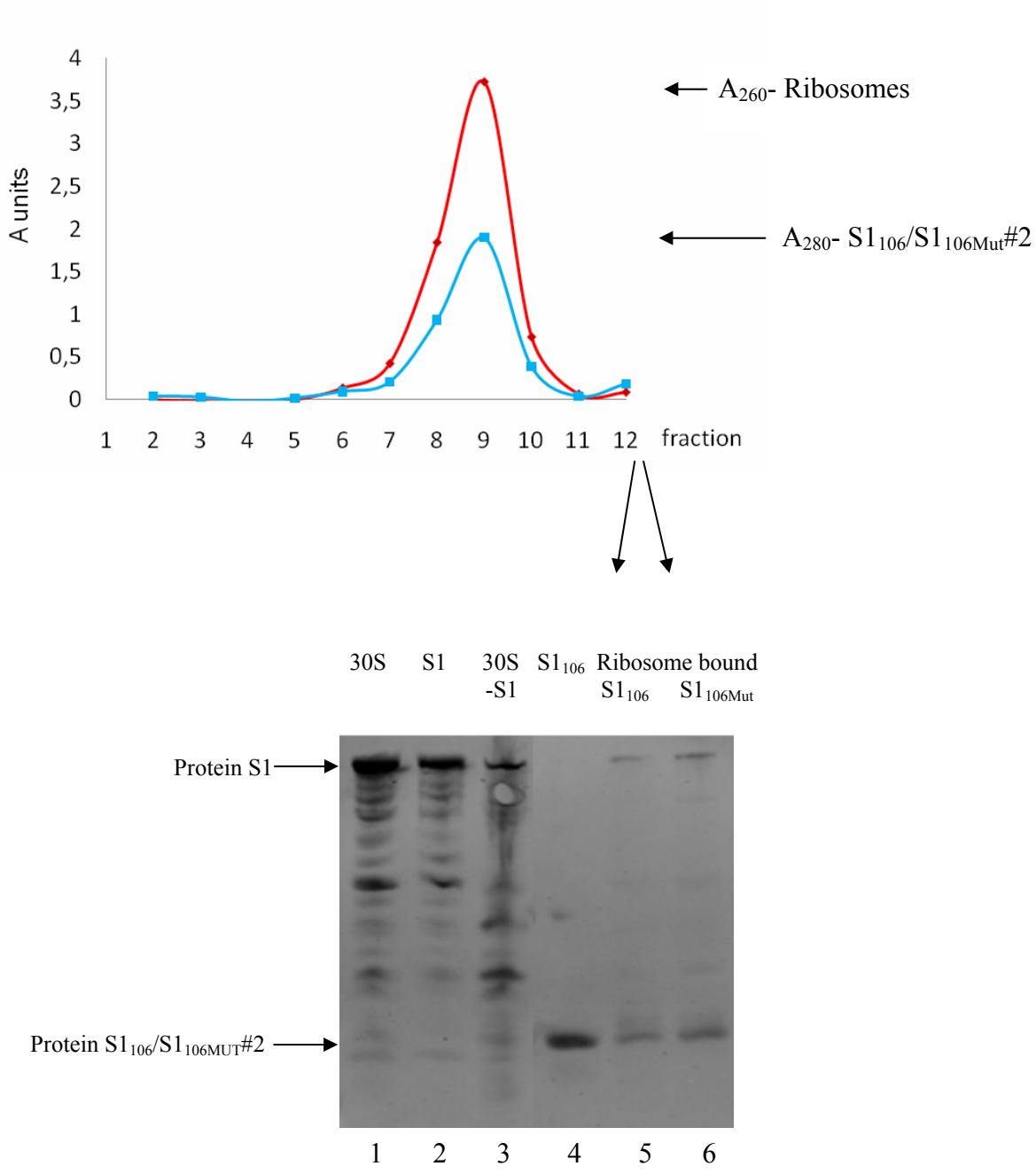


Fig. 4C


A units
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A260- Ribosomes
A280- S1106/S1106Mut#2
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Ribosome bound S1106Mut
Protein S1
Protein S1106/S1106MUT#2
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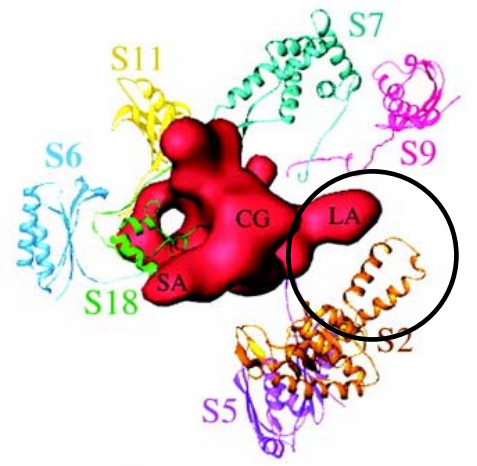
