THE DEVELOPMENT OF NOVEL ANTIMICROBIAL PEPTIDES WITH ACTIVITY AGAINST MRSA

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CONTENTS

ABBREVIATIONS

DNA	Deoxyribonucleic acid
2YT	2x yeast tryptone broth
Atl ami	Atl amidase
Atl GL	Atl glucosaminidase
bp	DNA base pair
C	Celsius
cMRSA	Community acquired MRSA
Da	Dalton
dATP	Deoxyadenosine 5'-triphosphate
dNTP	Deoxyribonucleoside 5'-triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
g (mass)	Gram
g (centrifugation)	Gravitational field
GFP	Green fluorescent protein
GL'	Truncated Atl glucosaminidase catalytic domain
IPTG	Isopropyl-β-thiogalactopyranoside
kbp	Kilo base pair
kDa	Kilo Dalton

l	Litre
LB	Luria-Bertani broth
LssT	Lysostaphin targeting domain
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimolar
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
ng	Nanogram
NICE	Nisin-controlled gene expression system
nm	Nanometre
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGRP	Peptidoglycan recognition protein
R12	Atl repeat regions 1 and 2
R123GL	Atl GL with additional repeat regions 1 and 2
R3	Atl repeat region 3

RLU	Relative light unit
SDS	Sodium dodecyl sulphate
u.v.	Ultraviolet
v/v	volume / volume
VISA	Vancomycin intermediately sensitive *S. aureus*
VRE	Vancomycin resistant enterococci
VRSA	Vancomycin resistant *S. aureus*
w/v	Weight / volume
µl	Microlitre
µM	Micromolar

ABSTRACT

MRSA is a significant pathogen, which can cause a range of minor and major infections both in the hospital and community environments. MRSA is developing resistance to many antibiotics, including vancomycin, which is now the first choice antibiotic to treat MRSA infections in the UK. This together with the dearth of new antibiotics being introduced could see the emergence of untreatable *S. aureus* strains. This has led to renewed interest in alternative antimicrobial agents.

Lysostaphin is an endopeptidase produced by *Staphylococcus simulans* biovar *staphylolyticus*, which cleaves the peptidoglycan cross-bridges of other staphylococcal species. Lysostaphin has been investigated as a potential therapeutic agent and has shown promise in *in vitro* and *in vivo* studies and in clinical trials. However, resistance to lysostaphin is likely to emerge and there will be a demand for second generation lysostaphins and/or other similar novel antimicrobials that can counteract this resistance.

This study describes the cloning, purification and assaying of an endolysin of the *S. aureus* P68 bacteriophage. Lys16 lysin has previously been shown to possess staphylolytic activity. This study demonstrates that the purified recombinant protein is poorly soluble and is inactive against live cells.

The Atl autolysin of *S. aureus* was also investigated as a potential antimicrobial. This study confirmed the hydrolytic profiles of the enzymes, and a chimeric peptide incorporating the lysostaphin targeting domain with the Atl glucosaminidase was designed. This did not confer greater activity against *S. aureus*, although the targeting domains of each enzyme were shown to utilise different cell surface receptors.

Finally, this study reports the development of a novel assay to measure the activity of antimicrobial peptides against *S. aureus*, using a bioluminescence reporter. This was shown to be a sensitive assay, able to distinguish small differences in the activity of antimicrobial peptides.

1. INTRODUCTION

1.1 THE THREAT OF MRSA

Staphylococcus aureus is a bacterium commonly found as a commensal organism living on the human skin and mucosa. It is found in up to 30% of the population living harmlessly on the skin, predominantly in the anterior nares (Livermore, 2000). However, it is a versatile organism and if it is able to gain access to the body it is able to colonise many other sites, often causing disease (Lowy, 1998). *S. aureus* is the most common cause of skin infections, entering either through a breach in the skin or as a result of blood borne spread. It can be responsible for conditions ranging from boils focussed around hair follicles, to scalded skin syndrome, which is caused by strains of *S. aureus* producing the exfoliatin toxin (Iwatsuki *et al.*, 2006). *S. aureus* can also colonise bones and joints, being the most common cause of osteomyelitis which can lead to permanent bone deformities, and of suppurative arthritis (Davis, 2005). In the respiratory tract *S. aureus* can cause pneumonia, where it is of particular concern for cystic fibrosis patients (Burns *et al.*, 1998). *S. aureus* is also a major cause of infective endocarditis. If a heart valve's endothelial lining becomes damaged, *S. aureus* is able to adhere to its surface, forming a vegetation. This is a serious condition which can lead to congestive heart failure. Even with aggressive antimicrobial therapy, the mortality rate of infective endocarditis remains at up to 40% (Bashore, Cabell & Fowler, 2006).

S. aureus is also the most common cause of surgical wound infections. The source of infection can be the patient's own microflora, a member of staff, or equipment within the hospital environment (Anon, 2007). Such hospital acquired infections may remain localised at the site of the surgical procedure, but *S. aureus* may also invade the bloodstream and disseminate, causing infections at other sites, such as those listed above. These infections are often difficult to treat due to the problems of administering suitable doses of the necessary antibiotics, to often deep seated infections.

The predicament of hospital acquired *S. aureus* infections is worsened by the ability of the organism to rapidly develop resistance to the antibiotics used against it. Antibiotics have been used in the treatment of *S. aureus* infections since the clinical introduction of penicillin in 1944. However, penicillin resistant strains were isolated only two years later (Barber & Rozwadowska-Dowenzko, 1948). Similarly, the introduction of methicillin, a semi-synthetic penicillin; in 1959 was shortly followed by the emergence of methicillin resistant *S. aureus* (MRSA) (Jevons, Coe & Parker, 1963).

MRSA has become a cause of great public concern and has received considerable media attention, due to the prospect of untreatable strains evolving and becoming entrenched within our hospitals. As a result, mandatory MRSA bacteraemia surveillance has been introduced to acute NHS Trusts in England. Currently, approximately forty percent of hospital acquired staphylococcal bacteraemias are now identified as MRSA. From October 2005 to March 2006 3,517 MRSA bacteraemia episodes were reported in England (Anon, 2006). Since mandatory surveillance was introduced the increase in MRSA incidence has been arrested, and thee has been a slight reduction in the incidence rate. However, this remains a dramatic increase from a rate of two percent in 1990 and poses a significant threat to public health (Reacher *et al.*, 2000). Between 2001 and 2005, the number of death certificates mentioning *S. aureus* rose from 1,211 to 2,083, with the percentage being specified as MRSA also increasing from 61% to 78% (Anon, 2007b).

It is difficult to determine precise figures of deaths due to MRSA, as patients who die with MRSA infections often have complex medical histories. A small scale national study has shown that in half the cases where MRSA is mentioned on the death certificate, it is probably not the main cause of death. Furthermore, MRSA was not mentioned on the death certificates of nearly half of the cases studied of patients with MRSA bacteraemia who subsequently died, where MRSA was considered to have caused or contributed to death (Anon, 2007c). There remains no doubt though, that MRSA is a considerable cause of morbidity in UK hospitals.

Resistance to methicillin is conferred by the *mecA* gene. This encodes an altered penicillin binding protein (PBP 2') with a low affinity for β-lactams, therefore allowing cross-linking of peptidoglycan to progress unaffected by their presence (Brown & Reynolds, 1980; Hartman & Tomasz, 1984). The *mecA* gene is carried on a mobile genetic element, the *Staphylococcal* chromosomal cassette (SCC) *mec* (Katayama, Ito & Hiramatsu, 2000). There are six main groups of SCC*mec* which insert into the *S. aureus* chromosome (Enright, 2003; Ito *et al.*, 2004; Oliveira, Milheirico & de Lencastre, 2006). These mobile cassettes are believed to have been transferred to *S. aureus* by a horizontal gene transfer event from another staphylococcal species, possibly *S. sciurii* (Enright, 2003; Wu *et al.*, 1996). Various strains of MRSA have been isolated, showing a range of antibiotic resistance profiles, many being multiple-drug resistant. The two most common strains in the UK have been designated epidemic MRSA (EMRSA) 15 and 16.

A recent development in the evolution of MRSA is the emergence of community acquired MRSA (cMRSA). Initial reports of community acquired MRSA were from the USA, and cases have since been reported elsewhere, including the UK (Baba *et al.*, 2002). Community acquired MRSA is associated with the Panton Valentine Leukocidin toxin and can cause rapidly progressing fatal infections such as necrotizing fasciitis (Boyle-Vavra & Daum, 2007; Miller *et al.*, 2005) and necrotizing pneumonia (Francis *et al.*, 2005). Fit, healthy young adults appear to be equally susceptible to cMRSA as the very old, or young, or infirm that are susceptible to hospital acquired MRSA (Fridkin *et al.*, 2005).

1.2 THE FAILURE OF CURRENT ANTIBIOTICS

Vancomycin is the antibiotic used most commonly in the treatment of MRSA infections, and is often the drug of last resort. However, resistance of *S. aureus* to vancomycin is also emerging. Vancomycin-intermediately-susceptible *S. aureus* (VISA) has a lowered susceptibility due to alterations in the peptidoglycan structure. The mechanisms for reduced susceptibility

are varied in different isolates and include thickened cell walls and altered peptidoglycan cross-linking. They confer the ability to sequester the drug and reduce the amount of it able to reach its target site (Cui *et al.*, 2000; Sieradzki & Tomasz, 2003).

Vancomycin resistant *S. aureus* (VRSA) have also been isolated (Chang *et al.*, 2003; Tenover *et al.*, 2004). The *vanA* gene responsible for resistance is believed to have been acquired by VRSA on a transposon from vancomycin resistant enterococci (VRE).

Linezolid was clinically introduced in 2001. This represented the first antibiotic from the oxazolidinones, the first new class of antibiotics for thirty years (Zurenko *et al.*, 2001). Linezolid resistant MRSA was isolated from a patient within one year of its clinical introduction (Tsiodras *et al.*, 2001).

The above examples highlight a pattern of resistance emerging amongst *S. aureus* within two years of the clinical introduction of a new antibiotic. This makes the development of novel conventional antibiotics an unattractive prospect to pharmaceutical companies. Any new antibiotics that are successfully introduced face the risk of becoming redundant before the high costs of bringing the drug to market have been recouped. As a result there is a lack of antibiotics in the drug development pipeline, at a time when MRSA and other infections demand new treatment options to be available.

1.3 A *S. AUREUS* VACCINE

Vaccines are routinely used in the prevention of infection of several bacterial infections. These include vaccinations against diseases caused by *Mycobacterium tuberculosis*, *Clostridium tetani* and some *Neisseria meningitidis* strains. The development of a vaccine against *S. aureus* holds particular challenges. *S. aureus* is a versatile pathogen, exploiting many environments within the human host. An ideal vaccine would need to provide safe long term protection against the organism and its toxins through comprehensive cell mediated and antibody responses. Currently

there are vaccines derived from the surface proteins of *S. aureus* under development which have shown some promise in animal studies (Kuklin *et al.*, 2006; Stranger-Jones, Bae & Schneewind, 2006). However, *S. aureus* has demonstrated an unparalleled ability to mutate and evade the actions of antibiotics. This adaptable organism may prove equally successful in altering its cell surface proteins included in a subunit vaccine. A polysaccharide conjugate vaccine, StaphVAX, recently failed a phase III clinical trial, with a group of haemodialysis patients showing no reduction in the incidence of *S. aureus* infections (Fattom *et al.,* 2004; Anon, 2005). Along with concerns over the quality of the vaccine preparation this failure was partly attributed to the emergence of *S. aureus* with increased virulence (Anon, 2006b). Future vaccines may be at risk of failing in a similar fashion as *S. aureus* continues to evolve.

Until a reliable vaccine is developed which *S. aureus* cannot evade there is a requirement for novel treatments to be developed. In the event of a successful *S. aureus* vaccine being introduced there will still remain unprotected individuals who will develop infections, especially in countries which cannot afford expensive protection campaigns. *S. aureus* will never be eradicated as it exists as a commensal organism of humans and animals. There will thus be a continuing demand on novel treatments for *S. aureus* infections.

1.4 RENEWED INTEREST IN NOVEL ANTIMICROBIALS

Prior to the discovery of antibiotics and their clinical introduction there was widespread interest in alternative antimicrobial therapies. These included bacteriophage therapy and antimicrobial peptides. Research into these alternatives waned as antibiotics proved to be successful in treating most bacterial infections (Nathan, 2004). However, now that conventional antibiotics are failing in the treatment of multiple-drug resistant MRSA and other organisms, there is renewed interested in developing alternative antimicrobials. There are currently enzybiotics against *S. aureus* under

development as well as vaccines. Enzybiotics are catalytic proteins with therapeutic potential as antimicrobial agents.

1.4.1 Sites of action of enzybiotics

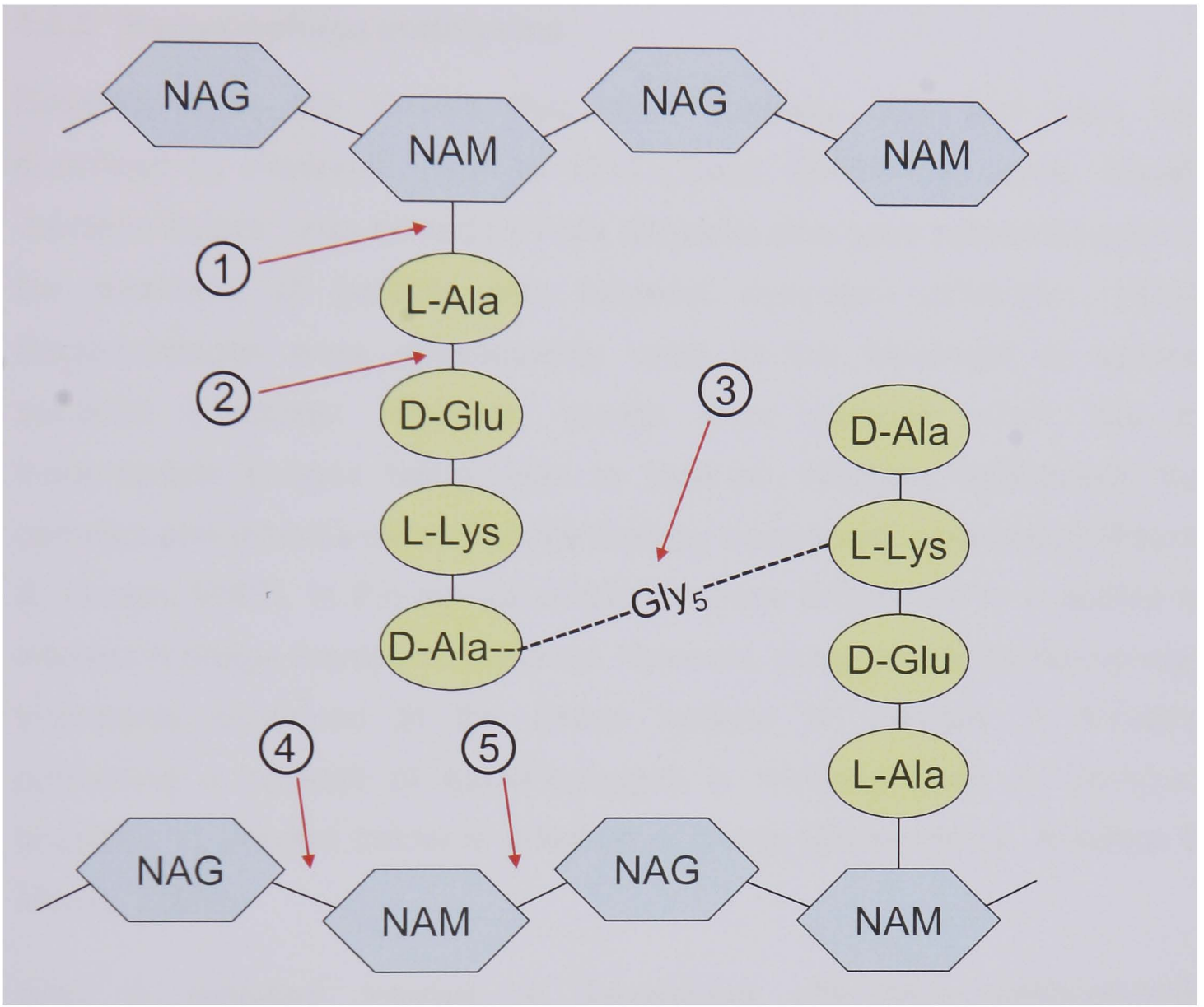


Figure 1.1 Structure of staphylococcal peptidoglycan and sites of enzymatic hydrolysis:

1. *N*-acetylmuramoyl-L-alanine amidase eg. Atl amidase, P68 Lys16.
2. L –alanoyl- D-glutamate endopeptidase eg *Listeria* phage endolysins
3. Glycylgycine endopeptidase eg. lysostaphin, LytM
4. *N*-acetyl-β-D-glucosaminidase eg Atl glucosaminidase
5. *N*-acetyl-β-D-muramidase eg. lysozyme

(Abbreviations: NAM - *N*-acetyl muramic acid; NAG - *N*-acetyl glucosamine)

Enzymes have evolved within bacteria and viruses able to hydrolyse nearly all of the bonds present in peptidoglycan, including that of *S. aureus*. The actions of these could be utilised in the development of enzybiotics active

against MRSA. The structure of staphylococcal peptidoglycan, and potential cleavage sites are shown in Figure 1.1 (Schleifer & Kandler, 1972; Loessner, 2005).

1.4.2 Bacteriophage endolysins

Bacteriophages are viruses that infect bacterial cells and were first described by Frederick Twort in 1915 (Twort, 1915). The name, literally "bacteria eaters", was coined by Felix d'Herelle who used bacteriophages in the treatment of patients with bacterial dysentery (d'Herelle, 1917). Bacteriophages were subsequently used in the treatment of several bacterial infections. However, results were variable, often due to inappropriate phages being used to treat an infection. Additionally the complex pharmacokinetics of phage therapy were poorly understood (Payne & Jansen, 2003). In the age of antibiotics these factors led to a decline in interest in phage therapy in the West. However, research into bacteriophage treatments continued at the Eliava Institute in Georgia. A dressing containing a cocktail of bacteriophages is routinely used in Georgian hospitals to prevent bacterial infection of burns (Sulakvelidze, Alavidze & Morris, 2001).

With a renewed interest in developing alternative antimicrobials bacteriophages have once again come into consideration. Several approaches to exploiting bacteriophages are being investigated, including phage therapy as discussed above, exploiting the viral endolysins and using bacteriophage genomics to identify bacterial target proteins susceptible to inhibition by small molecules (Liu *et al.*, 2004). Of particular interest is the utilisation of bacteriophage endolysins (Bradbury, 2004; Fischetti, 2001). Bacteriophages are obligate intracellular parasites and rely on their host cell for replication. Following replication, phage encoded endolysins are produced to hydrolyse the bacterial cell wall causing cellular lysis from within and the release of the progeny virions from the host, from where they can infect new cells. Bacteriophage endolysins thus are candidates as novel antimicrobials for treating bacterial infections. The replication cycle of

bacteriophages is very short and the lysis of host cells occurs rapidly. Such rapid lytic activity makes phage endolysins an attractive prospect as candidate antimicrobials.

There are also further benefits in the use of phage endolysins as therapeutic agents. Many bacteriophages have evolved to be highly species specific and their endolysins may have no enzymatic effect on patients' cells thus carrying a low risk of toxicity. Furthermore, they will also have a far lower effect on the natural microflora of patients than current broad spectrum antibiotics. This will help prevent conditions such as antibiotic associated diarrhoea caused by *Clostridium difficile,* a key problem in administering antibiotics to the elderly and one of increasing significance in hospitals (Thomas, Stevenson & Riley, 2003). In 2005 there were 51,690 reported incidences of *C. difficile* disease in people aged over 65 in England, a 17.2% increase on the previous year, and there is currently a significant focus on reducing infection rates (Anon, 2006).

If phage endolysins are clinically introduced this will create a selection pressure which may lead to *S. aureus* developing resistance. However, an effective solution for overcoming any resistance would be to utilise the mutability of the parent bacteriophage and select for phages able to cause lysis of the adapted *S. aureus.* These could then be used as a source from which new endolysins could be exploited.

Endolysins from bacteriophages of several bacterial species have been cloned and investigated for their antimicrobial activity. A key requirement of the endolysins, if they are to be used therapeutically, is that they are active extracellularly, as these enzymes have evolved to degrade cell walls from within the cell.

An early report of the activity of a recombinant phage endolysin was that of the phage lysin genes (*ply)* of *Listeria monocytogenes* bacteriophages (Loessner, Schneider & Scherer, 1996). These were shown to cause lysis of heat killed *L. monocytogenes* cells, although assays against live cells were not performed.

More recently the PlyG lysin from the γ phage of *Bacillus anthracis* has been shown to kill both vegetative cells and germinating spores of *B. anthracis* and closely related bacilli (Schuch, Nelson & Fischetti, 2002). In *in vitro* assays against *Bacillus cereus* RSVF1, PlyG resulted in a 17,000-fold decrease in cell viability after 20 seconds and near sterilisation after two minutes. In a mouse model, in which RSVF1 causes death within five hours, administering PlyG 15 minutes post-infection had a significant effect, with 13 out of 19 mice recovering fully. Efforts made to isolate PlyG resistant RSVF1 were unsuccessful and spontaneous mutants resistant to the γ phage remained sensitive to the PlyG endolysin. Further bacteriophage derived endolysins PlyL and PlyPH have been identified from the sequence of *B. anthracis* (Low *et al.*, 2005; Yoong *et al.*, 2006). These endolysins also show bacteriolytic activity with PlyL demonstrating activity against a wider range of Bacillus species. These are encouraging results for the development of further phage endolysin based antimicrobials.

Two phage endolysins have been identified with activity against *Streptococcus pneumoniae*. Pal has been used in the prevention and elimination of nasopharyngeal colonisation in mice (Loeffler, Nelson & Fischetti, 2001). Cpl-1 has also been shown to be effective in eliminating nasopharyngeal colonisation in mice, as well as prolonging survival in a model of *S. pneumoniae* bacteraemia (Loeffler & Fischetti, 2003b). Furthermore, rabbit serum raised against Cpl-1 had only a modest inhibitory effect on the endolysin, indicating that phage endolysins could be repeatedly administered in the treatment of infections. The Pal and Cpl-1 phage endolysins have been shown to act synergistically in *in vitro* studies (Loeffler *et al.*, 2003b). This synergy provides further positive encouragement in the use of phage endolysins as potential antimicrobials

Phage endolysins have also been identified with activity against a wide range of other species including Group B streptococci (Pritchard *et al.*, 2004; Cheng *et al.*, 2005), *Enterococcus faecalis* and *Enterococcus faecium* (Yoong *et al.*, 2004), and lactobacilli and lactococci (Deutsche *et al.*, 2004).

A number of phages of *S. aureus* have been studied and genes identified which encode potential endolysins. These include phage K, P68, Twort φ11, φ12 and φ13 (O'Flaherty *et al.*, 2004; Vybiral *et al.*, 2003; Loessner *et al.*, 1998; Navarre *et al.*, 1999b; Iandolo *et al.*, 2002). An endolysin from one of these bacteriophages with activity against *S. aureus*, including MRSA, would have significant potential as an antimicrobial agent. Recently there has been further research into the endolysins of *S. aureus* phages. LysK is an endolysin from the staphylococcal phage K which has a broad host spectrum. Cell lysates have demonstrated antimicrobial activity against a wide range of staphylococci in *in vitro* studies (O'Flaherty et al., 2005). Other lysins from the φ11, φ12 and P68 phages of *S. aureus* have been investigated and have been shown to have *in vitro* activity against *S. aureus* (Donovan, Lardeo & Foster-Frey, 2006b; Sass & Bierbaum, 2007; Takáč, Witte & Blasi, 2005b). These results suggest that a bacteriophage endolysin may provide an alternative antimicrobial for the treatment of *S. aureus* and that further research in the area is warranted.

1.4.3 Autolysins

Autolysins are enzymes produced by bacteria that cause hydrolysis of their own peptidoglycan. They are believed to be found in all bacterial species and are thought to have roles in many cellular processes including cell growth, cell wall turnover, protein secretion and cell division (Smith, Blackman & Foster, 2000). There must be transcriptional or post-translational regulation of autolysins to control their activity for these functions, as the uncontrolled expression of active enzyme could prove fatal to the cell. This presents the opportunity to develop recombinant autolysins as novel alternative antimicrobials. Purified recombinant autolysins could be used as specific, highly active bacteriolytic agents.

Autolysins have been identified from several staphylococcal species and their roles investigated. Many of these autolysins show sequence similarity, indicating a shared evolutionary history, and function.

The major autolysin of *S. aureus* is Atl (Oshida *et al.*, 1995; Foster, 1995). Atl is a bi-functional enzyme with *N*-acetyl-muramyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase activity as shown in Figure 1.1 (Oshida *et al.*, 1995; Sugai *et al.*, 1995). The regions encoding the active domains are separated by three repeat sequences which encode the cell wall targeting domain (Baba & Schneewind, 1998b). These repeat sequence regions are widespread, and named the LysM (lysin motif) domain (Bateman & Bycroft, 2000). The LysM domain of the AcmA autolysin of *Lactococcus lactis* has been demonstrated to bind to peptidoglycan (Steen *et al.*, 2003). Atl has been shown to localise to the septal ring and is thus believed to play a role in cell division (Yamada *et al.*, 1996). The enzymatic domains have been shown to have bacteriolytic activity against *S. aureus* and *Micrococcus luteus* (Oshida *et al.*, 1995; Sugai *et al.*, 1995).

AtlE is a closely related autolysin to Atl, identified in *S. epidermidis* (Heilmann *et al.*, 1997). The amino acid sequence of AtlE shares 61% identity with that of Atl and shows the same bi-functional modular structure, with enzymatic regions separated by three repeat sequences. AtlE deficient mutants form large clusters, indicating the role of the autolysin in cell division (Heilmann *et al.*, 1996). AtlE also shows bacteriolytic activity when tested against *M. luteus* and *Staphylococcus carnosus*. In addition to peptidoglycan hydrolase activity AtlE demonstrates a strong binding activity to the plasma protein vitronectin (Heilmann *et al.*, 1997). *S. epidermidis* is a major nosocomial pathogen of patients with indwelling catheters and other implanted medical devices. Host extracellular matrix and plasma proteins rapidly coat implanted medical devices and AtlE has been demonstrated to be involved in the attachment of *S. epidermidis* to polystyrene and is essential for biofilm formation on polystyrene surfaces (Rupp *et al.*, 2001). These results demonstrate that AtlE has a significant role in pathogenicity. An understanding of the multiple roles of an autolysin is thus required before it may be used as an antimicrobial therapy.

Another staphylococcal autolysin, Aas, with similarity to Atl has been identified in *Staphylococcus saprophyticus*, a urinary tract pathogen. The

amino acid sequences of the amidase and glucosaminidase active regions of Aas show 71% identity and 66% identity respectively with Atl (Hell, Meyer & Gatermann, 1998). The cell wall targeting regions show less similarity, with an 86 amino acid deletion in the second repeat region. Aas also has seven repeats of 40 amino acids located N-terminal to the amidase. However, the repeats between the active domains have been shown to be responsible for cell targeting rather than these seven N-terminal repeats (Hell *et al.*, 2003). Aas has been shown to be necessary for cell cluster dispersion and has bacteriolytic activity against heat inactivated *S. saprophyticus* cells (Hell *et al.*, 1998). Aas has also been shown to have adhesin properties. In contrast to AtlE, Aas does not bind vitronectin, but does bind to fibronectin and to erythrocytes. This provides further evidence alongside that of AtlE that autolysins can have a dual function with a role in pathogenicity as well as cell wall turnover.

Other staphylococcal autolysins that show a dual bacteriolytic and adhesin properties include Aae of *S. epidermidis*, which is lytic to *S. carnosus* and *S. epidermidis*, and binds fibrinogen, fibronectin and vitronectin (Heilmann *et al.*, 2003). Aae has an N-terminal three repeat sequence region homologous to the LysM cell wall binding domain of Atl and AtlE. The hydrolytic domain is likely to be an *N*-acetylmuramoyl-L-alanine amidase by homology with Aaa/Sle1 (Kajimura *et al.*, 2005). AtlC of *Staphylococcus caprae* is bacteriolytic against dried *S. caprae* and *M. luteus* cells and binds fibronectin (Allignet *et al.*, 2001; Allignet *et al.*, 2002).

In addition to Atl, another autolysin of *S. aureus* has been identified. This has been identified independently by Heilmann *et al.* and Kajimura *et al.*, and designated Aaa and Sle1 respectively (Heilmann *et al.*, 2005; Kajimura *et al.*, 2005). Aaa/Sle1 is 76% identical to Aae of *S. epidermidis*, has an N-terminal LysM and a C-terminal *N*-acetylmuramoyl-L-alanine amidase domain. a*aa/sle1* null mutants form cell clusters, indicating that the autolysin is involved in cell separation. Double *atl/sle1* mutants had significantly impaired growth and formed irregular cell clusters (Kajimura *et al.*, 2005). These results indicate that Atl and Sle1 are the only peptidoglycan

hydrolases involved in *S. aureus* cell separation and that Sle1 is important for splitting the septum during cell division. *sle1* null mutants also showed reduced pathogenicity in a mouse model (Kajimura *et al.*, 2005). This may be partly due to the formation of cell clusters and a reduced dissemination of daughter cells during an infection. Aaa/Sle1 has been shown to cause bacteriolysis to *S. aureus* and *S. carnosus* cell substrates by zymogram analysis (Heilmann *et al.*, 2005). In common with the closely related Aae, Aaa/Sle1 is able to bind fibrinogen, fibronectin and vitronectin, indicating that it may play a role in pathogenicity beyond the necessary cell separation (Heilmann *et al.*, 2005).

Another staphylococcal autolysin, LytM, shares significant homology with lysostaphin. LytM is discussed further later.

Staphylococcal autolysins therefore have multiple functions both during cellular functions and in pathogenicity. The use of an enzybiotic based on a staphylococcal autolysin may therefore have a profound effect on cells and be an effective antimicrobial. In designing an enzybiotic based on autolysins it would be necessary to eliminate the potential pathogenicity of the autolysin, for example, by altering the binding domain to prevent binding to plasma proteins and to address any potential immunological problems associated with the administering of a bacterial protein.

1.4.4 Antimicrobial peptides

Many organisms produce antimicrobial peptides to inhibit the growth of bacteria (Baba & Schneewind, 1998a). Animals and plants produce such proteins as a defence mechanism against infection. Bacteria often produce peptides active against closely related organisms, probably in order to gain an advantage over species with which they are in competition within an environment. These antimicrobial peptides provide another group of naturally occurring proteins which could be exploited as the basis of novel antimicrobials, and thus only prokaryotic antimicrobial peptides are discussed further. Several antimicrobial peptides have been investigated

including lysostaphin, enterolysin A, zoocin A and other peptides which all share homology.

Enterolysin A is a 34.5kDa antimicrobial peptide produced by *E. faecalis* (Nilsen, Nes & Holo, 2003), which shares 28% identity with lysostaphin, and 29% identity with zoocin A (Hickey *et al.*, 2003). The N-terminal region of enterolysin A is homologous to the M37 family of metallopeptidases, which includes lysostaphin and zoocin A which have Zn^{2+} in the catalytic site. Enterolysin A has been shown to target bacterial cell walls and probably acts as an endopeptidase (Hickey *et al.*, 2003; Nilsen *et al.*, 2003). The C-terminal region acts as a cell wall targeting domain and is not homologous with lysostaphin and zoocin A. Enterolysin A is active against a broader range of bacteria than other antimicrobial peptides, being bacteriolytic against lactococci, pediococci, lactobacilli, listeriae and some enterococci. Enterolysin A has previously been exploited to induce autolysis of *Lactococcus lactis*, which has potential applications in the dairy industry (Hickey, Ross & Hill, 2004). The broad range of the cell wall targeting domain of enterolysin A could be exploited as part of a novel enzybiotic. This would enable the development of a broader spectrum antimicrobial than would be possible with more specific antimicrobial peptides.

Zoocin A is an antimicrobial peptide identified from *Streptococcus equi* subspecies *zooepidemicus* strain 4881 (Simmonds *et al.*, 1995). Zoocin A has bacteriolytic activity against other *S. equi* ssp. *zooepidemicus* strains, *Streptococcus pyogenes*, *Streptococcus gordonii* and *Streptococcus mutans.* It is a cell wall hydrolytic enzyme with endopeptidase activity (Simmonds *et al.*, 1996). Zoocin A has been shown to be a penicillin binding protein, indicating that it is a D-alanyl endopeptidase active against the di- or tri-alanine cross-bridges of streptococcal peptidoglycan (Heath *et al.*, 2004). Zoocin A is a 30kDa modular protein, with an N-terminal catalytic domain homologous to that of lysostaphin and a C-terminal domain responsible for cell wall binding which shows limited homology with lysostaphin's C-terminal binding domain (Simmonds, Simpson & Tagg, 1997; Lai, Tran & Simmonds, 2002; Liang, Simmonds & Timkovich, 2004). The host *S. equi* ssp.

zooepidemicus strain carries a zoocin A immunity factor (*zif*) gene which protects itself against the activity of the antimicrobial peptide (Beatson, Sloan & Simmonds, 1998). Zoocin A therefore represents an antimicrobial peptide with a different spectrum of activity to both enterolysin and lysostaphin. These differences may be of interest in the development of enzybiotics active against *S. aureus* and other species.

Other antimicrobial peptides showing homology to lysostaphin have been identified in a range of species. *E. coli* produces MepA, a metallopeptidase with a homologous active site containing a Zn^{2+} ion that is involved in cell wall turnover (Marcyjaniak *et al.*, 2004). YibP, also named EnvC, is another *E. coli* zinc metallopeptidase with multiple cellular functions (Ichimura *et al.*, 2002; Hara *et al.*, 2002). Millericin B is an endopeptidase produced by *Streptococcus milleri* which cleaves peptidoglycan stem peptides and cross-bridges (Beukes *et al.*, 2000). The catalytic *milB* gene shares 36% identity with the lysostaphin gene at the DNA sequence level, with the protein sequences of their gene products sharing 62% identity (Beukes & Hastings, 2001).

ALE-1 is a close lysostaphin homologue produced by *Staphylococcus capitis* EPK1. It has bacteriolytic activity against *S. aureus* although it is inactive against *S. capitis*. It is a glycyl-glycine endopeptidase which cleaves peptidoglycan cross-bridges. ALE-1 share 50% overall identity with lysostaphin at the DNA sequence level, and 83% amino acid sequence identity of the C-terminal targeting domains (Sugai *et al.*, 1997a; Lu *et al.*, 2006). The crystal structure of the targeting domain has been elucidated (Lu *et al.*, 2006).

LytM is a peptidoglycan hydrolase of *S. aureus*, which has been shown to have autolytic activity, in addition to the autolysins Atl and Sle1/Aaa discussed previously (Ramadurai & Jayaswal, 1997). LytM is a 32kDa protein, with a C-terminal domain sharing 51% identity at the amino acid level with the N-terminal endopeptidase domain of lysostaphin. LytM is bacteriolytic against *S. aureus* and *S. carnosus* acting as a glycyl-glycine endopeptidase (Ramadurai *et al.*, 1997; Ramadurai *et al.*, 1999). LytM is

another zinc metallopeptidase, and the crystal structures of the latent and active forms of the protein have been determined (Odintsov *et al.*, 2004; Firczuk, Mucha & Böchtler, 2005).

There are a wide range of antimicrobial peptides which have been characterised. They display a range of specificities for different organisms and a number of target cleavage sites. Many of these may be suitable for the development of enzybiotics as novel antimicrobials. In addition to the antimicrobial peptides described above lysostaphin has been the subject of particular interest as an alternative antimicrobial, providing a paradigm for further studies, and is subsequently discussed in greater detail.

1.5 LYSOSTAPHIN

1.5.1 The discovery of lysostaphin

Lysostaphin was originally discovered by Schindler and Schuhardt in 1964. During transduction studies with staphylococci, a colony exerting a lytic effect on the surrounding *S. aureus* lawn was observed (Schindler & Schuhardt, 1964). Possible bacteriophage activity was excluded and the responsible protein was named lysostaphin. This protein was bacteriolytic against over 50 staphylococcal strains, but inactive against organisms from other genera. Lysostaphin is produced by the organism formerly known as *Staphylococcus staphylolyticus*, now designated S. *simulans* biovar *staphylolyticus* (Sloan, Robinson & Kloos, 1982). It is a glycyl-glycine endopeptidase active against the peptidoglycan cross-bridges of nearly all other staphylococcal species (Schindler & Schuhardt, 1965; Browder *et al.*, 1965; Zygmunt, Browder & Tavormina, 1967). Lysostaphin was identified as a zinc containing enzyme of around 25kDa, with an isoelectric point of 9.5 and a pH optimum of 7.5 (Browder *et al.*, 1965; Trayer & Buckley, 1970).

1.5.2 The expression, processing and secretion of lysostaphin

The gene encoding lysostaphin (*lss*) is carried on a 40kbp plasmid (pACK1) which also carries a β-lactamase gene (Recsei, Gruss & Novick, 1987; Heath, Heath & Sloan, 1987). The nucleotide sequence of *lss* and the amino acid sequence of lysostaphin have been determined (Heinrich *et al.*, 1987; Recsei *et al.*, 1987). The *lss* gene encodes a 493 amino acid preproenzyme, comprising a 36 aa signal peptide, and a 211 aa sequence of 15 tandem repeats and a mature protein of 246 aa which together comprise the propeptide (Thumm & Götz, 1997) (Figure 1.2).

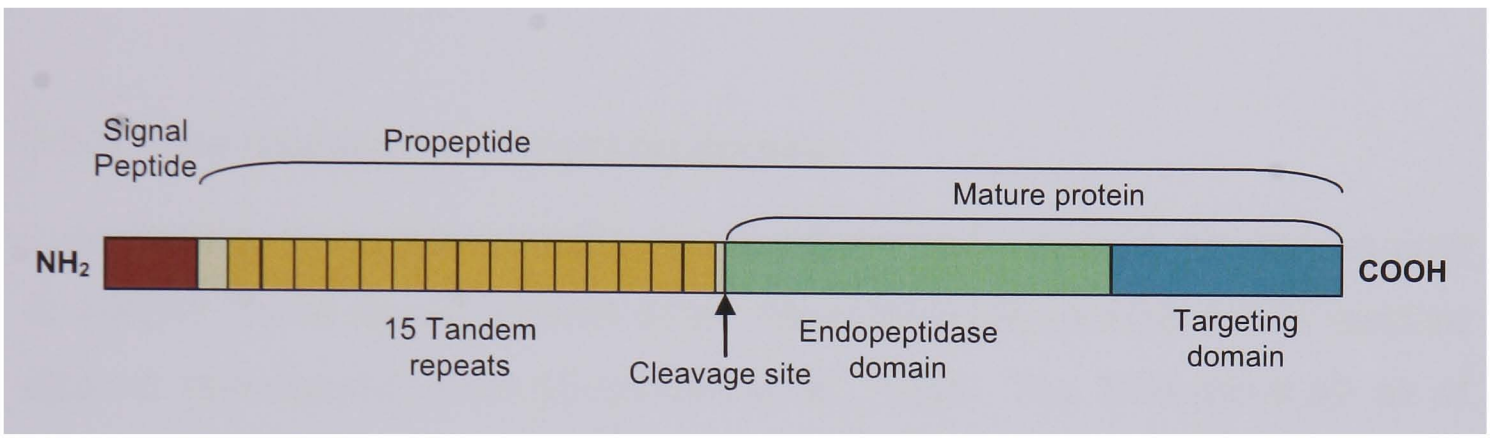


Figure 1.2 Organisation of the *lss* gene product

The preproenzyme is initiated into the secretory pathway by the 36 aa signal peptide which is then cleaved by leader peptidase, releasing the prolysostaphin into the extracellular environment (Recsei *et al.*, 1987; Heinrich *et al.*, 1987). The propeptide consists of 12 N-terminal aa followed by 15 tandem repeats of 13 aa and 4 C-terminal aa prior to a cysteine protease processing site (Thumm *et al.*, 1997). Fourteen of the tandem repeats are identical, whilst the fifteenth contains three substitutions. The propeptide sequence is cleaved by an extracellular cysteine protease, also secreted by *S. simulans* biovar staphylolyticus to leave the active mature protein (Neumann *et al.*, 1993). The mature form of lysostaphin has approximately 4.5-fold higher staphylolytic activity than the proenzyme form (Thumm *et al.*, 1997).

1.5.3 The structure of mature lysostaphin

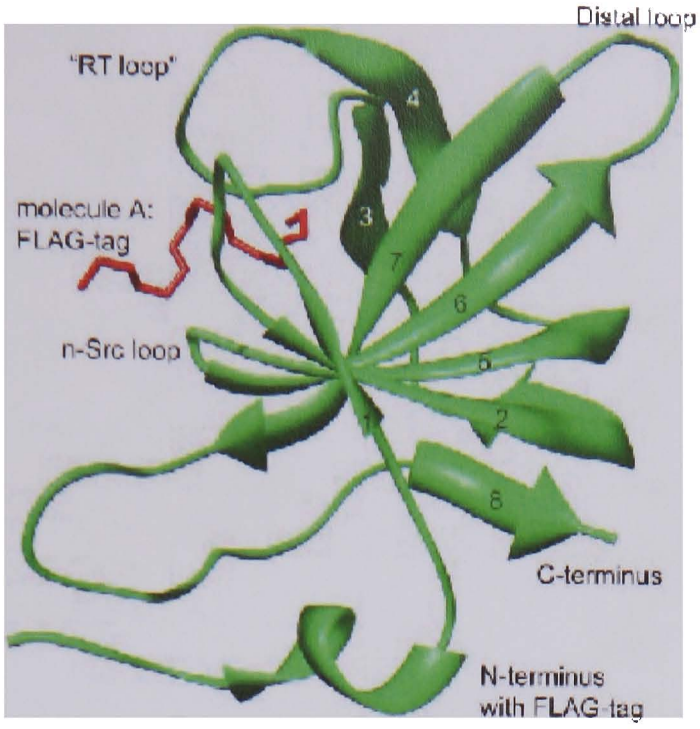
The active mature lysostaphin is a 246 aa peptide with a mass of approximately 27kDa. It has a modular structure consisting of an N-terminal catalytic domain and a 92 aa C-terminal cell wall targeting domain (Thumm *et al.*, 1997) (Figure 1.2). The crystal structure of lysostaphin has not yet been elucidated. However, structures of the active form of LytM and the cell wall targeting domain of ALE-1, close homologues of lysostaphin, have been reported and indicate possible structural features of lysostaphin (Firczuk *et al.*, 2005; Lu *et al.*, 2006).

1.5.4 The lysostaphin targeting domain

Lysostaphin is highly specific for staphylococci. It acts rapidly against *S. aureus*, more slowly against other staphylococcal species and is inactive against non-staphylococci (Schindler *et al.*, 1964). The C-terminal 92 aa of mature lysostaphin have been shown to be responsible for the targeting of the peptide to staphylococcal cell walls with approximately 10^6 receptors for the targeting domain on the surface of each *S. aureus* cell (Baba & Schneewind, 1996). Recently, a study investigating the binding of green fluorescent protein (GFP) tagged targeting domain to *S. aureus* cell walls and purified peptidoglycan has been reported (Grundling & Schneewind, 2006b). This demonstrates that exopolysaccharides, cell surface proteins, lipoproteins and teichoic acids, all components of the cell wall architecture, are not required for binding of the targeting domain to staphylococcal cells. Interestingly pentaglycine cross-bridges, the substrate for the endopeptidase, were found to be essential for cell wall binding. However, the addition of excess pentaglycine did not inhibit binding of the cell wall targeting domain. This indicates that the lysostaphin targeting domain binds to structural features of pentaglycine cross-linked peptidoglycan. It has not been determined whether the pentaglycine cross-bridge involved in targeting domain binding is also the substrate for the endopeptidase, or whether adjacent cross-bridges become substrate molecules.

The targeting domain of lysostaphin has been classified as an SH3b domain, a homologue of the src homology 3 (SH3) domain (Lu *et al.*, 2006). The SH3b domain in prokaryotes is believed to mediate the attachment of proteins to bacterial cell walls (Ponting *et al.*, 1999). The targeting domain of ALE-1 is another SH3b family member and has targeting activity to *S. aureus*. The targeting domain of ALE-1 is a close homologue of lysostaphin's with 83% identity at the amino acid level and therefore probably binds to the same surface receptor (Sugai *et al.*, 1997a). The three dimensional structure of a FLAG tagged ALE-1 targeting domain has been solved and consists of eight β-strands as shown in Figure 1.3 A (Lu *et al.*, 2006). This resembles the tertiary structure of eukaryotic SH3 domains although there are significant differences. The ALE-1 targeting domain is 30 aa larger than typical SH3 domains, and has an additional β-strand and other differences which block the binding groove of the SH3 substrate (Figure 1.3 B). These differences constitute a deep and narrow groove that can potentially accommodate a penta or hexapolypeptide, with the shape of the groove revealing a preference for glycine (Lu *et al.*, 2006) (Figure 1.4). The structure of the SH3b domain of ALE-1 appears to be adapted to fit the pentaglycine cross-bridges of *S. aureus.* Alterations to peptidoglycan cross-bridges would severely impair the binding of the ALE-1 targeting domain. The conserved tryptophan residue at position 358 is not located in the groove, although a W358A mutant reduced peptidoglycan binding by half, indicating a potential role for this region in substrate binding, possibly to another structural feature of peptidoglycan (Lu *et al.*, 2006). This provides further evidence that the targeting domains of ALE-1 and lysostaphin bind to pentaglycine cross-linked peptidoglycan, requiring both the cross-bridge and an additional structural feature of the *S. aureus* peptidoglycan.

A



B

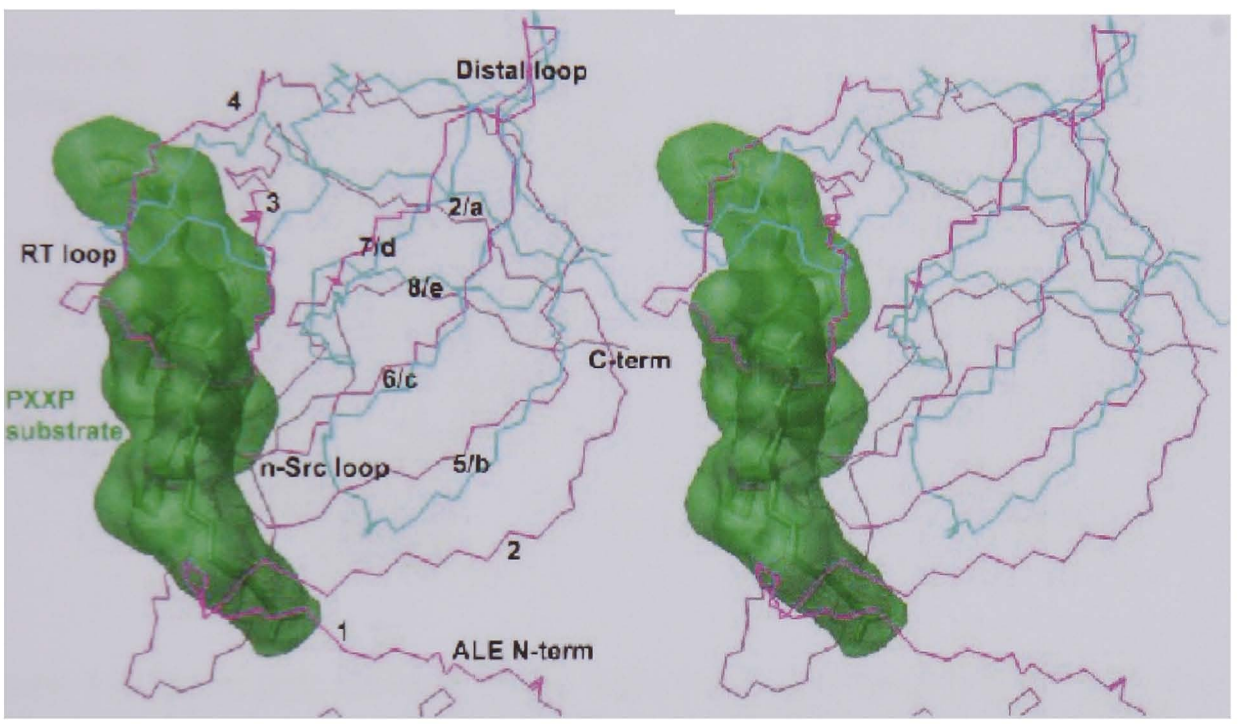


Figure 1.3 Structural comparison of ALE-1 targeting domain with Crk SH3 domain (from Lu *et al.*, 2006).

A; ALE-1 targeting domain with N-terminal FLAG-tag. Strands are numbered sequentially, and the loop regions are named after SH3 counterparts.

B; Stereo view of the superimposition of ALE-1 targeting domain (purple) and Crk-SH3 (cyan) main chain atoms. The β-strands of ALE-1 are numbered sequentially and those of Crk are in alphabetical order. The PXXP substrate of Crk is shown in green, showing the steric incompatibility of the substrate with the ALE-1 structure.