Fig. 5A


S7
S11
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S6
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Fig. 5B


Helix
Helix
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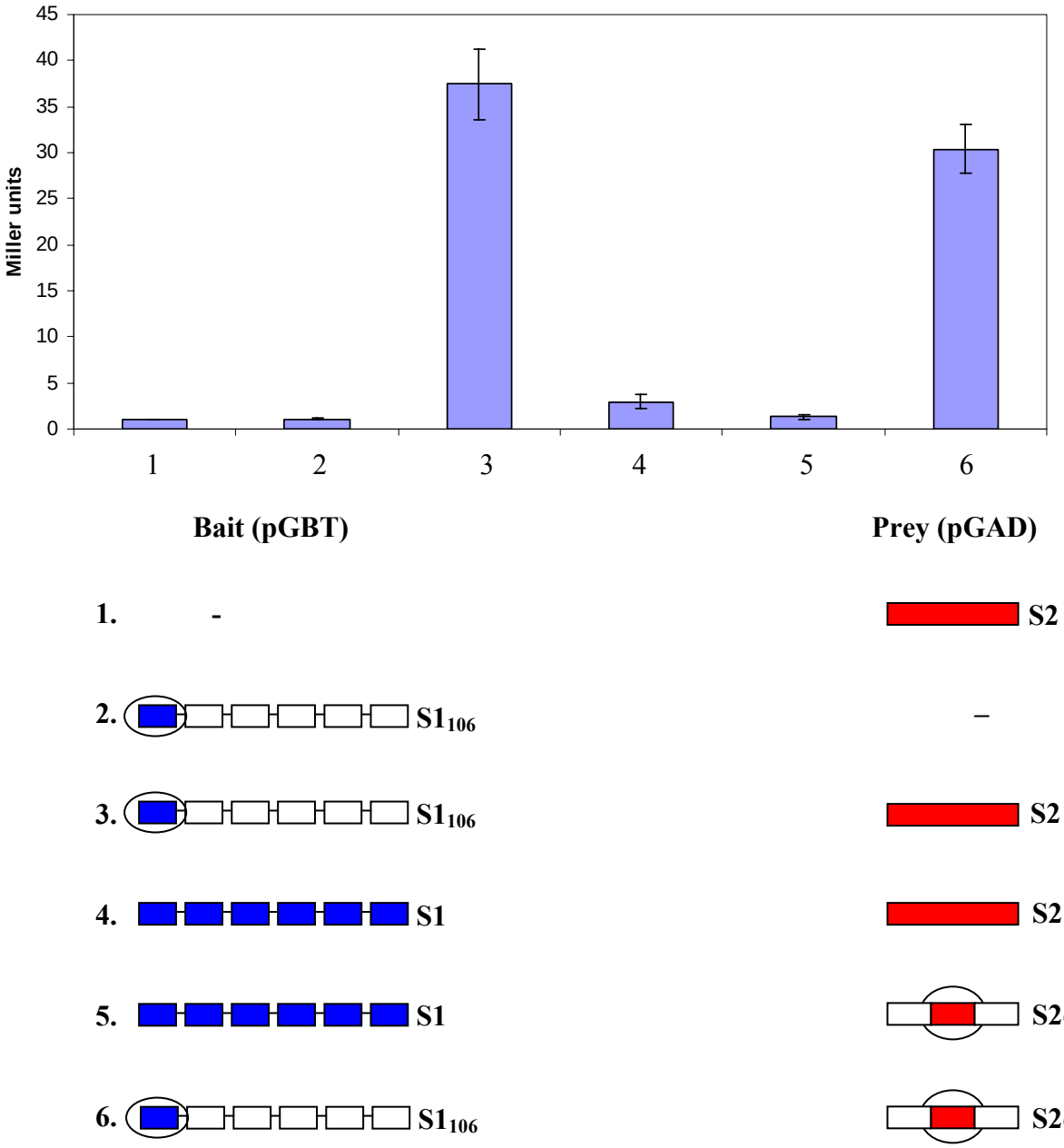
40
S2alpha2 E.coli MLTNWKTVRQSIKRLKDLETQSQDGTFDKLTKKEALMRTRELEKLENSLGG

Fig. 5C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2. S1106
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3. S1106
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4. S1
S2
5. S1
S2α2
6. S1106
S2α2

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Figure legends:

FIG.1. Schematic depiction of the different domains of ribosomal protein S1. The N-terminal repeats, required for binding to the ribosome (Giorginis and Subramanian, 1980) and the C-terminal RNA binding domains (Bycroft *et al*., 1997) are shown in grey and black, respectively. Numbers shown above indicate amino acid positions.

FIG. 2. Synthesis of $S1_{106}$ causes cell death by binding to the ribosome. (A). Growth of *E. coli* cells synthesizing $S1_{106}$. The cells carrying the corresponding plasmids (pS1, pS1106 and pET22b) harboring genes encoding for proteins S1, or $S1_{106}$, respectively, were induced with 50 μm IPTG. Growth was monitored by measuring the OD_{600}. 80 min upon induction of the gene encoding $S1_{106}$ and $S1_{194}$, cessation of growth was observation. Overexpression of the *rpsA* gene encoding for the full length S1 protein only slightly affected cell growth (B). Binding of protein $S1_{106}$ to the ribosome. Western blot analysis of the crude ribosome fraction reveals that 20', 40', 60' and 90' upon induction protein $S1_{106}$ was bound to the ribosome. The amount of $S1_{106}$ bound to the ribosome increase with time after induction of gene encoding $S1_{106}$ (lanes 2-6) compared to protein S2, which served as internal control. Lane 1: purified 70S ribosomes.

FIG. 3. Yeast two hybrid system indicating the interaction between S1 and its truncated variants with S2. The β-galactosidase activity given in Miller Units (MU) was used as reporter for the protein-protein interactions. Bars 1 and 2: controls lacking one interaction partner; bar 3: MU representing interaction between $S1_{106}$ and S2; bar 4: Interaction between $S1_{194}$ and S2; bar 5: Interaction between S1 and S2. Schematic representation of the protein domains of S1 and S2 used for the interaction studies in each experiment are indicated in blue (S1) and red (S2) below. The experiments were done in triplicates and error bars represent standard deviation.

FIG. 4. Yeast two hybrid system indicating interaction between $S1_{106Mut}$ with S2 (A) Interaction of $S1_{106Mut}$ towards S2 as detected by yeast two hybrid system. Bars 1 and 2: controls lacking one interaction partner; bar 3: MU representing interaction between $S1_{106}$ and S2; bar 4: Interaction between $S1_{106Mut}$#2 and S2; bars 5, 7 and 9: Interaction between $S1_{106Mut}$#13, $S1_{106Mut}$#18 and $S1_{106Mut}$#19 with protein S2; bars 6 and 9: Interaction between $S1_{106Mut}$#15 and $S1_{106Mut}$#20 with protein S2. Schematic representation of the protein domains of S1 and S2 used for the interaction studies in each experiment are indicated in blue (S1) and red (S2) below. Error bars represent standard deviation. The mutated amino acids are shown in red below. (B) Alignment showing the site of mutation on protein $S1_{106Mut}$#2 as compared to protein $S1_{106}$ from different bacteria. Sequence conservation of protein S1 in various Gram-negative bacteria is shown. (C) *In vitro* binding of $S1_{106}$ and $S1_{106Mut}$ to 30S (-S1). Lane 1: The 30S control; lane 2: protein S1; lane 3: 30S ribosomal subunit depleted of protein S1; lane 4: purified protein $S1_{106}$ used for *in vitro* binding studies; lane 5 and 6: 30S bound $S1_{106}$ and $S1_{106Mut}$ detected by western blot analysis.