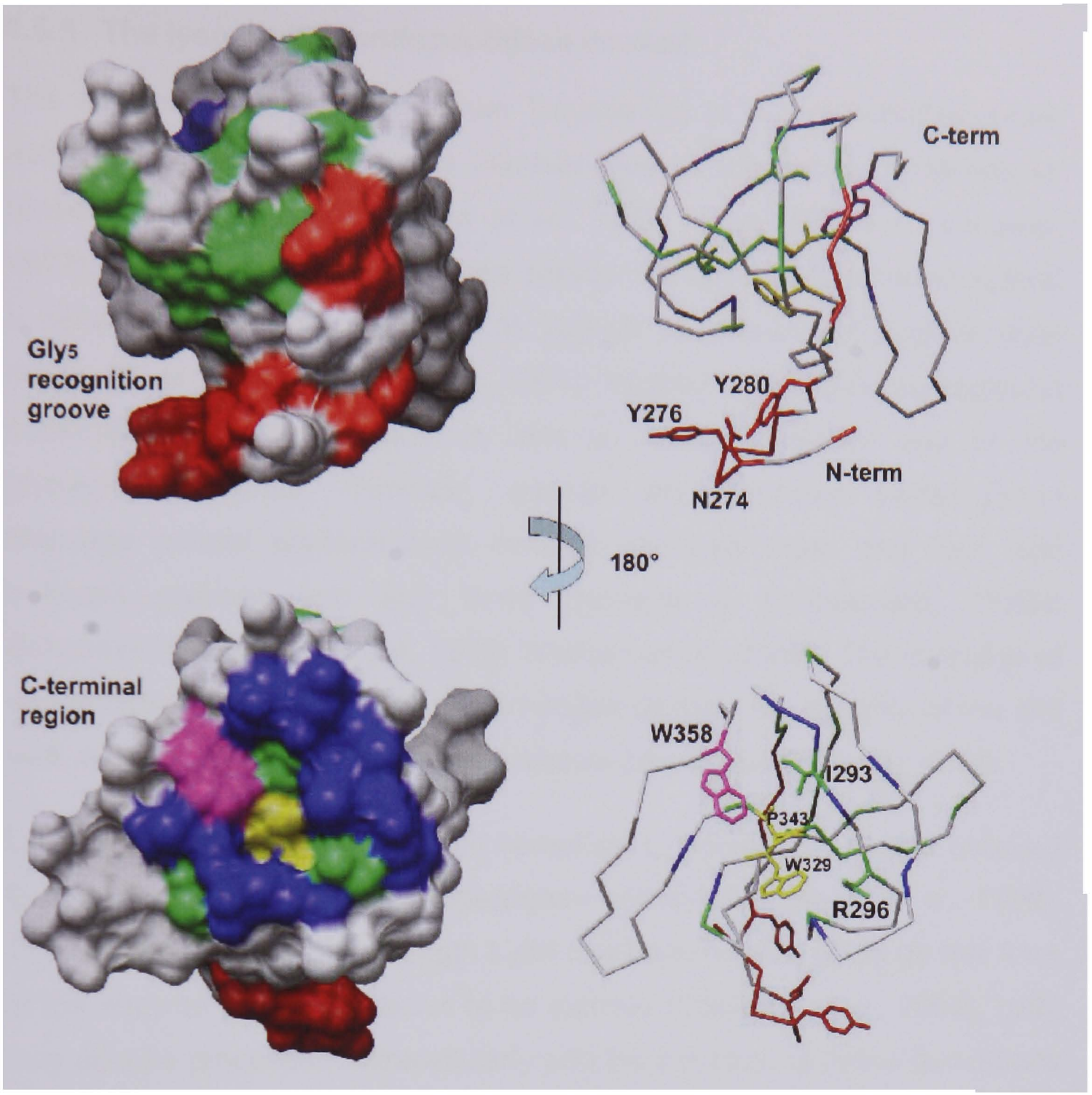


Figure 1.4 Molecular surface of the ALE-1 cell-wall targeting domain (from Lu *et al.*, 2006). Two orientations are shown through an 180° rotation. Highlighted in blue are the residues involved in the FLAG-tag interaction. Trp^{329} and Pro^{343} are shown in yellow at the bottom of the groove and are buried with no accessible surface. Trp^{358} located near the C-terminus is shown in magenta. Conserved residues in cell wall targeting domains are shown in green. In red are highly conserved residues of targeting domains that specifically target *S. aureus*.

1.5.5 The lysostaphin endopeptidase domain

The N-terminal domain of mature lysostaphin is a zinc-metalloprotease active against the pentaglycine cross-bridges of susceptible staphylococci (Browder *et al.*, 1965; Zygmunt *et al.*, 1967; Sloan, Smith & Lancaster, 1977). Glycylglycine endopeptidase activity has been demonstrated against a hexaglycine substrate, as well as against staphylococcal peptidoglycan (Kline, de la Harpe & Blackburn, 1994). Against a synthetic pentaglycine substrate the endopeptidase is able to cleave between any of the glycylglycine bonds. However, against staphylococcal peptidoglycan cleavage occurs predominantly between residues three and four, and between glycines two and three (Navarre & Schneewind, 1999a; Schneewind, Fowler & Faull, 1995; Warfield *et al.*, 2006). The cleavage of staphylococcal peptidoglycan cross bridges disrupts the integrity of the cell wall, with the high internal osmotic pressure causing cell lysis and death.

LytM is a *S. aureus* autolysin with homology to lysostaphin, that is believed to also have glycylglycine endopeptidase activity (Ramadurai *et al.*, 1999). The crystal structure of full length LytM has been solved, although this form of the enzyme has been shown to be inactive (Odintsov *et al.*, 2004). LytM may also be processed extracellularly and the structure of active truncations has also been determined (Firczuk *et al.*, 2005). LytM is also a bimodular protein. It has a C-terminal domain with highly conserved regions among lysostaphin-like proteins and an N-terminal domain with little similarity to other related proteins (Figure 1.5). The C-terminal domain contains the Zn^{2+} binding ligands anchored onto a central, six stranded anti-parallel β-sheet (Odintsov *et al.*, 2004). These ligands are histidine 210, aspartate 214 and histidine 293 as numbered in the sequence of full length LytM (Firczuk *et al.*, 2005) (Figure 1.6). Mutations of these residues results in the loss of activity of LytM, demonstrating their importance (Odintsov *et al.*, 2004). A phosphate acts as the fourth ligand, tetrahedrally co-ordinating the Zn^{2+} in the active site of the enzyme (Firczuk *et al.*, 2005). Histidine 293 is the second histidine in a highly conserved HXH motif. The first histidine of this motif although not involved in co-ordinating Zn^{2+} is essential for LytM activity

as shown by mutagenesis (Odintsov *et al.*, 2004). The active site of LytM is located at the bottom of an extended, long narrow groove, organised around the single Zn^{2+} ion. Data suggests that this is a binding cleft for the pentaglycine substrate (Firczuk *et al.*, 2005).

The active site of LytM, and by homology lysostaphin, is similar to that of D-Ala-D-Ala metallopeptidases such as VanX and the N-terminal cryptic peptidase domain of sonic hedgehog (Böchtler *et al.*, 2004). These proteins all have an arrangement of three zinc ligands located on or around a β-sheet scaffold. This group of families have been designated LAS enzymes, after the initials of **l**ysostaphin-type enzymes, D-**A**la-D-Ala metallopeptidases and **s**onic hedgehog proteins that they have been observed in (Böchtler *et al.*, 2004).

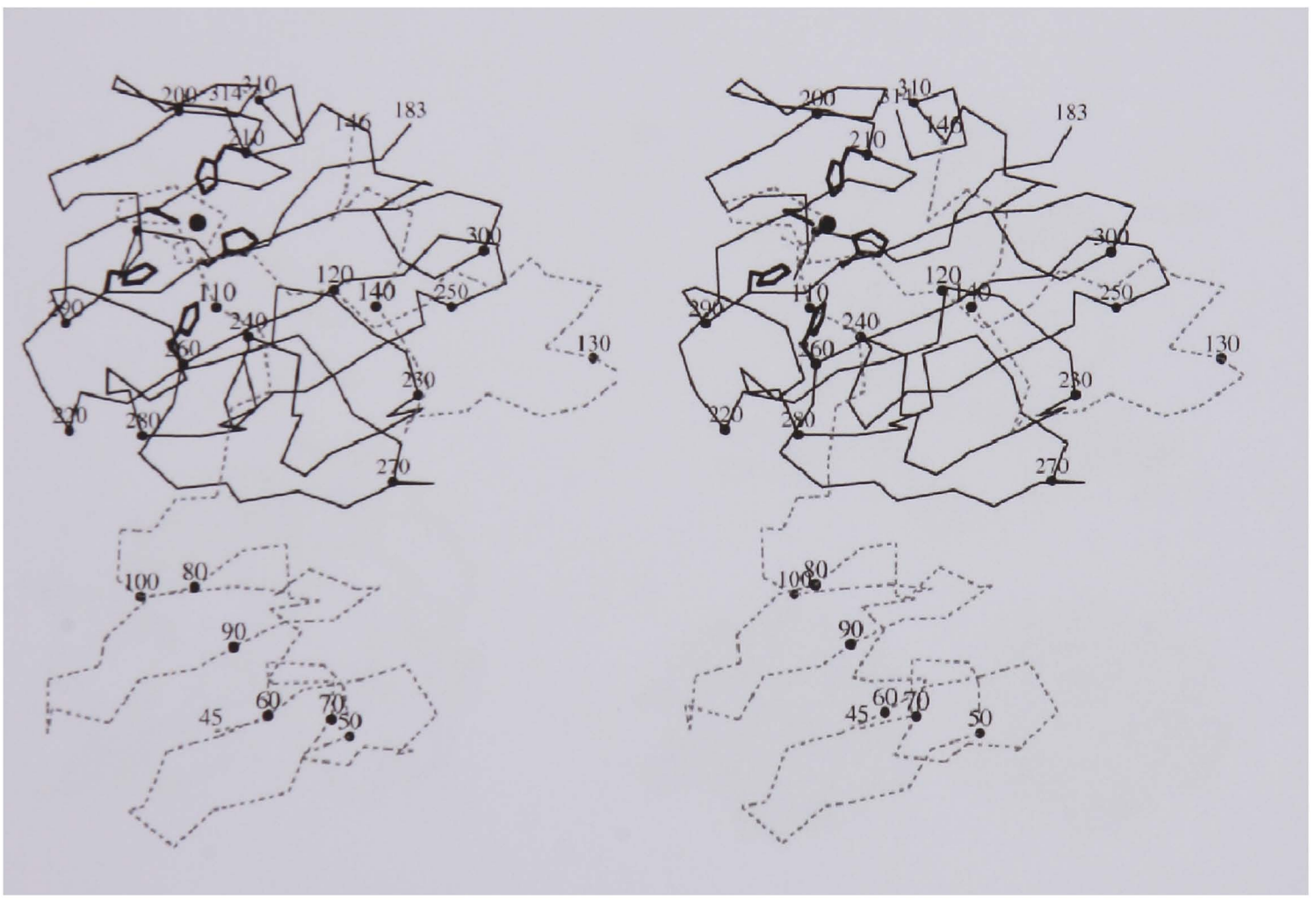


Figure 1.5 Stereo C^{α}-trace of the LytM structure (from Odintsov *et al.*, 2004). The N and C-domains are located at the bottom and top, respectively. Residues of the N-domain and of the C-domain upstream of the disordered segment are represented by dotted lines (45–146). The C^{α}-trace for residues downstream of the disordered segment (183–314) is presented as drawn lines and corresponds roughly to the active protease. Side-chains for some important active-site residues are also presented.

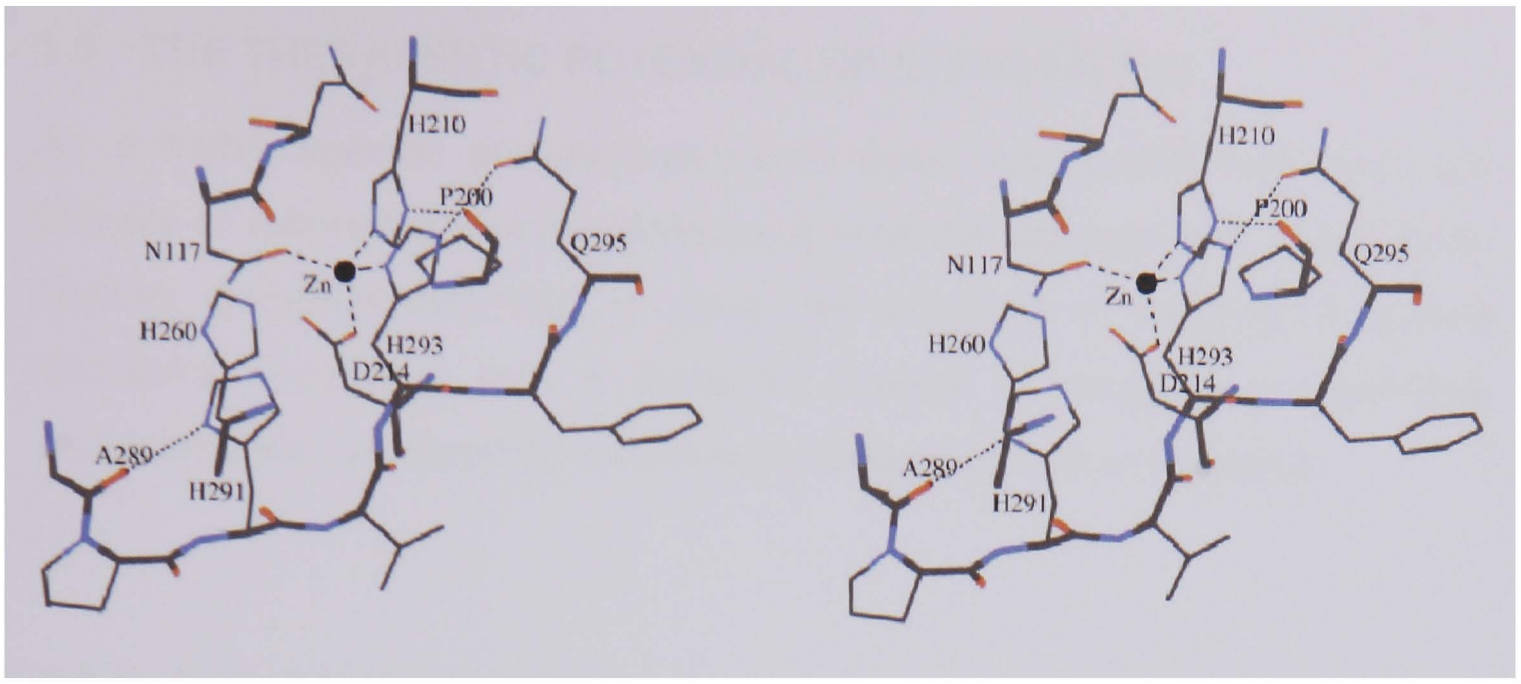


Figure 1.6 Stereo view of the Zn^{2+} -binding site in LytM (from Odintsov *et al.*, 2004). The main-chain trace is drawn with bold lines, side-chains are presented with thin lines. The signature sequence HxHxE/Q runs from left to right in the foreground, and contains H291, H293 and Q295. Interactions between the Zn^{2+} and its ligands are shown with broken lines, and hydrogen bonds that are donated from histidine residues 210 (211), 291 and 293 are presented as dotted lines.

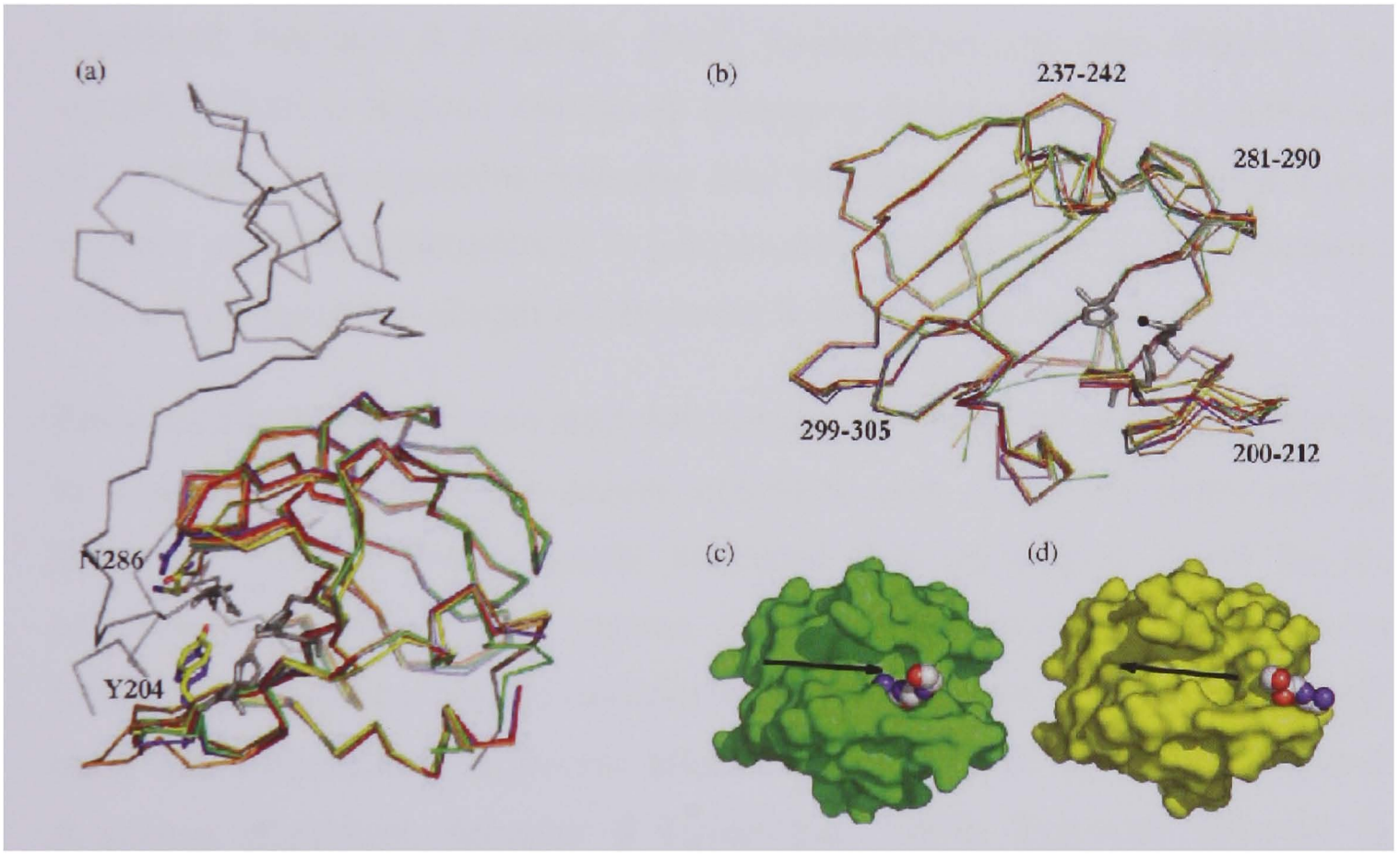


Figure 1.7 Superimposition of six models of active LytM with full-length LytM (from Firczuk *et al.*, 2005). The full-length LytM is shown in grey, the models for the active forms are in yellow, brown, blue, red, orange and green.

(a) Side view to illustrate the groove, and (b) top view to show the flexibility of the groove walls.
(c &d) Active forms surface representations. The arrows in (c) and (d) indicate the direction of the polypeptide chain in the tri-glycine loop used to demonstrate the substrate cleft.

1.6 THE THERAPEUTIC POTENTIAL OF LYSOSTAPHIN

As a highly specific anti-staphylococcal agent lysostaphin has been the subject of extensive investigations as a potential antimicrobial agent. Early studies demonstrated that it could be effective in treating *S. aureus* infections. Recently, with a renewed interest in developing alternative antimicrobials, lysostaphin has been the subject of further research.

1.6.1 Early lysostaphin trials

Shortly after its discovery lysostaphin was shown to be effective against 20 clinical isolates of *S. aureus*. These isolates were all resistant to penicillin G and ampicillin, and showed reduced susceptibility to methicillin. No incidence of lysostaphin resistance was observed and lysostaphin was found to be effective at lower concentrations than traditional antibiotics (Zygmunt, Harrison & Browder, 1965). Lysostaphin was also shown to be equally effective against the newly emerging threat of MRSA (Zygmunt *et al.*, 1968b). Investigations revealed that coagulase negative staphylococci showed variable susceptibility to lysostaphin, possibly due to differences in cell wall composition (Zygmunt, Browder & Tavormina, 1968a).

Early studies also demonstrated that lysostaphin could be used successfully to treat mice given intraperitoneal injections with *S. aureus* (Schuhardt & Schindler, 1964). These results indicated that lysostaphin could be an effective therapeutic agent against *S. aureus* including MRSA. This was further supported by results demonstrating that human sera and red blood cells had a minimal antagonistic effect on the action of lysostaphin against *S. aureus* (Zygmunt, Browder & Tavormina, 1966b; Zygmunt, Browder & Tavormina, 1966a). Lysostaphin was subsequently used in more sophisticated animal studies being effective in a model of *S. aureus* endocarditis in dogs and as a combined treatment with methicillin in the treatment of established staphylococcal abscesses in mice (Goldberg *et al.*, 1967; Dixon, Goodman & Koenig, 1968). Lysostaphin was also used in a mouse model of renal *S. aureus* infection where it was effective in

controlling the staphylococcal population of kidneys, reducing the mortality rate, and clearing high numbers of kidneys of infection (Harrison & Zygmunt, 1967).

Lysostaphin was also used in early human trials. Systemic lysostaphin treatment was showed to have an antimicrobial effect in a neutropoenic patient (Stark *et al.*, 1974). The patient had developed MRSA pneumonia and abscesses and had failed to respond to three weeks of anti-staphylococcal antibiotics. The patient was treated with a single intravenous dose of lysostaphin and experienced only a brief episode of flushing, hypotension, and tachycardia which was subsequently resolved. The patient died three days after lysostaphin treatment of progressive heart failure. The post-mortem revealed no evidence of staphylococcal infection, and previously infected sites were culture negative.

Lysostaphin was also investigated in a nasal spray and was shown to reduce the carriage rate of persistent *S. aureus* carriers within an institutional environment. This effect lasted up to eleven days after a five day treatment and indicated that lysostaphin could play a role in the clearance of *S. aureus* from a patient over the period of a hospital procedure (Quickel *et al.*, 1971). Other studies showed varying lengths of clearance of *S. aureus* following intranasal application in medical staff and in paediatric patient carriers (Martin & White, 1968; Harris, Nunnery & Riley, 1967). No immunological hypersensitivity was observed in these trial subjects, with the largest study only identifying a single wheal and flair reaction in one person after topical lysostaphin application, which had subsided within two hours post-exposure (Quickel *et al.*, 1971).

Although the results of these and other studies indicated that lysostaphin was a promising novel antimicrobial active against MRSA it was not at the time further developed as a therapeutic agent and clinically introduced. Reasons for this included the availability of effective anti-staphylococcal antibiotics, fears over the immunological response to a parentally administered protein antimicrobial, and problems with the purity of the available lysostaphin preparations (Climo *et al.*, 1998).

1.6.2 Renewed interest in lysostaphin

As techniques of producing recombinant proteins of high purity have been developed and as *S. aureus* has shown an increasing pattern of resistance to traditional antibiotics, the potential therapeutic value of lysostaphin has been re-evaluated.

Recent studies have tested the activity of recombinant lysostaphin against clinical isolates of *S. aureus*. A collection of 429 isolates was taken from German hospitals including 210 cultures from nasal swabs and 219 from patients with *S. aureus* bacteraemia. All isolates were susceptible to lysostaphin, with no difference observed between isolates from nasal carriage or from patients with bacteraemia, and no difference observed amongst the 23 isolates that were methicillin resistant (von Eiff *et al.*, 2003). A separate survey of 257 isolates from hospital patients in Beijing, China, also found all isolates of MRSA and methicillin sensitive *S. aureus* were susceptible to recombinant lysostaphin (Yang *et al.*, 2007).

The activity of lysostaphin has been investigated in several models of staphylococcal infection. A rabbit model of MRSA endocarditis was used to investigate the potential of lysostaphin, due to the frequent failure of vancomycin in treating such infections (Climo *et al.*, 1998). Lysostaphin was shown to retain bactericidal activity in rabbit serum, and was well tolerated, although some inhibition of activity by antibody production was observed. A single dose of lysostaphin caused an equal reduction in bacteraemia to vancomycin treatment, and following both, all animals were culture positive after 30 hours. However, in a three day treatment regime lysostaphin proved more effective than vancomycin, sterilising 91% of aortic valve vegetations. This was the highest reported clearance rate of any single agent therapy. Lysostaphin was also found to be effective in treating VISA vegetations, sterilising 83% in the rabbit endocarditis model (Patron *et al.*, 1999). Lysostaphin was less effective in the treatment of *S. epidermidis* in this model. However, lysostaphin was found to act synergistically with the β-lactam nafcillin in the rabbit endocarditis model against oxacillin resistant *S. epidermidis* (Kiri, Archer & Climo, 2002). The combination of lysostaphin

and nafcillin was as effective as vancomycin alone and significantly better than lysostaphin or nafcillin alone, and could provide a useful combination therapy in the event of vancomycin treatment failure.

Lysostaphin has been demonstrated to be effective in the elimination of MRSA in rabbit models of keratitis and endophthalmitis, both conditions which can lead to blindness (Dajcs *et al.*, 2000; Dajcs *et al.*, 2001). Resistance to the current antibiotic treatments is increasing amongst MRSA isolates. Lysostaphin was more effective than any other single agent, including vancomycin, at sterilising rabbit corneas in the keratitis model (Dajcs *et al.*, 2000). The effect of immunisation against lysostaphin in these models was also investigated (Dajcs *et al.*, 2002). Antibodies were raised in rabbits by subcutaneous, intranasal or topical exposure. Only following subcutaneous exposure was serum at a high titre able to inhibit the anti-staphylococcal activity of lysostaphin *in vitro*. Lysostaphin was effective in eliminating MRSA from rabbits previously exposed to it by all three routes in both models of eye infection, although a reduction in damage caused by disease was not observed. No adverse effects were observed in any rabbits exposed to lysostaphin by all three routes, or by treatment in the models of infection (Dajcs *et al.*, 2002).

Further *in vivo* studies have been carried out to investigate the potential of lysostaphin in veterinary medicine. Staphylococcal mastitis is the most prevalent disease of diary cattle. An early study showed that lysostaphin had some effect in the treatment of cattle infected with *S. aureus* and that improved lysostaphin formulations may have useful therapeutic potential (Oldham & Daley, 1991). In a mouse model, the expression of lysostaphin in the mammary glands of transgenic animals conferred resistance to staphylococcal mastitis (Kerr *et al.*, 2001). Subsequently, transgenic cattle carrying a mammary specific transgene encoding lysostaphin have also been shown to be resistant to infection by *S. aureus.* All six lines from these transgenic animals also express lysostaphin, including three founded by bulls (Wall *et al.*, 2005; Donovan, Kerr & Wall, 2005).

Nasal carriage is a key risk factor in infections by *S. aureus*, particularly in the hospital environment. Rates of infection are higher in carriers than non-carriers, and individuals are usually infected with their own carriage isolates (Peacock, de Silva & Lowy, 2001). The eradication of *S. aureus* nasal carriage in high risk individuals could therefore reduce the incidence of infections. This could have a particular role in the prevention of post-surgery wound infections by *S. aureus*. Mupirocin has been used to reduce nasal colonisation, but resistance to this agent is developing. Lysostaphin could be an effective alternative to eliminate nasal carriage in such patients. A cotton rat model of *S. aureus* colonisation of the anterior nares has been used to demonstrate the efficacy of lysostaphin in clearing nasal carriage (Kokai-Kun *et al.*, 2003). A cream formulation of lysostaphin was used in rats inoculated with the MRSA MBT5040 strain. A single dose of lysostaphin eradicated *S. aureus* in 93% of animals, and nasal colonisation was eradicated in all animals with a dosing regime of three doses over three days. Lysostaphin was tested against two MSSA strains, two MRSA and one mupirocin resistant *S. aureus* strain. All strains were sensitive to lysostaphin which was more effective than mupirocin, and nasal clearance lasted at least one week. No lysostaphin resistant *S. aureus* colonies were isolated, even when lysostaphin was added at sub-inhibitory concentrations. An improved cream formulation was subsequently reported to extend the nasal residency of lysostaphin (Walsh *et al.*, 2004). Lysostaphin remained in the anterior nares at inhibitory concentrations for at least 24 hours. In the clinical setting this cream could be applied daily and maintain lysostaphin above sub-inhibitory concentrations for the duration of a patient's hospital stay.

S. aureus is a major nosocomial pathogen of those with indwelling medical devices. Biofilms consisting of a multilayered community of sessile cells and an extracellular matrix often forms on the surface of such devices, which then acts as a source of further infections. Biofilms are often difficult to treat due to increased antibiotic resistance of cells in a biofilm, poor access to cells, and a slow growth rate once formed (Donlan & Costerton, 2002; Patel, 2005).

Lysostaphin has been shown to disrupt and eradicate *S. aureus* biofilms growing on polystyrene, polycarbonate and glass surfaces (Wu *et al.*, 2003). A rapid effect is seen, with a measurable disruption of the biofilm within 20 minutes, making lysostaphin treatment faster and more effective than treatment with oxacillin or vancomycin. Lysostaphin was also able to disrupt *S. epidermidis* biofilms growing on glass, although it had no affect on *Pseudomonas aeruginosa*. The mode of action of lysostaphin against *S. aureus* biofilms is not known, although it is suggested that there is sufficient lysis of sessile cells caused to destabilise the biofilm (Wu *et al.*, 2003). These results indicate that lysostaphin could be used in the eradication of biofilms from patients with indwelling medical devices. Lysostaphin has also been investigated for its potential to prevent the colonisation of catheters by *S. aureus* (Shah, Mond & Walsh, 2004). Lysostaphin was coated onto polystyrene and FEP polymer surfaces that are used in catheters, washed with phosphate buffered saline and then challenged with *S. aureus*. There was a 99.5% reduction in the bacterial count from lysostaphin-coated surfaces compared to untreated surfaces following bacterial challenge. Increased coating time led to increased killing activity up to an optimum time of 15 minutes, indicating that bound lysostaphin was responsible for the anti-staphylococcal activity. Lysostaphin retained activity against *S. aureus* for 96 hours with a change in buffer every 24 hours, indicating that a coated catheter could be used to prevent biofilm formation and protect a patient for several days. The presence of human serum had no adverse affect on the activity of lysostaphin coated to the surface. Lysostaphin coated polymers proved effective at preventing biofilm formation by several *S. aureus* strains including MRSA, and had significant activity against *S. epidermidis* (Shah *et al.*, 2004). These results demonstrate that as well as having potential as a treatment for biofilms growing on polymer surfaces, lysostaphin coated catheters could also be useful in preventing the colonisation of those devices.

As lysostaphin has been shown to have potential uses in the treatment of several staphylococcal infections work has also been carried out to improve the formulation of the protein, and to devise large scale production systems

that would be required for the clinical introduction of lysostaphin. One drawback in using lysostaphin as an intravenous treatment is the relatively short half life of the drug. One approach to resolving this has been to conjugate lysostaphin to polyethylene glycol (PEG) (Walsh, Shah & Mond, 2003). This reduces renal ultrafiltration by increasing the molecular size, and creates a molecular shield which can block access to antibodies. The PEG conjugated lysostaphin has slightly lower enzymatic activity than unconjugated protein, but in a mouse model the serum half life was extended from 1 hour to 24 hours, increasing the plasma drug concentration. The prolonged persistence of PEG conjugated lysostaphin, and its reduced antibody binding, enhance the therapeutic value of lysostaphin as an intravenous treatment.

Industrial scale production of lysostaphin has been achieved using the nisin-controlled gene expression system (NICE) in *Lactococcus lactis* (Mierau *et al.*, 2005a). A large scale culture of 3000L was grown, the cell filtrate collected and the protein purified by cation-exchange chromatography. The initial fermentation yielded 100mg L^{-1} of which 40% was recovered after downstream processing providing 120g from the 3000L fermentation. Although this is a fairly low yield for a bulk production the process has been further optimised on a small scale and could be used in an industrial scale production process (Mierau *et al.*, 2005b). Lysostaphin from the fermentation process described by Mierau et al (2005a) was used in subsequent phase I clinical trials.

1.6.3 Lysostaphin in clinical trials

Lysostaphin has been developed as a candidate therapeutic agent by Biosynexus Incorporated, a U.S. biotechnology company. It has been formulated as a topical anti-staphylococcal cream to eradicate *S. aureus* nasal carriage. Biosynexus have announced that their compound, BSYX-L210, has successfully completed phase I and phase I/II clinical trials to assess its safety and as an initial study of its efficacy (Anon, 2003). The phase I trial involved 18 volunteers including 7 *S. aureus* carriers. Four of

the carriers were completely cleared of *S. aureus* following treatment with BSYX-L210, whilst three carriers receiving a placebo remained unaffected. No significant adverse events were observed. Full details of the phase I/II trial and its results have not yet been described, although it is reported to validate results of pre-clinical studies in the cotton rat model (Kokai-Kun *et al.*, 2003). These results are encouraging for the further development of lysostaphin as a novel antimicrobial agent.

1.7 RESISTANCE TO LYSOSTAPHIN

The future clinical application of lysostaphin will introduce a selection pressure for the evolution of *S. aureus* mutants resistant to the antimicrobial peptide. This could occur through one of several possible mechanisms either blocking targeting of the peptide to staphylococcal peptidoglycan, or by altering the target of the endopeptidase. Lysostaphin resistant *S. aureus* mutants have previously been isolated in both *in vitro* and *in vivo* studies (Zygmunt *et al.*, 1967; Climo, Ehlert & Archer, 2001).

1.7.1 Host immunity to lysostaphin

S. simulans biovar *staphylolyticus* protects itself from the actions of lysostaphin by two mechanisms. The first is to express a propeptide with reduced lytic activity, which is only processed to the active, mature peptide extracellularly as described previously. It has been shown that this processing step is a significant contributory factor in the conferring of host resistance to recombinant *S. carnosus* (Thumm *et al.*, 1997).

Secondly, *S. simulans* biovar *staphylolyticus* produces a lysostaphin immunity factor (Lif) protein. The *lif* gene, also known as *epr* (endopeptidase resistance gene), is located on the pACK1 plasmid which had been shown to be required for lysostaphin resistance (DeHart *et al.*, 1995; Thumm *et al.*, 1997). The *lif* gene contains 413 codons and is transcribed in the opposite direction to the *lss* gene from which it is separated by a 208 non coding

base pair sequence (Thumm *et al.*, 1997). When *S. aureus* RN4220 is transformed with the *lss* and *lif* genes it produces and is resistant to lysostaphin (DeHart *et al.*, 1995). Lif was found to protect *S. simulans* biovar *staphylolyticus* by modifying the peptidoglycan cross-bridges, incorporating serine in place of glycine residues (DeHart *et al.*, 1995; Thumm *et al.*, 1997). Early studies on the susceptibility of coagulase negative staphylococci had previously shown that species with an increased presence of serine, at the expense of glycine, showed a reduced sensitivity to lysostaphin (Zygmunt *et al.*, 1968a).

The Lif protein is homologous to the FemA/B proteins of *S. aureus*. These proteins are responsible for the stepwise addition of glycine residues to staphylococcal peptidoglycan cross-bridges (Thumm *et al.*, 1997). The expression of Lif leads to the specific incorporation of serine residues at positions three and five of the cross-bridge (Ehlert *et al.*, 2000). However, Lif does not extend the interpeptide bridge, and FemA and FemB are still required for the cross-bridge formation. The interaction of Lif with FemA and, or FemB is thought to lead to the substitution of glycine with serine at these positions (Ehlert *et al.*, 2000). Lysostaphin has been shown to be unable to hydrolyse glycylserine or serylglycine bonds (Robinson, Hardman & Sloan, 1979). Therefore the expression of Lif protects the host organism from the enzymatic action of lysostaphin. Furthermore, as pentaglycine cross-linked peptidoglycan is believed to the receptor for the targeting domain of lysostaphin (Grundling *et al.*, 2006b; Lu *et al.*, 2006), Lif may have a dual protective effect inhibiting the binding of lysostaphin as well as the endopeptidase action.

Thumm and Götz (1997) suggest that *S. simulans* biovar *staphylolyticus* acquired the *lss* and *lif* genes through a horizontal gene transfer event. It remains possible that following the clinical introduction of lysostaphin, that *S. aureus* develop resistance by acquisition of the *lif* gene. A plasmid from *S. sciurii* strain DD4747 has been identified carrying a gene similar to *lif* and recombinant *S. aureus* RN4220 expressing this gene have a greater cell wall serine content and an increased resistance to lysostaphin (Heath *et al.*,

2005). This provides further evidence that such genetic transfers have previously occurred, and that there is another source of the *lif* gene which could become transferred to *S. aureus* in the clinical environment in the future. This would compromise the therapeutic value of lysostaphin, and should be considered in the development of lysostaphin based antimicrobials.

Fortunately the presence of the *lif* gene in *S. aureus* is reported to increase susceptibility to methicillin (DeHart *et al.*, 1995). Any infections caused by lysostaphin resistant mutants arising this way could therefore be simply treated with nafcillin. The co-administration of nafcillin with lysostaphin could prevent resistance developing through the acquisition of *lif*. However, if such mutants were able to develop nafcillin resistance in the future, they would pose a significant clinical threat.

1.7.2 Resistance to lysostaphin by shortened cross-bridges

Resistant *S. aureus* mutants have been isolated in both *in vitro* and *in vivo* studies following exposure to sub-inhibitory concentrations of lysostaphin (Climo *et al.*, 2001; Kusuma *et al.*, 2007). The isolates from both studies had shortened peptidoglycan cross-bridges.

The pentaglycine cross-bridge of staphylococci is formed by a sequential process involving several proteins. FmhB is involved in the addition of the first glycine residue to the stem peptide (Rohrer *et al.*, 1999). Glycines two and three are then added by the action of FemA (Kopp *et al.*, 1996; Stranden *et al.*, 1997). Finally, FemB is responsible for the addition of glycines four and five (Henze *et al.*, 1993). Mutation of *femB* results in a triglycine cross-bridge and *femA* mutations lead to a monoglycine cross-bridge.

The lysostaphin resistant mutants isolated both had *femA* mutations, resulting in monoglycine cross-bridges (Climo *et al.*, 2001; Kusuma *et al.*, 2007). This mutation confers resistance to lysostaphin by removing the

enzymatic target of the glycylglycine endopeptidase and also by altering the structure of the receptor site of the targeting domain.

From the studies carried out to date, the emergence of *femA/B* mutations is likely to be the most common form of resistance that may emerge following the clinical introduction of lysostaphin. In a rabbit model of endocarditis caused by oxacillin resistant *S. aureus* treated with a low dose of lysostaphin, resistant isolates were found after three days (Climo *et al.*, 2001). However, lysostaphin resistant mutants that have been isolated show a loss of resistance to β-lactams. The co-administration of lysostaphin with β-lactams *in vitro* prevented the emergence of lysostaphin resistance, and a joint lysostaphin and nafcillin therapy in the rabbit endocarditis model also prohibited resistance developing (Climo *et al.*, 2001).

Null *femAB* mutants of *S. aureus* have previously been shown to be hypersensitive to methicillin (Stranden *et al.*, 1997). It is thought that this is due to the substrate requirement of PBP2' being incompatible with the reduced peptidoglycan cross-bridge (Climo *et al.*, 2001). As well as having shortened cross-bridges *femAB* null mutants also had a reduced number of peptidoglycan cross-bridges, which is thought to explain their increased sensitivity to β-lactams (Stranden *et al.*, 1997).

Furthermore, lysostaphin resistant *femA* mutants also show a reduced fitness compared with their parent *S. aureus* strains. They demonstrate an impaired exponential growth rate and are out-competed by wild-type *S. aureus* (Stranden *et al.*, 1997; Kusuma *et al.*, 2007). They are also more susceptible to elevated temperatures and have been shown to be at least fivefold less virulent in a mouse model of kidney infection (Kusuma *et al.*, 2007). These mutants failed to develop compensatory mutations to restore fitness during a 14 day serial passage without selective pressure.

It therefore seems likely that lysostaphin resistant *femA/B* mutants do not pose a serious clinical threat and could easily be prevented in the hospital setting through combination therapy with another antibiotic. Lysostaphin also is reported to have a synergistic lytic activity in combination with β-

lactams, providing a dual therapeutic benefit to such a treatment (Climo *et al.*, 2001). However, there is currently no selective pressure in the hospital environment for the emergence of a methicillin, lysostaphin resistant *S. aureus*. If lysostaphin is introduced as a new antimicrobial such mutants may emerge and this event should be considered in the development of future lysostaphin based treatments.

1.7.3 Lysostaphin resistance in VISA

Lysostaphin resistance has been observed in vancomycin intermediately sensitive *S. aureus* strains, including clinical isolates. In one study six *S. aureus* blood cultures were taken from a patient receiving vancomycin therapy over a period of 16 days (Boyle-Vavra, Carey & Daum, 2001). Initially isolates were glycopeptide susceptible, but as the bacteraemia continued the vancomycin MIC increased. As vancomycin intermediate sensitivity developed the MIC of lysostaphin increased. This result concurs with VISA strains developed *in vitro*, although not all VISA strains showed increased resistance to lysostaphin (Pfeltz *et al.*, 2000; Boyle-Vavra *et al.*, 2001). This clinical VISA isolate had increased levels of peptidoglycan cross-linking. The composition of the cross-bridges was unaffected, indicating that the increased cross-linking may be responsible for the increase in the lysostaphin MIC. It is suggested that this could be due to the increased number of target sites for lysostaphin requiring a higher concentration to achieve the same lytic effect. However, it was noted that a VISA strain with a very high level of peptidoglycan cross linking had a low MIC of lysostaphin (Boyle-Vavra *et al.*, 2001).

A separate study characterising glycopeptide intermediately sensitive *S. aureus* has shown lysostaphin resistance in strains with reduced levels of peptidoglycan cross-linking (Koehl *et al.*, 2004). Again this strain shows no evidence of having shortened cross-bridges or any serine substitutions. Conversely peptidoglycan purified from this strain is highly sensitive to lysostaphin. It is suggested that the increased lysostaphin resistance may be related to defective autolysis in GISA strains, and that autolysins are

involved in the lysis caused by lysostaphin treatment (Koehl *et al.*, 2004). There is some evidence that *atl* null mutants show increased resistance to lysostaphin (Pfeltz *et al.*, 2000).

The emergence of lysostaphin resistant VISA strains in the clinical setting could lead to the failure of lysostaphin as a therapeutic agent. This mechanism of resistance is poorly understood, but efforts should be made to counteract its clinical emergence.

1.7.4 Other potential resistance mechanisms

Bacteria have evolved many different methods of evading the action of traditional antibiotics. The mechanisms described above have all been previously identified through *in vivo* and *in vitro* studies. Other mechanisms may evolve once the selection pressure of clinically administered lysostaphin is introduced.

A lysostaphin resistant mutant has been generated in a study of transposon mutants (Grundling, Missiakas & Schneewind, 2006a). Characterisation of the mutant identified that the transposon had inserted into a previously uncharacterised gene, subsequently named *lysostaphin resistance A* (*lyrA*). The inactivation of *lyrA* caused high level resistance to lysostaphin through apparently minor alterations to peptidoglycan structure. LyrA is predicted to be a multispanning membrane protein and contains an Abi domain. Abi containing proteins are metallo-dependent membrane proteases, although protease activity in LyrA does not seem to be required for lysostaphin resistance (Grundling *et al.*, 2006a). No change in lysostaphin targeting domain binding was observed to *lyrA* mutants, indicating that resistance is due to a non-peptidoglycan related attribute of *S. aureus*. Importantly *lyrA* mutants do not show a decrease in β-lactam resistance. If this resistance mechanism were to emerge in the hospital environment it would seriously compromise the therapeutic potential of lysostaphin.

Other resistance mechanisms that have not yet been observed could also emerge and threaten the efficacy of lysostaphin. The cellular receptor of lysostaphin is not fully understood, but is thought to be a structural feature of pentaglycine cross-linked peptidoglycan (Grundling *et al.*, 2006b; Lu *et al.*, 2006). Alterations to the cross-bridges have been shown to lead to lysostaphin resistance and other changes to the peptidoglycan structure could also lead to resistance emerging.

1.8 THE DEVELOPMENT OF SECOND GENERATION LYSOSTAPHINS

Results of *in vitro* and *in vivo* studies and of clinical trials provide encouragement that lysostaphin may be successfully introduced as a novel antimicrobial treatment for staphylococci including MRSA. However, once clinically introduced there will be a significant selection pressure for the evolution of lysostaphin resistant MRSA. This may occur by one or more of the several mechanisms discussed above and experience of traditional antibiotics suggests that this event is likely to occur at some point in time. This event may be delayed by the co-administration with other antibiotics, and by prudent prescription of lysostaphin. The likelihood of this event also justifies the investigation of second generation lysostaphin treatments. These second generation molecules should be developed with the aim of overcoming the likely resistance mechanisms that may evolve. Furthermore, they may also be designed to have a wider range of therapeutic applications, such as a broader spectrum of activity or have improved pharmacokinetics properties. Second generation lysostaphins may be developed by designing synthetic variations. Synthetic peptides based on the active site of insect defensins have been demonstrated to protect mice from a lethal MRSA challenge (Saido-Sakanaka *et al.*, 2005). Alternatively second generation lysostaphins may utilise features of other peptidoglycan hydrolases in the form of an enzybiotic. A lysostaphin based enzybiotic could incorporate features of bacteriophage endolysins, staphylococcal autolysins or other antimicrobial peptides previously discussed.

1.8.1 Exploiting the modular domain of lysostaphin

Lysostaphin and the other peptidoglycan hydrolases previously described are modular enzymes with discrete cell wall targeting and catalytic domains. This domain structure could be utilised in the development of second generation lysostaphin based enzybiotics by substituting either domain with that from another antimicrobial peptide. Such a chimeric peptide could be expected to demonstrate altered binding properties or substrate range. This could circumvent the likely resistance mechanisms and alter the spectrum of activity.

It has been hypothesised that peptidoglycan hydrolases could have evolved by the fusion of such modular domains (Garcia *et al.*, 1988; Oshida *et al.*, 1995). This provides encouragement that designing chimeric antimicrobial peptides could lead to highly active novel treatments for staphylococcal and other infections being developed, and could even mirror events that have occurred *in vivo*.

Previous studies have shown that peptidoglycan hydrolase fusions retain their parental activities. A fusion of the *Streptococcus agalactiae* B30 bacteriophage endolysin to mature lysostaphin lyses both *S. aureus* and *Streptococcus agalactiae* (Donovan *et al.*, 2006a). It is encouraging that being part of a fusion protein does not inhibit the activity of either domain, although this does not provide evidence that any additional activity is conferred to the fusion. A second fusion of lysostaphin to a truncation of the B30 phage endolysin was also lytic to both *S. aureus* and *S. agalactiae*, and it is suggested that the lysostaphin targeting domain could be contributing to the activity of the phage endolysin, which had lost its weakly homologous SH3b domain (Donovan *et al.*, 2006a). However, it was also noted that the truncated endolysin retained lytic activity against *S. agalactiae* on its own.

There have also been reports of multi-domain peptides developed with highly specific bacteriolytic activity. Colicin Ia kills *E. coli* cells by forming a channel in the cell membrane, causing lysis of the cell. Colicin Ia has been fused to the AgrD1 pheromone of *S. aureus*, which is secreted through the cell wall membrane initiating the cell signalling pathway, and is highly

specific for *S. aureus* (Qiu *et al.*, 2003). This multi-domain peptide, pheromonicin-AgrD1 (PMC-AgrD1), has been shown to form channels in lipid bi-layers. PMC-AgrD1 was shown to be bactericidal against *S. aureus* including MRSA, but not against *S. epidermidis* or *S. pneumoniae*. Neither of the domains alone had any inhibitory effect on growth of *S. aureus*, demonstrating that the multi-domain peptide had a novel activity. PMC-AgrD1 was also effective in a mouse model of MRSA sepsis and there was no evidence of toxicity. A subsequent peptide was engineered using a pheromone of *E. faecalis* fused to colicin Ia, which was effective in treating mice in a model of VRE sepsis (Qiu *et al.*, 2005). These results suggest that multi-domain peptide fusions could provide effective antimicrobials with novel activities and that this approach could lead to the development of second generation lysostaphins.

Several antimicrobial peptides, phage endolysins and staphylococcal autolysins display a degree of homology to either the targeting domain or endopeptidase domain of lysostaphin. Homologous peptides may be particularly suitable for the inclusion in a chimeric second generation lysostaphin, as the overall structure of the peptide may remain similar and retain its function. By finding peptidoglycan hydrolases with homology to lysostaphin, a group of candidate peptides can be identified for further study in the development of such novel multi-domain antimicrobials.

1.8.2 Screening activity of novel antimicrobial peptides and second generation lysostaphins

During the development of novel antimicrobial peptides their bacteriolytic activity must be assayed. There are a range of assay types available, including agar plate and liquid assays, and assays against live cells, killed cells and chemical substrates. These different assays have various attributes, including ease of use, sensitivity and reproducibility, although results between the different assays can often vary (Kusuma & Kokai-Kun, 2005). Enzymatic reactions to measure the hydrolysis of defined substrates can be used to produce reliable quantitative results of peptidoglycan

hydrolase activity (Kessler *et al.*, 2004). However, these reactions are not representative of the effect of these enzymes *in vivo*. Whilst developing novel antimicrobial peptides, including second generation lysostaphins, it would be invaluable to have access to a reliable, sensitive, quantitative assay of activity against live *S. aureus* cells.

1.9 PROJECT AIMS

The general aim of this project is to develop novel antimicrobial peptides with activity against MRSA. The approach described in this thesis is to design chimeric, second generation lysostaphin enzybiotics. This was through the testing of antimicrobial peptides domain functions, followed by the alteration of the peptides, and the use of assays to determine any improvement in activity and to screen for resistance. Changes in the activity of peptides were then analysed.

Peptidoglycan hydrolases with homology to lysostaphin were identified as candidate peptides. This thesis describes the cloning of candidate peptides and the investigation of their bacteriolytic activity against *S. aureus* and other bacteria. Other physical properties of the recombinant proteins are also considered.

The design of a chimeric lysostaphin based antimicrobial peptide is described, along with an analysis of its bacteriolytic properties, and a comparison to the parental properties of the component domains.

In addition to the investigation and development of novel antimicrobial peptides, the design of a novel assay for the screening of staphylolytic activity of these peptides is described.

2. MATERIALS AND METHODS

The general materials and methods that were used throughout chapters 3, 4 and 5, are described in this chapter. Further techniques used in the development of the bioluminescence reporter assay are described in chapter 5.

2.1 BACTERIAL STRAINS AND PLASMIDS

2.1.1 Media and growth conditions

Bacterial cultures were grown in LB or 2YT broth or on agar plates. Cultures grown during cloning procedures used LB media and recombinant clones were selected using medium supplemented with 100µg ml^{-1} ampicillin. Cultures grown for expression of recombinant proteins were grown in 2YT media supplemented with 100µg ml^{-1} ampicillin. Unless otherwise described, cultures were incubated at 37°C, and liquid cultures were subjected to orbital shaking at 200rpm.

2.1.1.1 *Luria-Bertani medium*

One litre of Luria-Bertani broth was made by adding 10g bacto-tryptone (Oxoid), 5g bacto-yeast extract (Oxoid) and 5g NaCl to 950ml of deionised water. The pH was adjusted to 7.0 with 5M NaOH, and the volume was made up to 1L using deionised water, prior to autoclaving at 121°C for 20 minutes.

To make LB agar, bacteriological agar (Oxoid) was added to the broth recipe to a final concentration of 1.5% w/v.

2.1.1.2 *2YT medium*

One litre of 2YT broth was made by adding 16g bacto-tryptone (Oxoid), 10g bacto-yeast extract (Oxoid) and 5g NaCl to 950ml of deionised water. The pH was adjusted to 7.0 with 5M NaOH, and the volume was made up to 1L using deionised water, prior to autoclaving at 121°C for 20 minutes.

To make 2YT agar, bacteriological agar (Oxoid) was added to the broth recipe to a final concentration of 1.5% w/v.

2.1.2 Bacterial Strains

Bacterial strains used are listed in Table 2.1 and were stored in LB broth with 10% (v/v) glycerol at -80°C.