FIG. 5. Yeast two hybrid system indicating interaction between α2 domain of S2 with S1 and $S1_{106}$. (A) The encircled region shows the close proximity of α2 domain of protein S2 to protein S1 (Sengupta *et al*., 2001). (B) The helix-turn-helix of α2 domain of protein S2 from *E. coli.* (C) The interaction between the α2 domain of S2 with whole length S1 and $S1_{106}$ were validated by yeast two hybrid analyses. Bars 1 and 2: controls lacking one interaction partner; bar 3: MU representing interaction between $S1_{106}$ and S2; bar 4: interaction between S1 and S2; bar 5: interaction between S1 and S2-α2 domain; bar 6: interaction between $S1_{106}$ and S2-α2 domain. Schematic representation of the protein domains of S1 and S2 used for the interaction studies in each experiment are indicated in blue (S1) and red (S2) below. The experiments were done in triplicates and error bars represent standard deviation.

Table 1. Strains and plasmids

	Genotype/ Relevant features	Source
Strains		
E. coli TUNER	F$^-$ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1*	Novagen, US
E. coli XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*Z M15* Tn*10* (Tet^r)]^c	Stratagene, US
Yeast HF7c	MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS 2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, URA3::(GAL 17mers) 3-Cyc1$_{TATA}$-lacZ	Clontech, US
Plasmids		
pET22b (+)	5.4 kb *E.coli* expression vector, His-tag at C-terminal T7 promoter, Amp^r , IPTG induction	Novagen, US
pGBT9	5.5 kb vector for yeast two hybrid system consisting binding domain. +*leu*, Amp^r	Clontech, US
pGAD424	6.6 kb vector for yeast two hybrid system consisting activation domain. +*trp*, Amp^r	Clontech, US
pS1	pET22b **::** 1671 bp PCR product containing the S1 gene under T7 promoter.	This work

PS1106	pET22b **::** 318 bp PCR product containing the $S1_{106}$ gene under T7 promoter.	This work
pPETMUTS1106	pET22b **::** 318 bp PCR product containing the mutant $S1_{106}$ gene under T7 promoter.	This work
pGBTS1	pGBT9 **::** 1671 bp PCR product containing the S1 gene.	This work
pGBTS1106	pGBT9 **::** 318 bp PCR product containing the $S1_{106}$ gene.	This work
pGBTS1194	pGBT9 **::** 582 bp PCR product containing the $S1_{194}$ gene.	This work
pGBTMUTS1106	pGBT9 **::** 318 bp PCR product containing the mutant $S1_{106}$ gene.	This work
pGADS2	pGAD424 **::** 750 bp PCR product containing the S2 gene.	This work
pGADS2helix	pGAD424 **::** 150 bp PCR product containing the S2 alpha helix gene.	This work

Table 2. Primers used for PCR amplification

Oligos	Sequence (5'-3')
FPS1	AATTCATATGATGACTGAATCTTTTGCTCA
FPY2HS1	AATTGGATCCGAATTCATGACTGAATCTTTTGCTCA
FPY2HS2	AATTGGATCCGAATTCATGGCAACTGTTTCCA
RPS1	AATTGGATCCCTGCAGTTACTCGCCTTTAGCTG
RPS1106	AAGCGTCGACTAACTCGAGTTCAGCATCTTCG
RPY2HS1	AATTGGATCCCTGCAGTTACTCGCCTTTAGCTG
RPY2HS1106	AATTGGATCCCTGCAGTTATTCAGCATCTTCGTAAG
RPY2HS1194	AATTGGATCCCTGCAGTTATTCCATGCCTTCC
RPY2HS2	AATTGGATCCCTGCAGTTACTCAGCTTCTACGAAGC

Underline represents the restriction sites

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