Table 2.1 List of strains used through this project with relevant features and sources

Strain	Relevant features / genotypes	Reference / Source
E. coli		
DH5α	LacZDM15 recA1	Invitrogen
ER2566	LacZ::T7λ (DE3)	New England Biolabs
BL21 (DE3)	araB::T7	Invitrogen
S. aureus		
Mu50	Sequenced VISA	(Kuroda *et al.*, 2001)
RN450	Laboratory strain	(Novick, 1991)
EMRSA16	Clinical isolate	QMC, Nottingham
MRSA SCC*mec* types IA, II, III and IV	Clinical isolates	QMC, Nottingham
RN6390	Laboratory strain	(Novick, 1991)
NTC 6571	Oxford staph	(Heatley, 1944)
Micrococcus luteus	Environmental isolate	University of Nottingham
S. epidermidis *S. pyogenes* *S. haemolyticus* *S. sciurii*	Clinical isolates	QMC, Nottingham

2.1.3 Plasmids

The full list of plasmids used in this study is presented in Table 2.2. pGEMT-Easy (Promega) was used as an intermediate vector during cloning. pET21a and pET21d (Studier & Moffatt, 1986) (Novagen) were used as expression vectors for genes of interest. Plasmid maps are shown in Figure 2.1 and Figure 2.2. The sequences of constructs generated as part of this project are shown in the appendices. These highlight the restriction sites used to make the clones and indicate the pET vector used. Expression is under the control of T7 bacteriophage transcription and translation signals. Reverse PCR primers were designed to remove the 3' stop codons and incorporate the vector sequence encoding a C-terminal hexahistidine tag, enabling the purification of recombinant protein by nickel-chelate chromatography (Garinot-Schneider *et al.*, 1996).

Table 2.2 List of plasmids used through this study

Plasmid	Description / Protein encoded	Reference
pGEMT-Easy	Intermediate cloning vector	Promega (Figure 2.1)
pET21a	Expression vector	(Studier *et al.*, 1986); Novagen; (Figure 2.2)
pET21d	Expression vector	(Studier *et al.*, 1986); Novagen; (Figure 2.2)
pKP1	Phage P68 Lys16	Appendix I
pUHHOLY1516	ORF15/16 from P68 genome	(Takáč *et al.*, 2005b)
pKP2	Atl glucosaminidase	Appendix II
pKP3	LssT: Atl GL chimera	Appendix III
pKP4	Truncated Atl GL	Appendix IV
pKP5	GFP tag in pGEMT	Appendix V
pKP6	GFP tagged Atl GL	Appendix VI
pKP7	GFP tagged GL'	Appendix VII
pKP8	R123GL	Appendix VIII
pKP9	Atl amidase	Appendix IX
pKP10	Atl R12	Appendix X
pKP11	Atl R3	Appendix XI
pEA3	Mature lysostaphin	(Antoniadou, 2004)
pLss-T-GFP	GFP tagged LssT	Appendix XII

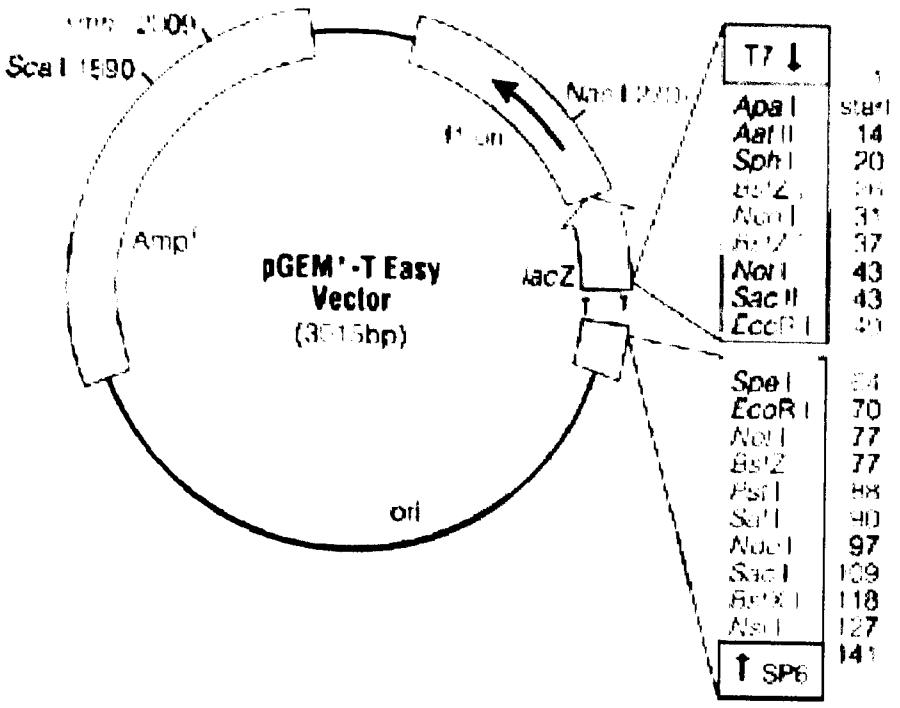


Figure 2.1 Plasmid map of pGEMT-Easy (Promega)

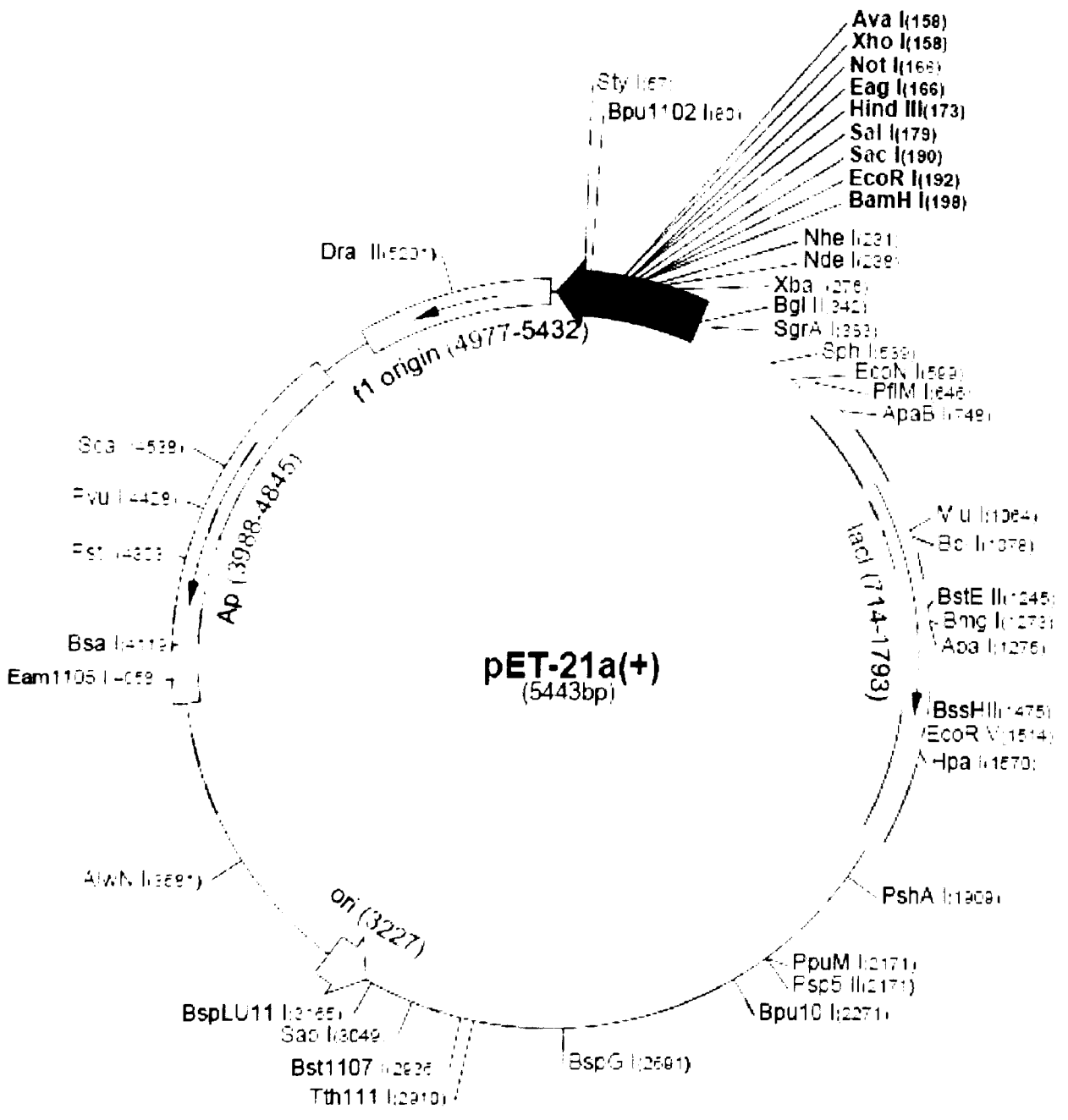


Figure 2.2 Plasmid map of pET21a (Novagen). pET21d is a 5440bp plasmid, based on pET21a with the removal of two base pairs following the *Bam*HI restriction site at position 198, and the replacement of the *Nde*I site with an *Nco*I site at position 238 with the loss of one base pair.

2.2 MANIPULATIONS OF DNA

2.2.1 Isolation of genomic DNA

Genomic DNA was extracted from overnight cultures grown in LB broth using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's protocol.

2.2.2 Isolation of plasmid DNA

Plasmid DNA was extracted from 3ml of overnight cultures of *E. coli* DH5α, grown in LB broth, using the Wizard Plus SV Miniprep kit (Promega) following the supplier's instructions. Larger stocks of plasmid DNA were isolated from 50ml of culture with the Qiagen Plasmid Midi kit, in accordance with the manufacturer's protocol. The concentration of DNA was measured using a NanoDrop ND1000 spectrophotometer.

2.2.3 Restriction and ligation of plasmids

Endonuclease digestion of plasmid DNA was carried out according to the methods of Sambrook *et al.* (Sambrook, Fritsch & Maniatis, 1989). Restriction enzymes and appropriate buffers from New England Biolabs were used. DNA fragments were ligated using the Rapid DNA Ligation kit (Roche) as directed by the supplier's instructions.

2.2.4 Agarose gel electrophoresis

DNA was analysed by agarose gel electrophoresis as described by Sambrook *et al.* (1989).

2.2.5 Purification of DNA from agarose gels

DNA was extracted from agarose gels using the QIAEX II gel purification kit (Qiagen) in accordance with the manufacturer's instructions.

2.2.6 Polymerase chain reaction

The polymerase chain reaction (PCR) (Saiki *et al.*, 1985) was performed in an Eppendorf Mastercycler Personal thermocycler. Pwo polymerase (Roche) was used during cloning procedures. Taq polymerase (Roche) was used for the PCR screening of recombinant clones. PCR reactions used to amplify sequences for subsequent cloning procedures comprised 1µl DNA polymerase, 2µl template DNA, 5µl x10 Buffer $+Mg^{2+}$, 5µl of each primer at 10mM, 5µl dNTPs at 10mM and 27µl ultrapure water (Sigma). Quantities were halved for 25µl volume screening reactions. The primers used through this project are detailed in Table 2.3.

Standard PCR reactions consisted of; 95°C for 2 minutes, followed by thirty cycles of 95°C for 30 seconds, 55°C for 40 seconds and 72°C for 40seconds. Finally, 72°C was held for seven minutes in order to ensure complete elongation of all PCR products.

Table 2.3 List of primers used in this project with their uses. Restriction sites are underlined. Positions of primers used to amplify regions of the Atl gene are from the published sequence of Oshida *et al.*, 1995, GenBank accession number D17366. Positions of primers for the cloning of P68 Lys16 are taken from GenBank accession number AF513033.

Primer	Sequence 5' – 3'	Use
KP4	GCTCGAGTTTTACAGCTGTTTTTGGTTG	Cloning. Reverse from R2 of Atl: pos. 4300
KP9	CCCATGGCTTATACTGTTACTAAACC	Cloning. Forward from R3 of Atl: pos. 4301
KP10	CCTCGAGTTTATATTGTGGGATGTCGAAG	Cloning. Reverse from Atl GL: pos. 5743
KP11	GGCCATGGGTTGGAAAACAAAC	Cloning. Forward from lysostaphin targeting.
KP12	GTGCAGTCTGCAGCTTTATAGTTCCCCAAAGAAC	Cloning. Reverse LssT overlaps KP13.
KP13	CTATAAAGCTGCAGACTGCACCAACTGCTGTG	Cloning. Forward GL' overlaps KP12: pos. 4769
KP14	CCATGGCAACAACTACCCCTACTACACC	Cloning. Forward from R1 of Atl: pos. 3238
KP15	GCCATGGGTACTGCACCAACTGCTGTG	Cloning. Forward from Atl GL': pos. 4769
KP16	GCTCGAGGGAGCCCGGGTACCGGTAG	Cloning. Forward from GFP.
KP17	GCATATTTAGCTGTACCTGCTGC	Sequencing of R123GL: pos. 4237
KP18	GTGTACCAGGTAAGTGGACAGATGC	Sequencing of R123GL: pos. 5107
KP19	CCTCGAGAGCAGTTGGTGCAGTTAAATC	Cloning. Reverse from R3 of Atl: pos. 4884
KP20	GGCATATGGCTTCAGCACAACCAAG	Cloning. Forward from Atl amidase: pos. 2570
PTB10	GCTCGAGTGAGAACACCCCCCAAGG	Cloning. Reverse from P68 Lys16: pos. 11647
PTB12	GGCCATGGCGAAATCACAACAACAAGC	Cloning. Forward from P68 Lys16: pos. 12394
PTB20	TGCTCGAGTTTGTAGAGCTCATCCATGCC	Cloning. Reverse from GFP.
T7 promoter	TAATACGACTCACTATAGGG	Sequencing from pET & pGEMT vectors.
T7 terminator	CTAGTTATTGCTCAGCGGTGG	Sequencing from pET vectors.
SP6 promoter	ATTTAGGTGACACTATAG	Sequencing from pGEMT vectors.

2.2.7 DNA sequencing

Samples of isolated plasmid DNA were sent to GeneService Ltd (Cambridge) for sequence analysis.

2.2.8 DNA sequence analysis

Lasergene DNAStar software was used to analyse sequencing trace data. DNA sequences in the published literature were found and compared using the NCBI BLAST server (www.ncbi.nlm.nih.gov).

2.3 TRANSFORMATION OF BACTERIA

2.3.1 Transformation of chemically competent *E. coli* DH5α

Aliquots of *E. coli* DH5α (Invitrogen) were made, and transformed as described by the manufacturer's protocol.

2.3.2 Preparation of calcium chloride competent *E. coli*

A 50ml culture of *E. coli* cells was grown in LB broth to an OD of 0.5 at 600nm and placed on ice for 10 minutes. Cells were collected by centrifugation at 2700g for 10 minutes at 4°C and resuspended in 10ml of ice cold 100mM $CaCl_2$, 20% (v/v) glycerol. Cells were left on ice for 15 minutes before pelleting as above. Cells were resuspended in 2ml of 100mM $CaCl_2$, 20% (v/v) glycerol, left on ice for two hours, divided into 100µl aliquots and stored at -80°C.

2.3.3 Transformation of calcium chloride competent *E. coli*

Approximately 50ng of DNA was added to 100µl of cells and incubated on ice for 30 minutes. The transformed cells were then heat shocked at 42°C

for 45 seconds, placed on ice for two minutes, and then incubated for 40 minutes at 37°C after the addition of 400µl of LB broth. 150µl of cells were then plated out onto selective plates.

2.4 CLONING PROCEDURES

2.4.1 Cloning into pGEMT-Easy

Purified PCR products were incubated at 72°C for one hour with 2µl Taq polymerase, 10µl PCR buffer, 10µl 2mM dATP and made up to 100µl with ultrapure water to introduce A' overhangs, based on the protocol given for the pGEMT vector (Promega). These were then ligated into pGEMT (Promega) as shown in Figure 2.1.according to the manufacturer's directions. Recombinant clones were identified as white colonies when grown on plates containing 40µg ml^{-1} X-Gal, due to the disruption of the β-galactosidase gene by insert DNA.

2.4.2 Cloning into pET21 vectors

Plasmid DNA isolated from pGEMT-Easy clones was digested with the appropriate restriction enzymes. Purified DNA fragments were ligated into pET21 vectors previously cut with the same restriction enzymes.

2.4.3 Screening of recombinant clones

A single colony of *E. coli* DH5α transformed with the relevant plasmid was used to inoculate 5ml of LB broth supplemented with 100µg ml^{-1} ampicillin and incubated at 37°C. Once the culture was turbid a 2µl sample was used as a template for PCR screening reactions as described in method 2.2.6.

2.5 EXPRESSION AND ANALYSIS OF RECOMBINANT PROTEINS

2.5.1 Small scale protein expression screening

A single colony of *E. coli* BL21 transformed with the relevant plasmid was used to inoculate 5ml of 2YT broth supplemented with ampicillin. At an $OD_{600\ nm}$ of 0.5, IPTG was added to a final concentration of 1.5mM. Following two hours incubation at 37°C with orbital shaking at 200rpm, the culture was analysed by SDS-PAGE for the presence of the over-expressed protein.

2.5.2 Large scale over-expression of recombinant proteins

500ml of 2YT broth was inoculated with a 5ml culture of *E. coli* BL21, transformed with the relevant plasmid, grown to an $OD_{600\ nm}$ of 0.25. Cultures were incubated at 37°C with orbital shaking at 200rpm until an $OD_{600\ nm}$ of 0.5 was reached after which they were moved from 37°C to 30°C. After 30 minutes incubation IPTG was added to a final concentration of 1mM and the culture incubated at 30°C with shaking at 200rpm for two hours. Cell pellets were harvested by centrifugation at 8000g for 10 minutes at 4°C in a Beckman Avanti J20I centrifuge.

2.5.3 SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were run using an Atto mini-gel system. Gels were poured with a 6% stacking layer and a 12% or 16% resolving gel, and run as described by Sambrook *et al.*, (1989). Protein samples were mixed with an equal volume of 2x protein loading buffer (50mM Tris-HCl pH6.8, 100mM dithiothreitol (DTT), 2% (w/v) lauryl sulphate, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol) denatured at 100°C for one minute, cooled and loaded into the gel. Protein molecular weight markers (New England Biolabs) were loaded alongside samples. SDS-PAGE gels were stained with Coomassie Blue stain (Coomassie Blue 1.25g/l, 50% (v/v) methanol, 10% (v/v) acetic

acid in distilled H_2O) for one hour and de-stained, initially in rapid de-stain (40% (v/v) methanol, 10% (v/v) acetic acid in distilled H_2O) for 30 minutes, and then in slow de-stain (10% (v/v) methanol, 7.5-10% (v/v) acetic acid in distilled H_2O) until the background stain was minimal, replacing the de-stain as necessary.

2.5.4 Purification of recombinant proteins by nickel-chelate chromatography

The purification of recombinant proteins by nickel-chelate chromatography was carried out by an adaptation of the protocol of Bardelang (Bardelang, 2002). Cell pellets from 500ml cultures collected as described above were gently resuspended in 30 ml cold Nic buffer (20mM imidazole pH 7.0, 50mM NaCl, 10% (v/v) glycerol, 1mM PMSF). Cells were lysed by three passages through a French Press (SIM-Aminco) on high ratio at 9000Psi, and the cell lysate was spun twice at 25,000 g, at 4°C in a Sigma 3k30 centrifuge for 30 minutes to remove cell debris and precipitated protein. A 5 ml HiTRAP affinity column (Amersham Biosciences) was used for nickel-chelate chromatography. The column was charged with a 12ml injection of 50mM $NiSO_4$, and equilibrated in 15 ml buffer B (25mM NaH_2PO_4 pH 7.0, 0.5 M NaCl, 5% (v/v) glycerol, 1 M imidazole) and 25 ml buffer A (25mM NaH_2PO_4 pH 7.0, 0.5M NaCl, 5% (v/v) glycerol, 0.2mM imidazole). NaCl and Triton X100 were added to the cleared lysate to final concentrations of 0.5 M and 0.1% (v/v), respectively, and the solution applied to the column at a flow-rate of 1 ml min^{-1}. Once loaded the column was washed with 20 ml of a 95% buffer A: 5% buffer B mix to elute all unbound proteins from the column. Bound protein was eluted from the column using a linear gradient of 5-85% buffer B in a total volume of 50 ml. The flow through was monitored using a UV absorbance meter. 2ml fractions eluted from the column were collected and stored on ice. Fractions eluted over the peak UV absorbance were analysed by SDS-PAGE to confirm protein size, pooled, and then transferred to BioDesign Dialysis Tubing (MWCO 8 kDa) and dialysed

overnight against 2L of buffer A supplemented with 0.05% (v/v) Triton X100. Proteins were then aliquoted and stored at -80°C.

2.5.5 Purification of recombinant proteins by pH based denaturing protocol

Cell pellets from 500ml cultures that were grown and induced to over-express recombinant proteins, as described in method 2.5.2, were gently resuspended in 10 ml Buffer B (8M urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, pH8.0) and incubated at room temperature for 30 minutes. Cell suspensions were kept on ice and disrupted by sonication using a Sanyo MSE Soniprep-150 at amplitude of 8 microns for 40 cycles of 20 seconds on and 30 seconds off. Where otherwise indicated, cells were lysed using the French Press as described in section 2.5.4. Cell debris was removed by two forty minute centrifugations at 25,000g, at 4°C using a Sigma 3k30 centrifuge. A 5ml HiTRAP affinity column (Amersham Biosciences) was charged with a 12 ml injection of 50mM $NiSO_4$, then equilibrated in 23ml Buffer C (8M urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, pH6.3). The soluble protein in Buffer B was loaded onto the column at a flow-rate of 1 ml min^{-1}, and the column was washed with 25ml of Buffer C. Protein was eluted with Buffer E (8M urea, 0.1M NaH_2PO_4, 0.01M Tris-HCl, pH4.5) and 2ml fractions were collected and stored on ice. The flow through was monitored using a UV absorbance meter. Fractions eluted over the peak UV absorbance were analysed by SDS-PAGE, and fractions containing the recombinant protein were pooled and transferred to BioDesign Dialysis Tubing (MWCO 8 kDa).

Proteins were re-natured by sequential dialysis steps at 4°C;

(1) Against 2L of 100mM NaH_2PO_4 (pH7), 1mM EDTA, 4M urea, 5% sucrose, 100mM NaCl, 100mM KCl, 100mM L-arginine, 4mM $MgCl_2$, for 48 hours without stirring.

(2) Against 2L of the buffer in step 1 diluted 1:1 with 100mM NaH_2PO_4 (pH7) for 24 hours without stirring.

(3) Against 2L of the buffer in step 1 diluted 1:1 with 100mM NaH_2PO_4 (pH7) for 12 hours without stirring.

(4) Against 2L of 100mM NaH_2PO_4 (pH7) for 48 hours with very slow stirring.

Insoluble precipitated protein was removed by centrifugation. The resulting soluble protein solution was aliquoted and stored at -80°C.

2.5.6 Determination of protein concentrations

Absorbance of protein samples at a wavelength of 280nm was measured using a NanoDrop ND1000 spectrophotometer. Protein concentrations were then calculated using the Beer - Lambert law:

$$Abs_{280} = \varepsilon \times \text{path length} \times \text{concentration}.$$

Equation 2.1 Beer - Lambert Law

Where path length =1:

$$\varepsilon = \frac{[(\text{number of tryptophan residues} \times 5690) + (\text{number of tyrosine residues} \times 1280) + (\text{number of cysteine residues} \times 120)]}{\text{molecular mass of protein}}$$

Equation 2.2 Calculation of molar extinction coefficients (ε)

Table 2.4 Molar extinction coefficients of proteins used in this study

Protein	**Molecular Mass (Da)**	**Number of tryptophans**	**Number of tyrosines**	**Number of cysteines**	**Molar extinction coefficient (cm -1 /mg ml -1)**
P68 Lys16	29651	10	23	5	2.93
Atl GL	54764	9	32	0	1.68
GL'	37538	6	24	0	1.73
LssT: GL	48145	10	29	0	1.95
GL: GFP	82635	10	42	2	1.34
GL': GFP	83590	7	34	2	1.00
R123GL	92700	15	51	0	1.62
Atl ami	64267	9	41	0	1.61
Atl R12	39150	6	19	0	1.49
Atl R3	18938	3	8	0	1.44

2.6 ASSAYS OF ANTIMICROBIAL ACTIVITY

2.6.1 The stab test

Single colonies of *E. coli* ER2566 or BL21 carrying the relevant plasmid were stab inoculated onto LB agar plates and incubated overnight at 37°C. 5ml LB broths were inoculated with the indicator organisms and incubated overnight at 37°C. Following incubation, the agar plates with lids removed were placed face down onto paper towel saturated with chloroform for 2 minutes within a fume hood. The plates were then placed face up under a Bunsen flame for 10 minutes to allow the residual chloroform to evaporate. 5ml of 0.7% (w/v) agar at 50°C was inoculated with 50µl of overnight culture of the indicator organism, gently mixed and poured over the surface of the agar plate whilst molten. The overlays were left to set before the plates were incubated overnight at 37°C. Stab tests were carried out in triplicate and negative controls were included by the inclusion of a stab of *E. coli* ER2566 or BL21 containing pET21a or pET21d without any insert.

2.6.2 Agar diffusion assay

5ml of 0.7% agar at 50°C was inoculated with 50µl of overnight culture of indicator organism, gently mixed, poured onto an LB agar plate and left to set. Antimicrobial proteins were diluted to a range of concentrations in buffer (25mM NaH_2PO_4 (pH7), 0.5M NaCl, 5% (v/v) glycerol) and 5µl of each dilution was dropped onto the surface of the agar plates. Buffer alone was also dropped onto plates as a negative control. Plates were left for 10 minutes, and then incubated at 37°C overnight.

2.6.3 Turbidity assay

The turbidity assay was based on that described by Kessler *et al.* (Kessler *et al.*, 1993). Cultures of indicator organisms were grown in LB broth to an OD_{600nm} of 0.6. Cells were collected by centrifugation at 8000g, for 8 minutes at 5°C in a Sigma 3K30 centrifuge. Cell pellets were washed with 25ml of ice cold 10mM Tris-HCl (pH7) followed by two washes with 25ml deionised water. The final cell pellet was resuspended in 5ml deionised water, frozen at –80°C and lyophilised overnight. Cells were resuspended in 0.5 ml of 25mM sodium phosphate (pH7) and 50µl aliquots were stored at -80°C.

Turbidity assay experiments were carried out in a Wallac Victor^2 1420 multi-channel counter. The quantity of lyophilised cell substrate, typically 5-10µl, required to reach an OD_{600nm} of ~0.4 in a total volume 200µl was determined, and this quantity was then aliquoted across the wells of a standard 96 well plate. Antimicrobial peptides were diluted in buffer (25mM NaH_2PO_4 (pH7), 0.5M NaCl, 5% (v/v) glycerol) to the concentrations of interest, allowing for the volume of cell substrate used. Buffer alone was added as a negative control. Peptides were added to cell substrate across all wells simultaneously using a multichannel pipette and immediately placed in the Victor^2 counter. The OD_{600nm} was measured at approximately 35 second intervals for 20-60 minutes, with shaking between each measurement. Triplicate measurements were made in all assays of peptide activity. All assays were performed twice, and plots shown are a representative plot from one of the assay runs.

2.7 PROTEIN BINDING STUDIES

2.7.1 Protection turbidity assay

The protection turbidity assay was based on the turbidity assay described above. It was used to investigate the binding activity of a protein when

through its effect on the known lytic activity of a similar protein, when both proteins were added to cell substrate together. To identify binding activity of a protein, it would be added to a final concentration tenfold higher than the chosen lytic protein, or the highest possible excess where this was lower. These were added to lyophilised cells and OD_{600nm} was monitored as described above.

2.7.2 Microscopic analysis of GFP tagged protein binding

Overnight cultures of the bacteria of interest were diluted in water to a dilution providing an even distribution of separate cells when viewed under the microscope. 10µl of cell suspension was mixed with 10µl of suitably diluted GFP tagged protein on a standard glass slide and covered with a cover slip. Cells were then viewed by light microscopy and under u.v. light at a magnification of x100 using a labophot-2 (Nikon) microscope. Images were taken of the same field of view under both conditions.

2.7.3 Larger scale analysis of GFP tagged protein binding

250µl of the overnight culture of interest was mixed with 250µl of GFP tagged protein, and incubated for 5 minutes at 37°C with gentle shaking. Cell pellets were collected by centrifugation at 13200rpm in a bench top microfuge (Eppendorf Centrifuge S415D), washed with 250µl water and viewed over a u.v. transilluminator (UVP) for evidence of GFP bound to cells.

3. INVESTIGATING A PUTATIVE AMIDASE FROM THE *S. AUREUS* BACTERIOPHAGE P68

3.1 INTRODUCTION

3.1.1 The P68 bacteriophage

The P68 bacteriophage is a lytic, double stranded DNA phage of *S. aureus*. Its 18,227 base pair nucleotide sequence has been published, GenBank accession number AF513033, and P68 has been classified as belonging to the order *Caudovirales*, family *Podoviridae*, genus 'Φ29-like phages' (Vybiral *et al.*, 2003). The genome of P68 contains 22 open reading frames, two of which encode proteins with lytic activity against *S. aureus*. Protein 17 is a virion associated murein hydrolase, thought to cause a localised rupture of the cell wall to facilitate penetration of the phage DNA at the time of infection. However, when subjected to the addition of purified protein 17, or of a high multiplicity of infection with P68, *S. aureus* cells undergo premature lysis (Takáč & Blasi, 2005a). The second lytic protein, Lys16, is responsible for host lysis following viral replication to release viral progeny, and is believed to have D-alanyl-glycyl endopeptidase, and possible *N*-acetylmuramoyl-L –alanine amidase activity. The initial description of Lys16 as a putative amidase from the genome sequence of P68 (Vybiral *et al.*, 2003) led to its identification as a potential anti-staphylococcal agent.

3.1.2 Structure of Lys16 of the P68 bacteriophage

The *lys16* gene encodes a 250 amino acid protein, with a predicted molecular mass of 28.5kDa and an isoelectric point of 8.6 (Vybiral *et al.*, 2003). The sequence of P68 *lys16* displays similarity to the endolysins of the *S. aureus* phages Twort and φ11 (Takáč *et al.*, 2005b). These endolysins have a modular structure comprising an N-terminal D-alanyl-glycyl endopeptidase, a central *N*-acetylmuramoyl-L-alanine amidase and a C-terminal SH3b cell-wall targeting domain (Loessner *et al.*, 1998; Navarre

et al., 1999b). The N-terminal 112 amino acids of Lys16 show 43% and 57% identity with the D-alanyl-glycyl endopeptidase domains of the φ11 and Twort endolysins, respectively, whilst the C-terminus shows similarity to each endolysin's cell-wall targeting domains. However, the central *N*-acetylmuramoyl-L –alanine amidase domain is missing in P68 Lys16 (Takáč *et al.*, 2005b). Double stranded DNA phages typically use a holin-endolysin system to cause lysis of their host cell (Takáč *et al.*, 2005b). Holins insert into the cytoplasmic membrane and cause localised disruption of the cell wall, regulating access of the endolysins from the cytoplasm to the peptidoglycan where they may cause cell lysis. Two predicted holin genes have been identified within the sequence of P68. *Hol12* is located downstream of *lys16,* and encodes a class II holin, having two predicted transmembrane domains (Takáč *et al.*, 2005b). A second holin gene, *hol15* lies embedded in the -1 reading frame at the 3' end of *lys16,* and the predicted protein has three transmembrane domains, characteristic of a class I holin. Evidence suggests that *hol15* has a dual start motif which can lead to either the synthesis of an anti-holin or the lysis-effector (Takáč *et al.*, 2005b).

3.1.3 Activity of Lys16 of the P68 bacteriophage

Muralytic activity of Lys16 has been demonstrated by zymogram against autoclaved *S. aureus* 68 and *E. coli* TOP10 cells (Takáč *et al.*, 2005b). Antimicrobial activity has also been shown by cell lysates of *E. coli* MC4100F' expressing the Lys16 protein against growing cultures of *S. aureus* clinical isolates. Twelve out of fifteen isolates were lysed following addition of Lys16 containing cell lysate, indicating that there is potentially a wide host spectrum that Lys16 could be used against (Takáč *et al.*, 2005b). Tests against identified *S. aureus* strains have not been reported. The similarity of Lys16 to the endolysins of φ11 and Twort suggests its muralytic activity against *S. aureus* is due to a D-alanyl-glycyl endopeptidase domain. However, this does not explain the observed activity against *E. coli*, which has different peptidoglycan cross-bridges to *S. aureus* and would not be

susceptible. It may therefore be possible that *N*-acetylmuramoyl-L –alanine amidase activity has been retained by Lys16 which could cleave the peptidoglycan backbone at the MurNAc-L-alanine link (Takáč *et al.*, 2005b).

3.1.4 Similar endolysins to Lys16 of the P68 bacteriophage

The activity of the endolysins of the Twort and φ11 bacteriophages has previously been characterised. The endolysin of the Twort phage, belonging to the *Myoviridea* family, has shown lytic activity against a *S. aureus* cell substrate overlaid onto chloroform-lysed *E. coli* JM109 (DE3) carrying a plasmid expressing the endolysin genes (Loessner *et al.*, 1998). Truncated enzyme fragments lacking the cell-wall targeting domain showed greater activity than the full length endolysin. Partially purified protein was used to demonstrate cleavage of staphylococcal cell wall substrate, and provided evidence of *N*-acetylmuramoyl-L-alanine amidase activity (Loessner *et al.*, 1998). The φ11 bacteriophage belongs to the *Siphoviridae* family. Initial studies assayed purified endolysin against cell wall substrate and peptidoglycan to show that it is a bi-functional enzyme with endopeptidase and amidase activity (Navarre *et al.*, 1999b). More recently the recombinant protein has been shown to lyse staphylococcal mastitis pathogens, including *S. aureus* and coagulase-negative staphylococci (Donovan *et al.*, 2006b). Activity of the φ11 endolysin has also been shown against heat killed staphylococci and staphylococcal biofilms, with results indicating that the intact SH3b cell-wall targeting domain is required for lysis of whole cells and that maximum activity is seen when the endopeptidase and amidase domains are combined (Sass *et al.*, 2007).

The LysK endolysin of the staphylococcal bacteriophage K has also been characterised. The phage has a wide host spectrum, and the activity has been demonstrated by zymogram and agar diffusion assay against several staphylococcal species including MRSA, VRSA, *S. epidermidis*, *S. capitis*, *S. caprae* and *Staphylococcus haemolyticus* (O'Flaherty *et al.*, 2005). The protein was obtained from lysates of *Lactococcus lactis* expressing the endolysin. It was not possible to purify the recombinant protein from *E. coli*

as it was found to be located in the insoluble fraction of cell lysate as inclusion bodies.

3.1.5 Aims of the chapter

This chapter describes efforts made to clone Lys16, express the recombinant protein in an *E. coli* host and purify the protein. Assays using cell lysates and purified protein have been used to investigate the extra-cellular activity of Lys16 against *S. aureus*, and a range of other staphylococci and other Gram-positive species. The results of these assays are used to determine the suitability of the candidate peptide Lys16 to be used either as an antimicrobial, or to be incorporated into a chimeric antimicrobial peptide with either the endopeptidase or cell-wall targeting domain of lysostaphin.

3.2 RESULTS

3.2.1 Validation of P68 Lys16 as a candidate peptide

The amino acid sequence of a putative amidase from the sequenced genome of the P68 bacteriophage was BLAST searched to identify homology to other peptides and conserved domains. This revealed an N-terminal CHAP domain and a C-terminal domain showing homology to a SH3b cell-wall targeting domain. The CHAP domain is a cysteine, histidine-dependent amidohydrolase/peptidase, which is often found in proteins with amidase activity, involved in peptidoglycan hydrolysis (Bateman & Rawlings, 2003; Rigden, Jedrzejas & Galperin, 2003). Other sequences were also identified as showing similarity to the putative amidase from the P68 bacteriophage, including the endolysins of the φ11 and Twort bacteriophages, and the SH3b cell-wall targeting domain of lysostaphin. The homologous regions of Lys16 and lysostaphin show 39% identity, making Lys16 a valid candidate peptide for incorporation into a chimeric peptide. The alignment of the homologous regions of Lys16 and recombinant mature lysostaphin is shown in Figure 3.1.

```
Lys16          156    WKRNQYGTYYRNENGTFTCGFLPIFARVGSP
                      WK N+YGT Y++E+ +FT     I  R   P
Lysostaphin    156    WKTNKYGTLYKSESASFTPN-TDIITRTTGP

Lys16          187    PNGYTPYNEVCLSDGYVWGYWFQEPNKLSWG
                        Y+EV   DG+VW    + P   SWG
Lysostaphin    186    AGQTIHYDEVMKQDGHVWSGVLKMPQFRSWG

Lys16          218    IGYNWQ-GTRYYLPVRQWNGKTGNSYSVGIP
                      +GY    G R YLPVR WN  T    ++G+
Lysostaphin    217    VGYTGNSGQRIYLPVRTWNKSTN---TLGVL
```

Figure 3.1 Alignment of homologous regions of Lys16 with lysostaphin

3.2.2 Cloning of P68 *lys16*

Plasmid pUHHOLY1516 (a gift from Marian Takáč, Vienna Biocenter), containing the P68 *lys16* gene was used as template DNA for the amplification of the *lys16* gene by PCR, using primers PTB10 and PTB12 (gifts from Philip Bardelang, University of Nottingham). This amplified a 780bp fragment with an N-terminal *NcoI* restriction site and a C-terminal *XhoI* restriction site, which when cloned into pET21d+ encoded Lys16 with a C-terminal hexahistidine tag. The PCR product was cloned via pGEMT-Easy into pET21d+ in *E. coli* DH5α, and plasmid DNA was isolated according to the methods described in chapter 2, generating the plasmid pKP1. The insert gene in purified pKP1 was sequenced and confirmed that *lys16* was in-frame and that there were no mutations present.

3.2.3 Purification of P68 Lys16

3.2.3.1 *Expression of Lys16 from pKP1*

Purified pKP1 was transformed into *E. coli* BL21, and a small scale expression screen was performed as described in chapter 2. Over-expression of Lys16 was induced with 1mM IPTG, and aliquots of the culture pre- and post-induction were analysed by SDS-PAGE (Figure 3.2). A band at ~28.5kDa corresponded with the expected size of Lys16.

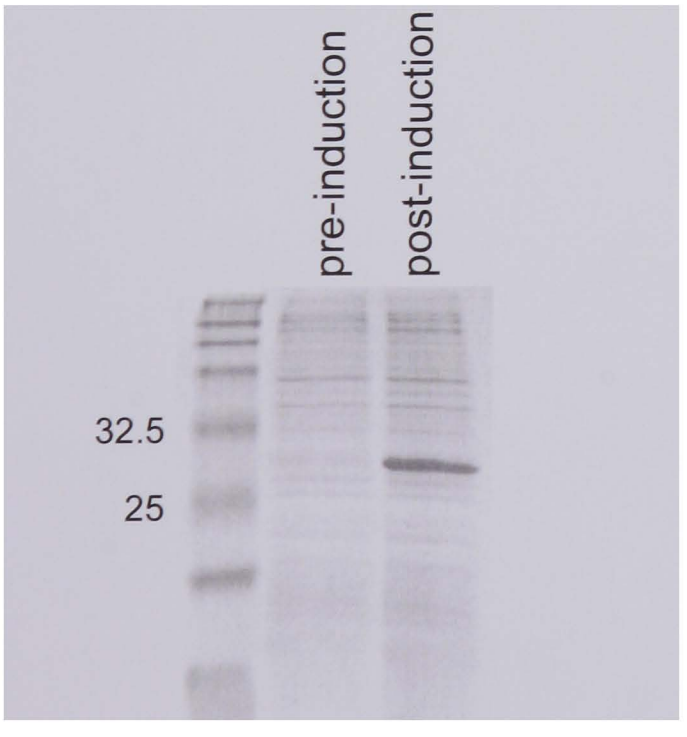


Figure 3.2 12% SDS-PAGE gel showing culture pre- and post-induction of Lys16

3.2.3.2 *Purification of Lys16 by nickel chelate chromatography*

A 500ml culture of *E. coli* BL21 carrying pKP1 was grown and over-expression of Lys16 was induced as described in chapter 2. Nickel chelate chromatography was used to purify Lys16 from the cell pellet. However, a large quantity of protein from the cell pellet precipitated out of solution during cell disruption, and there was no evidence of Lys16 in the fractions eluted by chromatography when analysed by SDS-PAGE (data not shown).

3.2.3.3 *Purification of Lys16 by pH based denaturing protocol*

A second cell pellet of *E. coli* BL21 (pKP1) induced with 1.5mM IPTG was collected and the pH based denaturing protocol (see section 2.5.5) was used to purify Lys16. SDS-PAGE analysis showed a protein of ~28.5kDa was eluted from the column over several fractions (Figure 3.4), of which fractions 7 to 13 were pooled together for the dialysis steps. Figure 3.4 shows the protein eluted in fraction 10. However, during dialysis a significant quantity of Lys16 was observed to precipitate out of solution. Efforts to re-solubilise Lys16 with the addition of glycerol to a final concentration of 5% (w/v) did not succeed in reducing the quantity of precipitated protein. The concentration of the remaining soluble Lys16 was determined as 22.8μM, which was stored at -80°C.

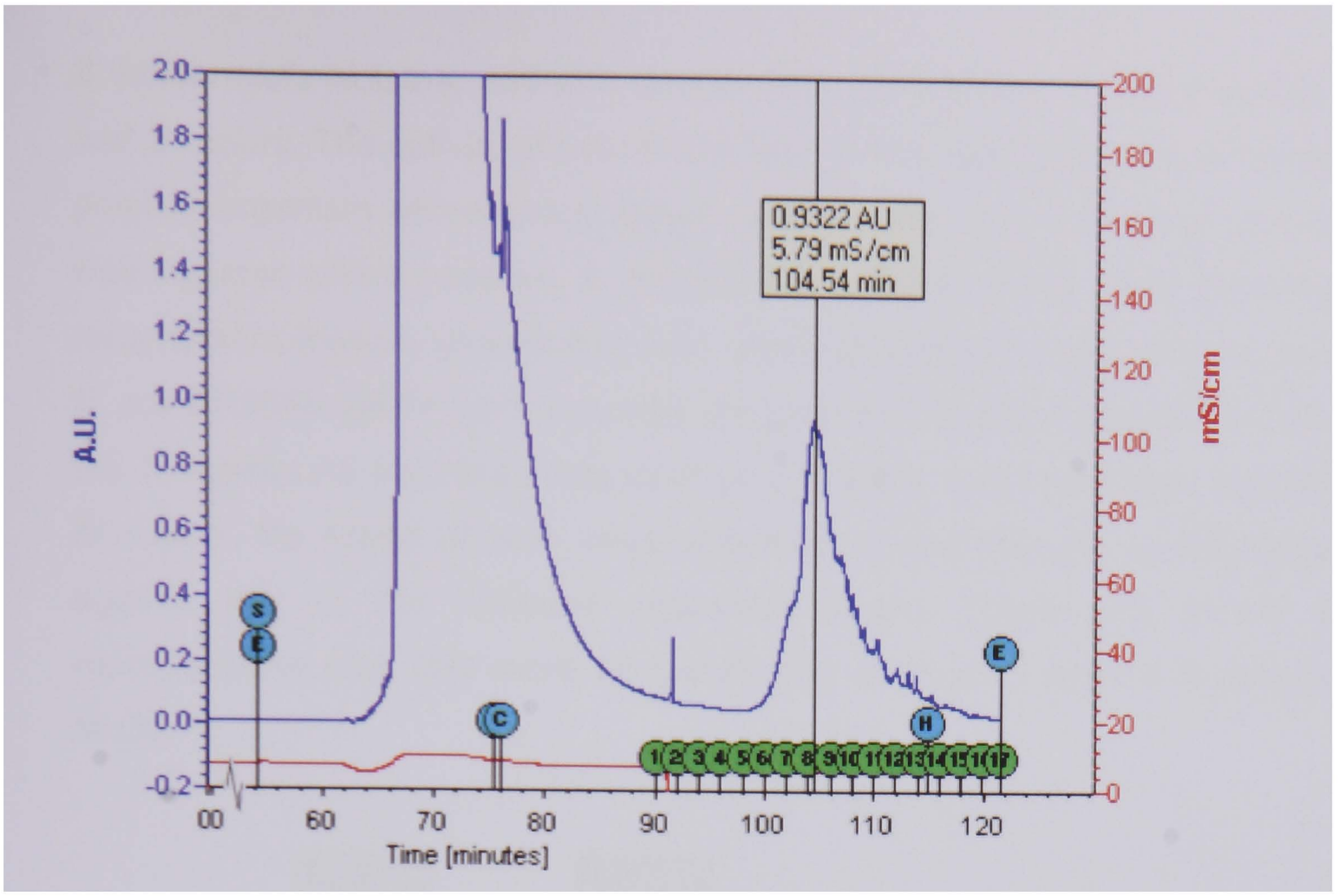


Figure 3.3 Column trace showing loading of cell lysate onto column and elution of protein. Fractions collected are shown in green.

Figure 3.4 16% SDS-PAGE gel of Lys16 contained in fraction 10, eluted by the pH denaturing protocol

3.2.4 Activity of P68 Lys16 in the stab test

The activity of Lys16 was tested in the stab test using pKP1 transformed into both *E. coli* ER2566 and BL21. Lys16 was tested against a range of staphylococcal laboratory strains and clinical isolates; Mu50, five MRSA isolates including EMRSA16 and representatives of SCC*mec* types IA, II, III and IV, the Oxford staphylococcus, two further *S. aureus* isolates, six

S. epidermidis isolates, and one isolate each of *S. capitis*, *S. haemolyticus*, and *S. sciurii*. The activity of Lys16 was also tested against the model Gram positive organism *Micrococcus luteus*. In each stab test a positive control was included where possible. *E. coli* ER2566 carrying pEA3, which encodes recombinant mature lysostaphin, was used for staphylococcal strains, and *E. coli* ER2566 (pKP4), which carries the gene for the glucosaminidase from the *S. aureus* Atl autolysin, was used as a positive control for tests against *M. luteus*. No zones of lysis were observed surrounding the Lys16 stabs against any of the indicator organisms tested. Figure 3.5 shows a representative stab test result for lysostaphin and Lys16 against *S. aureus* Mu50.

Figure 3.5 *E. coli* ER2566 stab tests of lysostaphin and Lys16 vs. *S. aureus* Mu50

3.2.5 Activity of P68 Lys16 in the turbidity assay

Lys16 was tested in the turbidity assay against lyophilised cell substrates of EMRSA16, the Oxford staphylococcus, and clinical *S. aureus* and *S. epidermidis* isolates as described in chapter 2. Lys16 was tested at 5μM and 2μM with lysostaphin included as a positive control. No lytic activity of Lys16 activity was observed against any of the lyophilised cell substrates tested (Figure 3.6). It was not possible to assay Lys16 at higher concentrations to determine weak lytic activity due to the low original concentration of the protein.

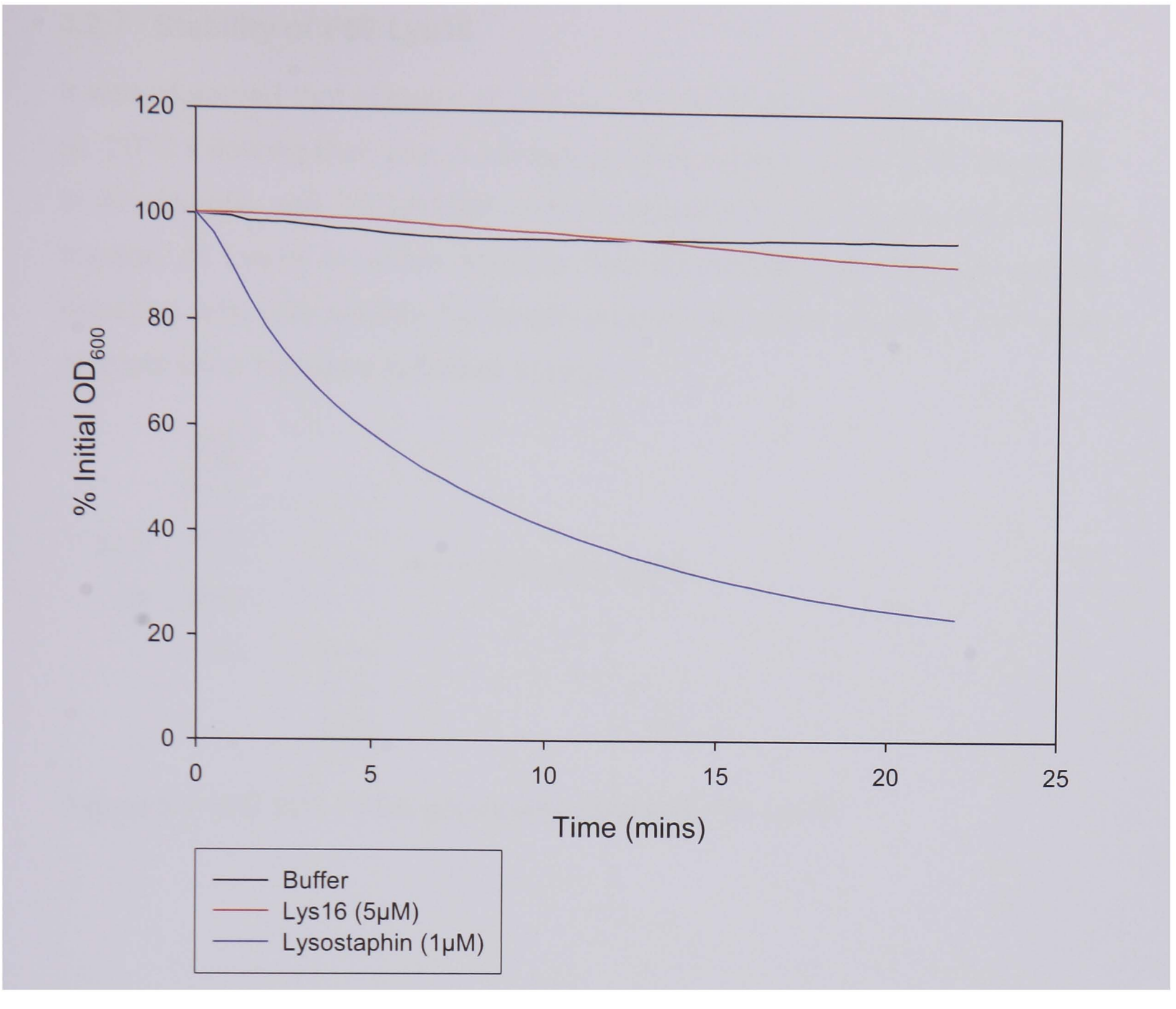


Figure 3.6 Turbidity assay testing activity of Lys16 and lysostaphin vs. EMRSA16

3.2.6 Activity of P68 Lys16 in the agar diffusion assay

The agar diffusion assay was used to assay the activity of Lys16 at higher concentrations than was possible in the turbidity assay. Lys16 was tested against the range of indicator organisms previously used in the stab test. It was dropped onto the plate in twofold serial dilutions; at 22.8μM, 11.4μM, 5.7μM and 2.85μM. Lysostaphin was used as a positive control. Plates were incubated overnight at 30°C and 37°C. No zones of lysis were observed in the lawns of any bacteria, at all dilutions, and at both temperatures, except by the lysostaphin controls (data not shown).

3.2.7 Stability of P68 Lys16

It was observed that aliquots of P68 Lys16 rapidly deteriorated when stored at -20°C following their use in assays. A white protein precipitate was visible in all aliquots, and SDS-PAGE analysis showed that the remaining soluble fraction of Lys16 in some aliquots had fragmented into several smaller peptides with little soluble full length protein remaining (Figure 3.7). These aliquots were not used in further assays.

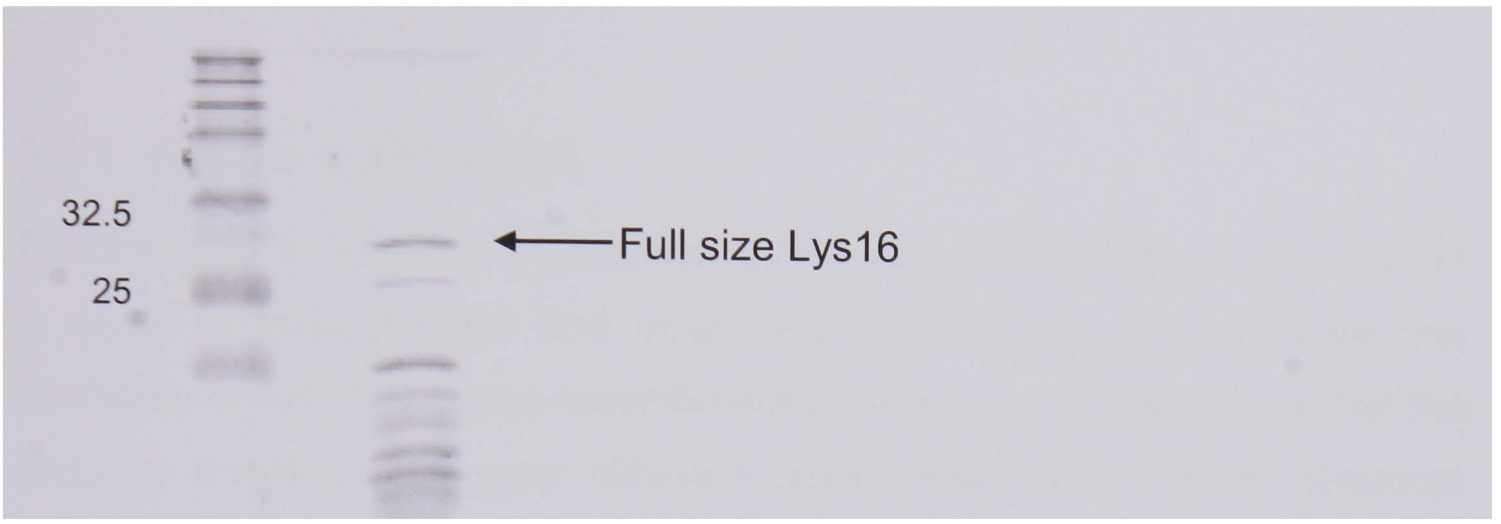


Figure 3.7 16% SDS-PAGE gel showing degraded P68 Lys16

3.3 DISCUSSION

This study has described the cloning, and for the first time, purification of the Lys16 endolysin of the P68 bacteriophage. A range of assays have been used to test the activity of Lys16 against a range of Gram positive organisms. The results of this study are compared and contrasted with those of related studies.

3.3.1 Activity of P68 Lys16

Following its cloning and purification, Lys16 was assayed for activity against a range of staphylococci and other Gram positive organisms by several methods. No activity of the recombinant protein was observed by either the turbidity assay or the agar diffusion assay when tested at a maximum concentration of 22.8μM. The stab test was also used to investigate the activity of Lys16 released by lysed *E. coli* cells expressing the recombinant protein. Again, no activity of Lys16 was observed. These results indicate that either the recombinant protein is itself inactive, that Lys16 is not active when used extracellularly, or that the recombinant protein is incorrectly folded or rapidly degraded following purification.

3.3.2 Comparison to results of other studies of P68 Lys16

3.3.2.1 *Zymogram assays*

A separate study into the functional analysis of the lysis genes of the P68 phage has been carried out by Takáč *et al.* (2005). The results of the study were published after the work described in this chapter was carried out. The aim of the work described in this chapter differed from the published study by investigating the potential of the endolysin of P68 as an antimicrobial agent. Takáč *et al.* (2005) demonstrated muralytic activity of P68 Lys16 using zymogram assays. Zymogram assays use a heat killed cell substrate incorporated into an SDS-PAGE gel. This is useful for determining the lytic activity of a specific polypeptide band on an SDS-PAGE gel, but is

inappropriate for investigating the antimicrobial potential of a peptide. The study described in this chapter used assays against live cells, providing a more representative model to investigate the antimicrobial activity of the endolysin of the P68 bacteriophage. The muralytic activity seen by zymogram analysis does provide evidence that the selection of Lys16 as a candidate antimicrobial was justified.

3.3.2.2 *Cell lysate assays*

The other assay of lytic activity used by Takáč *et al.* (2005b) involved IPTG induction of a culture of *E. coli* MC4100F' with the plasmid carrying the *lys16* gene, and sonication to produce a cell lysate which could be added to growing cultures of *S. aureus* clinical isolates. Results of this assay are of greater interest in assessing the potential antimicrobial activity of Lys16. In its format this assay is closest to the stab test of the assays used in this chapter, with the added effect of the addition of IPTG to induce over-expression of the *lys16* gene. The stab test described in this chapter relies on the "leaky" expression of plasmid encoded genes that is seen when using *E. coli* ER2566. The positive controls using pEA3 demonstrate that the leaky expression of the lysostaphin gene is sufficient to cause cell lysis in the overlay. Takáč *et al.* (2005b) observed lysis in 12 out of 15 cultures of clinical isolates from the Vienna General Hospital, whilst no lysis was observed in the study described in this chapter using clinical staphylococcal isolates from the Queen's Medical Centre, Nottingham, and against other *S. aureus* strains and Gram positives. The geographical variation of *S. aureus* strains between countries is well documented, especially amongst MRSA, with the incidence of MRSA in Austria being approximately a third of that of the UK, as recorded in 2001 (Enright *et al.*, 2002; Stefani & Varaldo, 2003). This difference could indicate the variation between the staphylococcal populations present in Vienna and Nottingham. Furthermore, as only 12 of 15 clinical isolates from Vienna were lysed with the addition of P68 Lys16 containing cell lysate, this provides further evidence that susceptibility amongst *S. aureus* is incomplete. The clinical application of

Lys16 alone would lead to the selection of resistant organisms, an event that would progress more rapidly if the resistance determinant were transferable.

3.3.2.3 *Purified recombinant Lys16*

The study published by Takáč *et al.* (2005) did not use any assays of purified protein. This chapter describes the purification of Lys16, the problems encountered with using the purified protein, and the lack of an observed activity of the purified recombinant Lys16.

3.3.3 Stability of P68 Lys16

Problems with the solubility of P68 Lys16 were experienced. Two different techniques were used to purify Lys16, and minor modifications to each of these were made in attempts to optimise the protocols. When using nickel-chelate chromatography, maintaining the protein in solution prior to running through the column was difficult, and no protein was found in the fractions eluted from the column. These difficulties were solved using the pH based denaturing method, as described in section 2.5.5, but Lys16 precipitated out of solution whilst removing urea from its buffer by dialysis. Small quantities of Lys16 were purified, but these rapidly deteriorated when removed from storage at -80°C. This suggests that the inactivity of P68 Lys16 observed in the agar diffusion assay and the turbidity assay may be due to the instability of the recombinant protein. Either Lys16 has been incorrectly folded during the purification process, or it is rapidly fragmenting or precipitating out of solution over the course of the assay. Aliquots in which the protein was observed to fragment may have become contaminated by a protease, and were not used in assays of activity. However, protein was observed to precipitate out of solution in all aliquots, demonstrating a separate, independent instability of Lys16.

LysK endolysin of the staphylococcal phage K was also found to be insoluble when over-expressed in *E. coli* (O'Flaherty *et al.*, 2005). The recombinant protein was found to be located in inclusion bodies. This demonstrates that the difficulties encountered with Lys16 are not unique, and that solubility of the recombinant protein is a key issue in identifying candidate endolysins.

3.3.3.1 *The role of cysteine residues in Lys16*

One explanation for the instability of the recombinant Lys16 is that the protein has not been folded correctly. Cysteine residues often play a structural role within proteins, with disulphide bridges forming between two cysteines. The sequence of the recombinant Lys16 contains five cysteine residues. One cysteine residue belongs to the conserved CHAP domain, which confers amidase activity to P68 Lys16. The other cysteines may be involved in the structure of the protein, and the conditions required for the purification of Lys16, and when assaying its activity may be inappropriate for the correct folding of the protein. Reviewing the literature relating to the most similar phage endolysins to P68 Lys16, reveals that the sequence of Lys16 shares three conserved cysteine residues with the Twort protein, and only one with that of φ11. The study published on the Twort endolysin does not include results showing activity of a purified protein against live cells (Loessner *et al.*, 1998). However, recombinant φ11 endolysin has shown lytic activity against live staphylococci (Donovan *et al.*, 2006b; Sass *et al.*, 2007). The absence of the additional cysteine residues in the sequence of the φ11 endolysin may enable its purification in an active form. It may be possible to exploit the antimicrobial potential of Lys16 if a recombinant protein lacking the additional cysteine residues could be designed and purified without the loss of endolysin activity.

3.3.4 The therapeutic potential of P68 Lys16

The results of this study do not support the selection of recombinant P68 Lys16 as an antimicrobial agent. The difficulties in purifying the protein and maintaining it in solution are significant barriers to its practical application, regardless of whether it has extracellular activity against *S. aureus* and other Gram-positive organisms. The results of Takáč *et al.* (2005b), in showing activity against 12 out of 15 clinical isolates, demonstrate that even if these purification problems are resolved, further work will be required to determine the spectrum of susceptible *S. aureus* and understand the pattern of resistance amongst other strains, prior to the promotion of P68 Lys16 as a potential antimicrobial agent. Likewise, Lys16 is not a suitable candidate peptide for the incorporation into a chimeric peptide alongside a domain from lysostaphin.

3.3.4.1 *A role for the P68 bacteriophage in phage therapy?*

Although Lys16 has not been shown to be an appropriate antimicrobial, this does not preclude the use of P68 in phage therapy. Use of the whole bacteriophage would circumvent the problem of instability of the recombinant protein. A study into synonymous codon usage of *S. aureus* bacteriophages suggests that the lysis genes of P68 are likely to be expressed rapidly by the host's translation machinery, and that P68 may be useful in phage therapy (Sau *et al.*, 2005). Incomplete susceptibility amongst *S. aureus* strains would not prevent the use of P68 as part of a "cocktail" of phages.

3.4 SUMMARY

The P68 bacteriophage is a lytic phage of *S. aureus* which causes lysis of its host cell with the endolysin Lys16. This chapter has shown that P68 Lys16 is a poorly soluble and unstable protein. This is possibly due to incorrect protein folding as a result of cysteine residues not interacting in the necessary conformation during protein purification, and under assay conditions. No lytic activity of Lys16 has been observed against staphylococcal clinical isolates, laboratory *S. aureus* strains or other Gram-positive organisms. These results show that Lys16 is not a suitable candidate peptide to be used either as an antimicrobial peptide on its own, or as part of a chimeric peptide with a domain from lysostaphin. If the protein were to be purified and its solubility maintained, further work would be required to determine the spectrum of activity against *S. aureus* strains, and investigate potential resistance mechanisms. The P68 bacteriophage itself may be useful as a phage therapy treatment, where a high level of expression of Lys16 could lead to an effective treatment.

The principle of using a bacteriophage endolysin as an antimicrobial remains attractive. This has been previously demonstrated by studies, including *in vivo* models, of endolysins active against *S. pneumoniae* (Loeffler *et al.*, 2001; Loeffler *et al.*, 2003b) and *B. anthracis* (Schuch *et al.*, 2002; Low *et al.*, 2005; Yoong *et al.*, 2006). As more phages of *S. aureus* are isolated in the future, and their sequences determined, it may be possible to predict the solubility and stability of their endolysins, enabling the selection of active and stable candidate antimicrobials.

4. ASSESSING THE POTENTIAL OF STAPHYLOCOCCAL AUTOLYSINS AS ANTIMICROBIALS

4.1 INTRODUCTION

4.1.1 The autolysins of *S. aureus*

As previously discussed, bacteria use autolysins to cause localised cell wall hydrolysis as part of the cell cycle. Other proteins identified as autolysins also seem to play a role in pathogenicity and adhesion. *S. aureus* produces several known autolysins including Atl, Aaa, LytM and Sle1 (Oshida *et al.*, 1995; Heilmann *et al.*, 2005; Ramadurai *et al.*, 1997; Kajimura *et al.*, 2005). Autolysins are tightly regulated *in vivo*, as the uncontrolled expression of these proteins could prove fatal to the cell. Producing a recombinant autolysin which could be added at externally at high concentration provides a potential source of novel antimicrobial agents.

4.1.2 Atl is the major autolysin of *S. aureus*

The predominant peptidoglycan hydrolase of *S. aureus* is Atl (Oshida *et al.*, 1995; Foster, 1995). The Atl autolysins have been shown to localize at the septal ring, indicating its involvement in the cell division process (Yamada *et al.*, 1996). The characterisation of *atl* null mutants has shown inhibition of cell-cell separation, causing cells to form large clusters, and sediment out if grown in liquid media overnight without shaking (Sugai *et al.*, 1995; Takahashi *et al.*, 2002; Biswas *et al.*, 2006).

4.1.3 The structure of Atl

Atl is a bifunctional modular enzyme. The *atl* open reading frame is 3768bp, encoding a 1256 amino acid protein of 137kDa (Oshida *et al.*, 1995; Foster, 1995). The *atl* gene product is arranged with an N-terminal signal sequence,

pro-peptide and amidase domain, three central homologous repeat regions and a C-terminal glucosaminidase. An N-terminal signal sequence exports pro-Atl to the cell surface. The pro-peptide is proteolytically cleaved from the amidase, and a further cleavage separates the amidase from the glucosaminidase (Figure 4.1) (Baba *et al.*, 1998b). The mature amidase is a 62kDa peptide which contains repeats one and two at the C-terminus. The glucosaminidase is 51kDa and retains repeat three at its N-terminus (Oshida *et al.*, 1995).

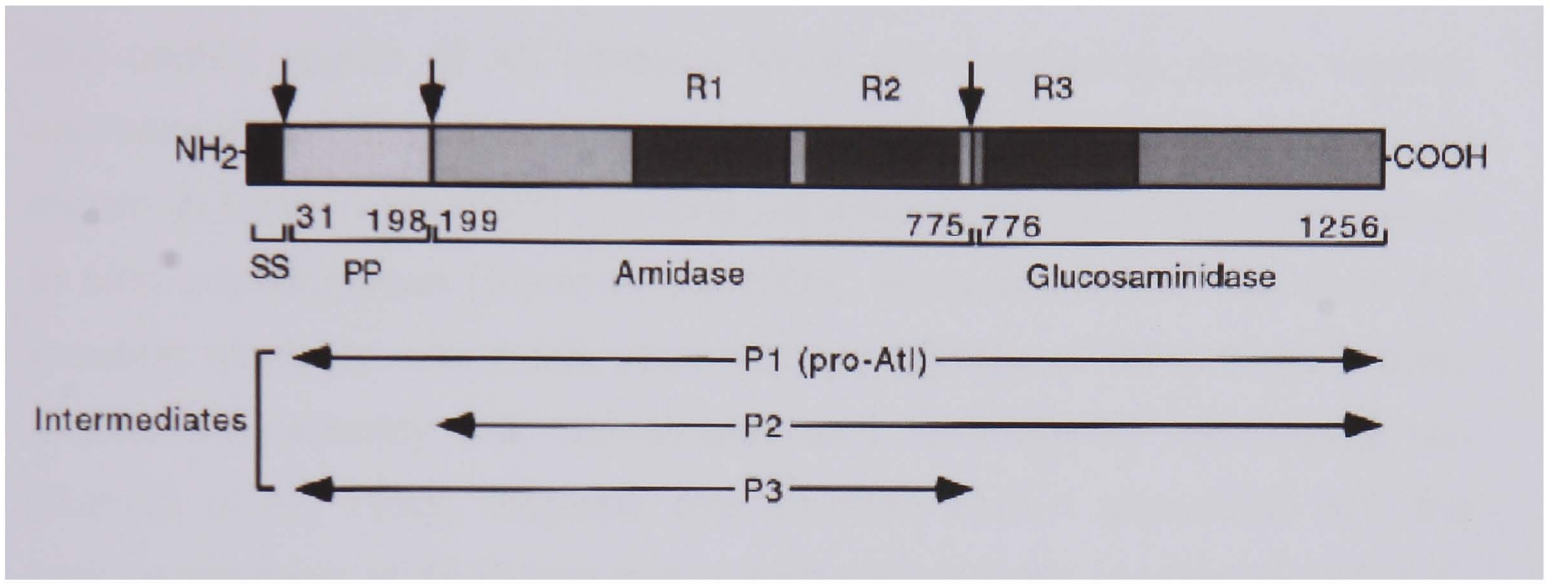


Figure 4.1 Processing of Atl (from Baba & Schneewind, 1998): The Atl precursor is exported by an N-terminal signal sequence (SS) to leave intermediate P1 (pro-Atl). Proteolytic cleavage at residues 198 and 775 generate the intermediates P2 and P3, as well as the mature pro-peptide, amidase and glucosaminidase. The repeat domains (R1, R2 and R3) are shown as dark segments. Amino acid positions are relative to the first methionine of Atl.

4.1.4 Activity of the Atl amidase and glucosaminidase

The muralytic activity of the amidase and glucosaminidase of Atl has been assayed by zymogram analysis and a turbidity assay using heat killed cells against *M. luteus* and *S. aureus* (Oshida *et al.*, 1995; Sugai *et al.*, 1995). Both techniques have shown similar results. The amidase has shown moderate lytic activity against *S. aureus* and relatively low activity against *M. luteus*. The glucosaminidase has shown a high degree of activity against *M. luteus*, but no observed activity against *S. aureus* activity. It is hypothesised that the inactivity against *S. aureus* reflects a regulation mechanism, and that the glucosaminidase targets altered peptidoglycan at the septal ring, preventing uncontrolled lysis of the cell (Sugai *et al.*, 1995). If the high activity seen against *M. luteus* could be more effectively targeted

to staphylococcal peptidoglycan the glucosaminidase could be an attractive prospect as a candidate antimicrobial peptide. The modular structure of the glucosaminidase presents the opportunity to substitute its targeting domain with that of lysostaphin with the aim of designing a highly active, targeted chimeric anti-staphylococcal agent.

4.1.5 The role of the repeat regions of Atl

The central region of Atl contains three GW containing repeat regions, approximately 150 amino acids in length. These repeat sequence regions known as LysM (lysin motif) domains are widespread and have been shown to bind peptidoglycan (Steen *et al.*, 2003). Repeats one and two show the greatest similarity with each other with an identity of 76%. Repeat three shares 34% identity with repeat one, and 35% identity with repeat two (Oshida *et al.*, 1995). Repeats one and two remain associated with the mature amidase at its C-terminus, whilst repeat three is situated at the N-terminus of the glucosaminidase. The difference in the sequence of repeat three from the other repeat regions perhaps provides an explanation for the difference in activity between the amidase and glucosaminidase seen in the bacteriolytic assays. The repeat regions have been shown to be necessary and sufficient for cell binding to *S. aureus*, and there are approximately 10^8 receptors on the cell surface, predominantly at the septal region (Baba *et al.*, 1998b). The precise nature of the cell surface receptor has not yet been determined, although studies on the closely related AtlE of *S. epidermidis* appear to rule out teichoic acid and the *O*-acetylation of peptidoglycan (Biswas *et al.*, 2006).

4.1.6 Effect of antibiotics on autolytic activity of Atl

Several studies have investigated the effect of antibiotics on the *in vivo* autolytic activity of Atl. It has been shown that cell lysis following treatment of *S. aureus* with penicillin G is due to the activity of Atl at the septal region, where the synthesis of new peptidoglycan is inhibited (Sugai *et al.*, 1997b).

Autolysis also increases with exposure of the cells to oxacillin, whilst MRSA strains show no increase in autolysis (Ledala, Wilkinson & Jayaswal, 2006). This indicates a synergistic effect of Atl with cell wall synthesis inhibiting antibiotics, which could be exploited by the use of the amidase or glucosaminidase from Atl as antimicrobial agents.

Glycopeptide-intermediate *S. aureus* (GISA) show reduced expression of *atl* and susceptibility to autolysis. This may contribute to the thickening of the cell walls in these strains, with the thicker walls themselves contributing to the resistance to autolysis (Koehl *et al.*, 2004; Wootton *et al.*, 2005). GISA strains often show reduced susceptibility to lysostaphin (Boyle-Vavra *et al.*, 2001). A recombinant Atl hydrolase could be used to weaken staphylococcal cell walls in tandem with lysostaphin.

S. aureus cells treated with the protein synthesis inhibiting antibiotics tetracycline and chloramphenicol show decreased levels of autolysis (Yamada *et al.*, 2001; Ledala *et al.*, 2006). Tetracycline has been shown to inhibit the localisation of Atl, at high concentrations, to the septal region (Yamada *et al.*, 2001). A recombinant Atl hydrolase lysostaphin's targeting domain may show greater binding to the whole cell surface, and provide an effective novel antimicrobial.

4.1.7 Aims of the chapter

This chapter describes the cloning of the recombinant Atl amidase and glucosaminidase. The activities of these enzymes are investigated using a range of assays. The potential of a chimeric peptide incorporating the lysostaphin targeting domain is explored, and the results are compared with those of the parent peptides. Results of assays of the chimeric peptide lead to a further exploration of the role of the central repeat regions of Atl.

4.2 RESULTS

4.2.1 The glucosaminidase of Atl

The glucosaminidase domain of Atl was investigated first, as the literature suggests that it has a higher degree of activity than the amidase, which could be exploited if effectively targeted to staphylococci.

4.2.1.1 *Cloning of the Atl glucosaminidase*

S. aureus RN450 (a gift from Alan Cockayne, University of Nottingham) was grown in LB broth overnight. Genomic DNA was isolated from this culture as described in chapter 2. This was used as template DNA for the amplification by PCR of the sequence encoding the glucosaminidase of Atl, described in GenBank accession number D17366. Primers KP9 and KP10 were designed to amplify repeat 3 and the glucosaminidase with 5' *Nco*I and 3' *Xho*I restriction sites as shown in Figure 4.2. When the PCR product was cloned into pET21a a hexahistidine tag sequence was added to the C-terminus of the encoded gene product. This 1473bp sequence was cloned via pGEMT-Easy into pET21a using *E coli* DH5α, generating plasmid pKP2. Plasmid DNA was isolated and the correct sequence of Atl confirmed according to the methods described in chapter 2.

Figure 4.2 Organisation of the Atl GL construct, showing primers used and restriction sites introduced.

4.2.1.2 *Purification of the Atl glucosaminidase*

E. coli BL21 cells were transformed with pKP2 and a 500ml culture grown in 2YT broth was induced to over-express the recombinant Atl

glucosaminidase (GL) by the addition of 1mM IPTG. The cell pellet was collected and the GL was purified by nickel-chelate chromatography. Cell pellets had a viscous consistency and required immediate resuspension and overnight storage at 4°C in an addition to the method described in 2.5.4. A protein band of approximately 51kDa was eluted off the column over several fractions which were then subjected to dialysis. SDS-PAGE demonstrated the presence of a highly concentrated GL preparation (Figure 4.3). The concentration of GL was determined as 266μM. Aliquots were stored at -80°C.

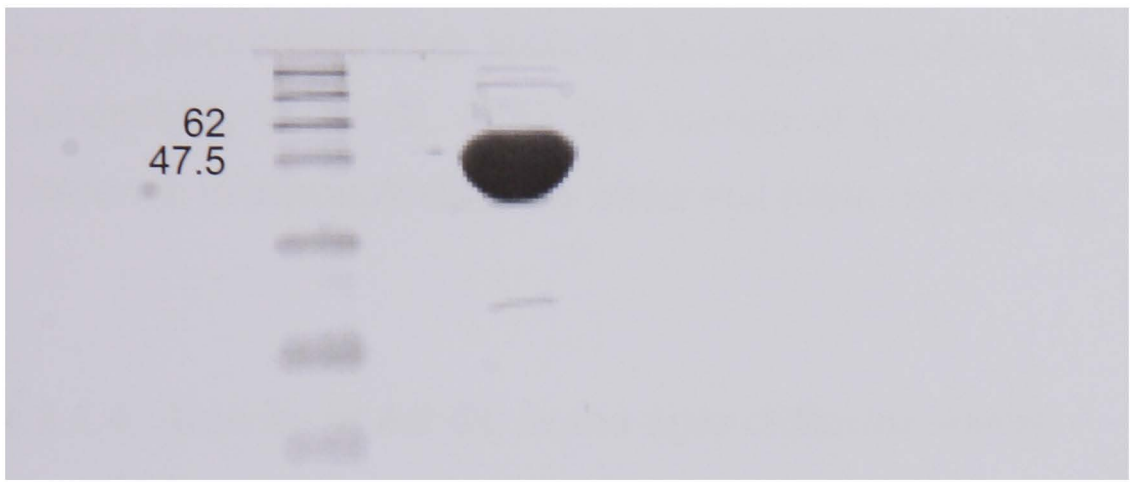
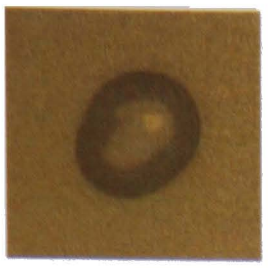


Figure 4.3 16% SDS-PAGE gel of purified recombinant Atl GL

4.2.1.3 *Activity of Atl GL in the stab test*

E. coli ER2566 were transformed with pKP2. The stab test was carried out against *S. aureus* Mu50, clinical staphylococcal isolates including EMRSA16, and *M. luteus*. Plates were incubated overnight at both 30°C and 37°C. When testing GL against staphylococci, *E. coli* ER2566 (pEA3), which encodes lysostaphin, was also stabbed onto the plate as a positive control. *E. coli* ER2566 carrying an empty pET21a vector was stabbed onto plates as a negative control. No activity of Atl GL against staphylococci was observed. Clear zones of lysis were observed in *M. luteus* lawns (Figure 4.4). No lytic zones against *M. luteus* were observed with either control stab. Zones of lysis appeared slightly larger following overnight incubation at 30°C than at 37°C.



pKP2 pET21a pEA3

Figure 4.4 *E. coli* ER2566 stab tests of lysostaphin and Atl GL vs. *M. luteus*

4.2.1.3.1 Atl resistance of *M. luteus* in the stab test

M. luteus growing at the edge of the clear lytic zone were subcultured and used in successive stab tests to investigate whether they showed reduced susceptibility to Atl GL. After five rounds of subculture and stab testing no difference in susceptibility was observed (data not shown).

4.2.1.4 *Activity of Atl GL in the agar diffusion assay*

The purified recombinant Atl GL was tested against *M. luteus* in the agar diffusion assay. Atl GL was tested in twofold serial dilutions from undiluted protein at ~266µM to ~0.25µM. Protein buffer was also added as a control. Atl caused clear zones of lysis from 266µM down to 2µM (Figure 4.5). The MIC in this assay was at 2µM, where a clear zone of lysis was observed.

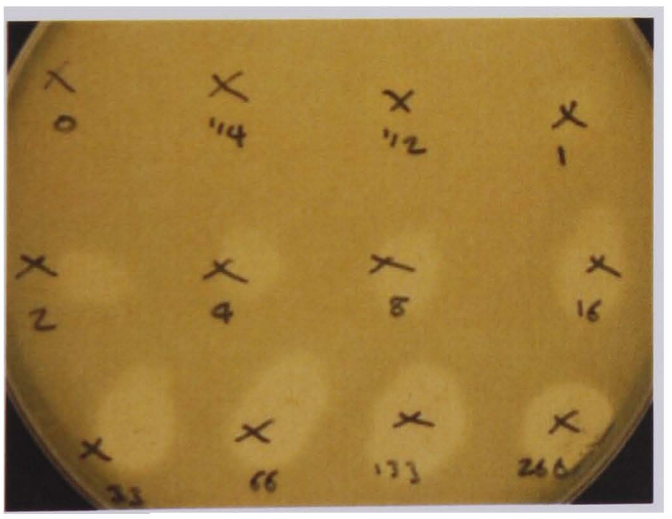


Figure 4.5 Agar diffusion assay testing Atl GL against *M. luteus*

4.2.1.5 *Activity of Atl GL in the turbidity assay*

Purified recombinant Atl GL was tested against lyophilised *M. luteus* and *S. aureus* cell substrates in the turbidity assay. The protein was assayed at

a range of concentrations from 0.5μM to 32μM. Assay buffer alone was also tested as a control. No activity was seen against *S. aureus*. Activity of Atl GL was observed against *M. luteus* at all concentrations tested (Figure 4.6). At 0.5μM Atl GL a slight decline in the turbidity of the cell substrate was observed. At all other concentrations the drop in turbidity was rapid.

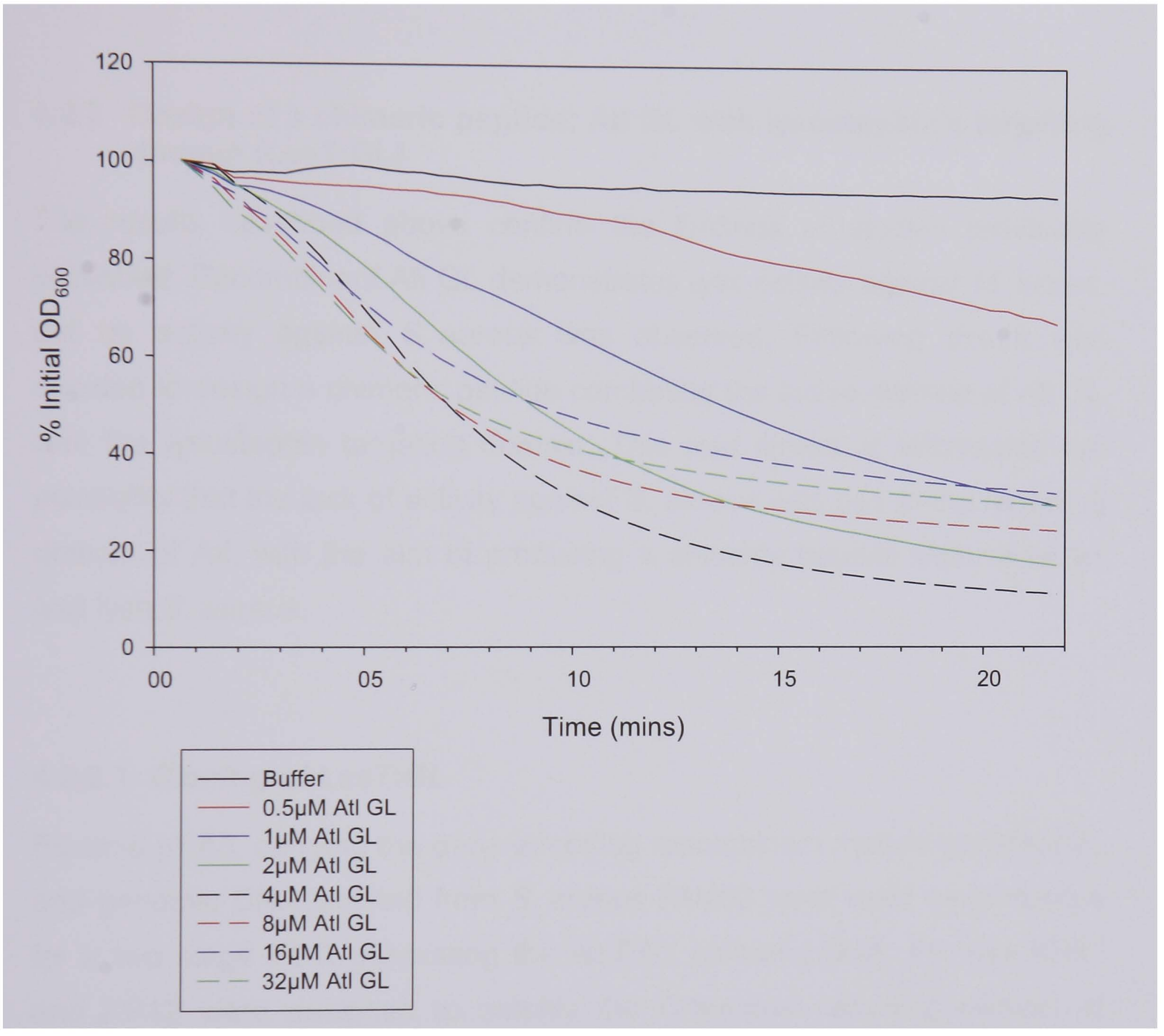


Figure 4.6 Turbidity assay testing activity of Atl GL vs. *M. luteus*

4.2.1.6 *Activity of Atl GL in the bioluminescence reporter assay*

Atl GL was tested against *S. aureus* RN6390 in the bioluminescence reporter assay described in chapter 5. Briefly, this assay is a measure of live *S. aureus* cells through a bioluminescent growth reporter. Peptide solutions are added to cells in a liquid assay format and the effect on

bioluminescence, relative to the effect of a buffer control sample, is recorded. Chapter five contains a further description of the bioluminescence reporter assay, with the protocol described in section 5.3.1.7.

No activity of Atl GL was observed when assayed up to 10μM in the bioluminescence reporter assay (data not shown).

4.2.2 Design of a chimeric peptide; Atl GL with lysostaphin's targeting domain (LssT:GL)

The results described above confirm the findings of studies previously published. Recombinant Atl GL demonstrates lytic activity against *M. luteus*, but no activity against *S. aureus* was observed. Following this it was decided to design a chimeric peptide combining the active domain of Atl GL with the lysostaphin targeting domain. This was aimed at addressing the possibility that the lack of activity against *S. aureus* was due to the targeting domain of Atl, with the aim of producing a chimeric peptide able to target and lyse *S. aureus*.

4.2.2.1 *Cloning of LssT:GL*

Plasmid pEA3, carrying the gene encoding recombinant mature lysostaphin, and genomic DNA isolated from *S. aureus* RN450 were used as templates for a two stage PCR generating the *lssT:GL* chimeric DNA. Primers KP11 and KP12 were designed to amplify the C-terminal targeting domain of lysostaphin with a 5' *Nco*I and a 3' *Pst*I restriction site. Primers KP13 and KP10 were used to amplify the sequence encoding the C-terminal glucosaminidase of GL without the R3 binding domain. This incorporated a 5' *Pst*I and a 3' *Xho*I restriction site, in frame with a C-terminal hexahistidine tag sequence from pET21(d). The two amplified sequences shared a 21bp overlap at the 3' end of the lysostaphin targeting domain sequence and the 5' end of the glucosaminidase. A second stage PCR was then carried out to anneal the two sequences together using primers KP10 and KP11, as shown in Figure 4.7. The chimeric sequence encodes a 429 amino acid

protein comprising the lysostaphin targeting domain at the N-terminus, the glucosaminidase and a C-terminal hexahistidine tag. The chimeric sequence was cloned via pGEMT-Easy into pET21(d)+ in *E. coli* DH5α and plasmid DNA was isolated as described in chapter 2. The presence of the correct chimeric sequence was confirmed by sequence analysis of plasmid DNA and the plasmid was designated pKP3. Both the glucosaminidase and lysostaphin targeting domains are situated at the C terminus of their host peptides. The lysostaphin targeting domain was chosen to be situated at the N-terminus as the domain had previously been shown to be active independently, allowing the best possible opportunity for each domain to remain active within the chimeric peptide.

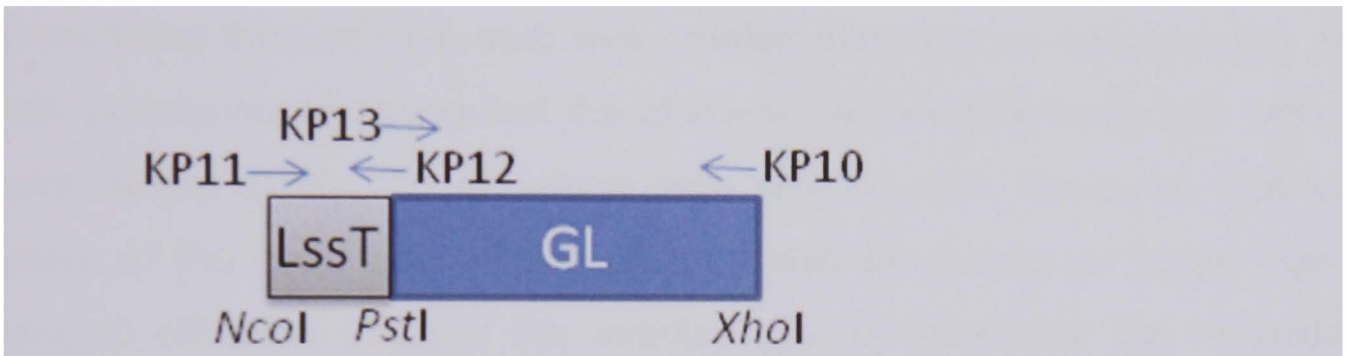


Figure 4.7 Organisation of the LssT:GL construct, showing primers used and restriction sites introduced.

4.2.2.2 *Activity of LssT:GL in the stab test*

The stab test was used as to investigate whether the chimeric protein showed activity against *S. aureus* prior to optimising conditions for the purification of the protein. *E.* coli ER2566 cells were transformed with pKP3, and LssT:GL was tested against *S. aureus* isolates including EMRSA16 and Mu50, *S. epidermidis,* and also against other Gram-positive organisms *S. pyogenes* and *M. luteus*. No lytic activity was observed against the staphylococci or the streptococci. Clear lytic zones were observed in the lawn of *M. luteus* (Figure 4.8). This indicates that the chimeric peptide is able to bind and kill *M. luteus* despite lacking the R3 region believed to be responsible for cell wall targeting. However, as with Atl GL, the chimeric

peptide is either unable to bind to *S. aureus* or the GL is unable to lyse *S. aureus cells* under the conditions of the stab test.

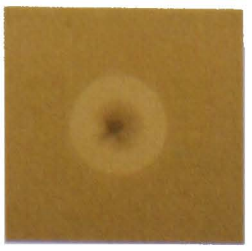


Figure 4.8 *E. coli* ER2566 stab test of LssT:GL vs. *M. luteus*

Further stab tests were carried out alongside stabs of the recombinant Atl GL, to provide a comparison of the relative levels of activity, under identical growth conditions of *E. coli* ER2566 carrying the plasmids. The zone of lysis surrounding the LssT:GL stab was smaller than that surrounding the Atl GL stab, possibly suggesting that the chimeric peptide has lower lytic activity or lower ability to bind to *M. luteus* cells (Figure 4.9). However, due to the nature of the stab test, this result may also be due to its larger size and reduced diffusion through the overlay, poorer growth of the recombinant clone, or reduced release of the protein following chloroform lysis.

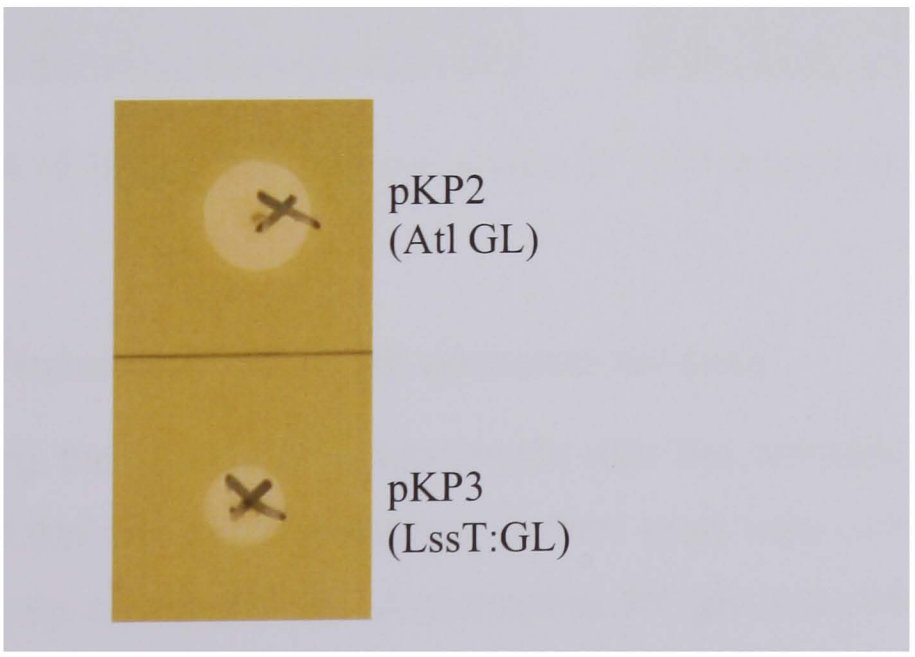


Figure 4.9 Comparison of activity of Atl GL and LssT:GL in the stab test vs. *M. luteus*

4.2.2.3 *Binding of LssT to* M. luteus

The LssT:GL chimera demonstrated the ability to bind to and lyse *M. luteus* but was unable to lyse *S. aureus.* Further studies were performed to investigate the binding of the lysostaphin targeting domain to each of these organisms. A recombinant lysostaphin targeting domain with a GFP tag (a gift from Philip Bardelang, University of Nottingham) was used in binding studies with live cells as described in chapter 2. The lysostaphin targeting domain was shown to bind to *S. aureus* cells as anticipated (Figure 4.10). However, no GFP tagged lysostaphin targeting domain was observed to bind to the surface of *M. luteus*. The inactivity of the LssT:GL chimera against *S. aureus* may be due either to a lack of hydrolytic activity of the Atl GL, or an inhibition of the binding of the lysostaphin targeting domain.

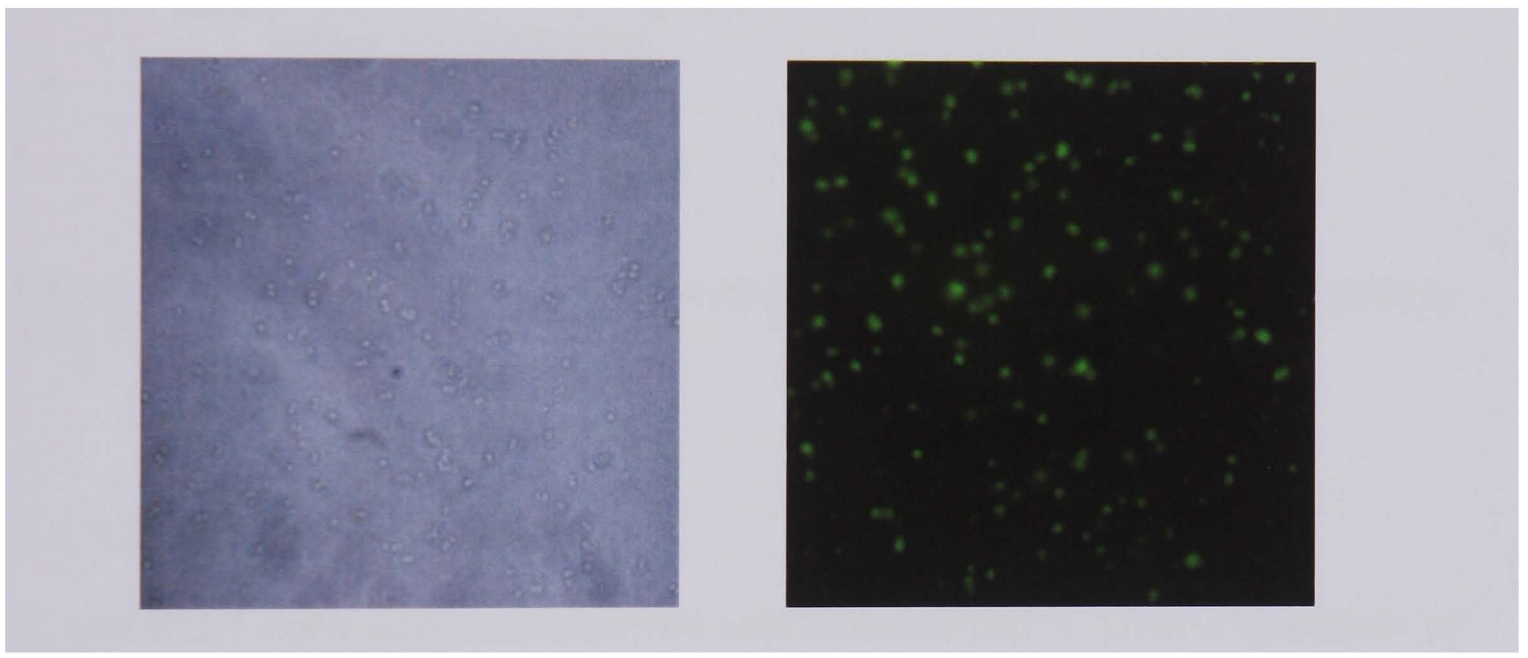


Figure 4.10 Bright field and u.v. image of GFP tagged LssT binding to *S. aureus* cells

4.2.3 Truncation of the Atl glucosaminidase

Following the results of experiments with the chimeric LssT:GL in the stab test and the cell binding studies, further work was carried out to investigate the activity of the Atl GL. A truncated Atl glucosaminidase, lacking the N-terminal repeat 3 binding domain was cloned. This was used to investigate whether the lysostaphin targeting domain was responsible for the binding of LssT:GL to *M. luteus*, or whether the glucosaminidase domain alone was capable of binding to and lysing the cell wall.

4.2.3.1 *Cloning of the truncated Atl GL*

Genomic DNA isolated from *S. aureus* RN450 was used as template DNA for the PCR amplification of the Atl glucosaminidase active domain (GL'). Primers KP10 and KP15 were used to amplify the *gl'* sequence with a 5' *Nco*I restriction site and a 3' *Xho*I restriction site as shown in Figure 4.11. The 1005bp fragment was cloned via pGEMT-Easy into pET21(d)+ using the methods described in chapter 2, leaving the sequence in frame with a C-terminal hexahistidine tag. The plasmid was designated pKP4 and the DNA sequence was confirmed to be correct by sequence analysis.

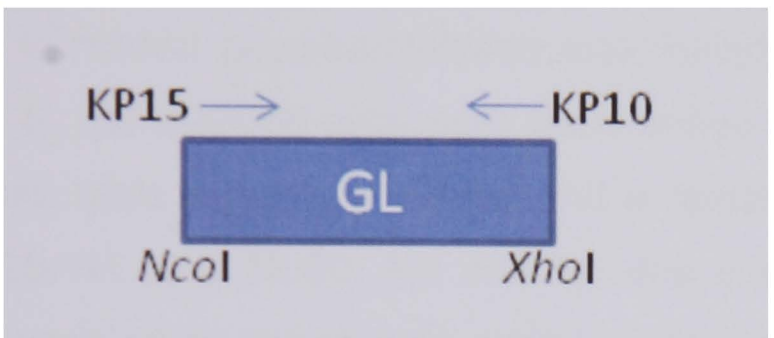


Figure 4.11 Organisation of the GL' construct, showing primers used and restriction sites introduced.

4.2.3.2 *Purification of GL'*

E. coli BL21 cells were transformed with isolated pKP4. Over-expression of GL' with IPTG was demonstrated on a small scale using 5ml cultures. Cell pellets were then collected from 500ml cultures grown in 2YT induced to over-express the recombinant truncated glucosaminidase. Again, cell pellets were particularly viscous and were immediately resuspended prior to overnight storage, and purification of GL' by nickel-chelate chromatography as described in chapter 2. Protein running close to the anticipated size of hexahistidine tagged GL', ~37.5kDa, was eluted off the column over several fractions which were collected and subjected to dialysis. Pure recombinant GL' was analysed by SDS-PAGE (Figure 4.12). The concentration of the truncated glucosaminidase was determined as 50μM.

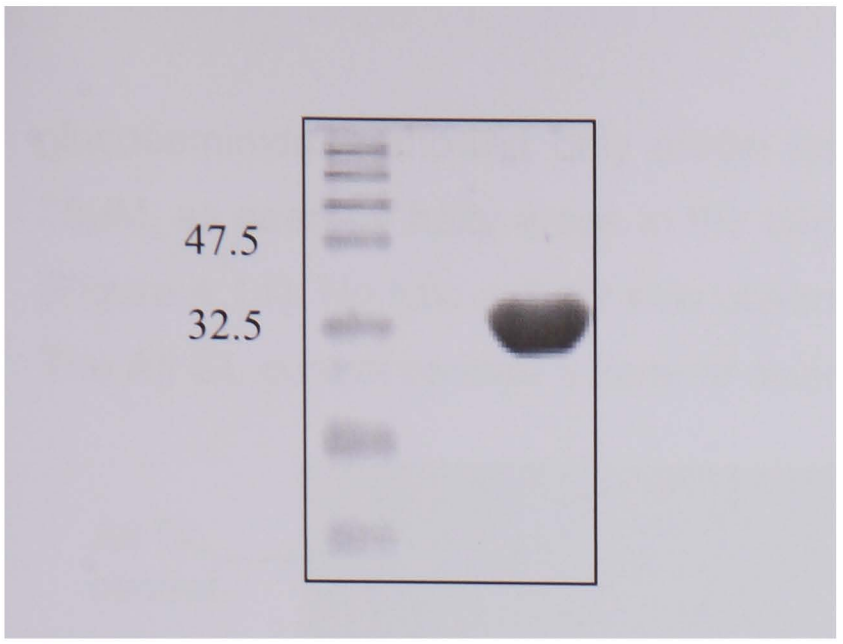


Figure 4.12 16% SDS-PAGE gel of purified GL'

4.2.3.3 *Activity of GL' in the stab test*

The truncated glucosaminidase was initially tested for activity in the stab test. *E. coli* ER2566 cells were transformed with pKP4 and these were used in stab tests against *M. luteus* and a range of *S. aureus* isolates including EMRSA16 and Mu50. No activity was observed in the stab test against *S. aureus* (data not shown), whilst clear lytic zones in the lawn of *M. luteus* were observed (Figure 4.13). There was no obvious size difference between the zones of lysis caused by Atl GL or GL'.

Figure 4.13 Stab test showing activity of GL' against *M. luteus*

4.2.3.4 *Activity of GL' in the agar diffusion assay*

Following the results of the stab test, the purified recombinant GL' was tested in the agar diffusion assay against *S. aureus* isolates and *M. luteus*. The truncated GL was tested at a range of concentrations from undiluted at 50μM through to 0.4μM by twofold serial dilutions. Lysostaphin was used as a positive control in assays against *S. aureus* isolates whilst purified recombinant Atl GL was used as a control when testing against *M. luteus*. No lytic activity of GL' was observed against *S. aureus.* The truncated

glucosaminidase showed only partial lysis against *M. luteus* at 50μM and 25μM, as seen by hazy areas in the bacterial lawn around the protein drop (Figure 4.14). No lytic activity was observed at lower concentrations of GL'. The Atl GL control caused a zone of clear lysis at 20 μM.

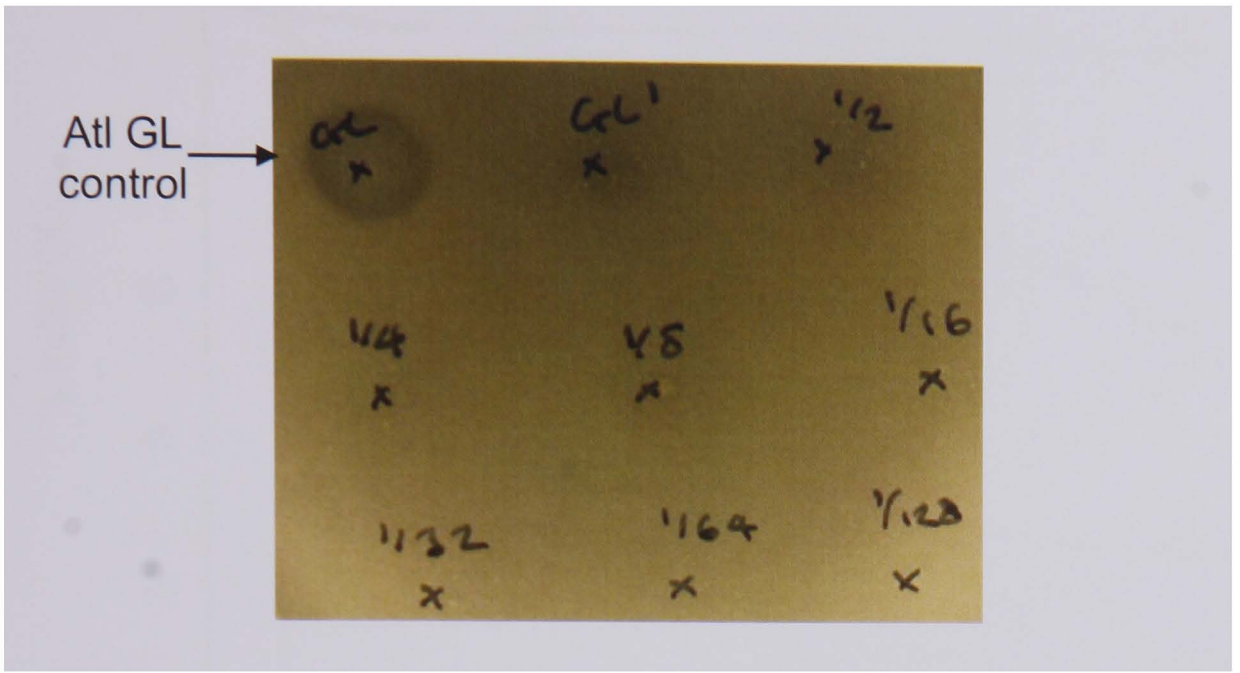


Figure 4.14 Agar diffusion assay testing GL' against *M. luteus*

4.2.3.5 *Activity of GL' in the turbidity assay*

The lytic activity of the truncated glucosaminidase was tested in the turbidity assay against *S. aureus* and *M. luteus*. The GL' was added at 0.5μM, 1μM, 5μM and 10μM. The recombinant Atl GL complete with the repeat 3 domain was added at 1μM as a positive control. There was no lytic activity of GL' against *S. aureus*. A decline in the optical density of the *M. luteus* substrate was observed at 5μM and 10μM (Figure 4.15). The activity of GL' was significantly lower than that of the whole glucosaminidase. The difference was such that the addition of Atl GL at 1μM causes more rapid lysis of *M. luteus* than the addition of GL' at 5μM.

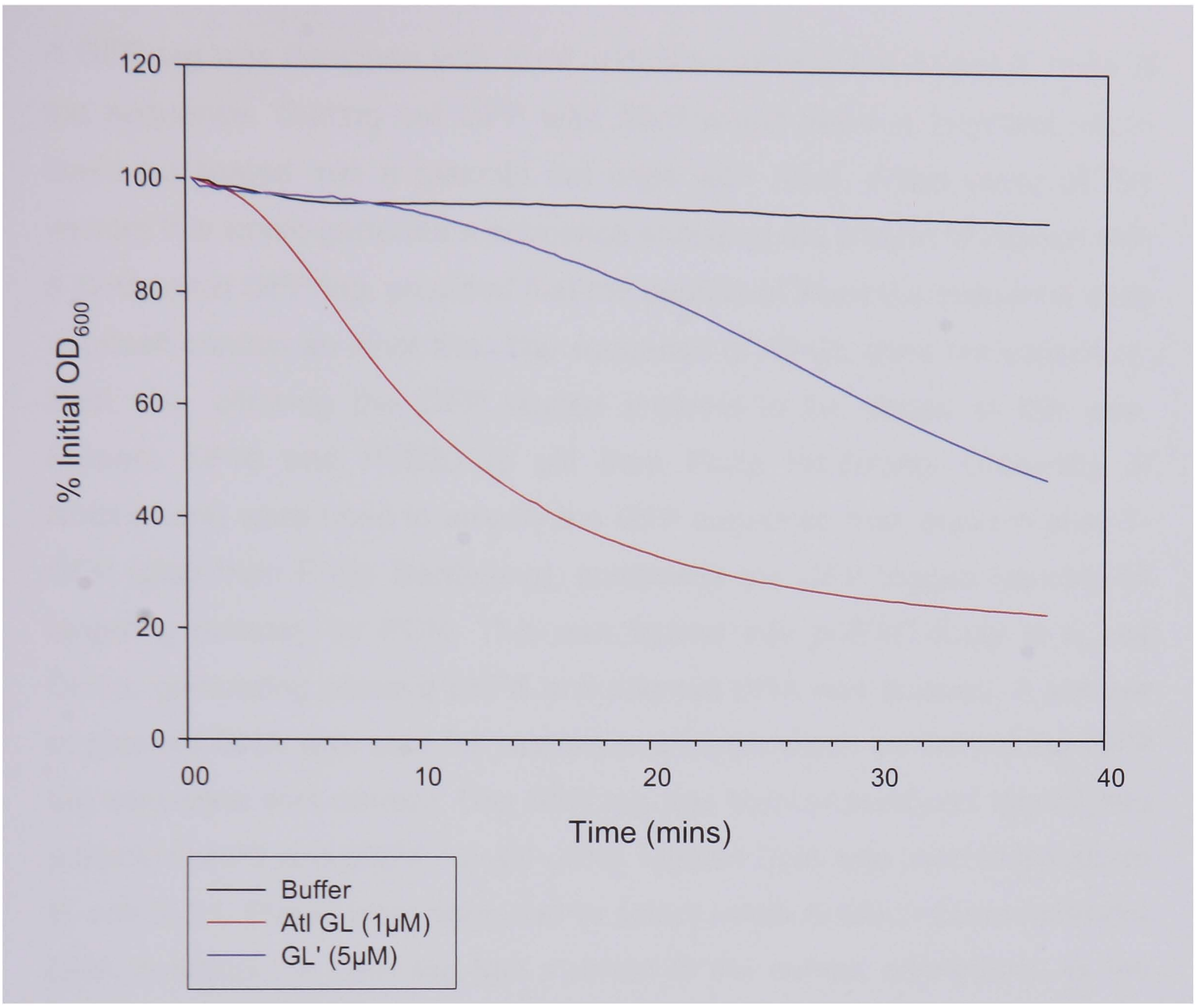


Figure 4.15 Turbidity assay testing activity of GL' vs. *M. luteus*

4.2.3.6 *Activity of GL' in the bioluminescence reporter assay*

GL' was tested against *S. aureus* RN6390 in the bioluminescence reporter assay described in chapter 5. The protein was tested up to a concentration of 10μM. No activity was observed against *S. aureus* RN6390.

4.2.4 Binding of Atl GL and GL' to *S. aureus* and *M. luteus*

Following the results of the assays of activity of Atl GL and the truncated glucosaminidase, further work was carried out to investigate the binding of these peptides to cells. GFP tagged proteins were designed to compare the binding of Atl GL and GL', and also to determine whether the recombinant proteins were able to bind to the whole cell surface.

4.2.4.1 *Cloning of GFP tagged Atl GL and GL'*

A GFP tag was designed with *Xho*I restriction sites at the 5' and 3' ends of the sequence. Cutting out GFP with *Xho*I would leave a fragment which could be ligated into a plasmid cut once with *Xho*I. When using pET21 vectors this would generate a sequence encoding the protein of interest with a C–terminal GFP tag, provided that the peptide of interest's sequence does not itself contain an *Xho*I site. The sequence of Atl GL does not contain an *Xho*I site, allowing the GFP tagged proteins to be cloned in this way. Primers KP16 and PTB20 (a gift from Philip Bardelang, University of Nottingham) were used to amplify the GFP sequence from plasmid pLss-T-GFP (also from Philip Bardelang), containing the GFP tagged lysostaphin targeting domain, by PCR. This was ligated into pGEMT-Easy in *E. coli* DH5α, generating plasmid pKP5 and plasmid DNA was isolated. A sample of plasmid DNA was sent for sequence analysis which confirmed the GFP tag sequence was correct. The GFP tag was then excised and ligated into plasmids pKP2 and pKP4 cut with *Xho*I. Ligated DNA was used to transform *E. coli* BL21. Plates were screened to select colonies which carried plasmid DNA in which the GFP tag had inserted in the correct orientation. As the sequence has both 5' and 3' *Xho*I sites it may ligate into the singly cut plasmid in either direction. Screening was performed by viewing plates over u.v. light and selecting colonies which fluoresced green. Several green colonies were observed (Figure 4.17), and these were picked and used in stab tests against *M. luteus* to confirm that the tagged proteins retained lytic activity. Both the GFP tagged Atl GL and GL' caused zones of clear lysis in the bacterial lawn (Figure 4.18). The zones of lysis were observed to be smaller than those caused by the untagged proteins, possibly due to the larger GFP tagged peptides diffusing into a smaller area. Colonies of *E. coli* BL21 that both fluoresced and demonstrated lytic activity were subcultured and plasmid DNA was isolated. The organisation of the GFP tagged glucosaminidases is shown in Figure 4.16. The plasmids encoding the GFP tagged Atl GL and GL' were designated pKP6 and pKP7 respectively. *E. coli* DH5α were transformed with plasmids pKP6 and pKP7, and a stock of plasmid DNA was isolated from these.

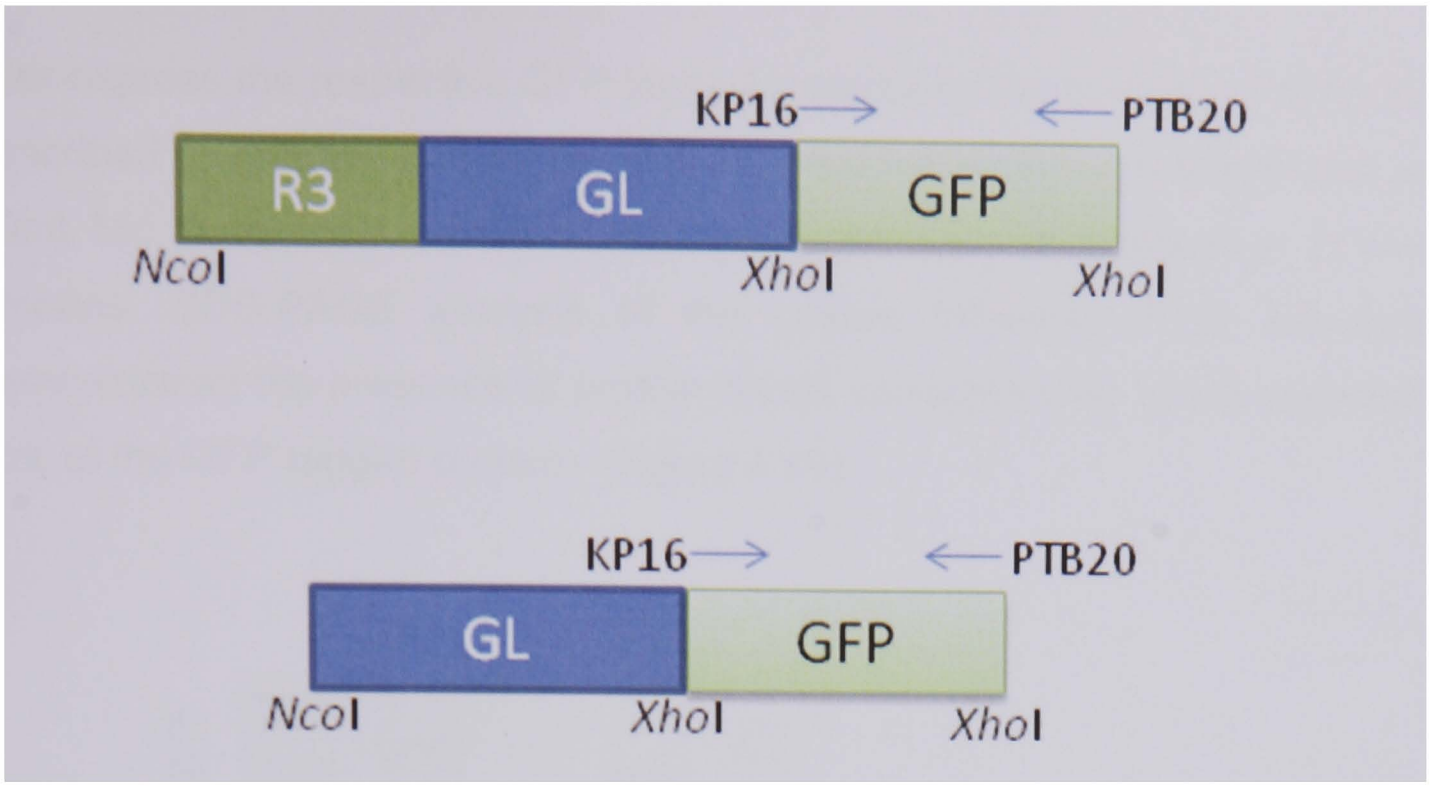


Figure 4.16 Organisation of the GL:GFP (top) and GL':GFP (bottom) constructs, showing primers used and restriction sites introduced.

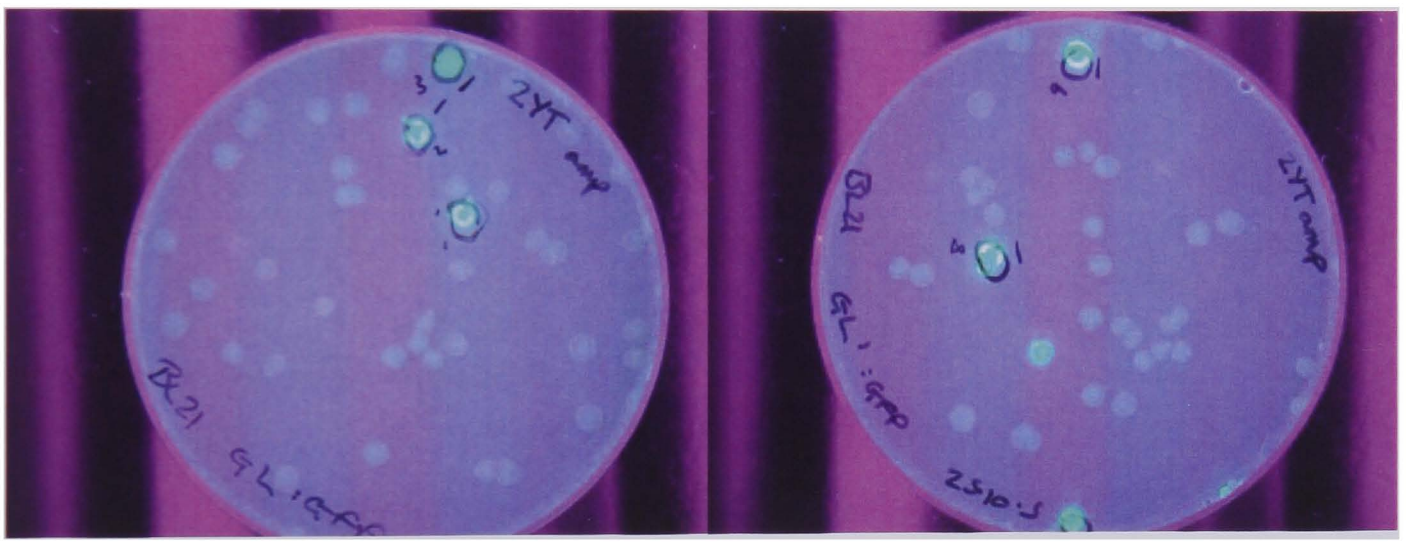


Figure 4.17 Screening plates for fluorescence of GFP tagged GL and GL'

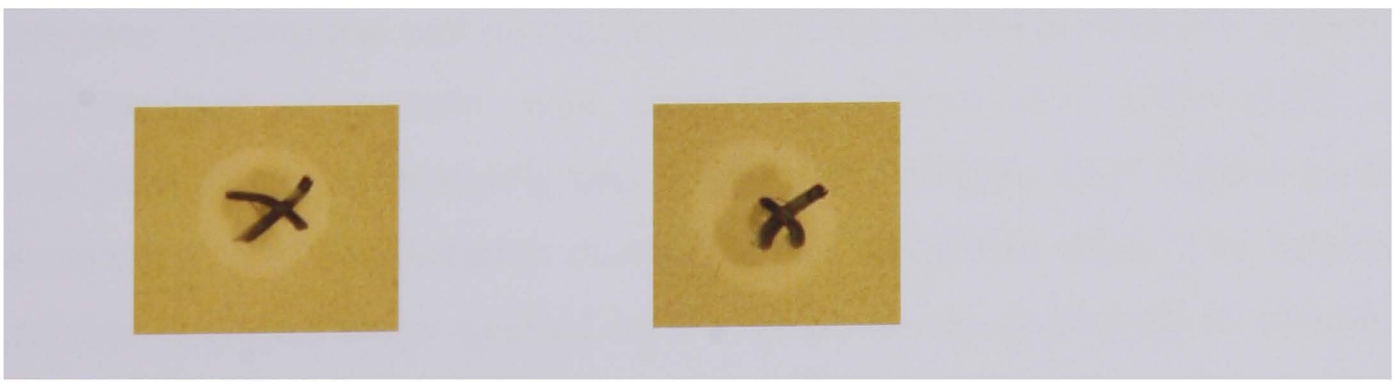


Figure 4.18 Stab tests of GFP tagged GL and GL' vs. *M. luteus*

4.2.4.2 *Purification of GFP tagged Atl GL and GL'*

E. coli BL21 was transformed with plasmids pKP6 and pKP7, and cell pellets were collected from 400ml cultures grown in 2YT and induced to

over-express the respective GFP tagged proteins by the addition of IPTG as described in chapter 2. The cell pellets were immediately resuspended in 30ml Nic buffer and stored at 4°C overnight prior to purification of the proteins. SDS-PAGE analysis of the culture following IPTG induction demonstrated the presence of protein bands corresponding to the expected size of the GFP tagged proteins (Figure 4.19).

Figure 4.19 12% SDS-PAGE gel showing over-expression of GFP tagged Atl GL and GL' in *E. coli* BL21. *E. coli* BL21 expressing Atl GL:GP after (Lane 1) and before (Lane 2) IPTG induction. *E. coli* BL21 expressing GL':GFP after (Lane 3) and before (Lane 4) IPTG induction.

The resuspended cell pellets were subjected to nickel-affinity chromatography to purify the recombinant GFP tagged Atl GL and GL' proteins. During the cell disruption step of the protein purification, significant precipitation of protein was observed. Subsequent SDS-PAGE gels indicated that approximately two thirds of the soluble GFP tagged proteins was lost due to precipitation during the cell disruption steps. The remaining soluble proteins were applied to the columns as described in chapter 2. Small quantities of proteins of the anticipated size were eluted off the columns and the fractions containing these were selected for dialysis. Following dialysis, samples of the proteins were run on an SDS-PAGE gel, and showed that small amounts of each protein had been purified (Figure 4.20). Other contaminant bands were also present, although at a significantly lower intensity than for the full size GFP tagged proteins. The

concentrations of the proteins were determined as 7.2μM and 7.0μM for the GFP tagged Atl GL and GL' respectively. Aliquots of each protein were stored at -80°C. Further efforts made to increase the yield of the GFP tagged glucosaminidases were unsuccessful.

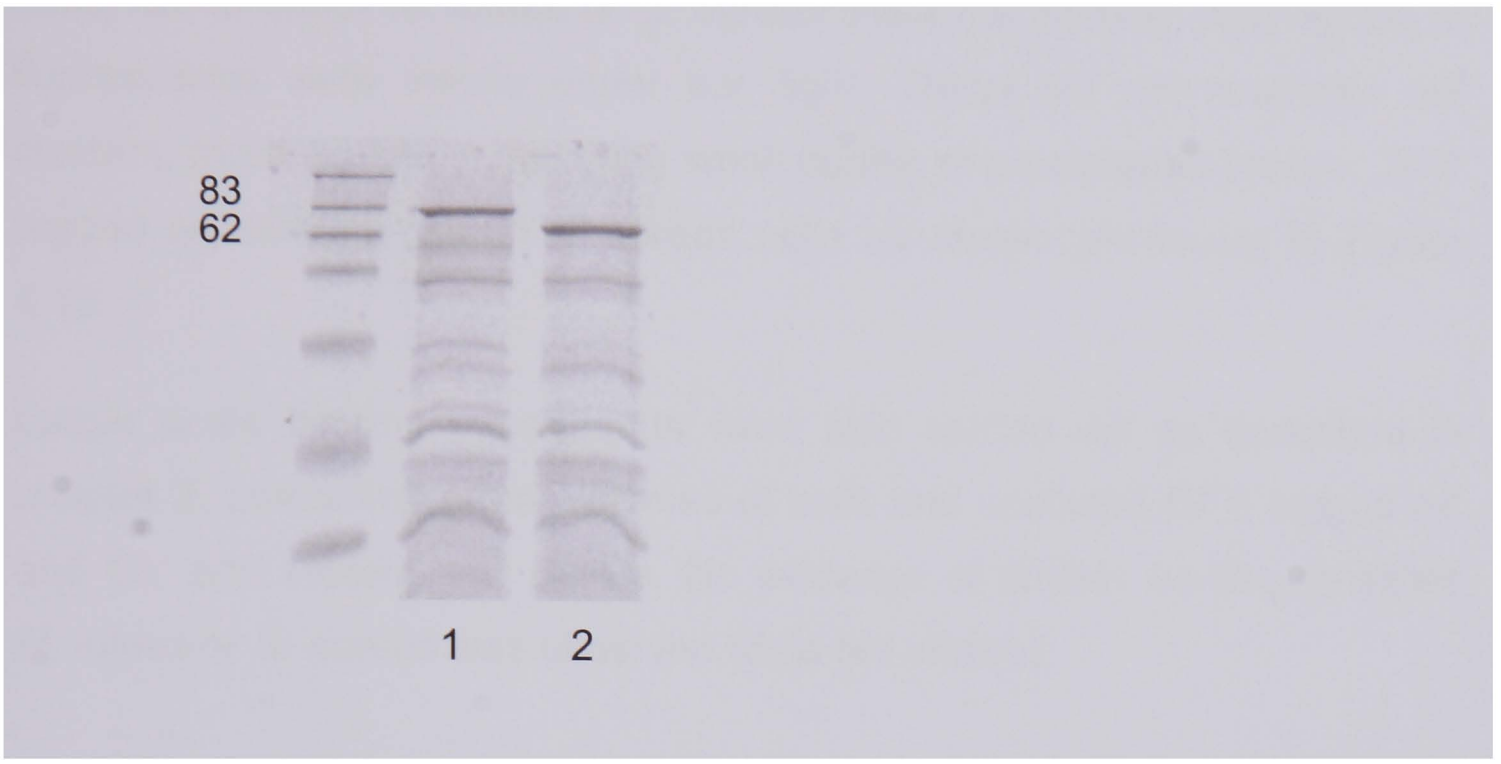


Figure 4.20 12% SDS-PAGE gel showing purified GFP tagged Atl GL (Lane 1) and GL' (Lane 2)

4.2.4.3 *Visualisation of GFP tagged Atl GL and GL'*

Aliquots of GFP tagged Atl GL and GL' were viewed over u.v. light. The samples fluoresced green, indicating the presence of correctly folded GFP in the samples. Despite the low concentration of each protein the fluorescence was intense, and as only small quantities are required for the cell binding experiments, these proteins were used in further studies.

4.2.4.4 *Binding of GFP tagged Atl GL and GL' to* M. luteus *and* S. aureus

The GFP tagged glucosaminidases were used in binding studies, as described in chapter 2, with *M. luteus* and *S. aureus*. An overnight culture of each organism was suitably diluted to the optimum cell density of 100 to 200 cells per field visible under the light microscope at a magnification of x100. A range of ratios of protein to cells were made to test binding of the tagged

proteins. 5µl of cells were mixed with 1µl, 5µl and 10µl of the undiluted GFP tagged glucosaminidases and viewed by phase contrast microscope. The GFP tagged lysostaphin targeting domain was used as a control in assays against *S. aureus*. No binding of the GFP tagged Atl GL or GL' was observed to either *M. luteus* or *S. aureus* (data not shown). Aggregates of fluorescence were visible under u.v. light. These did not resemble cell clusters, more probably indicating small bodies of precipitated protein. GFP tagged lysostaphin bound *S. aureus* cells as shown previously in Figure 4.10.

Larger scale binding experiments were also carried out as described in chapter 2, combining equal volumes of cells and undiluted GFP tagged Atl and GL' and viewing cell pellets. No evidence of protein binding to either *M. luteus* or *S. aureus* was observed (data not shown).

4.2.5 Addition of R1 and R2 binding domains to Atl GL

In vivo the amidase and glucosaminidase of Atl separate between repeat regions R2 and R3, leaving R3 with the glucosaminidase and R12 with the amidase where they act as binding domains. A recombinant glucosaminidase with the R1 and R2 binding domains at the N-terminus may show improved binding properties, or a wider range of cell targets. The extended Atl GL (R123GL) was cloned to investigate its activity and binding.

4.2.5.1 *Cloning of* r123gl

S. aureus RN450 genomic DNA was used as template DNA for the PCR amplification of the Atl glucosaminidase sequence with the additional R1 and R2 binding domains. Primers KP10 and KP14 were used to amplify the *r123gl* sequence with a 5' *Nco*I restriction site and a 3' *Xho*I restriction site as shown in Figure 4.21. The 2537bp fragment was cloned via pGEMT-Easy into pET21(d)+ in *E. coli* DH5α, as described in chapter 2, in frame with a sequence encoding a C-terminal hexahistidine tag from the vector. The

plasmid was designated pKP8. Plasmid DNA was isolated and DNA sequence analysis was used to confirm the correct sequence of the insert DNA. Due to the size of the DNA insert, and the ability of the sequencing service to reliably sequence approximately 1000bp, the internal primers KP17 and KP18 were designed to enable the sequencing of the whole *r123gl* gene.

Figure 4.21 Organisation of the R123GL construct, showing primers used and restriction sites introduced.

4.2.5.2 *Expression of R123GL*

E. coli ER2566 were transformed with pKP8 and used in a small scale expression screen as described in chapter 2. Low levels of growth of the culture and expression of the extended glucosaminidase were observed. Little improvement was gained despite varying the growth medium or temperature. A protein running slightly lower than 93kDa, the anticipated size of R123GL, was over-expressed as shown by SDS-PAGE (Figure 4.22). However, the poor growth and expression of the culture was also observed on a large scale and a cell pellet suitable for the purification of R123GL could not be collected. *E. coli* BL21 transformed with pKP8 showed similar levels of growth and expression.

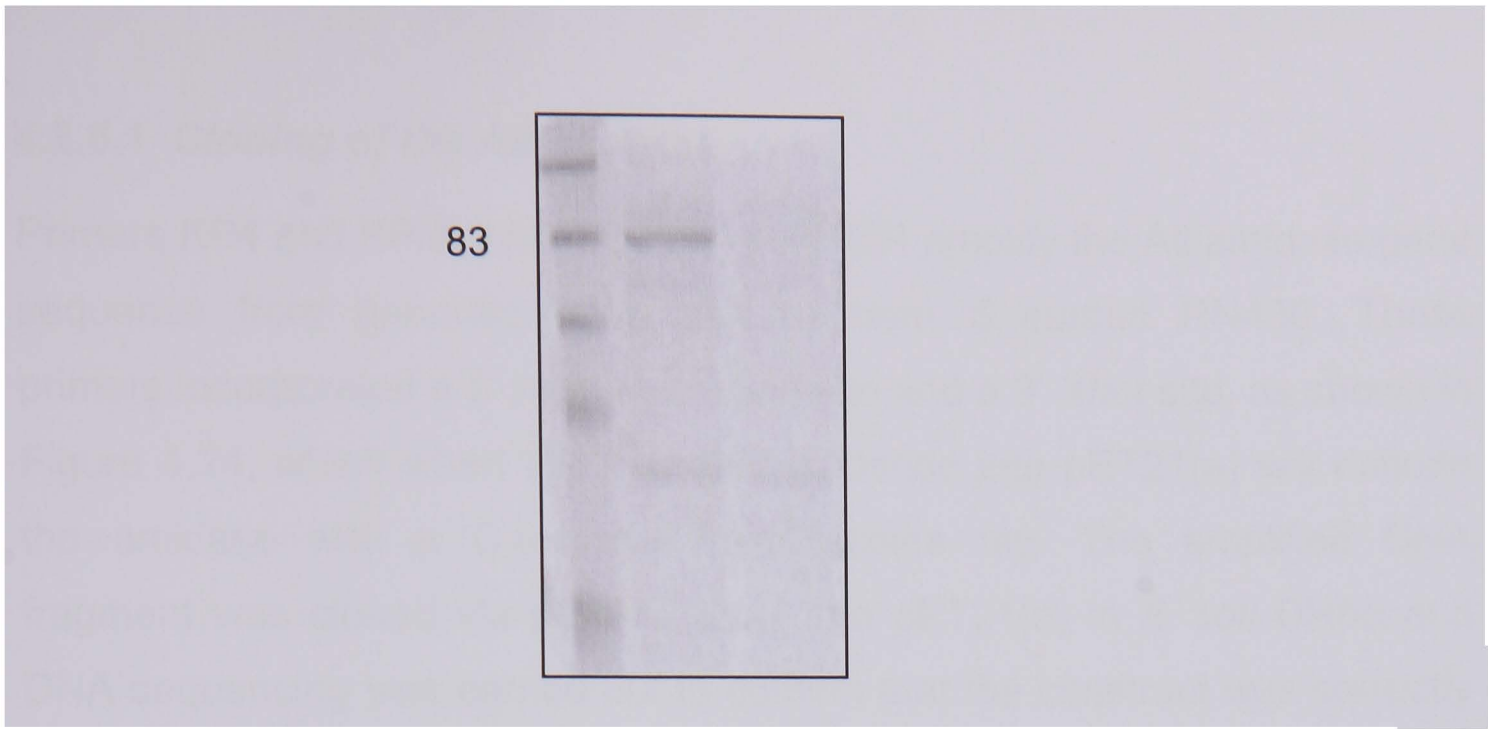


Figure 4.22 12% SDS-PAGE gel showing over-expression of R123GL in *E. coli* ER2566

4.2.5.3 *Activity of R123GL in the stab test*

E. coli ER2566 transformed with pKP8 were used in the stab test against *M. luteus* and *S. aureus*. R123GL was tested against *S. aureus* Mu50, *S. aureus* RN6390 and a range of clinical isolates including EMRSA16. As positive controls Atl GL and lysostaphin containing stabs were also included on plates. No clear zones of lysis were observed in the lawns of any of the staphylococcal isolates tested. Zones of lysis were observed against *M. luteus* (Figure 4.23). These zones were smaller than those seen in stab tests of both Atl GL and the truncated glucosaminidase.

Figure 4.23 Stab test of R123GL in ER2566 against *M. luteus*

4.2.6 The amidase of Atl

Following the investigation of the glucosaminidase of Atl, and the unsuccessful attempts at targeting its activity to staphylococci, the amidase of Atl was investigated.

4.2.6.1 *Cloning of the Atl amidase*

Primers KP4 and KP20 were designed to PCR amplify the Atl amidase gene sequence from genomic DNA isolated from *S. aureus* RN450. These primers incorporated a 5' *Nde*I restriction site and a 3' *Xho*I site, as shown in Figure 4.24, which when the fragment is cloned into pET21(a) will encode the amidase with a C-terminal hexahistidine tag. The amplified DNA fragment was cloned via pGEMT-Easy into pET21(a) in *E. coli* DH5α and DNA sequencing was carried out to confirm that the construct was correctly cloned without mutations. A stock of the plasmid DNA, which was labelled pKP9, was isolated according to the methods described in chapter 2.

Figure 4.24 Organisation of the Atl amidase construct, showing primers used and restriction sites introduced.

4.2.6.2 *Purification of the Atl amidase*

E. coli BL21 were transformed with pKP9. 400ml cultures were grown in 2YT medium and induced to over-express the amidase by the addition of IPTG. Cell pellets were collected and protein purified by nickel chelate chromatography. Again, a large quantity of the protein was observed to precipitate during cell disruption and only a small amount remained to apply to the column. Soluble protein was eluted off the column, and following dialysis the concentration of the protein was determined as 9μM. Aliquots of the Atl amidase were stored at -80°C.

4.2.6.3 *Activity of the Atl amidase in the stab test*

E. coli ER2566 were transformed with pKP9 and used in the stab test against *M. luteus* and *S. aureus*. Stabs of *E. coli* ER2566 expressing lysostaphin and Atl GL were also made on the same plates as positive controls. Atl amidase demonstrated weak lytic activity against *S aureus* compared to the activity of lysostaphin (Figure 4.25). The Atl amidase showed no activity against *M. luteus*.

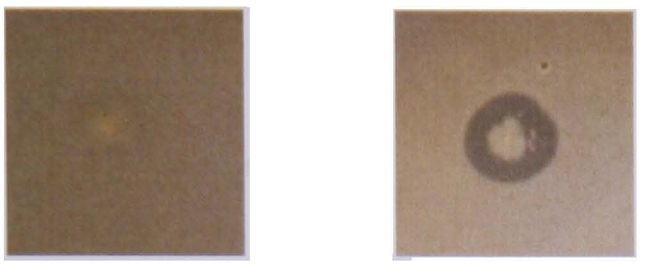


Figure 4.25 Stab test showing effect of Atl amidase and lysostaphin against *S. aureus* Mu50

4.2.6.4 *Activity of the Atl amidase in the turbidity assay*

The purified recombinant Atl amidase was tested in the turbidity assay against the Oxford staphylococcus and EMRSA16 according to the method in chapter 2. To maximise the concentration of the Atl amidase, undiluted protein was used giving a final concentration of 8.5μM. Lysostaphin was included in assay runs as a positive control. No drop in optical density of the staphylococcal substrate was observed (data not shown).

4.2.6.5 *Activity of the Atl amidase in the bioluminescence reporter assay*

Atl amidase was also tested in the more sensitive bioluminescence reporter assay against S. *aureus* RN6390 as described in chapter 5. Undiluted protein was again used, making a final concentration of 2.7μM. A slight reduction in the rate of increase of bioluminescence was observed following the typical lag seen at the start of the assay (Figure 4.26). However, this indicates only very weak activity, as more active proteins cause a decline in

bioluminescence. Lysostaphin was also included in the assay run at a concentration of 0.5μM as a positive control.

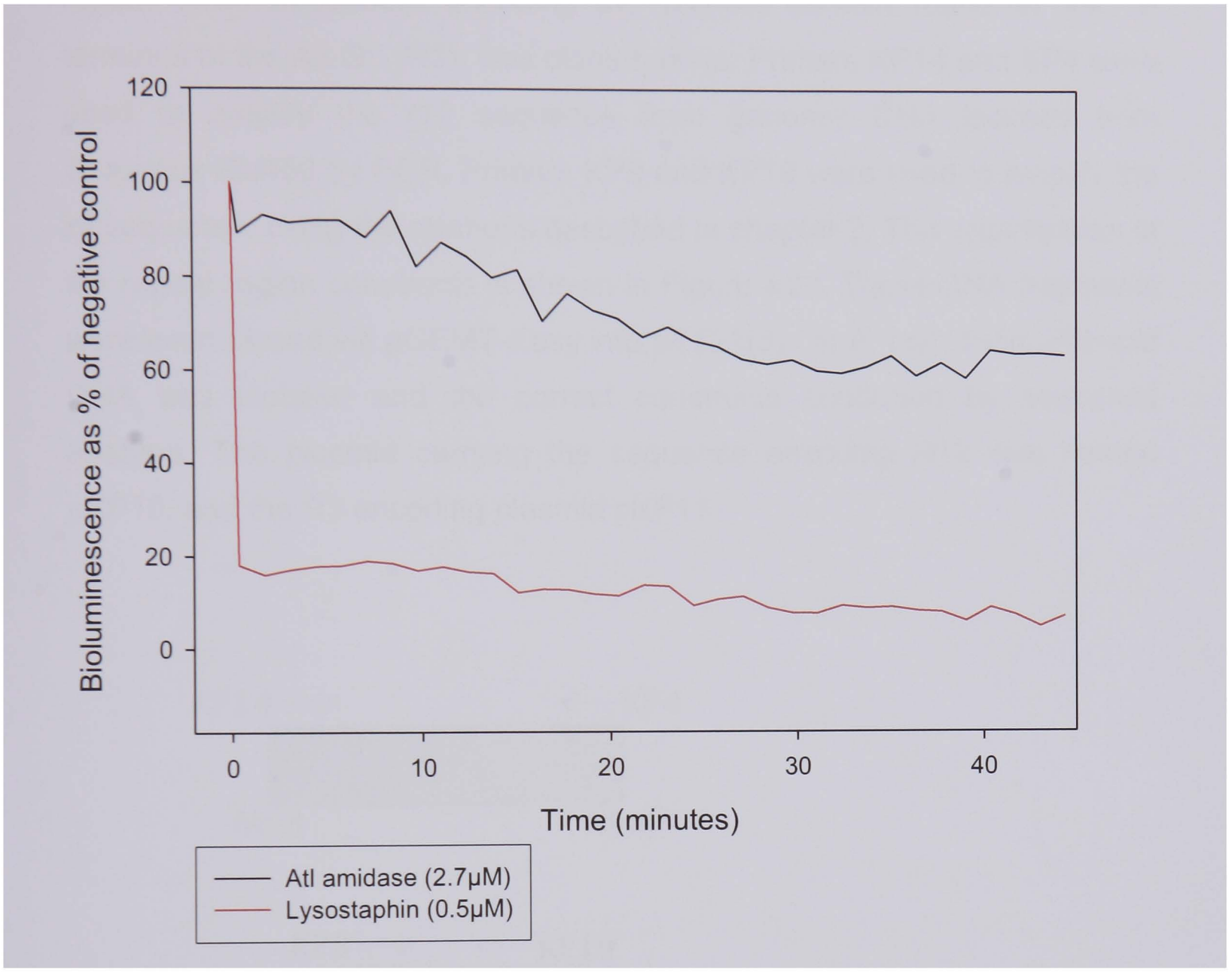


Figure 4.26 Bioluminescence reporter assay showing activity of Atl amidase against *S. aureus* RN6390

4.2.7 The repeat regions of Atl

To understand further the binding of the Atl amidase and glucosaminidase, in the absence of results using GFP tagged proteins, the binding properties of the repeat regions of Atl alone were investigated. The repeats associated with each active domain of Atl were cloned alone and tested for activity alone, and for binding in competition with other peptides.

4.2.7.1 *Cloning of the repeat regions of Atl*

A construct comprising the sequence encoding repeats one and two of Atl (R12), as are located at the C-terminus of the Atl amidase, was cloned. The repeat three sequence, encoding the binding domain found at the N-terminus of the Atl GL (R3), was cloned alone. Primers KP14 and KP4 were used to amplify the *r12* sequence from genomic DNA isolated from *S. aureus* RN450 by PCR. Primers KP9 and KP19 were used to amplify the *r3* sequence, using the methods described in chapter 2. The organisation of the repeat region constructs is shown in Figure 4.27. These DNA fragments were each cloned via pGEMT-Easy into pET21(d)+ in *E. coli* DH5α. Plasmid DNA was isolated and the correct constructs confirmed by sequence analysis. The plasmid carrying the sequence encoding R12 was named pKP10, and the R3 encoding plasmid pKP11.

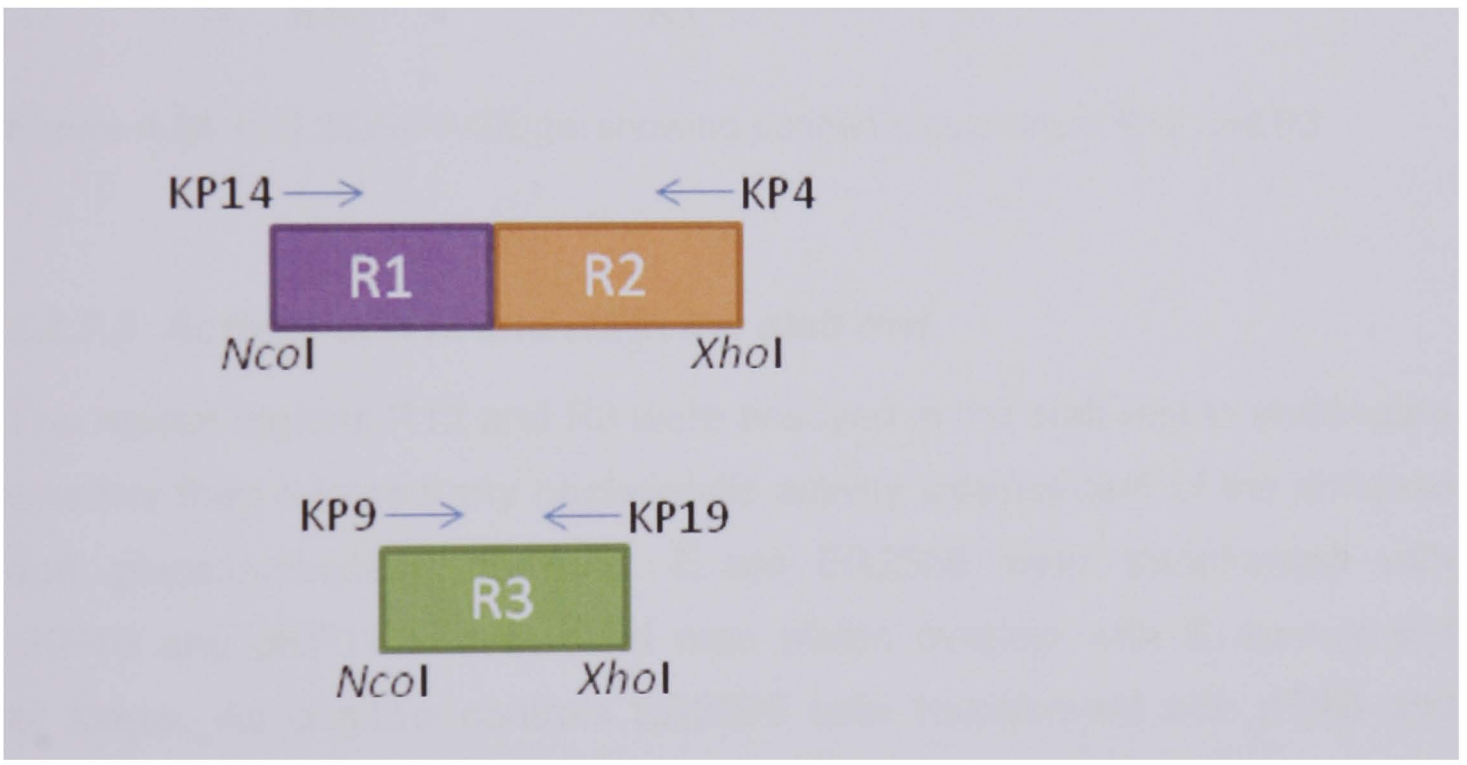


Figure 4.27 Organisation of the Atl R12 (top) and R3 (bottom) constructs, showing primers used and restriction sites introduced.

4.2.7.2 *Purification of R12 and R3*

E. coli BL21 was transformed with pKP10 and pKP11. Pellets of each were collected from 400ml cultures grown in 2YT medium and induced to over-express protein with the addition of IPTG. The recombinant proteins were purified by the pH based denaturing method described in chapter 2. A significant quantity of both proteins was observed to precipitate during cell

disruption by sonication. The remaining soluble proteins were applied to the columns. Soluble R12 and R3 were eluted off the columns and collected for dialysis. SDS-PAGE analysis confirmed that proteins were purified of the anticipated sizes of 39kDa and 19kDa for R12 and R3 respectively (Figure 4.28). The concentrations of the purified recombinant R12 and R3 were determined as 15μM and 20μM respectively. Aliquots of the proteins were stored at -80°C.

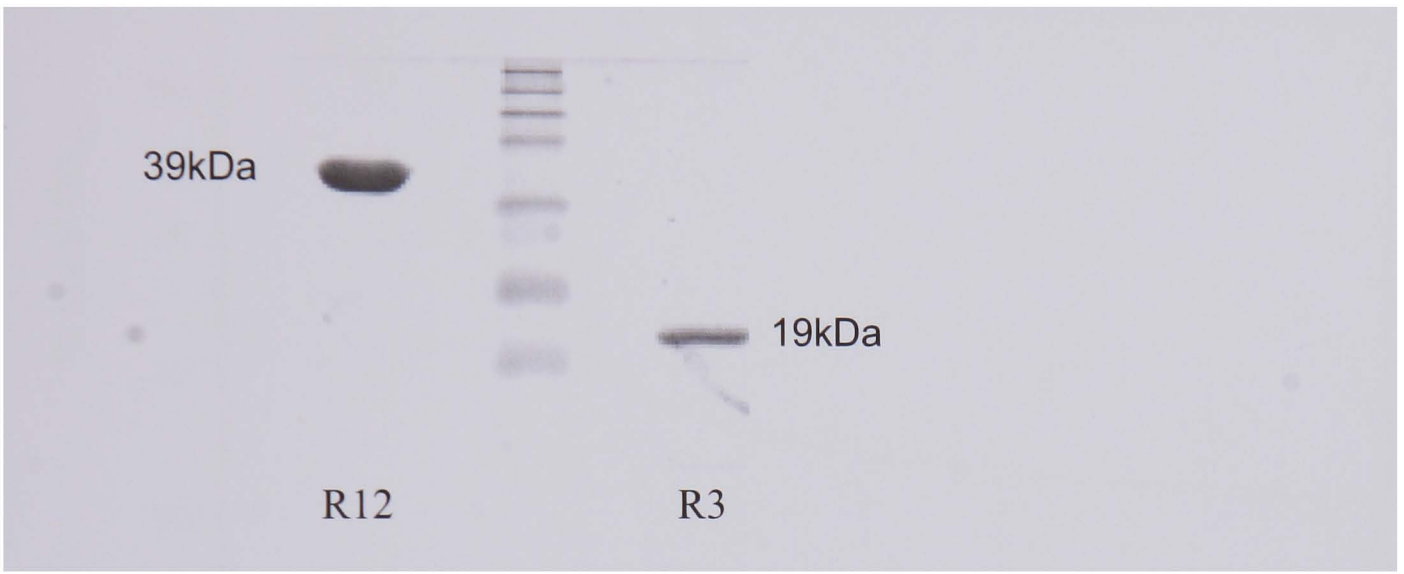


Figure 4.28 16% SDS-PAGE gel showing purified recombinant R12 and R3

4.2.7.3 *Activity of R12 and R3 in the stab test*

The repeat regions R12 and R3 were assayed in the stab test to investigate whether they retained any bacteriolytic activity independent of the amidase and glucosaminidase domains. *E. coli* ER2566 were transformed with pKP10 and pKP11 and stabbed onto plates overlaid with *S. aureus* and *M. luteus*. As positive controls ER2566 cells transformed with pEA3 and pKP2 to express lysostaphin and Atl GL were also stabbed onto the plates. No activity of either binding domain was observed against *S. aureus* or *M. luteus* (data not shown).

4.2.7.4 *Activity of R12 and R3 in the turbidity assay*

To confirm the binding domains of Atl have no independent bacteriolytic activity R12 and R3 were tested in the turbidity assay against *S. aureus* and *M. luteus*. Each of the recombinant proteins was tested at a concentration of 10μM. Neither protein showed any bacteriolytic activity against *S. aureus*

(Figure 4.29) or *M. luteus* (Figure 4.30). Lysostaphin and Atl GL were included as positive controls.

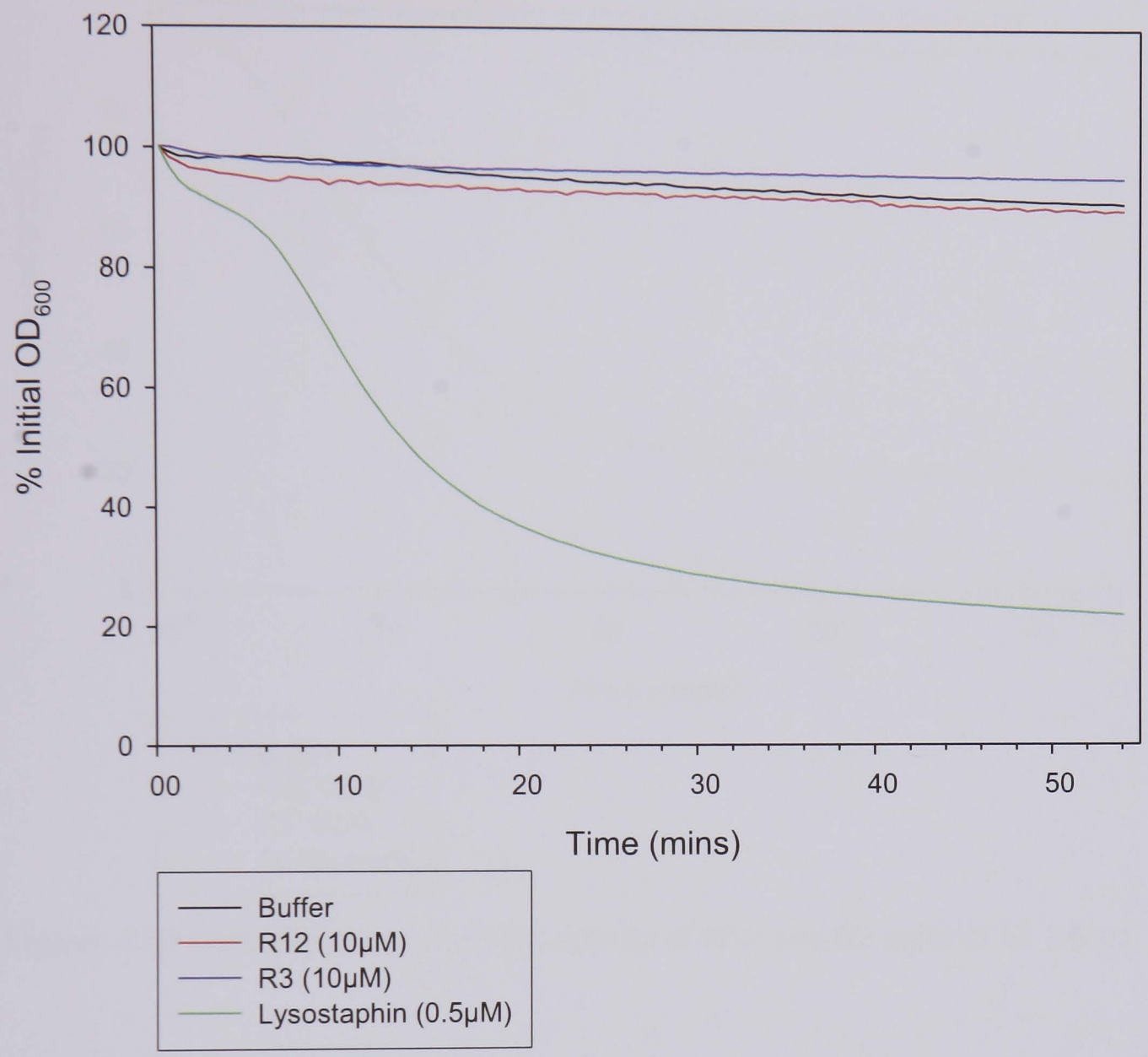


Figure 4.29 Turbidity assay showing activity of R12 and R3 against *S. aureus*

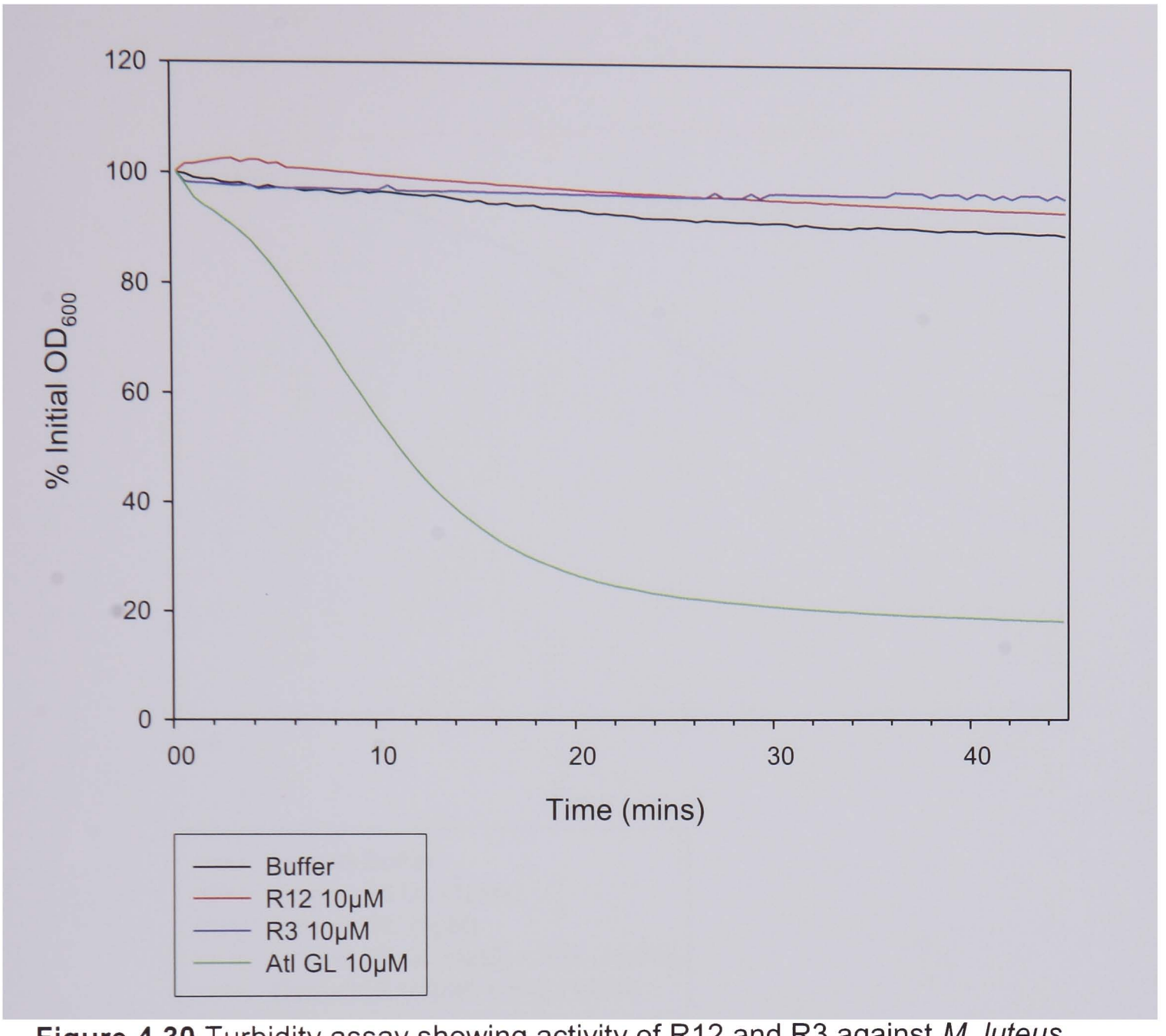


Figure 4.30 Turbidity assay showing activity of R12 and R3 against *M. luteus*

4.2.7.5 *Binding of R12 and R3 to* M. luteus *in competition with Atl GL and GL'*

The purified recombinant binding domains of Atl were tested in the turbidity assay in combination with Atl GL and GL' to investigate binding of the repeat regions to *M. luteus*. In this protection assay variation of the turbidity assay Atl GL was tested at 1μM and GL' at 5μM, the lowest concentrations at which activity could be reliably detected. The binding domains R12 and R3 were added to the same wells at 10μM. Figure 4.31 shows the effect of R12 on the activity of Atl GL and GL'. In the presence of 10μM R12, neither of the recombinant glucosaminidases causes any drop in the optical density of the *M. luteus* cell substrate. R12 provides complete protection to *M. luteus* from both Atl GL and GL'.

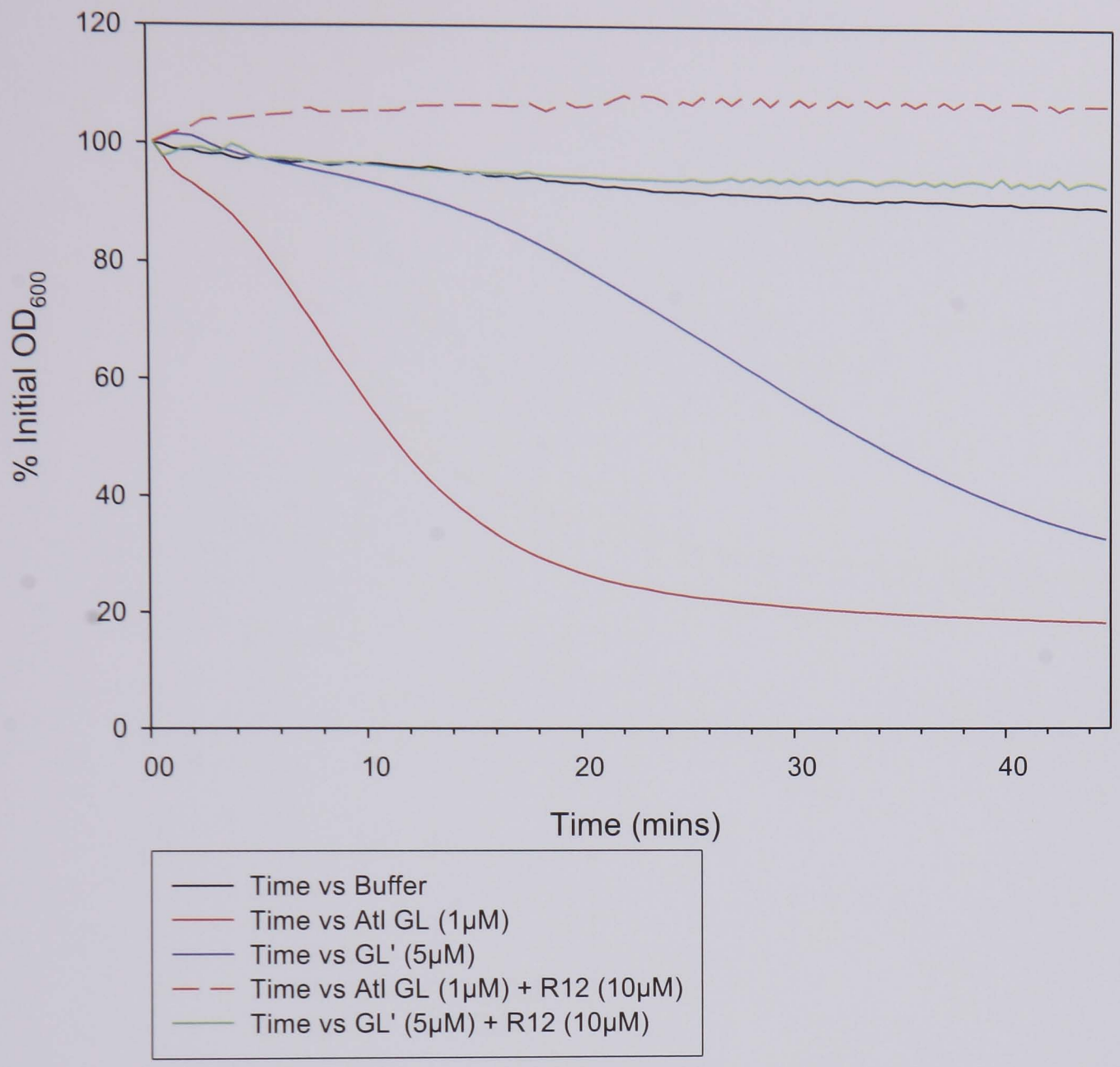


Figure 4.31 Protection turbidity assay showing effect of R12 on activity of Atl GL and GL' against *M. luteus*

Figure 4.32 shows the effect of R3 on the activity of Atl GL and GL' against *M. luteus*. There is no significant difference between the effect of Atl GL on the optical density traces of the cell substrate with or without the presence of 10µM R3. The R3 binding domain therefore provides no protection from the activity of Atl GL to *M. luteus*. The truncated glucosaminidase appears to cause a more rapid drop in the turbidity of the cell substrate in the presence of 10µM R3 than on its own. The R3 domain thus does not protect *M. luteus* from GL', but conversely confers greater bacteriolytic activity to the truncated glucosaminidase.

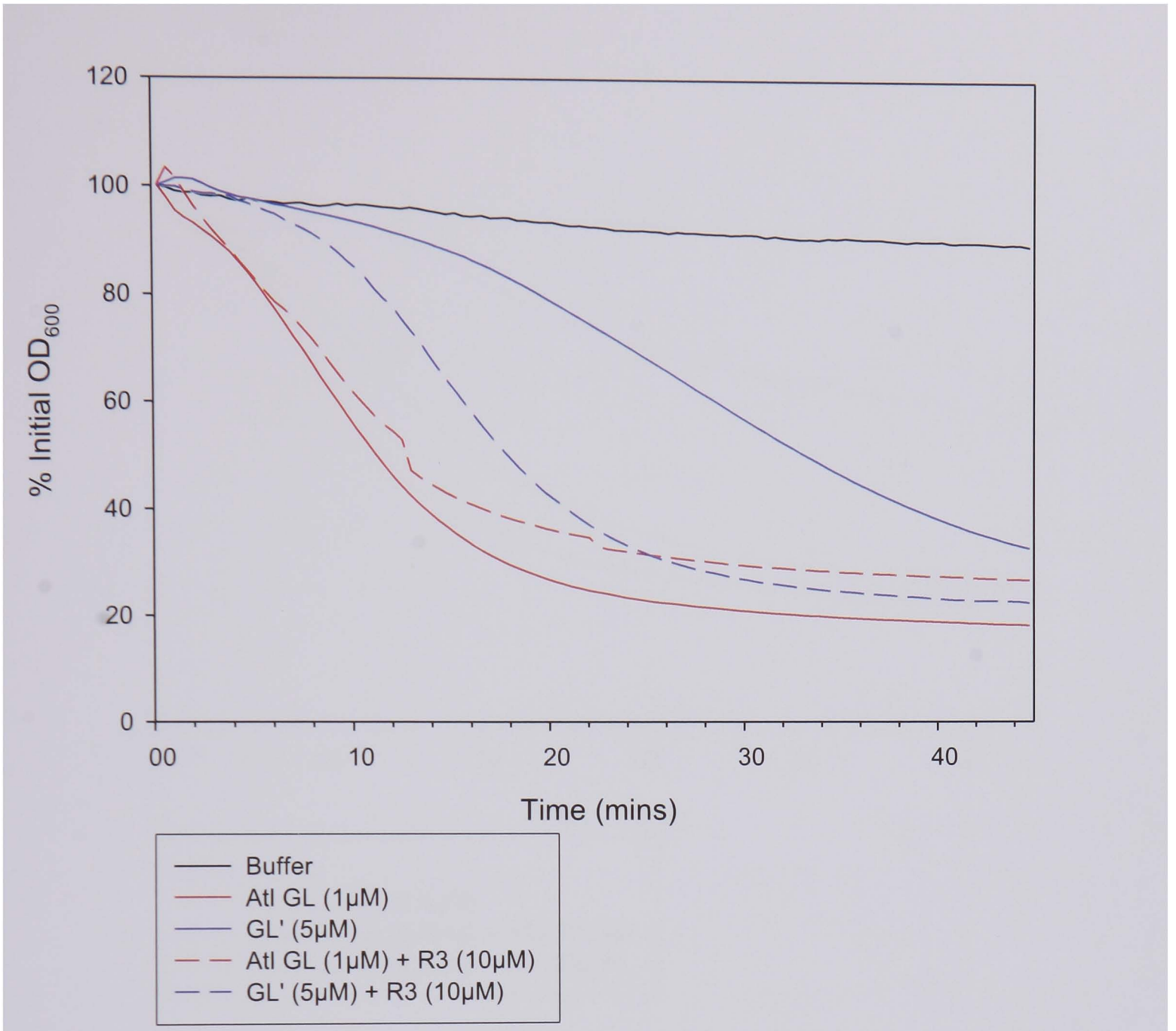


Figure 4.32 Protection turbidity assay showing effect of R3 on activity of Atl GL and GL' against *M. luteus*

4.2.7.6 *Binding of R12 and R3 to* S. aureus *in competition with lysostaphin*

The binding domains of Atl were also tested in a protection assay with lysostaphin to investigate their effect on its activity against *S. aureus*. Lysostaphin was used at 0.5μM in combination with R12 and R3, each at a concentration of 10μM. The R3 domain showed little protective effect for the cell substrate against the activity of lysostaphin (Figure 4.33). The R12 binding domain reduced the rate of decrease in optical density of *S. aureus*. These results were reproduced in a subsequent repeat of the assay. This indicates a partial protective effect of the R12 binding region of Atl against the activity of lysostaphin on *S. aureus*.

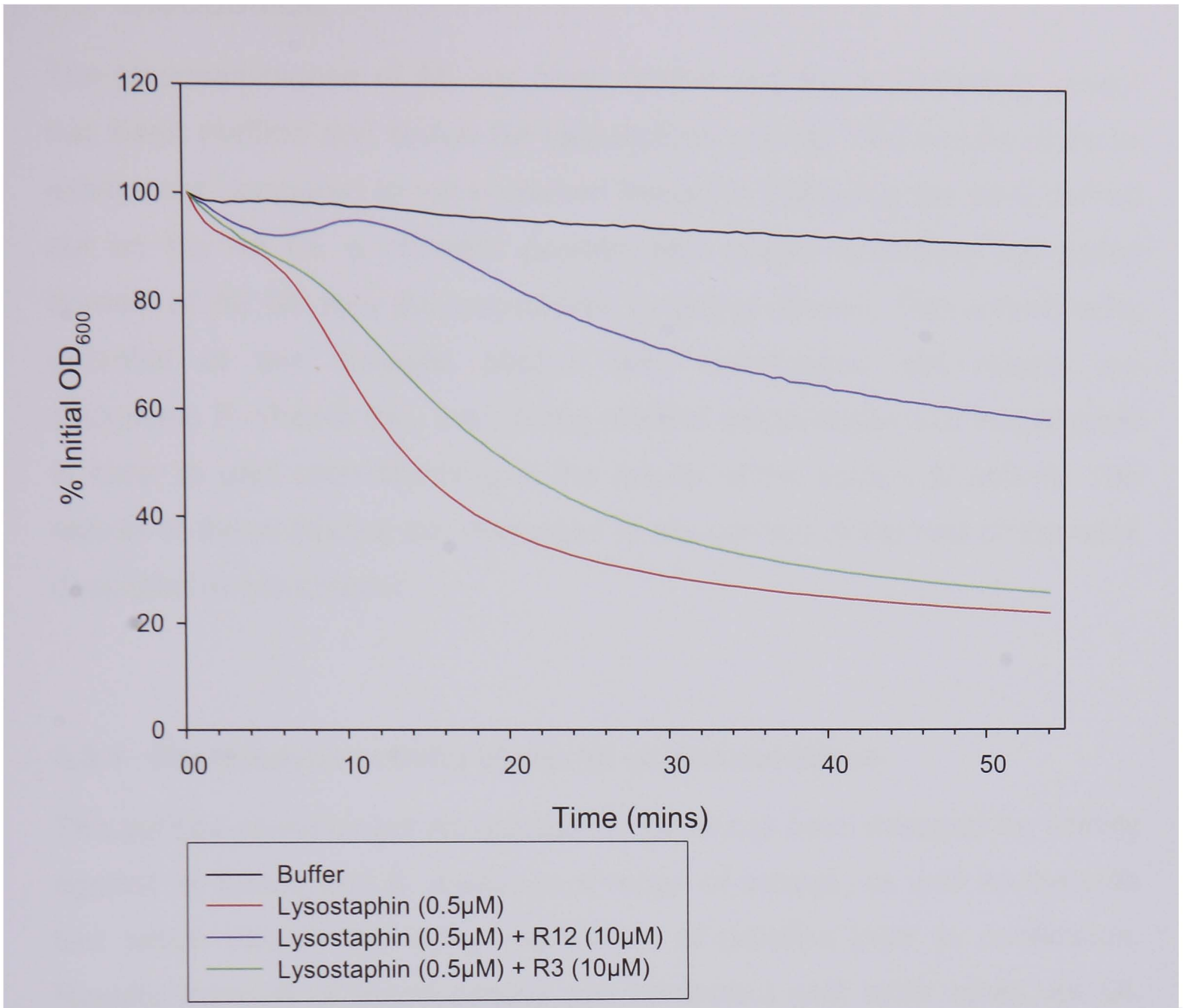


Figure 4.33 Protection turbidity assay showing affect of R12 and R3 on activity of lysostaphin against *S. aureus*

4.2.7.7 *Activity of R12 and R3 in the bioluminescence reporter assay*

The Atl binding domains were also used in the bioluminescence reporter assay described in chapter 5. Neither protein showed any bacteriolytic activity against *S. aureus* RN6390 at the highest possible concentrations of 2.25μM for R12 and 3μM for R3. When tested in a protection assay in combination with 0.5μM lysostaphin neither had any inhibitory effect on its activity (data not shown), suggesting different binding mechanisms for Atl and lysostaphin.

4.3 DISCUSSION

The glucosaminidase of Atl has been cloned and the recombinant protein has been purified and tested for bacteriolytic activity. The results of these assays are compared to the published literature. Following the work carried out on the Atl GL a chimeric peptide was cloned combining the active domain of Atl GL with the lysostaphin targeting domain. The antimicrobial potential of this chimeric peptide was investigated and results are discussed. Further to this, the binding of Atl to cell surfaces was investigated in order to gain understanding of the results of the assays of activity. The results of these studies are discussed in the context of the rest of the work described in this chapter.

4.3.1 Bacteriolytic activity of the Atl glucosaminidase

The purified recombinant Atl glucosaminidase has been assayed for activity against *M. luteus* and *S. aureus* in a range of assays, as well as the stab test which utilises the leaky expression of proteins prior to purification. Results from all of these assays are consistent with each other. Atl GL demonstrates lytic activity against *M. luteus*. In the stab test Atl GL caused a similar sized zone of lysis in the *M. luteus* overlay to that caused in lawns of *S. aureus* by lysostaphin, a highly active anti-staphylococcal peptide. Using the plate based agar diffusion assay, lysis of live cells can be observed at a minimum concentration of 1μM. In the turbidity assay, a liquid assay format, the lytic effect of Atl GL against a *M. luteus* cell substrate can be detected as low as 0.5 μM. These results demonstrate a high degree of activity of the Atl GL against *M. luteus.*

No activity was detected in any of the assay formats against *S. aureus*. Several isolates of *S. aureus* were used in each assay; Mu50, RN6390, and clinical isolates including EMRSA16 and the Oxford staphylococcus. Other staphylococcal isolates including *S. epidermidis*, *S. haemolyticus*, *S. capitis* and *S. sciurii* were also used in the stab test, and no activity of Atl GL was

recorded. The high level of activity seen against *M. luteus* is not observed against *S. aureus*.

These results are consistent with the results of other studies published in the literature. Oshida *et al.* (1995) demonstrated the activity of the glucosaminidase of Atl against heat-inactivated *M. luteus* cells by zymogram analysis, and also showed no activity against *S. aureus*. Activity of Atl GL against heat-killed *M. luteus* and *S. aureus* cells has also been assayed in a turbidity assay with results consistent with those reported in this assay (Sugai *et al.*, 1995). The agar diffusion assay and the stab test described in this chapter represent the first report of the activity of Atl GL against live cells, with the agar diffusion assay result being the first quantifiable assay of the purified recombinant protein.

The bacteriolytic activity of Atl GL against *M. luteus* confirms that it is a suitable candidate antimicrobial peptide for use against Gram positive organisms. However, the lack of activity against *S. aureus* is an obstacle. If the protein has no activity against staphylococcal peptidoglycan then it is of little further interest. Atl GL has been shown to have endo-β-*N*-acetylglucosaminidase activity and will cleave between sugar residues in the peptidoglycan backbone at bonds present in staphylococcal peptidoglycan (Oshida *et al.*, 1995; Sugai *et al.*, 1995). Therefore, if the Atl GL can be correctly targeted to staphylococcal cell walls it may provide an effective antimicrobial agent. Tests against purified staphylococcal peptidoglycan could aid in the determination of this activity.

4.3.2 The chimeric peptide LssT:GL

The approach of fusing two hydrolytic proteins has previously been shown to be successful using lysostaphin and an endolysin from the *Streptococcus agalactiae* bacteriophage B30 (Donovan *et al.*, 2006a). This demonstrates

that separate hydrolases can maintain their activity within a chimeric peptide. This approach was taken a step further by combining lysostaphin's targeting domain with the Atl GL hydrolytic domain, with the aim of targeting the glucosaminidase to staphylococcal cell walls.

In the stab test LssT:GL demonstrated a reduced degree of activity against *M. luteus* and no bacteriolytic activity against *S. aureus*. Reduced hydrolysis of *M. luteus* was anticipated, as the chimeric peptide was cloned without the R3 binding domain from Atl GL. However the presence of the lysostaphin targeting domain failed to confer activity against *S. aureus*. This could be for one or more reasons. As discussed above it is unlikely, but possible, that the Atl glucosaminidase does not have hydrolytic activity against staphylococcal peptidoglycan, and that the presence of the glucosaminidase in *S. aureus* is the result of an evolutionary event from which the amidase of Atl was acquired. However, as discussed earlier the site of the proposed activity of the glucosaminidase is present in *S. aureus*, and furthermore the presence of anti-GL IgG inhibits the penicillin G induced lysis of cells (Sugai *et al.*, 1997b). It may also be possible that the amidase and glucosaminidase of Atl act synergistically *in vivo*, and that alone the activity of Atl GL is insufficient to cause hydrolysis of the host peptidoglycan. This may be part of a regulatory system which has evolved to prevent uncontrolled autolysis of the cell. The study of Sugai *et al.* (1997) on the inhibition of penicillin G induced lysis of *S. aureus* by anti-Ami and anti-GL IgG indicates that the two peptidoglycan hydrolases act synergistically in causing the lysis of cells weakened by penicillin G.

Another explanation for the inactivity of LssT:GL against *M. luteus* is that the lysostaphin targeting domain within the chimeric peptide is incorrectly folded, or that it binds to the staphylococcal cell wall in such a way as the glucosaminidase does not have access to its site of action. If the lysostaphin targeting domain was non-functional due to this, the bacteriolytic activity

observed against *M. luteus* would not be due to the chimeric design of the peptide. Subsequent results of binding studies using GFP-tagged lysostaphin targeting domain demonstrated that the LssT is unable to bind to *M. luteus* cells, and also that the truncated glucosaminidase alone, lacking any recognised cell binding domain, is able to cause a similar degree of bacteriolysis against *M. luteus.*

A positive outcome from this work is that the attachment of the lysostaphin targeting domain to the N-terminus of the Atl GL does not prevent the correct folding of the glucosaminidase into its active form. This indicates that the future design of chimeric peptides combining a peptidoglycan hydrolase with lysostaphin's targeting domain remains a viable strategy for the design of novel antimicrobial peptides.

4.3.3 The truncated glucosaminidase

The truncated glucosaminidase, comprising only the C-terminal active hydrolytic domain, was cloned to investigate whether the N-terminal R3 binding domain is essential for bacteriolytic activity against *M. luteus*. In the stab test GL' was found to have a similar degree of activity against *M. luteus* to the LssT:GL chimera. The purified protein was also shown to be active against *M. luteus* in all of the assays tested, although less active than the full size glucosaminidase. Using the agar diffusion assay the minimum concentration of GL' which caused an inhibition in the growth of the bacterial overlay was 25μM.The turbidity assay required a concentration of 5μM GL' to cause a notable effect. These results show that without the R3 binding domain the Atl GL is 5-10 fold less active against *M. luteus*. The residual activity of GL' in the absence of a recognised cell binding domain may be explained by the action of a highly active peptidoglycan hydrolase forming interactions with the cell surface. A study on the AtlE autolysin of *S. epidermidis,* which is closely related to Atl, has shown that the cell wall lytic activity of the amidase domain is only slightly reduced with the removal

of the R1and R2 repeat regions, whilst the R1 and R2 domains exhibit a good binding affinity to peptidoglycan (Biswas *et al.*, 2006). This mirrors the activity of the truncated glucosaminidase observed in the work described in this chapter. This work also shows that this binding is able to cause cell death in the absence of a separate binding domain.

GL' shows no bacteriolytic activity against *S. aureus*. This is consistent with previous results, as the lack of a recognised cell binding domain could not be expected to improve binding to staphylococcal cell surfaces.

The full size Atl GL and the truncated protein are both soluble proteins which are stable when stored at -80°C. The solubility and stability of these proteins is an important feature in confirming the suitability of Atl GL as a candidate peptide, as previously discussed in chapter 3. However, before Atl GL could be further considered as a suitable candidate, greater understanding of its cell binding is required, and this knowledge exploited to better target the peptide to staphylococcal peptidoglycan.

4.3.4 Binding of Atl GL and GL' to cells

Atl GL and the truncated glucosaminidase were each tagged with GFP to enable the visualisation of their binding to cells, and determine any differences between the two proteins. Problems of protein solubility and stability were experienced, but sufficient soluble, fluorescent protein was purified to allow binding assays to be carried out. However, no fluorescent protein was observed to bind to either *M. luteus* or *S. aureus* cells. Either the GFP tagged protein was unable to bind to the cells or the number of receptors on the cell surface is insufficient for enough protein to bind to the cell to be visualised by phase contrast microscopy.

Previous electron microscopy studies have shown that Atl binds at the site of nascent septal rings, prior to cell division *in vivo*, and that the repeat regions of Atl are responsible for targeting the peptidoglycan hydrolases to this region (Yamada *et al.*, 1996; Baba *et al.*, 1998b). This may indicate that the GFP tagged GL is binding to the site of cell division and that too little protein is binding to the cell to be visualised in this way. The truncated glucosaminidase, lacking the repeat region that is believed to target it to the septal ring, may also have less specific, reduced binding which cannot be visualised in this way.

The activity of the GFP tagged proteins against *M. luteus* in the stab test demonstrate that the tagged proteins are able to bind to cell surfaces, and that the GFP tag does not impede the attachment of the proteins to their target site. However, the purified proteins were found to be poorly soluble. The observation of fluorescent aggregates under the microscope suggests that the tagged proteins were precipitating out of solution during the cell binding assays. The precipitation of protein during the assay thus reduces the amount of protein available to bind to cells, and this could also explain the lack of binding observed. Alternative experiments were thus carried out to gain an understanding of the binding of the Atl amidase.

4.3.5 The amidase of Atl

The amidase domain of Atl, complete with R1 and R2, was cloned and tested for activity against *M. luteus* and *S. aureus* in the stab test. No activity was observed against *M. luteus*. The amidase was active against *S. aureus*, although the small zone of lysis indicates that the bacteriolytic activity is significantly weaker than that of lysostaphin, and the level of activity observed for the Atl GL against *M. luteus*. These results concur with studies previously published in the literature (Oshida *et al.*, 1995; Sugai *et al.*, 1995). Work on the amidase of the closely related autolysin AtlE from *S. epidermidis* has also demonstrated activity of the purified recombinant

protein against *S. aureus* peptidoglycan (Biswas *et al.*, 2006). The purification of the Atl amidase described in this chapter demonstrated that the protein is poorly soluble, and as a result could only be tested at low concentrations in the turbidity assay and the bioluminescence reporter assay. The bioluminescence reporter assay showed that a 2.7μM concentration of Atl amidase caused a slight inhibition in the metabolism of live, growing *S. aureus* RN6390 cells. However, in comparison with lysostaphin, the inhibition of *S. aureus* was very weak. Lysostaphin at 0.5μM caused a near complete abolition of bioluminescence.

The amidase of Atl was not investigated further as a candidate antimicrobial peptide due its poor solubility, and its relatively low level of bacteriolytic activity. Its ability to target *S. aureus* cells is of interest, and may be exploited in the design of alternative antimicrobials.

4.3.6 The extended glucosaminidase R123GL

As the amidase of Atl has been shown to be active against *S. aureus,* the R1 and R2 binding domains of Atl, which remain at the C-terminus of the amidase *in vivo*, were cloned with the Atl GL to generate R123GL. The aim was to investigate whether the addition of these binding domains would enable the glucosaminidase to bind to *S. aureus* and cause cell lysis. The R123GL was tested in the stab test against *M. luteus* and *S. aureus*. No activity was observed against *S. aureus,* showing that the presence of the additional binding domains is insufficient to confer activity of GL against *S. aureus*. This suggests that the inactivity of Atl GL may not be due to poor cell binding, but that the glucosaminidase alone is unable to cause lysis of staphylococcal cell walls.

The R123GL showed reduced activity against *M. luteus* compared to the Atl GL in the stab test. A possible explanation for this is that the large size of the protein inhibits diffusion of the released protein through the overlay. Alternatively, the reduced zone of lysis may be due to insolubility of the

protein. Poor expression of the protein was observed in liquid cultures and it was not possible to purify the recombinant protein. Without purified protein the activity of R12GL could not be investigated further in the other assays. Due to these problems R123GL was not considered further as a candidate antimicrobial peptide.

4.3.7 The repeat regions of Atl

The repeat regions of Atl, shown to target the enzymes to the cell surface (Baba *et al.*, 1998b), were cloned as a method of investigating the binding of the amidase and glucosaminidase. The R1 and R2 repeat regions were cloned together to represent the binding domain of the Atl amidase to *S. aureus* cells, and R3 was cloned alone as the binding domain of the glucosaminidase to *M. luteus* cells. Initial tests of the purified proteins confirmed that the repeat regions have no bacteriolytic activity. Protection assays were then performed to investigate whether they would compete for binding sites with the glucosaminidases and lysostaphin. Similar work on the related autolysin Aas of *Staphylococcus saprophyticus* has shown that its repeat regions are able to inhibit the lysis of a heat-killed cell substrate by the amidase domain of Aas (Hell *et al.*, 2003).

The R12 binding domains demonstrated a protective effect for *M. luteus* against lysis by Atl GL and GL'. This indicates that the R12 repeat regions target the same cell surface receptor as the R3 repeat region and the glucosaminidase. This may be anticipated from the high degree of similarity between all three of the repeat regions. The inactivity of the glucosaminidase alone against *S. aureus* is therefore unlikely to be due to the inability of the R3 binding domain to bind to its cell surface. The lower activity of Atl GL may be due to the *O*-acetylation of *N*-acetyl-muramic acid residues which occurs in *S. aureus* but not *M. luteus.* This has been shown previously for other hydrolases with glucosaminidase activity (Schleifer & Steber, 1974). The results of this assay and those of previous studies suggest that the role of Atl GL is in synergy with the amidase domain during the regulated localised hydrolysis of peptidoglycan during cell division.

Interestingly, the R3 repeat region did not have a protective effect from the activity of the glucosaminidases on *M. luteus*. Conversely, it appeared to restore lytic to the truncated glucosaminidase. This suggests that the R3 domain is able to form an interaction with GL' and increase the degree of binding of the truncated glucosaminidase to the cell surface. Any interaction of R3 with the full size Atl GL does not promote a higher degree of bacteriolytic activity. Although R12 and R3 target the same cell surface receptors as shown above, R3 appears to be able to form a positive interaction with the glucosaminidase, whilst the R12 does not. This may reflect the organisation of the Atl domains *in vivo*.

The results of the protection assays of R12 and R3 against the effect of lysostaphin on *S. aureus* provide contradictory results. R3 provides no protection to the *S. aureus* cells whereas R12 appeared to afford some protection against the activity of lysostaphin. The lack of protection by R3 suggests that lysostaphin and the Atl hydrolases target different cell surface receptors or have different recognition factors and bind to the same receptor in a different way. As R12 and R3 are likely to share a common receptor, the partial protection against lysostaphin by R12 seems unusual, and may be explained by R12 and R3 also having different recognition factors. Lysostaphin's and R12's recognition factors may be similar, and this could explain the partial protection by R12 against lysostaphin. Alternatively, it may be that the larger size of the two repeat regions of R12 plays a role in this protection effect by steric hindrance.

As the above evidence indicates that lysostaphin and R3 have different cell surface receptors or recognition factors, the design of the LssT:GL chimeric peptide is likely to have altered the binding of the glucosaminidase to staphylococcal cell surfaces. However, either the glucosaminidase was unable to access its site of action, or the protein is not able to hydrolyse staphylococcal peptidoglycan alone, as discussed earlier.

4.4 SUMMARY AND FUTURE EXPERIMENTS

The activity and binding of the Atl amidase and glucosaminidase to *M. luteus* and *S. aureus* has been investigated. The results of this study show that the glucosaminidase has bacteriolytic activity against live *M. luteus* cells, and the amidase has a lower degree of activity against *S. aureus*. These results concur with the published literature and also support the hypothesis that the GL acts in synergy with the amidase *in vivo*. The R12 binding domain of the amidase and the glucosaminidase's R3 domain are targeted to the same receptor on the cell surface, and this is a different receptor or has different recognition factors to that of lysostaphin. Due to its low level of activity against *S. aureus* the Atl glucosaminidase is not a suitable candidate as a novel antimicrobial peptide. The repeat regions may be suitable binding domains to be incorporated into a chimeric peptide, although further work is needed to investigate whether the localisation of the peptide at the septal region might limit the lytic activity of the chimeric peptide.

There are several further experiments that could be carried out to investigate the activity and binding of Atl GL further. The nature of the resistance of *S. aureus* to Atl GL could be examined to determine whether an altered Atl GL may be an appropriate candidate peptide. This could be through the cloning of *S. aureus* genomic DNA into *M. luteus*, and screening the recombinant clones for resistance. Alternatively a *S. aureus* transposon library could be screened for sensitivity to Atl GL. These methods could enable the identification of genes responsible for resistance, and provide useful information in the development of an altered GL.

Experiments could also be performed to create alternative chimeric peptides incorporating the Atl glucosaminidase. Peptidoglycan recognition proteins (PGRPs) are proteins produced by many organisms in the response to bacterial infections. These may provide suitable alternative targeting domains, and may broaden the spectrum of hydrolases such as Atl GL, if combined in a chimeric peptide.

Experiments may also be carried out to provide further understanding of the nature of the binding of the Atl repeat regions. The combination of R12 with the lysostaphin or LytM endopeptidase may reveal altered cell specificity, and may also provide further evidence that by altering the targeting domain of an antimicrobial peptide, the bacteriolytic activity can be changed. An R12:LssE chimera could also be screened in the bioluminescence assay to confirm whether R12 and the lysostaphin targeting domain have different cell surface receptors.

In addition to the assays of antimicrobial activity used throughout this assay, experiments should be performed using purified staphylococcal peptidoglycan. This would help to determine the mechanisms of activity, and the target sites of the Atl hydrolases, and such information would be valuable in the exploitation of these proteins as potential novel antimicrobials.

5. Development of a bioluminescence reporter assay to measure the effect of antimicrobial peptides on the growth of *S. aureus*

5.1 INTRODUCTION

5.1.1 Assays of antimicrobial activity used in the literature

Several assays of activity of peptidoglycan hydrolases have been used through chapters 3 and 4 of this thesis, and others have been used in other studies published in the literature. These have been developed with various aims including understanding mechanisms and sites of action as well as assessing the use of potential antimicrobials. These assays each have relative merits and disadvantages for the aims of this thesis in the investigation of novel antimicrobial peptides as potential antimicrobial agents. Some of these are discussed below, and the ideal assay format is considered.

5.1.1.1 *Zymogram assays*

Many of the studies reported in the literature use zymogram analysis to demonstrate the activity of peptidoglycan hydrolases. These included many of the published studies of the Atl autolysin of *S. aureus* previously discussed in chapter 4 (Oshida *et al.*, 1995; Sugai *et al.*, 1995; Foster, 1995; Biswas *et al.*, 2006). Briefly, the zymogram comprises running proteins of interest down an SDS-PAGE gel containing heat killed cells as enzyme substrates. Gels are then washed and incubated in phosphate buffer to re-nature the electrophoresed proteins (Heilmann *et al.*, 1997). Lysis of the bacterial cell wall substrate leaves a clear area in the opaque gel, indicating which protein bands have lytic activity against that substrate. This assay format is very useful in the identification of hydrolytic proteins amongst several proteins isolated from a bacterial culture of interest. It is also useful as a relatively simple assay format that can assess activity from the lysate of

cells over-expressing the protein without the need for purifying the recombinant protein. However, the assay is not useful in the screening of peptides as potential therapeutic antimicrobial agents. The assay utilises heat-killed cells, thus not being representative of either their *in vivo* activity or as a model of antimicrobial therapy. Furthermore, the assay is not quantitative, and thus differences in the level of activity of peptides are difficult to distinguish. Zymogram assays were not used in the course of this project for these reasons.

5.1.1.2 *Turbidity assays*

Liquid turbidity assays have also been used in several of the studies previously discussed, as well as a variant being used in this project. These may use peptidoglycan preparations (Biswas *et al.*, 2006), heat killed cells (Sugai *et al.*, 1995), or live cells (Donovan *et al.*, 2006b). The variation used in this study uses a lyophilised cell substrate and is described in chapter 2. This is a versatile assay in which purified recombinant proteins or cell lysates can be tested. If purified protein is available the amount added can be quantified, and the effect on turbidity can also be measured. The use of live cells is commonly reported in the analysis of bacteriophage enzymes, often showing the percentage survival of cultures after a set period of incubation (Takáč *et al.*, 2005a; Yoong *et al.*, 2006), although studies showing the decline of turbidity against time have been published (Schuch *et al.*, 2002; Pritchard *et al.*, 2004; Grundling *et al.*, 2006b). However, the use of live cells in turbidity assays has been shown to produce variable results between assays (Donovan *et al.*, 2006b; Kessler *et al.*, 2004; Kusuma *et al.*, 2005). Live cells were not used in turbidity assays through this project. In common with using peptidoglycan or heat killed cells, the use of heat killed cells may not be representative of the therapeutic activity of a protein.

5.1.1.3 *The stab test*

The stab test was used in the work described in this thesis as a rapid initial assay to detect antimicrobial activity of recombinant clones. The leaky expression of plasmid encoded recombinant proteins by *E. coli* ER2566 is utilised by chloroform lysing the cells, which releases these proteins onto the surrounding agar, where their activity against overlaid cells can be detected. This provides a tool for detecting whether a protein has antimicrobial activity before the over-expression and purification of the protein has been carried out. This is useful for the analysis of proteins that are difficult to over-express or purify. This assay is also a test of activity against live cells, demonstrating that the level of peptidoglycan hydrolysis is sufficient to cause cell lysis and death. However, the stab test is only used as an initial test as it is hard to quantitate and may not detect low levels of peptidoglycan hydrolysis. Large proteins may also provide artificially reduced zones of lysis, due to a lower rate of diffusion through the overlay agar. Furthermore, proteins are also released from the *E. coli* host. Controls show that *E. coli* ER2566 carrying a pET21 plasmid does not cause any cell lysis alone in the stab test, but it is not possible to discount the possible effect of any interaction between host proteins and the recombinant protein of interest. This is a problem not only with the stab test but also with other assays using unseparated proteins from cell lysate.

5.1.1.4 *The agar diffusion assay*

The agar diffusion assay is a plate based assay against live cells that has been used in this project. Use of a similar assay has been reported in the literature by Schuh *et al.* (2002). It can be used to rapidly screen for bacteriolytic activity against a range of organisms. The assay is semi-quantifiable, as a wide range of concentrations can be tested simultaneously, providing an initial indication of the level of activity of a protein. This information may then be used in choosing the concentration range for a liquid assay format. The use of a liquid assay format is required as the effects of proteins are poorly quantifiable, with little detectable

difference in the zones of lysis at concentrations above the minimum inhibitory concentration. A study comparing methods of assaying lysostaphin reports that a similar disc-diffusion assay produces the most reliable simple assay results, but that further measurements are required, as increases in concentration fail to produce correlating increase in zones of lysis due to poor diffusion of the protein (Kusuma *et al.*, 2005). Unlike the stab test, which is a similar assay format, any bacteriolytic activity observed may be attributed to the protein solution dropped onto the plate, providing the correct controls have been performed.

5.1.1.5 *Microscopic studies of activity*

The visible effect on cells exposed to peptidoglycan hydrolases can be performed using microscopic analysis. This has shown the dispersal of cluster forming mutant cells by autolysins (Sugai *et al.*, 1995), and the effect of producing null mutants of autolysins (Takahashi *et al.*, 2002; Biswas *et al.*, 2006). These studies provide valuable information about the *in vivo* role of proteins, particularly autolysins which do not cause host cell lysis. However, they are neither rapid nor simple methods suitable for testing the bacteriolytic activity of recombinant proteins against a range of bacterial species.

5.1.1.6 In vivo *assays*

The use of animal models in *in vivo* assays of antimicrobial activity provides the most representative assay format for the study of potential therapeutic agents. These assays are suitable for well characterised, highly active antimicrobial peptides such as lysostaphin and bacteriophage hydrolases such as PlyG active against *Bacillus anthracis* (Patron *et al.*, 1999; Schuch *et al.*, 2002). However, the use of animal studies in the early stages of investigating a thus far poorly characterised candidate antimicrobial peptide is unjustified. Furthermore, such assays are unsuitable for measuring small

differences whilst optimising the activity of recombinant antimicrobial peptides.

5.1.1.7 *Features of an ideal assay format*

The ideal assay for measuring the activity of candidate antimicrobial peptides will be quantifiable, reliable, simple, rapid and representative of the activity of the protein against live cells. A liquid assay format is most easily quantified as both the concentration of proteins can be controlled, and their effect measured. Live cells provide a representative assay of activity, but as has previously been discussed provide variable results in the turbidity assay. Therefore, the ideal assay will be a liquid assay using live cells and utilising a sensitive reliable reporter of the effect of added proteins on cells.

5.1.2 A bioluminescent growth reporter

A dual GFP – lux reporter under the control of a growth dependent promoter has been developed in *S. aureus* (Qazi *et al.*, 2001). The *luxCDABE* operon of *Photorhabdus luminescens* is poorly expressed in *S. aureus*, and was modified to introduce the *lux* sequences *luxABCDE* downstream of the *gfp* gene under the control of the *xylA* promoter from *Bacillus megaterium*. Bacterial luciferase uses reduced flavin mononucleotide as an energy source and requires live cells for signal generation. The Lux proteins also have a short half life, allowing emitted bioluminescence to be measured in real time. The *xylA* promoter of the reporter is only expressed in actively growing, log phase cultures, and bioluminescence readings fall after this phase (Qazi *et al.*, 2001). The P_{xylA}*::gfp-luxABCDE* reporter in *S. aureus* has been used to visualise the invasion of cells by *S. aureus* and to monitor in real time their intracellular replication (Qazi *et al.*, 2001; Qazi *et al.*, 2004). This reporter presents an opportunity to develop a novel assay to measure the antimicrobial activity of proteins. Novel peptides could be added to growing cultures of *S. aureus* and the bioluminescence of the culture measured as an indication of the effect of the added peptide on the

metabolic state of the cells. This may provide a sensitive assay of antimicrobial activity against live cells in a liquid format. Such an assay would be a valuable tool in the development of novel antimicrobial peptides.

5.1.3 Aims of the chapter

This chapter describes the development of a novel assay to investigate the antimicrobial activity of proteins against *S. aureus*. The assay is used to screen several previously defined peptides and results compared with those from other assays. Other novel peptides are assayed to determine whether the assay is sensitive enough to detect small, previously unquantified differences in activity. Future applications of the assay are considered.

5.2 METHODS

Additional general methods to those described in chapter 2 are described below. Specific methods used during the course of developing the assay are discussed in the results section.

5.2.1 Bacterial strain and plasmid

S. aureus RN6390 was used in all assays (Novick, 1991).

Plasmid pSB2030 (P_{xylA}::*gfp-luxABCDE*) was used in developing the assay (Qazi *et al.*, 2001). The plasmid also confers resistance to chloramphenicol.

S. aureus RN6390 (pSB2030) was a gift from Saara Qazi, University of Nottingham.

5.2.2 Growth media and conditions

S. aureus RN6390 carrying pSB2030 was grown in LB broth or on agar plates supplemented with 5μg ml^{-1} chloramphenicol. Plates and liquid cultures were incubated at 37°C, with broths being shaken at 200rpm. Cells were stored in 10% glycerol at -80°C.

5.2.3 Selection of bioluminescent cells

Fresh cultures were made from glycerol stocks for each experiment. Colonies were screened for bioluminescence to ensure maintenance of the reporter using a Hamamatsu VIM3 intensified video camera (Hamamatsu Photonics).

5.2.4 Measurement of bioluminescence in assays

Cultures of *S. aureus* RN6390 (pSB2030) were grown, and bioluminescence measured, in black sided 96-well plates in a Wallac Victor^2 multi-channel

counter at 37°C. During the initial lag phase of culture growth bioluminescence was measured at 8 minute intervals. 200µl starting volumes were used during assays of antimicrobial activity and bioluminescence recordings were taken at intervals of approximately 35 seconds, with a shaking step between measurements. All measurements were made in triplicate.

5.3 RESULTS

5.3.1 Development of the bioluminescence reporter assay

5.3.1.1 *Validation of use of* S. aureus *RN6390 in assays*

Prior to the development of the bioluminescence reporter assay *S. aureus* RN6390 had not been used in the assays of antimicrobial activity described in this thesis, although it had previously been used in assays of activity of antibiotics against internalized *S. aureus*, and lysostaphin had been used remove planktonic cells in these assays (Qazi *et al.*, 2001; Qazi *et al.*, 2004). *S. aureus* RN6390 was tested using the agar diffusion assay, demonstrating that is susceptible to lysostaphin and resistant to the Atl glucosaminidase in common with *S aureus* Mu50, EMRSA16 and the other strains tested (Figure 5.1). A 5µl drop of 1µM lysostaphin produced a similar sized zone of lysis against *S. aureus* RN6390 and *S. aureus* Mu50.

Figure 5.1 Agar diffusion assay showing activity of lysostaphin and Atl GL against *S. aureus* RN6390

5.3.1.2 *Initial inoculum of cultures*

As cultures are grown in 96-well plates it is not appropriate to inoculate the broth in each well with a single colony as this would be both impractical and, introduce variation between wells. LB broth was inoculated with 16 hour overnight cultures of *S. aureus* (pSB2030) and grown in the Victor^2 multi-channel counter at 37°C over a period of 7½ hours. A range of inoculum sizes were used and bioluminescence was monitored to determine the optimum conditions under which the log growth phase is reached as quickly as possible, whilst ensuring a reliable emission of bioluminescence (Figure 5.2). As anticipated cultures receiving higher initial inocula emitted bioluminescence earlier than cultures from a low inoculum. However, a tenfold dilution causes a high initial spike of bioluminescence, followed by a second peak of 630 CPS after approximately 90 minutes. A one in one hundred dilution of the overnight culture reached mid-log phase after 4 hours reaching a slightly lower peak of 550CPS after 4 hours. Further assays were performed using an initial dilution of overnight culture of one in one hundred.

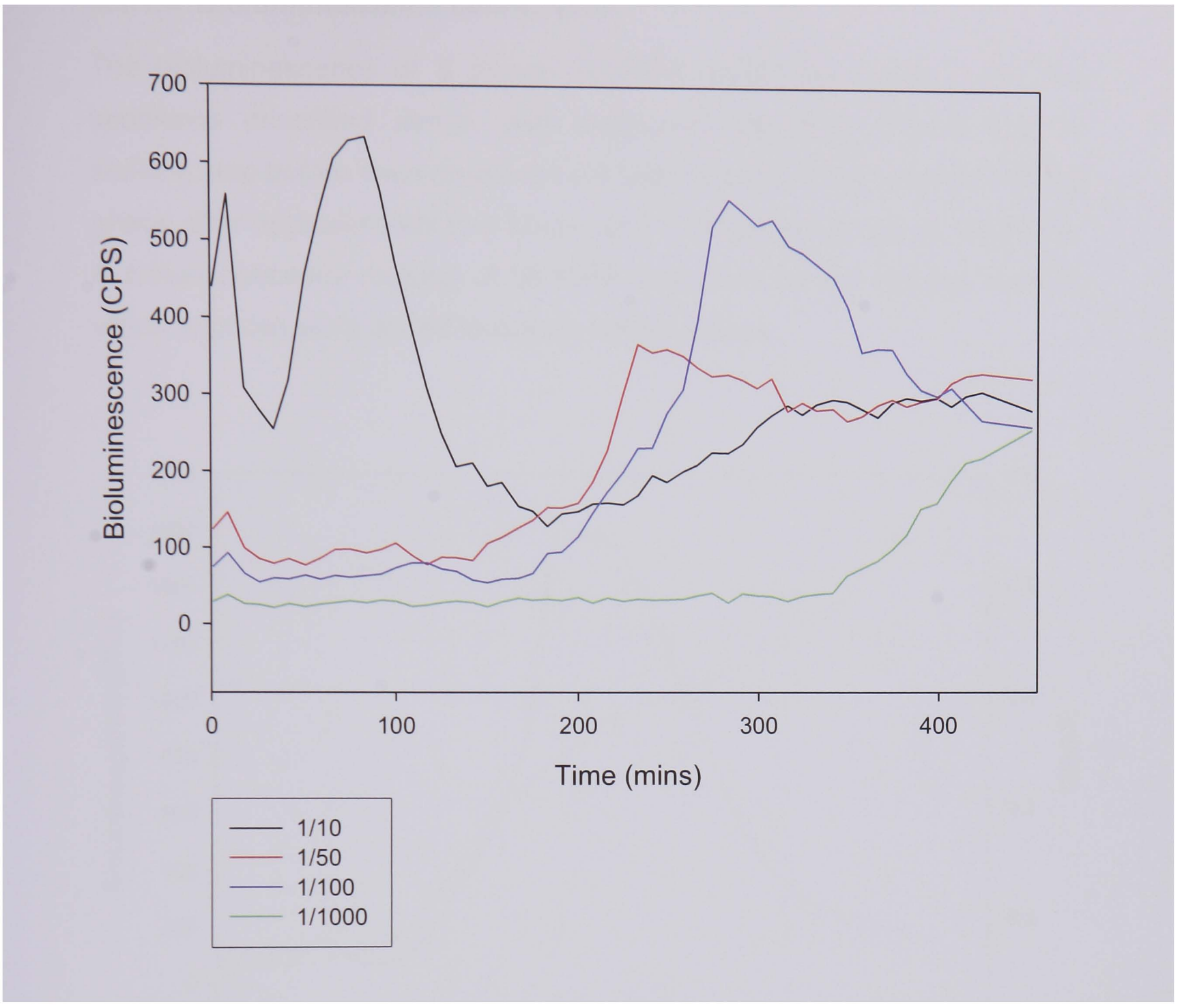


Figure 5.2 Effect of initial dilution of inoculum on bioluminescence of S. aureus RN6390 (pSB2030)

5.3.1.3 *Antibiotic concentration*

The effect of the concentration of chloramphenicol in the medium was investigated. The pSB2030 plasmid is selected for using a concentration of 5μg ml^{-1}. This was used as the highest concentration tested, alongside concentrations of 1μg ml^{-1} and 3μg ml^{-1} to determine whether lower concentrations of chloramphenicol affected the rate at which cells entered the log growth phase, and also whether the level of expression was altered. No differences were observed between the bioluminescence of cultures grown under any of these antibiotic concentrations (data not shown). Further assays were performed using a chloramphenicol concentration of 5μg ml^{-1}.

5.3.1.4 *Bioluminescence time-course*

The bioluminescence of *S. aureus* RN6390 (pSB2030), grown under the conditions described above, was measured over time (Figure 5.3). A shaking step before each measurement was included. Cells reached mid log phase after approximately four hours, and a bioluminescence of ~500CPS. A bioluminescence reading of 500CPS was used as the mid-log point at which peptides were added to cells in further assays.

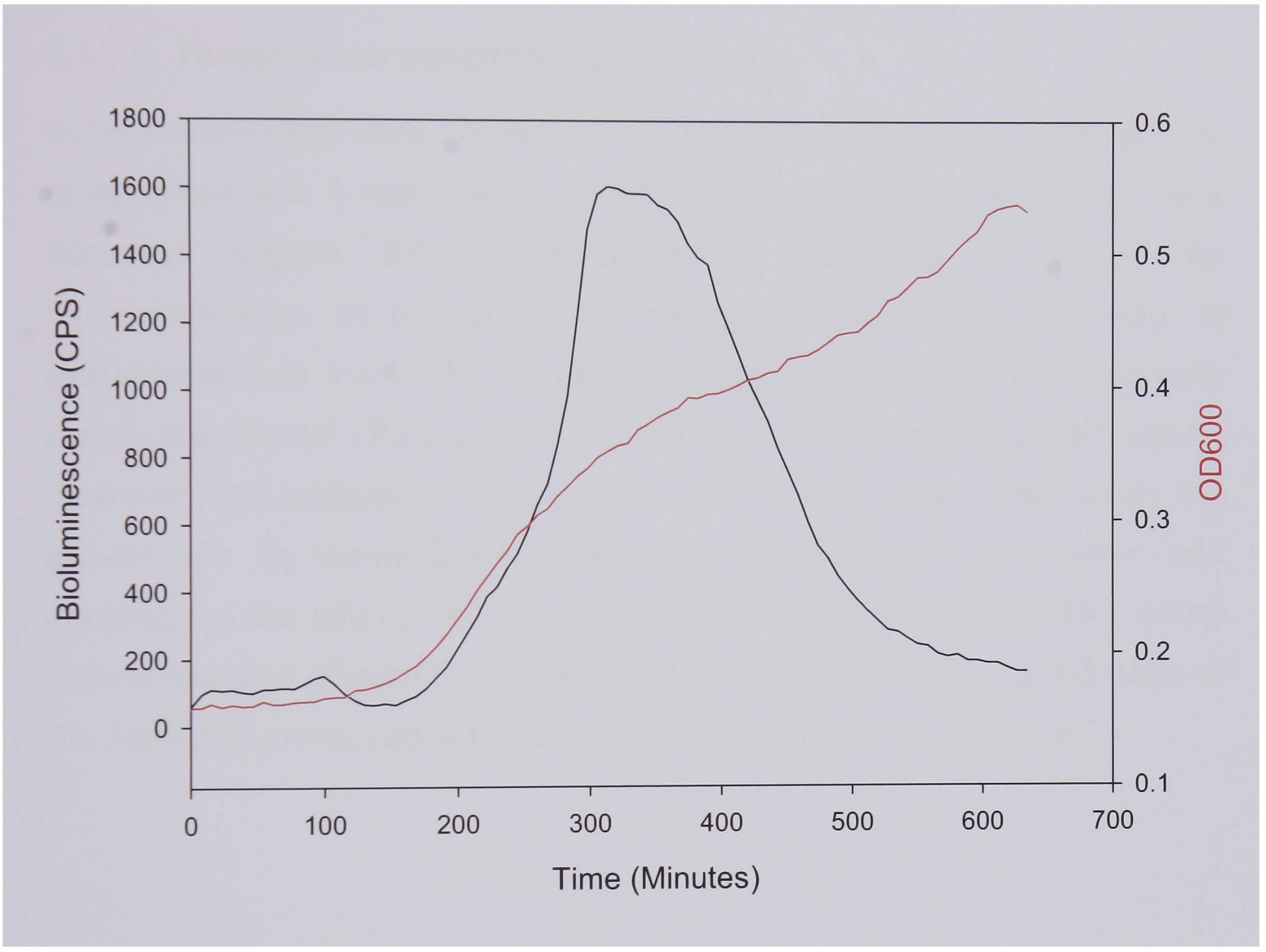


Figure 5.3 Time-course of *S. aureus* RN6390 (pSB2030) bioluminescence

5.3.1.5 *Addition of peptides to cells*

The lux reporter is only expressed in actively growing, log phase cultures. It is therefore important to minimise any non-specific effects of adding peptides to the cultures.

In order to accommodate the addition of recombinant peptides to the cells experiments were carried out to determine the maximum volume of protein

and buffer that may be added to cells without causing a significant effect on bioluminescence. As recombinant proteins to be tested may be at low concentrations, this could represent a significant dilution of the culture. An addition of 30µl of buffer in a final volume of 200µl was found to be the optimum volume (data not shown), allowing a significant quantity of protein to be added without a total loss of lux activity.

5.3.1.6 *Temperature sensitivity of the assay*

Initial experiments were carried out adding cold solutions of lysostaphin to growing cultures. A rapid decline in the bioluminescence of the cultures was observed (Figure 5.4). However, there was also a lag in the bioluminescence of the buffer control cells. This background drop in bioluminescence masks the full effect of the added protein. This is partially due to the dilution effect seen in the addition of peptides discussed above. However, the addition of cold peptides to the cells also slows down the growth rate. By warming the proteins to 37°C prior to their addition, and carrying out the addition of proteins *in situ* at 37°C the lag in buffer control cells is reduced (Figure 5.5). Bioluminescence is shown as a percentage of the initial bioluminescence to correct for slight variation between wells.

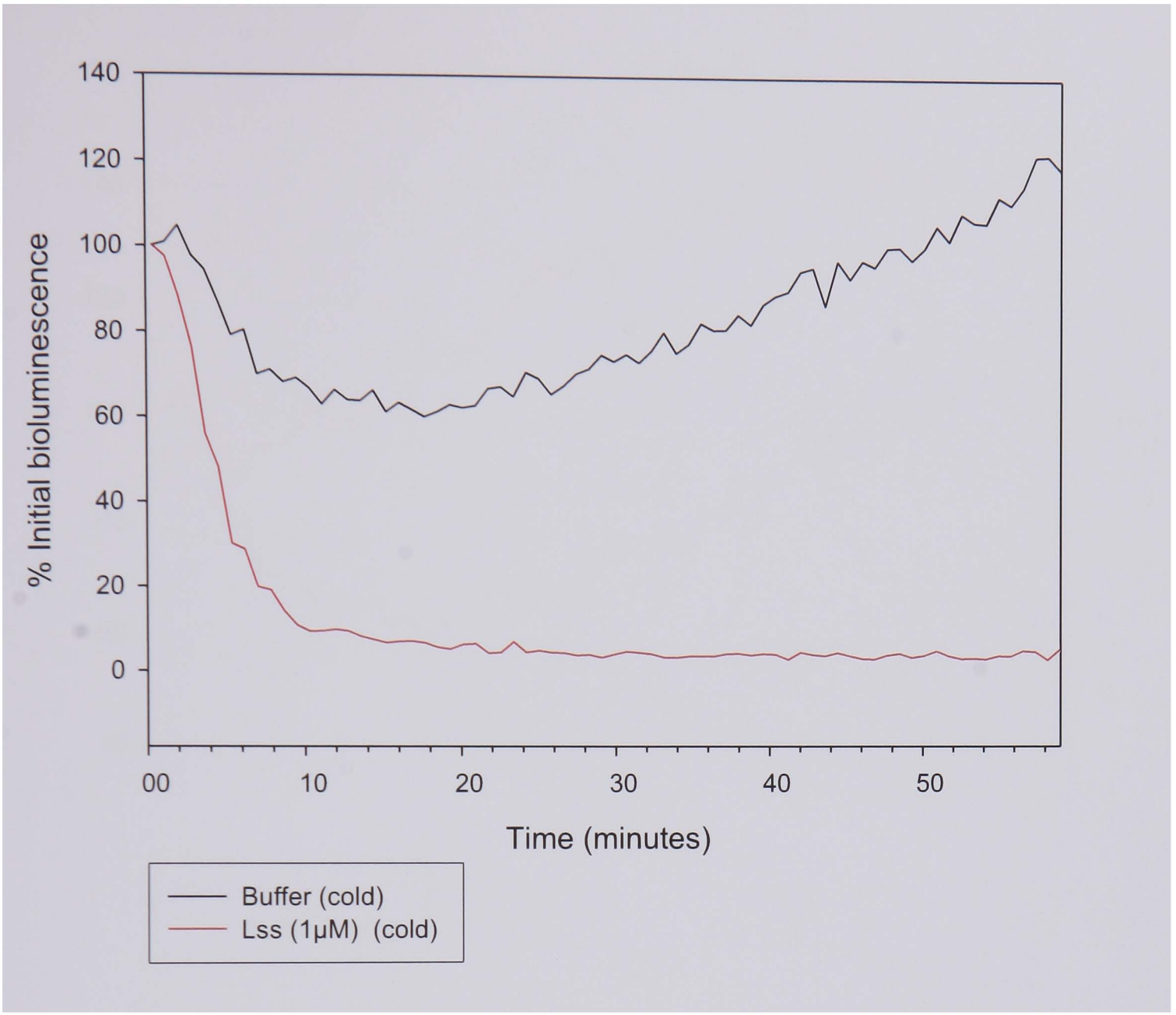


Figure 5.4 Effect of adding a cold solution of lysostaphin on bioluminescence of *S. aureus* RN6390

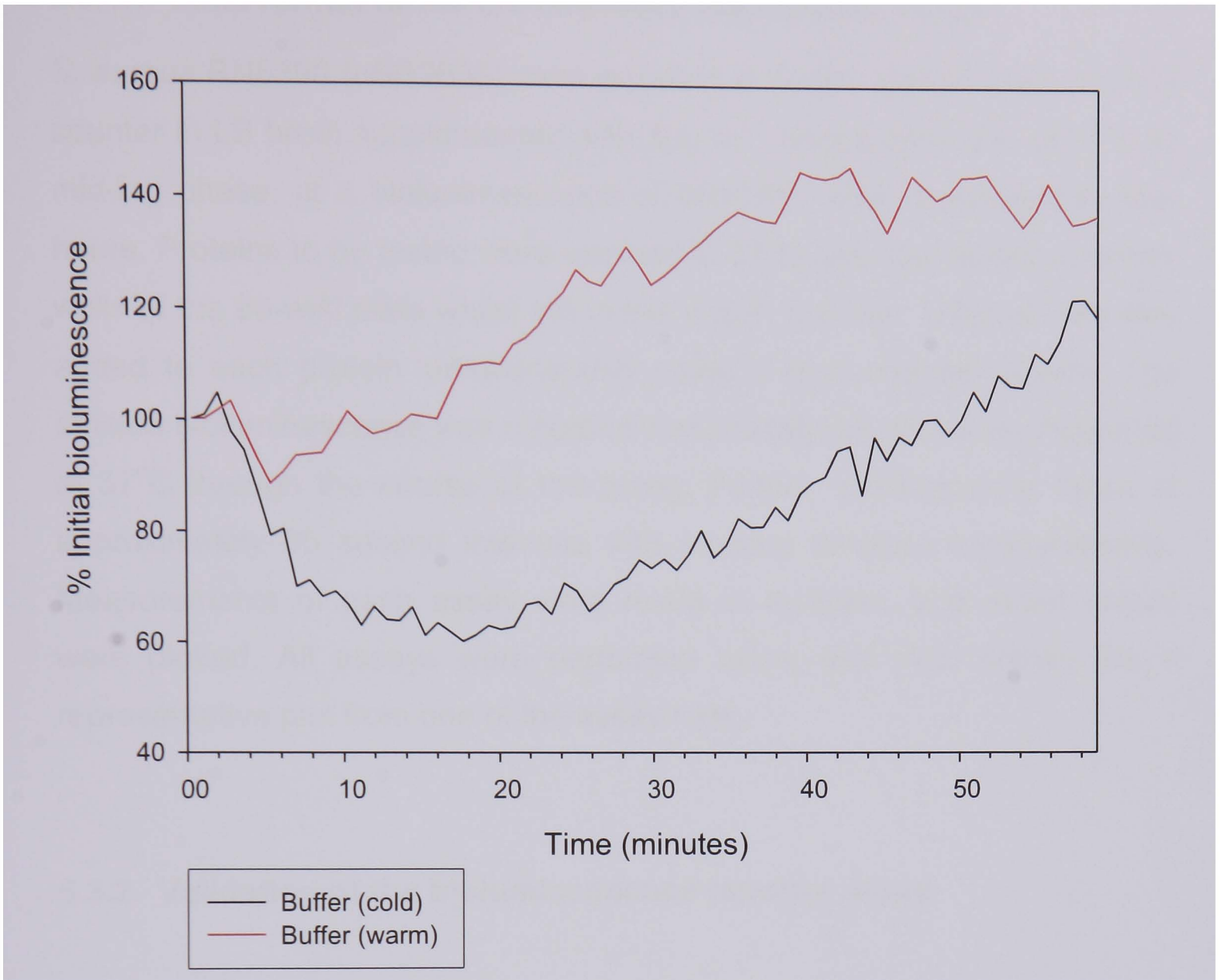


Figure 5.5 Comparison of effects of adding cold and warm buffer to bioluminescence of *S. aureus* RN6390

With the initial lag period in the bioluminescence of buffer control cells minimised, data from the assay may be expressed in relative light units (RLU). The relative bioluminescence of cells subjected to proteins is calculated as a percentage of the bioluminescence of the buffer control cells at each timepoint. This provides a measurement of the effect of the protein over time in RLU as a percentage of the negative control. All further assays are presented in this format. Further explanation of the effect of plotting the effect of peptides as a percentage of the negative control is presented in Appendix XIII.

5.3.1.7 *Final format of the bioluminescence reporter assay*

S. aureus RN6390 (pSB2030) were grown in a Wallac Victor^2 multi-channel counter in LB broth supplemented with 5μg ml^{-1} chloramphenicol at 37°C to mid-log phase, at a bioluminescence of 500CPS, after approximately four hours. Proteins to be tested were warmed to 37°C and 30μl added to empty wells of the 96-well plate whilst still in the Victor^2 counter. 170μl of cells was added to each protein simultaneously using a multi-channel pipette. The emitted bioluminescence was recorded immediately, as cells were incubated at 37°C through the course of the assay. Further readings were taken at approximately 35 second intervals with shaking between measurements. Measurements of each assay were made in triplicate, and mean values were plotted. All assays were performed twice, and plots shown are a representative plot from one of the assay runs.

5.3.2 Validation of the bioluminescence reporter assay

5.3.2.1 *Activity of lysostaphin in the assay*

The activity of lysostaphin, a well characterised antimicrobial peptide was investigated in the bioluminescence reporter assay. Lysostaphin was added to final concentrations of 0.1μM, 0.25μM, 0.5μM and 1μM. All concentrations of lysostaphin caused decrease in the bioluminescence of *S. aureus* RN6390 (pSB2030) relative to the effect of buffer alone (Figure 5.6). Concentrations equal and greater than 0.25μM caused a rapid abolition of bioluminescence. This indicates that the bioluminescence reporter is a highly sensitive assay.

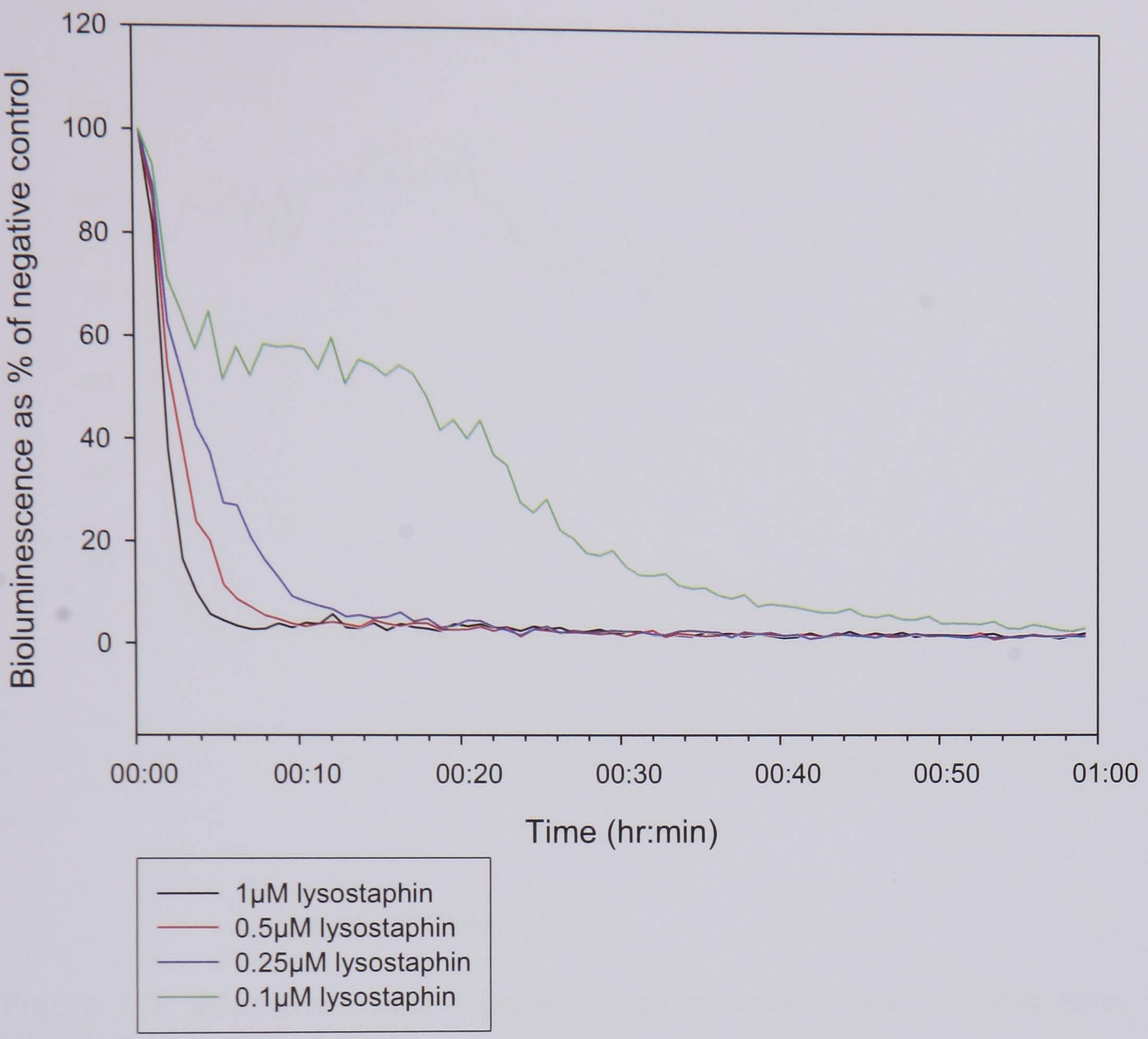


Figure 5.6 Affect of lysostaphin at different concentrations, on bioluminescence of *S. aureus*

5.3.2.2 *Activity of a mutant lysostaphin in the assay*

The activity of a mutant lysostaphin, previously characterised by the turbidity assay (Jarvis, 2005), was investigated in the bioluminescence reporter. This protein has a histidine residue at position 114 of mature lysostaphin substituted with alanine. This mutation renders the peptide inactive against *S. aureus.* Lysostaphin H114A was tested against *S. aureus* RN6390 (pSB2030) at a concentration of 10μM. As a control, lysostaphin was tested at 0.25μM. No activity of lysostaphin H114A was observed, with bioluminescence maintained around 100RLU (Figure 5.7). This correlates with results previously seen in the turbidity assay.

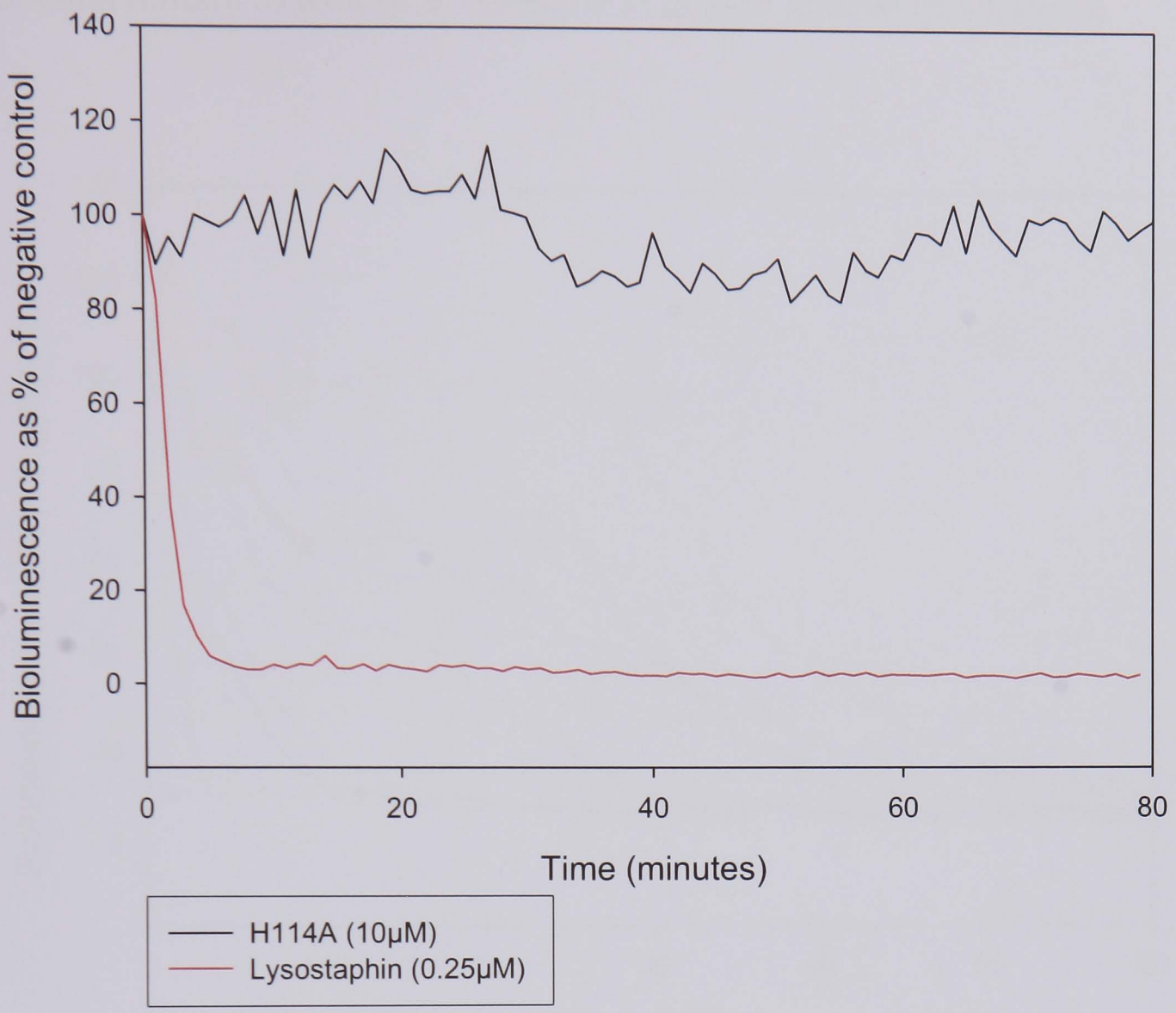


Figure 5.7 Bioluminescence reporter assay showing effect of lysostaphin and H114A mutant on *S. aureus*

5.3.3 Applications of the bioluminescence reporter assay

5.3.3.1 *Screening lysostaphin mutants in a protection assay*

Having developed the bioluminescence reporter a protection assay variant was employed, similar to the protection turbidity assay described in chapter 2. Lysostaphin was added to cells in combination with an excess of the mutant peptides to investigate their protective effect. Previously, protection turbidity assays were used to demonstrate which mutant peptides were competitively binding to *S. aureus* cells. The assay had not distinguished major differences between the level of protection provided by each protein, with one mutant, H83A, appearing to provide partial protection, rather than complete or no protection (Jarvis, 2005). The bioluminescence

reporter was used to investigate whether there were differences in the ability of each mutant to bind to *S. aureus* and protect against lysostaphin.

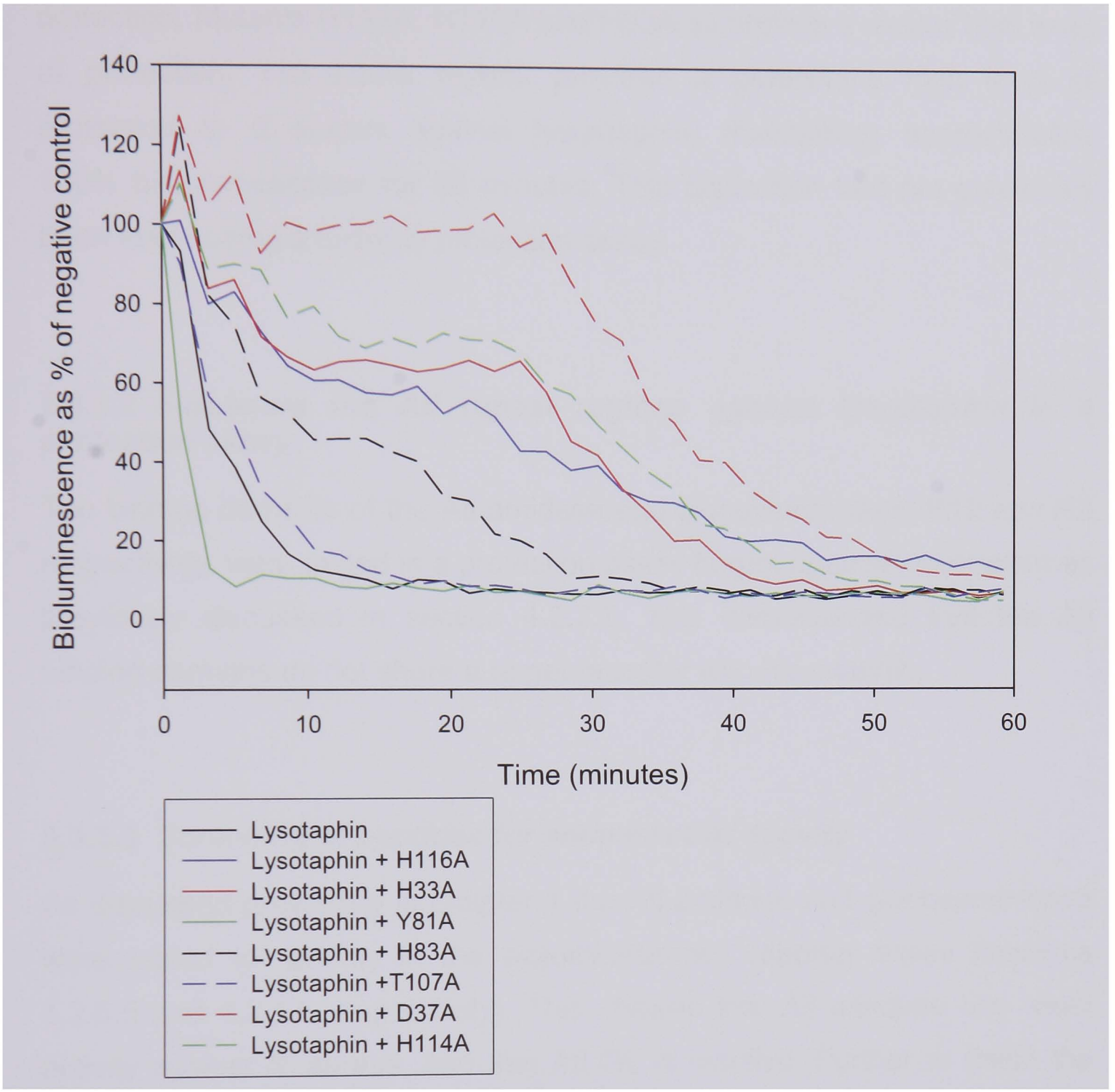


Figure 5.8 Bioluminescence protection assay showing effect of 0.25μM lysostaphin in combination with 10μM mutant lysostaphins

Lysostaphin was added to *S. aureus* RN6390 (pSB2030) at 0.25μM. Mutant lysostaphins were added to a final concentration of 10μM (gifts from Hannah Jarvis, University of Nottingham). A range of levels of protection was observed (Figure 5.8). The addition of mutant Y81A caused a more rapid decrease in bioluminescence than lysostaphin alone. This mutant has been shown to be active in the turbidity assay. Mutant T107A had little effect and this protein has been shown previously to be partially active. The remaining

peptides were all inactive when used alone in the turbidity assay, and all provided protection to *S. aureus* against lysostaphin. Correlating with results from a protection turbidity assay H83A was shown to provide partial protection. Mutants H114A, H116A and H33A all provide a similar high level of protection. The D37A mutant provided a particularly high level of protection to *S. aureus* against lysostaphin, maintaining approximately 100% bioluminescence for 30 minutes. This distinction had not previously been made using a turbidity protection assay.

5.3.3.2 *Screening the Atl repeat regions against lysostaphin in a protection assay*

The binding domains of the Atl amidase and glucosaminidase, R12 and R3 respectively, were tested in a protection assay format against lysostaphin as previously discussed in section 4.2.7.7. This demonstrated that the Atl binding domains do not share a target receptor with lysostaphin.

5.3.3.3 *Screening of peptides for antimicrobial activity*

As discussed previously in chapter 4 the Atl amidase and glucosaminidase were tested for activity in the bioluminescence reporter assay (sections 4.2.6.5 and 4.2.1.6 respectively). This showed that Atl amidase has weak activity against *S. aureus*, and that Atl GL is inactive. Further to these the bioluminescence reporter assay was used to measure the activity of other candidate antimicrobial peptides. These included putative amidases identified by sequence homology from *S. aureus* Mu50, and *Staphylococcus haemolyticus* (from Philip Bardelang, University of Nottingham).

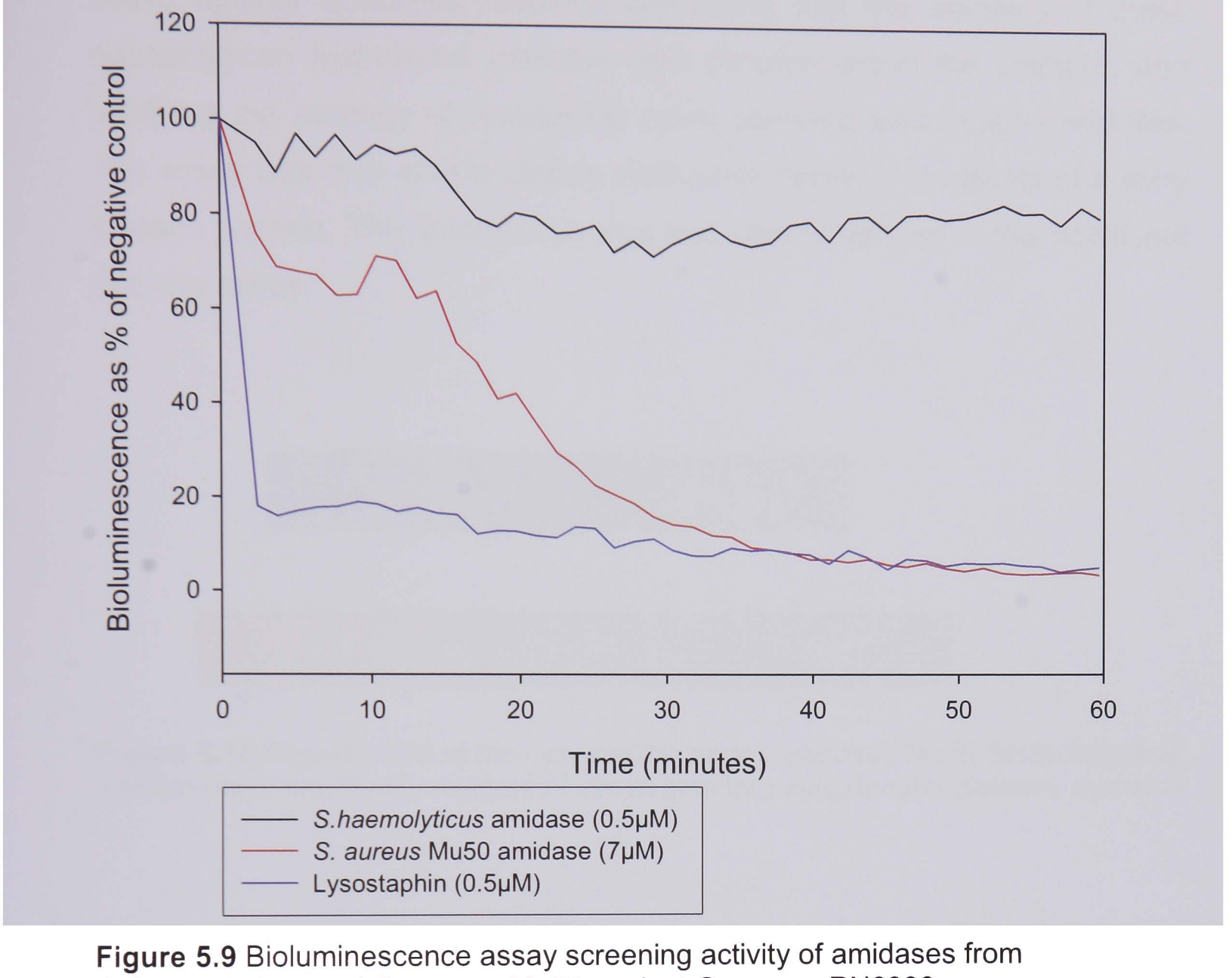


Figure 5.9 Bioluminescence assay screening activity of amidases from *S. haemolyticus* and *S. aureus* Mu50 against *S. aureus* RN6390

The assay demonstrated that the recombinant amidase derived from *S. aureus* Mu50 had activity against *S. aureus* RN6390, and may be further considered as a candidate peptide, whereas the *S. haemolyticus* amidase was inactive at the maximum possible concentration (Figure 5.9). The peptides were screened alongside lysostaphin as a positive control.

5.3.3.4 *Screening of chimeric peptides*

The bioluminescence reporter assay was also used to measure the activity of two chimeric peptides against *S. aureus* RN6390. These chimeras each comprised either the enzymatic or targeting domain from the *S. aureus* Mu50 amidase described above with the complementary domain from lysostaphin as shown in Figure 5.10 (from Philip Bardelang, University of

Nottingham). The assay demonstrated that both chimeric peptides were active against *S. aureus* RN6390, confirming that the domains of these peptidoglycan hydrolases maintain their function within the chimera, and justifying the strategy of developing novel chimeric antimicrobial peptides. The assay was also able to clearly distinguish between the levels of activity of each peptide. This information was less clearly defined in the traditional turbidity assay.

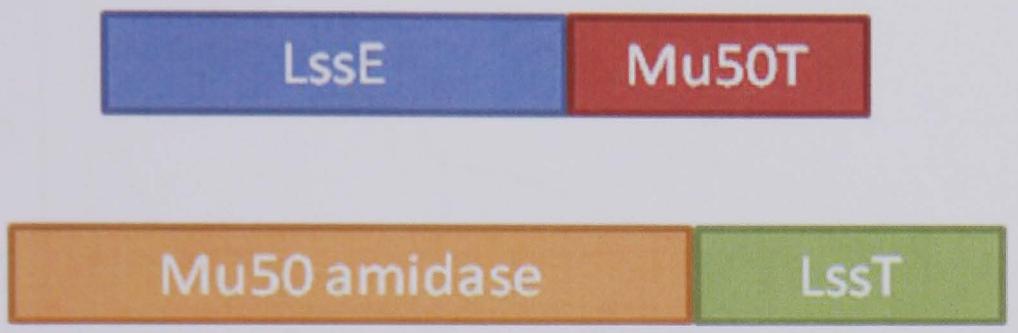


Figure 5.10 Organisation of the Lysostaphin endopeptidase / Mu50 SH3b targeting domain (top), and Mu50 amidase / Lysostaphin targeting domain (bottom) chimeras.

Each chimeric peptide was assayed alongside the *S. aureus* Mu50 amidase and lysostaphin as comparisons (Figure 5.11). Lysostaphin was tested at 0.5μM and other peptides at 1μM. Neither peptide caused as rapid a decrease in bioluminescence as the highly active lysostaphin. However, both chimeras showed greater activity against *S. aureus* RN6390 than the parent *S. aureus* Mu50 amidase. This demonstrates that the chimeric peptide strategy has potential for improving the activity of candidate antimicrobial peptides. Furthermore, the assay clearly distinguishes between the two chimeric peptides. The chimera comprising the lysostaphin endopeptidase with the Mu50 targeting domain shows greater activity than the Mu50 amidase with lysostaphin's targeting domain. The level of activity of the first chimera is close to that of wild type lysostaphin. This is an encouraging result, showing that in the event of resistance developing to lysostaphin due to an altered binding site, a second generation, highly active lysostaphin based peptide may be developed utilising the targeting domain

from another antimicrobial peptide which targets different cell surface receptors.

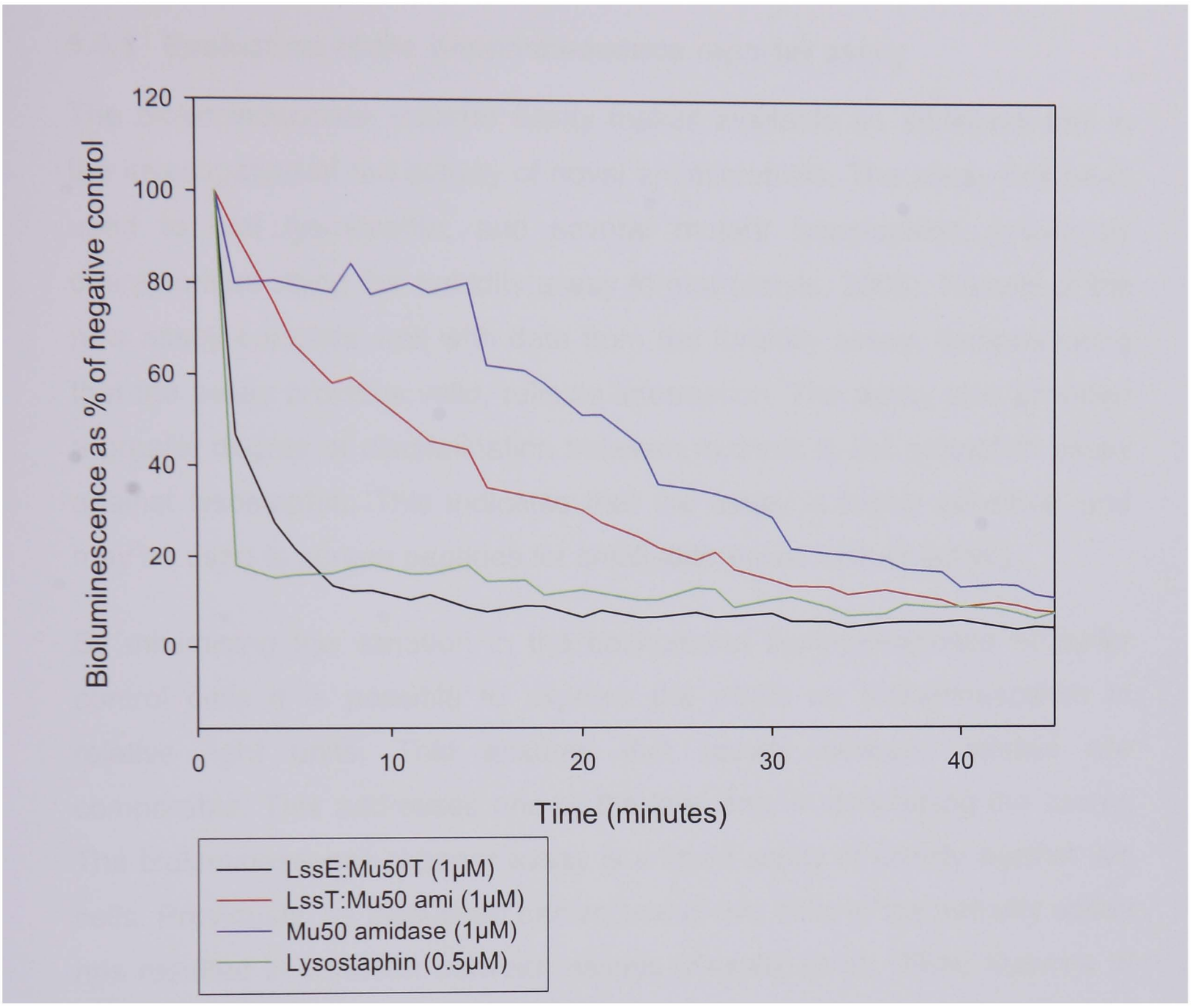


Figure 5.11 Bioluminescence reporter assay showing activity of chimeric Mu50: lysostaphin peptides

5.4 DISCUSSION

5.4.1 Evaluation of the bioluminescence reporter assay

The bioluminescence reporter assay makes available an additional tool in the investigation of the activity of novel antimicrobials. The assay has been used to test lysostaphin, and several mutant lysostaphins, previously characterised using the turbidity assay format (Jarvis, 2005). Results of the new assay correlate well with data from the turbidity assay, demonstrating that the assay provides valid, reliable information. The assay also provided a greater degree of discrimination between mutants in the protection assay against lysostaphin. This indicates that the assay is highly sensitive, and may be used to screen peptides for small differences in their activity.

By minimising the variation in the background bioluminescence of buffer control cells it is possible to express the effect on bioluminescence in relative light units. This ensures that results between assays are comparable. This addresses one of the key aims in developing the assay.

The bioluminescence reporter assay is a liquid assay of activity against live cells. Previously, as discussed earlier, use of live cells in the turbidity assay has resulted in variation between assays (Kessler *et al.*, 2004; Kusuma *et al.*, 2005; Donovan *et al.*, 2006b).

The bioluminescence reporter assay should not be used as a sole screen of activity of novel antimicrobials. Only live, actively growing *S. aureus* RN6390 (pSB2030) cells emit bioluminescence (Qazi *et al.*, 2001). Any reduction of bioluminescence is thus a measure of inhibition of cell growth. Further assays, such as the turbidity assay, or the agar diffusion assay should be used to investigate cell death and lysis. The bioluminescence reporter assay should be used as an additional, highly sensitive assay to determine either low levels of antimicrobial activity, or differences of activity between antimicrobials with similar profiles as seen by other assay formats.

5.4.2 Potential adaptations to the bioluminescence reporter assay

The bioluminescence reporter assay described in this chapter has been developed as a measure of antimicrobial activity against the laboratory strain *S. aureus* RN6390 containing the plasmid (pSB2030) of Qazi *et al.* (2001). The assay could be used to investigate the activity of antimicrobials against other *S. aureus* strains by transforming them with the reporter plasmid pSB2030. The assay could also be adapted to measure the effect on other organisms if a bioluminescent reporter plasmid suitably expressed in that species is available or could be designed. Such alterations to the assay would require that the assay conditions were optimised for that strain or species, although the basic format may require little alteration.

The bioluminescence reporter assay has been developed as a liquid assay format. There may be future interest in a variation of the assay based on agar plates or other solid media. This could be used, for example, in a model of biofilm formation. The bioluminescence reporter plasmid would be suitable in screening antimicrobials designed to inhibit biofilm formation, where there will be actively growing cells. The reporter would not be appropriate in models of mature biofilms, as it is only expressed in actively growing cells. In order for a solid assay format to be developed a reliable method of measuring the bioluminescence would be required. This would demand a highly sensitive camera system. It would also be necessary to calibrate the assay against background bioluminescence from surrounding regions on the media. The liquid assay format is carried out in black sided 96-well plates to prevent the transfer of bioluminescence between wells.

5.4.3 Future applications of the bioluminescence reporter assay

The bioluminescence reporter assay will be used in future screens of antimicrobial activity using novel candidate peptides. It can be used as a secondary screen to determine small differences in activity or detect low levels of antimicrobial activity against *S. aureus*, as discussed above. It may

also be used in further protection assays to investigate the binding properties of novel antimicrobial peptides.

For example, as previously discussed in chapter 4, the bioluminescence assay could be used to determine the binding profile and activity of a novel R12:LssE chimeric peptide. This could confirm whether Atl and lysostaphin have different cell surface receptors

An interesting potential role for the bioluminescence reporter assay is in the development of mutant peptides. The assay has been shown to have the ability to discriminate between the binding properties of mutant lysostaphins. Future candidate antimicrobial peptides, such as the amidase identified from *S. aureus* Mu50 may be subjected to targeted mutagenesis with the aim of increasing their binding or hydrolytic activity. The assay may be used to identify those peptides with even slightly improved activity. Through further sequential mutagenesis and screening steps a novel antimicrobial peptide with superior activity to its parent peptide may be developed. This approach may be used on a single domain from such a candidate peptide in the development of a chimeric antimicrobial peptide. The bioluminescence reporter assay could thus play a significant role in the development of a novel, second generation, lysostaphin based antimicrobial peptide.

5.5 SUMMARY

A bioluminescence reporter assay was developed to investigate the activity of novel antimicrobial peptides against *S. aureus* RN6390 utilising the reporter plasmid of Qazi *et al.* (2001). It is a liquid assay format against live cells and results of different assays are reliable and comparable. Results of the assay correlate with data previously obtained by the turbidity assay. The bioluminescence reporter assay is sensitive and may be used in the discrimination of peptides with similar levels of antimicrobial activity. The assay adds another tool to those previously used to the investigation of novel antimicrobial peptides active against MRSA.

6. GENERAL DISCUSSION

6.1 INTRODUCTION

MRSA is an important nosocomial pathogen capable of causing a wide range of infections. As multi-drug resistant MRSA strains develop resistance to the drug of last resort, vancomycin and new antibiotics such as Linezolid, the threat of untreatable *S. aureus* emerging in hospitals and the community increases. In order to meet this challenge there has been renewed interest in alternative antimicrobial agents. One such agent is the antimicrobial peptide lysostaphin. Lysostaphin is an endopeptidase produced by *S. simulans* biovar *staphylolyticus* which cleaves the pentaglycine cross-bridges of other staphylococci, disrupting the cell wall and subjecting the cell to osmotic pressure leading to lysis and death. Lysostaphin has been investigated in *in vitro* and *in vivo* studies and has successfully completed phase I/II clinical trials. These results indicate that it is a promising antimicrobial which could play a significant role in the treatment of staphylococcal infections. However, the clinical introduction of lysostaphin will lead to a selection pressure for resistance emerging. This demands that measures are taken to address the future emergence of lysostaphin resistant MRSA.

The work described in this thesis was aimed at developing second generation novel lysostaphins that have altered activity profiles and could counteract any future resistance that may evolve. This work focussed on a phage endolysin from the P68 bacteriophage of *S. aureus*, and the glucosaminidase and amidase of the major autolysin of *S. aureus*; Atl. A chimeric peptide combining the targeting domain of lysostaphin and the Atl glucosaminidase was designed. The activity of this was investigated and compared with the activity profiles of the parent peptides.

The activity of these proteins was investigated in a range of assays, including a novel bioluminescence reporter assay that was developed during the course of the work described in this thesis.

6.2 THE LYS16 ENDOLYSIN OF THE P68 BACTERIOPHAGE

The genome sequence of the P68 *S. aureus* bacteriophage contains a gene with homology to endolysins from the *S. aureus* phages Twort and φ11 (Vybiral *et al.*, 2003; Takáč *et al.*, 2005b). As a peptidoglycan hydrolase with *in vivo* activity against *S. aureus* P68 Lys16 was identified as a candidate peptide for the development of second generation lysostaphins.

6.2.1 Activity of Lys16

No bacteriolytic activity of recombinant Lys16 was observed against *S. aureus* or *M. luteus* in any of the assays used. The maximum concentration of Lys16 that could be used in the turbidity assay was 22.8μM. Lysostaphin shows activity at concentration lower than 1μM, indicating that the Lys16 purified was not a promising candidate peptide. The results described in chapter three contrasts with those of other studies, reported after this work was completed. Lys16 was shown to have extracellular bacteriolytic activity against *S. aureus* by zymogram assay, and in a turbidity assay using *E. coli* cell lysate overexpressing the protein (*et al.*, 2005b). However, as the focus of this thesis is to exploit peptidoglycan hydrolases as potential enzybiotics it is important that active purified recombinant proteins can be obtained.

6.2.2 Stability of Lys16

The recombinant Lys16 was found to be poorly soluble and unstable. It is possible that this led to the apparent inactivity of the protein, due to its precipitation during assays. Similar problems of solubility have been reported for other staphylococcal phage endolysins (O'Flaherty *et al.*, 2005). This is a significant obstacle when considering Lys16 as a candidate antimicrobial peptide and further work concentrated on other candidates with greater solubility and stability.

6.3 THE STAPHYLOCOCCAL AUTOLYSIN ATL

Autolysins are peptidoglycan hydrolases produced by bacteria to cause highly regulated localised cell wall disruption during cell cycle processes. Atl is considered to be the major autolysin of *S. aureus* and has been the subject of much research interest (Oshida *et al.*, 1995; Foster, 1995; Yamada *et al.*, 1996; Baba *et al.*, 1998b). Atl has been shown to be involved in the cell separation process, and autolysins have been demonstrated to have multiple cellular roles. None of the previously reported research has considered the potential of Atl as a novel antimicrobial, and this was the subject of the work described in chapter four.

6.3.1 Activity of the Atl peptidoglycan hydrolases

The glucosaminidase and amidase domains of Atl were cloned separately, each with the repeat regions with which they are associated *in vivo*. Purified recombinant protein was obtained and tested in a range of assays against *S. aureus* and *M. luteus*. Results of these assays confirmed that the glucosaminidase had lytic activity against *M. luteus*, whilst the amidase had weaker activity against *S. aureus*, which had previously been reported by other researchers (Oshida *et al.*, 1995; Sugai *et al.*, 1995). The work described in chapter four extends this information and shows that the purified recombinant protein is active against live cells, essential if the autolysin is to be considered as a novel antimicrobial.

6.3.2 Binding of the Atl repeat regions

This study has also confirmed the role of the repeat regions in binding to the staphylococcal cell surface, previously described by Baba & Schneewind (1998). The repeats attached to each hydrolytic domain have been shown to target the same cell surface receptor, and this has been shown to differ from the surface receptor of lysostaphin or to have different recognition factors.

This led to the development of a chimeric peptide containing the lysostaphin targeting domain and the glucosaminidase domain of Atl.

6.4 THE DESIGN OF A CHIMERIC ANTIMICROBIAL PEPTIDE

The Atl glucosaminidase was cloned in a fusion with the lysostaphin targeting domain, with the aim of conferring greater activity against *S. aureus*. The LssT:GL chimera retained activity against *M. luteus*, but no activity against *S. aureus* was observed. It was shown that the activity against *M. luteus* was due to residual binding ability of the truncated glucosaminidase, rather than the binding ability of the lysostaphin targeting domain. Although this was a disappointment in the process of developing a novel antimicrobial peptide active against MRSA it provides encouragement to the strategy of developing multi-domain novel antimicrobials. The result demonstrates that the domains within the chimeric peptide retain parental properties, as has been shown by other researchers (Donovan *et al.*, 2006a; Qiu *et al.*, 2003). Therefore if compatible catalytic and cell wall binding domains can be identified, they may be fused to result in a chimeric novel antimicrobial peptide with activity against MRSA, and therefore this is a valid strategy to continue in the future.

6.5 A BIOLUMINESCENCE REPORTER ASSAY

A number of assays of bacteriolytic activity have previously been used to study peptidoglycan hydrolases (Kusuma *et al.*, 2005). These assays have varied benefits and there is no single ideal assay with which to test a potential novel antimicrobial peptide. The development of a bioluminescent *S. aureus* growth reporter plasmid presented the opportunity to develop a novel assay measuring the viability of live *S. aureus* cells (Qazi *et al.*, 2001).

6.5.1 Development of the assay

Using the *S. aureus* bioluminescence reporter of Qazi *et al.* (2001) a liquid assay format was developed to measure in real time the effect of recombinant antimicrobial peptides on cellular metabolism. The assay was able to distinguish between novel chimeric peptides that had apparently similar levels of activity when assayed by other tests. This highly discriminatory assay could be a valuable tool in the future development of novel antimicrobials. Minor modifications could be made to existing candidate peptides and the bioluminescence reporter assay could be used to screen for differences in antimicrobial activity.

The assay does require purified recombinant protein to be available and should not be used in isolation. Other assays, such as the stab test, should be used alongside the bioluminescence reporter assay, for initial experiments to identify bacteriolytic activity. Furthermore, as bioluminescence is a reporter of cell growth additional assays will be required to determine whether cells are being killed and lysed.

6.6 EVALUATION OF ALTERNATIVE ANTIMICROBIAL AGENTS

The continuing emergence of antibiotic resistance in *S. aureus* requires novel antimicrobial agents to be developed. These will include traditional antibiotics and also alternative antimicrobial agents. No outstanding candidate antimicrobials were identified through the work described in this thesis. However, there are a number of other candidates available, and hopefully these will provide our continuing protection against *S. aureus* infections.

The ideal method of reducing the clinical impact of staphylococcal infections would be to prevent infections developing in the first instance. With a view to this there is significant interest in developing a *S. aureus* vaccine (Kuklin *et al.*, 2006; Stranger-Jones *et al.*, 2006). These have shown some promise in animal studies. The high mutability of *S. aureus* poses the threat that a vaccine may become ineffective shortly after its introduction. Counteracting

this threat will be a major challenge in the development of a successful vaccine. If an effective vaccine is developed, protection programmes will be costly to developing nations, and *S. aureus* will never be eradicated as there is an animal reservoir as well as human carriers. There will therefore always be a requirement for antimicrobials active against *S. aureus*. In any event, until an effective vaccine is introduced it is of the highest importance to pursue multiple lines of defence against *S. aureus* infections, and other alternative treatments should be investigated.

Lysostaphin is a promising novel antimicrobial peptide active against MRSA. Results of *in vitro* and *in vivo* studies and phase I/II clinical trials indicate that it is an effective anti-staphylococcal agent and will be of particular interest as a topical intranasal treatment to eradicate nasal carriage pre-surgery, and as a coating for catheters and other indwelling medical devices (Anon, 2003; Wu *et al.*, 2003). Resistance to lysostaphin is likely to emerge by one of several mechanisms and this should be considered with its future clinical introduction.

There are further concerns over the application of lysostaphin as a treatment for a wider range of infections. If lysostaphin is to have a role in treating *S. aureus* infections such as pneumonia and endocarditis then its safety as an intravenous treatment needs to be assessed. Previous studies have not demonstrated any significant toxicity of lysostaphin and have only detected modest inhibition of its anti-staphylococcal activity by antibodies (Dajcs *et al.*, 2002). Long term studies of the toxicity and immunological response to lysostaphin are required, as patients may receive prolonged treatment regimes, and may require treatment over several episodes during their life. Concerns over the immunological effect of a bacterial protein treatment led to the initial decline in interest in lysostaphin following promising experimental results in the 1960s and 1970s. It may be possible to overcome any potential immunological problems by engineering a "humanised" second generation lysostaphin. If the sequence of lysostaphin could be altered to resemble more closely that of a human protein, whilst retaining its activity against *S. aureus*, it should provoke a lesser immune

response, which would improve and prolong the efficacy of such a treatment.

If these concerns over future resistance and immunological response to lysostaphin can be addressed, then lysostaphin remains a highly attractive alternative antimicrobial. Research into the development of enzybiotics based on lysostaphin, including multi-domain chimeric peptides should continue. There are several candidates which may become components of such a multi-domain peptide, or could be used as novel antimicrobials in their own right.

Staphylococcal autolysins have been considered as potential candidates as they have evolved to be highly specific peptidoglycan hydrolases. Exposure to excessive concentrations of autolysins may cause rapid lysis, and thus be an effective anti-staphylococcal treatment. However, autolysins have not evolved to cause host cell lysis and play wide and varied roles in cellular processes, the regulation of which is not fully understood (Smith *et al.*, 2000). The clinical implication of using a staphylococcal autolysin with multiple functions may be complicated, and this could compromise the efficacy and safety of such an antimicrobial. Furthermore, many of the staphylococcal autolysins investigated are poorly soluble and unstable proteins. For an antimicrobial peptide to have potential as a therapeutic agent it needs to be a stable protein with extracellular activity. Taking into consideration these drawbacks, there are stronger candidate peptides than staphylococcal autolysins.

Bacteriophage endolysins have been the subject of recent research interest. Encouraging results have been reported, demonstrating effective antimicrobial activity *in vitro* and *in vivo*. Phage endolysins active against *B. anthracis* and *S. pneumoniae* have been developed (Schuch *et al.*, 2002; Loeffler, Djurkovic & Fischetti, 2003a). These could provide highly specific antimicrobial agents for treating specific infections. Narrow spectrum agents may play an important role in the treatment of defined infections as they will cause a reduced disruption to a patient's natural microflora. Broadly active phage endolysins have also been identified, and these may play a role in

the treatment of other undefined infections (Yoong *et al.*, 2004). Many bacteriophages of most bacterial species exist in nature, and their endolysins provide an extensive pool of potential novel antimicrobials. To exploit this, phages with the host range of interest, including *S. aureus*, need to be isolated and identified. It is then key to identify which endolysins are stable proteins with extracellular activity. An additional benefit of phage endolysins is that the phages themselves could be used to develop second generation endolysins in the event of resistance emerging. Phage endolysins therefore present a strong case for further investigation as alternative antimicrobials active against MRSA.

Other candidates include bacteriophage therapy itself, although concerns may persist regarding the pharmacokinetics and immune response to such a treatment. Other animal, plant or bacterial antimicrobial peptides may also be identified with activity against *S. aureus* and could provide novel alternative agents. Novel synthetic antimicrobial peptides with activity against *S. aureus* have been designed, based on the active sites of insect defensins (Saido-Sakanaka *et al.*, 2005). As our knowledge and understanding of lysostaphin, phage endolysins and other antimicrobial peptides increases, it may be possible to develop more novel antimicrobial agents in this way.

6.7 FUTURE DIRECTIONS

Further work is required to identify and develop novel antimicrobial peptides with activity against *S. aureus*, including MRSA. The most promising source of candidate peptides could prove to be bacteriophage endolysins. Once further bacteriophages of *S. aureus* have been identified and isolated it will be possible to clone and purify recombinant endolysins. These may be screened for activity in a range of assays, including the bioluminescence reporter activity for activity against *S. aureus* and other organisms.

Endolysins that show promising activity may be developed as novel antimicrobials or combined in a multi-domain chimeric peptide. If lysostaphin shows continuing success as it progresses through the clinical trial phases then second generation lysostaphin/phage endolysin chimeric peptide could be of great therapeutic value. The lysostaphin endopeptidase could be specifically targeted to staphylococci with a phage endolysin targeting domain, or vice versa. Such combinations could improve and prolong the efficacy of novel antimicrobial peptides active against MRSA.

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APPENDICES

```
   M  A  K  S  Q  Q  Q  A  K  E  W  I  Y  K  H  E  G  A  G
gccatggcgaaatcacaacaacaagcaaaagaatggatatataagcatgaggggcaggt
 NcoI
V  D  F  D  G  A  Y  G  F  Q  C  M  D  L  S  V  A  Y  V  Y
gttgactttgatggtgcatatggatttcaatgtatggacttatcagttgcttatgtgtat

Y  I  T  D  G  K  V  R  M  W  G  N  A  K  D  A  I  N  N  D
tacattactgacggtaaagttcgcatgtggggtaatgctaaagacgcgataaataatgac

F  K  G  L  A  T  V  Y  K  N  T  P  S  F  K  P  Q  L  G  D
tttaaaggtttagcgacggtgtataaaaatacaccgagctttaaacctcaattaggggac

V  A  V  Y  T  N  G  Q  Y  G  H  I  Q  C  V  L  S  G  N  L
gttgctgtatatacaaatggacaatatggacatattcaatgtgtgttaagtggaaatctt

D  Y  Y  T  C  L  E  Q  N  W  L  G  G  G  F  D  G  W  E  K
gattattatacatgcttagaacaaaactggttaggcggcggttttgacggttgggaaaaa

A  T  I  R  T  H  Y  Y  D  G  V  T  H  F  I  R  P  K  F  S
gcaaccattagaacacattattatgacggtgtaactcactttattagacctaaattttca

G  S  N  S  K  A  L  E  T  S  K  V  N  T  F  G  K  W  K  R
ggtagtaatagcaaagcattagaaacatcaaaagtaaatacatttggaaaatggaaacga

N  Q  Y  G  T  Y  Y  R  N  E  N  G  T  F  T  C  G  F  L  P
aaccaatacggcacatattatagaaatgaaaatggtacatttacatgtggtttttacca

I  F  A  R  V  G  S  P  K  L  S  E  P  N  G  Y  W  F  Q  P
atatttgcacgtgtcggtagtccaaaattatcagaacctaatggctattggttccaacca

N  G  Y  T  P  Y  N  E  V  C  L  S  D  G  Y  V  W  I  G  Y
aacggttatacaccatataacgaagtttgtttatcagatggttacgtatggattggttat

N  W  Q  G  T  R  Y  Y  L  P  V  R  Q  W  N  G  K  T  G  N
aactggcaaggcacacgttattatttaccagtgcgccaatggaatggaaaaacaggtaat

S  Y  S  V  G  I  P  W  G  V  F  S  L  E  H  H  H  H  H  H
agttacagtgttggtattccttgggggtgttctcactcgagcaccaccaccaccaccac
                                    XhoI
```

Appendix I. The sequence of the P68 bacteriophage Lys16 DNA and protein from pKP1. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence is shown in blue upper case font.

```
   M  A  Y  T  V  T  K  P  Q  T  T  Q  T  V  S  K  I  A  Q
gccatggcttatactgttactaaaccacaaacgactcaaacagttagcaagattgctcaa
 NcoI
V  K  P  N  N  T  G  I  R  A  S  V  Y  E  K  T  A  K  N  G
gttaaaccaaacaacactggtattcgtgcttctgtttatgaaaaaacagcgaaaaacggt

A  K  Y  A  D  R  T  F  Y  V  T  K  E  R  A  H  G  N  E  T
gcgaaatatgcagaccgtacgttctatgtaacaaaagagcgtgctcatggtaatgaaacg

Y  V  L  L  N  N  T  S  H  N  I  P  L  G  W  F  N  V  K  D
tatgtattattaaacaatacaagccataacatcccattaggttggttcaatgtaaaagac

L  N  V  Q  N  L  G  K  E  V  K  T  T  Q  K  Y  T  V  N  K
ttaaatgttcaaaacctaggcaaagaagttaaaacgactcaaaaatatactgttaataaa

S  N  N  G  L  S  M  V  P  W  G  T  K  N  Q  V  I  L  T  G
tcaaataacggcttatcaatggttccttggggtactaaaaaccaagtcattttaacaggc

N  N  I  A  Q  G  T  F  N  A  T  K  Q  V  S  V  G  K  D  V
aataacattgctcaaggtacatttaatgcaacgaaacaagtatctgtaggcaaagatgtt

Y  L  Y  G  T  I  N  N  R  T  G  W  V  N  A  K  D  L  T  A
tatttatacggtactattaataaccgcactggttgggtaaatgcaaaagatttaactgca

P  T  A  V  K  P  T  T  S  A  A  K  D  Y  N  Y  T  Y  V  I
ccaactgctgtgaaaccaactacatcagctgccaaagattataactacacttatgtaatt

K  N  G  N  G  Y  Y  Y  V  T  P  N  S  D  T  A  K  Y  S  L
aaaaatggtaatggttattactatgtaacaccaaattctgatacagctaaatactcatta

K  A  F  N  E  Q  P  F  A  V  V  K  E  Q  V  I  N  G  Q  T
aaagcatttaatgaacaaccattcgcagttgttaaagaacaagtcattaatggacaaact

W  Y  Y  G  K  L  S  N  G  K  L  A  W  I  K  S  T  D  L  A
tggtactatggtaaattatctaacggtaaattagcatggattaaatcaactgatttagct

K  E  L  I  K  Y  N  Q  T  G  M  T  L  N  Q  V  A  Q  I  Q
aaagaattaattaagtataatcaaacaggtatgacattaaaccaagttgctcaaatacaa

A  G  L  Q  Y  K  P  Q  V  Q  R  V  P  G  K  W  T  D  A  N
gctggtttacaatataaaccacaagtacaacgtgtaccaggtaagtggacagatgctaac

F  N  D  V  K  H  A  M  D  T  K  R  L  A  Q  D  P  A  L  K
tttaatgatgttaagcatgcaatggatacgaagcgtttagctcaagatccagcattaaaa

Y  Q  F  L  R  L  D  Q  P  Q  N  I  S  I  D  K  I  N  Q  F
tatcaattcttacgcttagaccaaccacaaaatatttctattgataaaattaatcaattc

L  K  G  K  G  V  L  E  N  Q  G  A  A  F  N  K  A  A  Q  M
ttaaaaggtaaaggtgtattagaaaaccaaggtgctgcatttaacaaagctgctcaaatg

Y  G  I  N  E  V  Y  L  I  S  H  A  L  L  E  T  G  N  G  T
tatggcattaatgaagtttatcttatctcacatgccctattagaaacaggtaacggtact

S  Q  L  A  K  G  A  D  V  V  N  N  K  V  V  T  N  S  N  T
tctcaattagcgaaaggtgcagatgtagtgaacaacaaagttgtaactaactcaaacacg


K  Y  H  N  V  F  G  I  A  A  Y  D  N  D  P  L  R  E  G  I
aaataccataacgtatttggtattgctgcatatgataacgatcctttacgtgaaggtatt
```

```
K  Y  A  K  Q  A  G  W  D  T  V  S  K  A  I  V  G  G  A  K
aaatatgctaaacaagctggttgggacacagtatcaaaagcaatcgttggtggtgctaaa

F  I  G  N  S  Y  V  K  A  G  Q  N  T  L  Y  K  M  R  W  N
ttcatcggcaactcatatgtaaaagctggtcaaaatacactttacaaaatgagatggaat

P  A  H  P  G  T  H  Q  Y  A  T  D  V  D  W  A  N  I  N  A
cctgcacatccaggaacacaccaatatgctacagatgtagattgggctaacatcaatgct

K  I  I  K  G  Y  Y  D  K  I  G  E  V  G  K  Y  F  D  I  P
aaaatcatcaaaggctactatgataaaattggcgaagtcggcaaatacttcgacatccca

Q  Y  K  L  E  H  H  H  H  H  H
caatataaactcgagcaccaccaccaccaccac
         XhoI
```

Appendix II. The sequence of the *S. aureus* Atl GL DNA and protein from pKP2. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence is shown in blue upper case font.

```
   M  G  W  K  T  N  K  Y  G  T  L  Y  K  S  E  S  A  S  F
gccatgggttggaaaacaaacaaatatggcacactatataaatcagagtcagctagcttc
 NcoI
T  P  N  T  D  I  I  T  R  T  T  G  P  F  R  S  M  P  Q  S
acacctaatacagatataataacaagaacgactggtccatttagaagcatgccgcagtca

G  V  L  K  A  G  Q  T  I  H  Y  D  E  V  M  K  Q  D  G  H
ggagtcttaaaagcaggtcaaacaattcattatgatgaagtgatgaaacaagacggtcat

V  W  V  G  Y  T  G  N  S  G  Q  R  I  Y  L  P  V  R  T  W
gtttgggtaggttatacaggtaacagtggccaacgtatttacttgcctgtaagaacatgg

N  K  S  T  N  T  L  G  V  L  W  G  T  I  K  L  Q  T  A  P
aataaatctactaatactttaggtgttctttggggaactataaagctgcagactgcacca
                                          PstI
T  A  V  K  P  T  T  S  A  A  K  D  Y  N  Y  T  Y  V  I  K
actgctgtgaaaccaactacatcagctgccaaagattataactacacttatgtaattaaa

N  G  N  G  Y  Y  Y  V  T  P  N  S  D  T  A  K  Y  S  L  K
aatggtaatggttattactatgtaacaccaaattctgatacagctaaatactcattaaaa

A  F  N  E  Q  P  F  A  V  V  K  E  Q  V  I  N  G  Q  T  W
gcatttaatgaacaaccattcgcagttgttaaagaacaagtcattaatggacaaacttgg

Y  Y  G  K  L  S  N  G  K  L  A  W  I  K  S  T  D  L  A  K
tactatggtaaattatctaacggtaaattagcatggattaaatcaactgatttagctaaa

E  L  I  K  Y  N  Q  T  G  M  T  L  N  Q  V  A  Q  I  Q  A
gaattaattaagtataatcaaacaggtatgacattaaaccaagttgctcaaatacaagct

G  L  Q  Y  K  P  Q  V  Q  R  V  P  G  K  W  T  D  A  N  F
ggtttacaatataaaccacaagtacaacgtgtaccaggtaagtggacagatgctaacttt

N  D  V  K  H  A  M  D  T  K  R  L  A  Q  D  P  A  L  K  Y
aatgatgttaagcatgcaatggatacgaagcgtttagctcaagatccagcattaaaatat

Q  F  L  R  L  D  Q  P  Q  N  I  S  I  D  K  I  N  Q  F  L
caattcttacgcttagaccaaccacaaaatatttctattgataaaattaatcaattctta

K  G  K  G  V  L  E  N  Q  G  A  A  F  N  K  A  A  Q  M  Y
aaaggtaaaggtgtattagaaaaccaaggtgctgcatttaacaaagctgctcaaatgtat

G  I  N  E  V  Y  L  I  S  H  A  L  L  E  T  G  N  G  T  S
ggcattaatgaagtttatcttatctcacatgccctattagaaacaggtaacggtacttct

Q  L  A  K  G  A  D  V  V  N  N  K  V  V  T  N  S  N  T  K
caattagcgaaaggtgcagatgtagtgaacaacaaagttgtaactaactcaaacacgaaa

Y  H  N  V  F  G  I  A  A  Y  D  N  D  P  L  R  E  G  I  K
taccataacgtatttggtattgctgcatatgataacgatcctttacgtgaaggtattaaa

Y  A  K  Q  A  G  W  D  T  V  S  K  A  I  V  G  G  A  K  F
tatgctaaacaagctggttgggacacagtatcaaaagcaatcgttggtggtgctaaattc

I  G  N  S  Y  V  K  A  G  Q  N  T  L  Y  K  M  R  W  N  P
atcggcaactcatatgtaaaagctggtcaaaatacactttacaaaatgagatggaatcct


A  H  P  G  T  H  Q  Y  A  T  D  V  D  W  A  N  I  N  A  K
gcacatccaggaacacaccaatatgctacagatgtagattgggctaacatcaatgctaaa
```

```
I  I  K  G  Y  Y  D  K  I  G  E  V  G  K  Y  F  D  I  P  Q
atcatcaaaggctactatgataaaattggcgaagtcggcaaatacttcgacatcccacaa

Y  K  L  E  H  H  H  H  H  H
tataaactcgagcaccaccaccaccaccac
      XhoI
```

Appendix III. The sequence of the lysostaphin targeting domain: Atl GL chimera DNA and protein from pKP3. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence from the lysostaphin targeting domain is shown in blue upper case font, and that of the Atl GL in green.

```
   M  G  T  A  P  T  A  V  K  P  T  T  S  A  A  K  D  Y  N
gccatgggtactgcaccaactgctgtgaaaccaactacatcagctgccaaagattataac
 NcoI
Y  T  Y  V  I  K  N  G  N  G  Y  Y  Y  V  T  P  N  S  D  T
tacacttatgtaattaaaaatggtaatggttattactatgtaacaccaaattctgataca

A  K  Y  S  L  K  A  F  N  E  Q  P  F  A  V  V  K  E  Q  V
gctaaatactcattaaaagcatttaatgaacaaccattcgcagttgttaaagaacaagtc

I  N  G  Q  T  W  Y  Y  G  K  L  S  N  G  K  L  A  W  I  K
attaatggacaaacttggtactatggtaaattatctaacggtaaattagcatggattaaa

S  T  D  L  A  K  E  L  I  K  Y  N  Q  T  G  M  T  L  N  Q
tcaactgatttagctaaagaattaattaagtataatcaaacaggtatgacattaaaccaa

V  A  Q  I  Q  A  G  L  Q  Y  K  P  Q  V  Q  R  V  P  G  K
gttgctcaaatacaagctggtttacaatataaaccacaagtacaacgtgtaccaggtaag

W  T  D  A  K  F  N  D  V  K  H  A  M  D  T  K  R  L  A  Q
tggacagatgctaactttaatgatgttaagcatgcaatggatacgaagcgtttagctcaa

D  P  A  L  K  Y  Q  F  L  R  L  D  Q  P  Q  N  I  S  I  D
gatccagcattaaaatatcaattcttacgcttagaccaaccacaaaatatttctattgat

K  I  N  Q  F  L  K  G  K  G  V  L  E  N  Q  G  A  A  F  N
aaaattaatcaattcttaaaaggtaaaggtgtattagaaaaccaaggtgctgcatttaac

K  A  A  Q  M  Y  G  I  N  E  V  Y  L  I  S  H  A  L  L  E
aaagctgctcaaatgtatggcattaatgaagtttatcttatctcacatgccctattagaa

T  G  N  G  T  S  Q  L  A  K  G  A  D  V  V  N  N  K  V  V
acaggtaacggtacttctcaattagcgaaaggtgcagatgtagtgaacaacaaagttgta

T  N  S  N  T  K  Y  H  N  V  F  G  I  A  A  Y  D  N  D  P
actaactcaaacacgaaataccataacgtatttggtattgctgcatatgataacgatcct

L  R  E  G  I  K  Y  A  K  Q  A  G  W  D  T  V  S  K  A  I
ttacgtgaaggtattaaatatgctaaacaagctggttgggacacagtatcaaaagcaatc

V  G  G  A  K  F  I  G  N  S  Y  V  K  A  G  Q  N  T  L  Y
gttggtggtgctaaattcatcggcaactcatatgtaaaagctggtcaaaatacactttac

K  M  R  W  N  P  A  H  P  G  T  H  Q  Y  A  T  D  V  D  W
aaaatgagatggaatcctgcacatccaggaacacaccaatatgctacagatgtagattgg

A  N  I  N  A  K  I  I  K  G  Y  Y  D  K  I  G  E  V  G  K
gctaacatcaatgctaaaatcatcaaaggctactatgataaaattggcgaagtcggcaaa

Y  F  D  I  P  Q  Y  K  L  E  H  H  H  H  H  H
tacttcgacatcccacaatataaactcgagcaccaccaccaccaccac
                          XhoI
```

Appendix IV. The sequence of the truncated *S. aureus* Atl GL DNA and protein from pKP4. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence is shown in blue upper case font.

gctcgagggagcccgggtaccggtagaaaaaatgagtaaaggagaagaacttttcactgg
 XhoI
agttgtcccaattcttgttgaattagatggtgatggtaatgggcacaaattttctgtcag

tggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatttgcactac

tggaaaactacctgttccgtggccaacacttgtcactactttctcttatggtgttcaatg

cttttcccgttatccggatcacatgaaacggcatgacttttcaagagtgccatgcccga

aggttatgtacaggaacgcactatatctttcaaagatgacgggaactacaagacgcgtgc

tgaagtcaagtttgaaggtgataccttgttaatcgtatcgagttaaaaggtattgattt

taaagaagatggaaacattctcggacacaaactagagtacaactttaactcacacaatgt

atacatcacgacagacaaacaaaagaatggaatcaaagctaacttcaaaattcgccacaa

cattgaagatggatccgttcaactagcagaccattatcaacaaaatactccaattggcga

tggccctgtccttttaccagacaaccattacctgtcgacacaatctgcccttcgaaaga

tcccaacgaaaagcgtgaccacatggtccttcttgagtttgtaactgctgctgggattac

acatggcatggatgagctctacaaactcgagca
                          XhoI

Appendix V. The DNA sequence of the GFP tag of pKP5 with 5' and 3' *Xho*I restriction sites highlighted in red.

```
   M  A  Y  T  V  T  K  P  Q  T  T  Q  T  V  S  K  I  A  Q
gccatggcttatactgttactaaaccacaaacgactcaaacagttagcaagattgctcaa
 NcoI
V  K  P  N  N  T  G  I  R  A  S  V  Y  E  K  T  A  K  N  G
gttaaaccaaacaacactggtattcgtgcttctgtttatgaaaaaacagcgaaaaacggt

A  K  Y  A  D  R  T  F  Y  V  T  K  E  R  A  H  G  N  E  T
gcgaaatatgcagaccgtacgttctatgtaacaaaagagcgtgctcatggtaatgaaacg

Y  V  L  L  N  N  T  S  H  N  I  P  L  G  W  F  N  V  K  D
tatgtattattaaacaatacaagccataacatcccattaggttggttcaatgtaaaagac

L  N  V  Q  N  L  G  K  E  V  K  T  T  Q  K  Y  T  V  N  K
ttaaatgttcaaaacctaggcaaagaagttaaaacgactcaaaaatatactgttaataaa

S  N  N  G  L  S  M  V  P  W  G  T  K  N  Q  V  I  L  T  G
tcaaataacggcttatcaatggttccttggggtactaaaaaccaagtcattttaacaggc

N  N  I  A  Q  G  T  F  N  A  T  K  Q  V  S  V  G  K  D  V
aataacattgctcaaggtacatttaatgcaacgaaacaagtatctgtaggcaaagatgtt

Y  L  Y  G  T  I  N  N  R  T  G  W  V  N  A  K  D  L  T  A
tatttatacggtactattaataaccgcactggttgggtaaatgcaaaagatttaactgca

P  T  A  V  K  P  T  T  S  A  A  K  D  Y  N  Y  T  Y  V  I
ccaactgctgtgaaaccaactacatcagctgccaaagattataactacacttatgtaatt

K  N  G  N  G  Y  Y  Y  V  T  P  N  S  D  T  A  K  Y  S  L
aaaaatggtaatggttattactatgtaacaccaaattctgatacagctaaatactcatta

K  A  F  N  E  Q  P  F  A  V  V  K  E  Q  V  I  N  G  Q  T
aaagcatttaatgaacaaccattcgcagttgttaaagaacaagtcattaatggacaaact

W  Y  Y  G  K  L  S  N  G  K  L  A  W  I  K  S  T  D  L  A
tggtactatggtaaattatctaacggtaaattagcatggattaaatcaactgatttagct

K  E  L  I  K  Y  N  Q  T  G  M  T  L  N  Q  V  A  Q  I  Q
aaagaattaattaagtataatcaaacaggtatgacattaaaccaagttgctcaaatacaa

A  G  L  Q  Y  K  P  Q  V  Q  R  V  P  G  K  W  T  D  A  N
gctggtttacaatataaaccacaagtacaacgtgtaccaggtaagtggacagatgctaac

F  N  D  V  K  H  A  M  D  T  K  R  L  A  Q  D  P  A  L  K
tttaatgatgttaagcatgcaatggatacgaagcgtttagctcaagatccagcattaaaa

Y  Q  F  L  R  L  D  Q  P  Q  N  I  S  I  D  K  I  N  Q  F
tatcaattcttacgcttagaccaaccacaaaatatttctattgataaaattaatcaattc

L  K  G  K  G  V  L  E  N  Q  G  A  A  F  N  K  A  A  Q  M
ttaaaaggtaaaggtgtattagaaaaccaaggtgctgcatttaacaaagctgctcaaatg

Y  G  I  N  E  V  Y  L  I  S  H  A  L  L  E  T  G  N  G  T
tatggcattaatgaagtttatcttatctcacatgccctattagaaacaggtaacggtact

S  Q  L  A  K  G  A  D  V  V  N  N  K  V  V  T  N  S  N  T
tctcaattagcgaaaggtgcagatgtagtgaacaacaaagttgtaactaactcaaacacg

K  Y  H  N  V  F  G  I  A  A  Y  D  N  D  P  L  R  E  G  I
aaataccataacgtatttggtattgctgcatatgataacgatcctttacgtgaaggtatt
```

K Y A K Q A G W D T V S K A I V G G A K
aaatatgctaaacaagctggttgggacacagtatcaaaagcaatcgttggtggtgctaaa

F I G N S Y V K A G Q N T L Y K M R W N
ttcatcggcaactcatatgtaaaagctggtcaaaatacactttacaaaatgagatggaat

P A H P G T H Q Y A T D V D W A N I N A
cctgcacatccaggaacacaccaatatgctacagatgtagattgggctaacatcaatgct

K I I K G Y Y D K I G E V G K Y F D I P
aaaatcatcaaaggctactatgataaaattggcgaagtcggcaaatacttcgacatccca

Q Y K L E G A R V P V E K M S K G E E L
caatataaactcgaggagcccgggtaccggtagaaaaaatgagtaaaggagaagaactt
XhoI

F T G V V P I L V E L D G D V N G H K F
ttcactggagttgtcccaattcttgttgaattagatggtgatggtaatgggcacaaattt

S V S G E G E G D A T Y G K L T L K F I
tctgtcagtggagagggtgaaggtgatgcaacatacggaaaacttacccttaaatttatt

C T T G K L P V P W P T L V T T F S Y G
tgcactactggaaaactacctgttccgtggccaacacttgtcactactttctcttatggt

V Q C F S R Y P D H M K R H D F F K S A
gttcaatgcttttcccgttatccggatcacatgaaacggcatgacttttcaagagtgcc

M P E G Y V Q E R T I S F K D D G N Y K
atgcccgaaggttatgtacaggaacgcactatatctttcaaagatgacgggaactacaag

T R A E V K F E G D T L V N R I E L K G
acgcgtgctgaagtcaagtttgaaggtgataccttgttaatcgtatcgagttaaaaggt

I D F K E D G N I L G H K L E Y N F N S
attgattttaaagaagatggaaacattctcggacacaaactagagtacaactttaactca

H N V Y I T T D K Q K N G I K A N F K I
cacaatgtatacatcacgacagacaaacaaaagaatggaatcaaagctaacttcaaaatt

R H N I E D G S V Q L A D H Y Q Q N T P
cgccacaacattgaagatggatccgttcaactagcagaccattatcaacaaaatactcca

I G D G P V L L P D N H Y L S T Q S A L
attggcgatggccctgtccttttaccagacaaccattacctgtcgacacaatctgccctt

S K D P N E K R D H M V L L E F V T A A
tcgaaagatcccaacgaaaagcgtgaccacatggtccttcttgagtttgtaactgctgct

G I T H G M D E L Y K L E H H H H H H
gggattacacatggcatggatgagctctacaaactcgagcaccaccaccaccaccac
XhoI

Appendix VI. The sequence of the GFP tagged Atl GL DNA and protein from pKP6. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence of Atl GL is shown in blue upper case font, and that of the GFP tag in green.

```
   M  G  T  A  P  T  A  V  K  P  T  T  S  A  A  K  D  Y  N
gccatgggtactgcaccaactgctgtgaaaccaactacatcagctgccaaagattataac
 NcoI
Y  T  Y  V  I  K  N  G  N  G  Y  Y  Y  V  T  P  N  S  D  T
tacacttatgtaattaaaaatggtaatggttattactatgtaacaccaaattctgataca

A  K  Y  S  L  K  A  F  N  E  Q  P  F  A  V  V  K  E  Q  V
gctaaatactcattaaaagcatttaatgaacaaccattcgcagttgttaaagaacaagtc

I  N  G  Q  T  W  Y  Y  G  K  L  S  N  G  K  L  A  W  I  K
attaatggacaaacttggtactatggtaaattatctaacggtaaattagcatggattaaa

S  T  D  L  A  K  E  L  I  K  Y  N  Q  T  G  M  T  L  N  Q
tcaactgatttagctaaagaattaattaagtataatcaaacaggtatgacattaaaccaa

V  A  Q  I  Q  A  G  L  Q  Y  K  P  Q  V  Q  R  V  P  G  K
gttgctcaaatacaagctggtttacaatataaaccacaagtacaacgtgtaccaggtaag

W  T  D  A  K  F  N  D  V  K  H  A  M  D  T  K  R  L  A  Q
tggacagatgctaactttaatgatgttaagcatgcaatggatacgaagcgtttagctcaa

D  P  A  L  K  Y  Q  F  L  R  L  D  Q  P  Q  N  I  S  I  D
gatccagcattaaaatatcaattcttacgcttagaccaaccacaaaatatttctattgat

K  I  N  Q  F  L  K  G  K  G  V  L  E  N  Q  G  A  A  F  N
aaaattaatcaattcttaaaaggtaaaggtgtattagaaaaccaaggtgctgcatttaac

K  A  A  Q  M  Y  G  I  N  E  V  Y  L  I  S  H  A  L  L  E
aaagctgctcaaatgtatggcattaatgaagtttatcttatctcacatgccctattagaa

T  G  N  G  T  S  Q  L  A  K  G  A  D  V  V  N  N  K  V  V
acaggtaacggtacttctcaattagcgaaaggtgcagatgtagtgaacaacaaagttgta

T  N  S  N  T  K  Y  H  N  V  F  G  I  A  A  Y  D  N  D  P
actaactcaaacacgaaataccataacgtatttggtattgctgcatatgataacgatcct

L  R  E  G  I  K  Y  A  K  Q  A  G  W  D  T  V  S  K  A  I
ttacgtgaaggtattaaatatgctaaacaagctggttgggacacagtatcaaaagcaatc

V  G  G  A  K  F  I  G  N  S  Y  V  K  A  G  Q  N  T  L  Y
gttggtggtgctaaattcatcggcaactcatatgtaaaagctggtcaaaatacactttac

K  M  R  W  N  P  A  H  P  G  T  H  Q  Y  A  T  D  V  D  W
aaaatgagatggaatcctgcacatccaggaacacaccaatatgctacagatgtagattgg

A  N  I  N  A  K  I  I  K  G  Y  Y  D  K  I  G  E  V  G  K
gctaacatcaatgctaaaatcatcaaaggctactatgataaaattggcgaagtcggcaaa

Y  F  D  I  P  Q  Y  K  L  E  G  A  R  V  P  V  E  K  M  S
tacttcgacatcccacaatataaactcgaggggagcccgggtaccggtagaaaaaatgagt
                          XhoI
K  G  E  E  L  F  T  G  V  V  P  I  L  V  E  L  D  G  D  V
aaaggagaagaacttttcactggagttgtcccaattcttgttgaattagatggtgatggt

N  G  H  K  F  S  V  S  G  E  G  E  G  D  A  T  Y  G  K  L
aatgggcacaaattttctgtcagtggagagggtgaaggtgatgcaacatacggaaaactt

T  L  K  F  I  C  T  T  G  K  L  P  V  P  W  P  T  L  V  T
acccttaaatttatttgcactactggaaaactacctgttccgtggccaacacttgtcact
```

```
T  F  S  Y  G  V  Q  C  F  S  R  Y  P  D  H  M  K  R  H  D
actttctcttatggtgttcaatgcttttcccgttatccggatcacatgaaacggcatgac

F  F  K  S  A  M  P  E  G  Y  V  Q  E  R  T  I  S  F  K  D
tttttcaagagtgccatgcccgaaggttatgtacaggaacgcactatatctttcaaagat

D  G  N  Y  K  T  R  A  E  V  K  F  E  G  D  T  L  V  N  R
gacgggaactacaagacgcgtgctgaagtcaagtttgaaggtgataccttgttaatcgt

I  E  L  K  G  I  D  F  K  E  D  G  N  I  L  G  H  K  L  E
atcgagttaaaaggtattgattttaaagaagatggaaacattctcggacacaaactagag

Y  N  F  N  S  H  N  V  Y  I  T  T  D  K  Q  K  N  G  I  K
tacaactttaactcacacaatgtatacatcacgacagacaaacaaaagaatggaatcaaa

A  N  F  K  I  R  H  N  I  E  D  G  S  V  Q  L  A  D  H  Y
gctaacttcaaaattcgccacaacattgaagatggatccgttcaactagcagaccattat

Q  Q  N  T  P  I  G  D  G  P  V  L  L  P  D  N  H  Y  L  S
caacaaaatactccaattggcgatggccctgtccttttaccagacaaccattacctgtcg

T  Q  S  A  L  S  K  D  P  N  E  K  R  D  H  M  V  L  L  E
acacaatctgcccttttcgaaagatcccaacgaaaagcgtgaccacatggtccttcttgag

F  V  T  A  A  G  I  T  H  G  M  D  E  L  Y  K  L  E  H  H
tttgtaactgctgctgggattacacatggcatggatgagctctacaaactcgagcaccac
                                                 XhoI

H  H  H  H
caccaccaccac
```

Appendix VII The sequence of the GFP tagged GL' DNA and protein from pKP7. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence of the truncated GL is shown in blue upper case font, and that of the GFP tag in green.

```
   M  A  T  T  T  P  T  T  P  S  K  P  T  T  P  S  K  P  S
gccatggcaacaactacccctactacaccatcaaaaccaacaacaccgtcgaaaccatca
 NcoI
T  G  K  L  T  V  A  A  N  N  G  V  A  Q  I  K  P  T  N  S
actggtaaattaacagttgcagcaaacaatggtgtcgcacaaatcaaaccaacaaatagt

G  L  Y  T  T  V  Y  D  K  T  G  K  A  T  N  E  V  Q  K  T
ggtttatatactactgtttacgacaaaactggtaaagcaactaatgaagttcaaaaaaca

F  A  V  S  K  T  A  T  L  G  N  Q  K  F  Y  L  V  Q  D  Y
tttgctgtatctaaaacagctacattaggtaatcaaaaattctatcttgttcaagattac

N  S  G  N  K  F  G  W  V  K  E  G  D  V  V  Y  N  T  A  K
aattctggtaataaatttggttgggttaaagaaggcgatgtggtttacaacacagctaaa

S  P  V  N  V  N  Q  S  Y  S  I  K  P  G  T  K  L  Y  T  V
tcacctgtaaatgtaaatcaatcatattcaatcaaatctggtacgaaactttatacagta

P  W  G  T  S  K  Q  V  A  G  S  V  S  G  S  G  N  Q  T  F
ccttggggtacatctaaacaagttgctggtagcgtgtctggctctggaaaccaaacattt

K  A  S  K  Q  Q  Q  I  D  K  S  I  Y  L  Y  G  S  V  N  G
aaggcttcaaagcaacaacaaattgataaatcaatttatttatatggctctgtgaatggt

K  S  G  W  V  S  K  A  Y  L  V  D  T  A  K  P  T  P  T  P
aaatctggttgggtaagtaaagcatatttagttgatactgctaaacctacgcctacacca

T  P  K  P  S  T  P  T  T  N  N  K  L  T  V  S  S  L  N  G
atacctaagccatcaacacctacaacaaataataaattaacagtttcatcattaaacggt

V  A  Q  I  N  A  K  N  N  G  L  F  T  T  V  Y  D  K  T  G
gttgctcaaattaatgctaaaaacaatggcttattcactacagtttatgacaaaactggt

K  P  T  K  E  V  Q  K  T  F  A  V  T  K  E  A  S  L  G  G
aagccaacgaaagaagttcaaaaaacatttgctgtaacaaaagaagcaagtttaggtgga

N  K  F  Y  L  V  K  D  Y  N  S  P  T  L  I  G  W  V  K  Q
aacaaattctacttagttaaagattacaatagtccaactttaattggttgggttaaacaa

G  D  V  I  Y  N  N  A  K  S  P  V  N  V  M  Q  T  Y  T  V
ggtgacgttatttataacaatgcaaaatcacctgtaaatgtaatgcaaacatatacagta

K  P  G  T  K  L  Y  S  V  P  W  G  T  Y  K  Q  E  A  G  A
aaaccaggcactaaattatattcagtaccttggggcacttataaacaagaagctggtgca

V  S  G  T  G  N  Q  T  F  K  A  T  K  Q  Q  Q  I  D  K  S
gtttctggtacaggtaaccaaacttttaaagcgactaagcaacaacaaattgataaatct

I  Y  L  F  G  T  V  N  G  K  S  G  W  V  S  K  A  Y  L  A
atctatttatttggaactgtaaatggtaaatctggttgggtaagtaaagcatatttagct

V  P  A  A  P  K  K  A  V  A  Q  P  K  T  A  V  K  A  Y  T
gtacctgctgcacctaaaaaagcagtagcacaaccaaaaacagctgtaaaagcttatact

V  T  K  P  Q  T  T  Q  T  V  S  K  I  A  Q  V  K  P  N  N
gttactaaaccacaaacgactcaaacagttagcaagattgctcaagttaaaccaaacaac

T  G  I  R  A  S  V  Y  E  K  T  A  K  N  G  A  K  Y  A  D
actggtattcgtgcttctgtttatgaaaaaacagcgaaaaacggtgcgaaatatgcagac
```

R T F Y V T K E R A H G N E T Y V L L N
cgtacgttctatgtaacaaaagagcgtgctcatggtaatgaaacgtatgtattattaaac

N T S H N I P L G W F N V K D L N V Q N
aatacaagccataacatcccattaggttggttcaatgtaaaagacttaaatgttcaaaac

L G K E V K T T Q K Y T V N K S N N G L
ctaggcaaagaagttaaaacgactcaaaaatatactgttaataaatcaaataacggctta

S M V P W G T K N Q V I L T G N N I A Q
tcaatggttccttggggtactaaaaaccaagtcattttaacaggcaataacattgctcaa

G T F N A T K Q V S V G K D V Y L Y G T
ggtacatttaatgcaacgaaacaagtatctgtaggcaaagatgtttatttatacggtact

I N N R T G W V N A K D L T A P T A V K
attaataaccgcactggttgggtaaatgcaaaagatttaactgcaccaactgctgtgaaa

P T T S A A K D Y N Y T Y V I K N G N G
ccaactacatcagctgccaaagattataactacacttatgtaattaaaaatggtaatggt

Y Y Y V T P N S D T A K Y S L K A F N E
tattactatgtaacaccaaattctgatacagctaaatactcattaaaagcatttaatgaa

Q P F A V V K E Q V I N G Q T W Y Y G K
caaccattcgcagttgttaaagaacaagtcattaatggacaaacttggtactatggtaaa

L S N G K L A W I K S T D L A K E L I K
ttatctaacggtaaattagcatggattaaatcaactgatttagctaaagaattaattaag

Y N Q T G M T L N Q V A Q I Q A G L Q Y
tataatcaaacaggtatgacattaaaccaagttgctcaaatacaagctggtttacaatat

K P Q V Q R V P G K W T D A K F N D V K
aaaccacaagtacaacgtgtaccaggtaagtggacagatgctaactttaatgatgttaag

H A M D T K R L A Q D P A L K Y Q F L R
catgcaatggatacgaagcgtttagctcaagatccagcattaaaatatcaattcttacgc

L D Q P Q N I S I D K I N Q F L K G K G
ttagaccaaccacaaaatatttctattgataaaattaatcaattcttaaaaggtaaaggt

V L E N Q G A A F N K A A Q M Y G I N E
gtattagaaaaccaaggtgctgcatttaacaaagctgctcaaatgtatggcattaatgaa

V Y L I S H A L L E T G N G T S Q L A K
gtttatcttatctcacatgccctattagaaacaggtaacggtacttctcaattagcgaaa

G A D V V N N K V V T N S N T K Y H N V
ggtgcagatgtagtgaacaacaaagttgtaactaactcaaacacgaaataccataacgta

F G I A A Y D N D P L R E G I K Y A K Q
tttggtattgctgcatatgataacgatcctttacgtgaaggtattaaatatgctaaacaa

A G W D T V S K A I V G G A K F I G N S
gctggttgggacacagtatcaaaagcaatcgttggtggtgctaaattcatcggcaactca

Y V K A G Q N T L Y K M R W N P A H P G
tatgtaaaagctggtcaaaatacactttacaaaatgagatggaatcctgcacatccagga

```
T  H  Q  Y  A  T  D  V  D  W  A  N  I  N  A  K  I  I  K  G
acacaccaatatgctacagatgtagattgggctaacatcaatgctaaaatcatcaaaggc

Y  Y  D  K  I  G  E  V  G  K  Y  F  D  I  P  Q  Y  K  L  E
tactatgataaaattggcgaagtcggcaaatacttcgacatcccacaatataaactcgag
                                                      XhoI
H  H  H  H  H  H
caccaccaccaccaccac
```

Appendix VIII. The sequence of the R12 extended AtI GL DNA and protein from pKP8. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence of R1 is shown in green, of R2 is shown in orange, and that of AtI GL is shown in blue upper case font.

```
     M  A  S  A  Q  P  R  S  V  A  A  T  P  K  T  S  L  P
ggcatatggcttcagcacaaccaagatcagttgctgcaacaccaaaaacgagtttgcca
  NdeI
K  Y  K  P  Q  V  N  S  S  I  N  D  Y  I  R  K  N  N  L  K
aaatataaaccacaagtaaactcttcaattaacgattacattcgtaaaaataacttaaaa

A  P  K  I  E  E  D  Y  T  S  Y  F  P  K  Y  A  Y  R  N  G
gcacctaaaattgaagaagattatacatcttacttccctaaatacgcataccgtaacggc

V  G  R  P  E  G  I  V  V  H  D  T  A  N  D  R  S  T  I  N
gtaggtcgtcctgaaggtatcgtagttcatgatacagctaatgatcgttcgacgataaat

G  E  I  S  Y  M  K  N  N  Y  Q  N  A  F  V  H  A  F  V  D
ggtgaaattagttatatgaaaaataactatcaaaacgcattcgtacatgcatttgttgat

G  D  R  I  I  E  T  A  P  T  D  Y  L  S  W  G  V  G  A  V
ggggatcgtataatcgaaacagcaccaacggattacttatcttggggtgtcggtgcagtc

G  N  P  R  F  I  N  V  E  I  V  H  T  H  D  Y  A  S  F  A
ggtaaccctagattcatcaatgttgaaatcgtacacacacacgactatgcttcatttgca

R  S  M  N  N  Y  A  D  Y  A  A  T  Q  L  Q  Y  Y  G  L  K
cgttcaatgaataactatgctgactatgcagctacacaattacaatattatggtttaaaa

P  D  S  A  E  Y  D  G  N  G  T  V  W  T  H  Y  A  V  S  K
ccagacagtgctgagtatgatggaaatggtacagtatggactcactacgctgtaagtaaa

Y  L  G  G  T  D  H  A  D  P  H  G  Y  L  R  S  H  N  Y  S
tatttaggtggtacggaccatgccgatccacatggatatttaagaagtcataattatagt

Y  D  Q  L  Y  D  L  I  N  E  K  Y  L  I  K  M  G  K  V  A
tatgatcaattatatgacttaattaatgaaaaatatttaataaaaatgggtaaagtggcg

P  W  G  T  Q  F  T  T  T  P  T  T  P  S  K  P  T  T  P  S
ccatggggtacgcaatttacaactacccctactacaccatcaaaaccaacaacaccgtcg

K  P  S  T  G  K  L  T  V  A  A  N  N  G  V  A  Q  I  K  P
aaaccatcaactggtaaattaacagttgcagcaaacaatggtgtcgcacaaatcaaacca

T  N  S  G  L  Y  T  T  V  Y  D  K  T  G  K  A  T  N  E  V
acaaatagtggtttatatactactgtttacgacaaaactggtaaagcaactaatgaagtt

Q  K  T  F  A  V  S  K  T  A  T  L  G  N  Q  K  F  Y  L  V
caaaaaacatttgctgtatctaaaacagctacattaggtaatcaaaaattctatcttgtt

Q  D  Y  N  S  G  N  K  F  G  W  V  K  E  G  D  V  V  Y  N
caagattacaattctggtaataaatttggttgggttaaagaaggcgatgtggtttacaac

T  A  K  S  P  V  N  V  N  Q  S  Y  S  I  K  S  G  T  K  L
acagctaaatcacctgtaaatgtaaatcaatcatattcaatcaaatctggtacgaaactt

Y  T  V  P  W  G  T  S  K  Q  V  A  G  S  V  S  G  S  G  N
tatacagtaccttggggtacatctaaacaagttgctggtagcgtgtctggctctggaaac

Q  T  F  K  A  S  K  Q  Q  Q  I  D  K  S  I  Y  L  Y  G  S
caaacatttaaggcttcaaagcaacaacaaattgataaatcaatttatttatatggctct

V  N  G  K  S  G  W  V  S  K  A  Y  L  V  D  T  A  K  P  T
gtgaatggtaaatctggttgggtaagtaaagcatatttagttgatactgctaaacctacg
```

P T P I P K P S T P T T N N K L T V S S
cctacaccaatacctaagccatcaacacctacaacaaataataaattaacagtttcatca

L N G V A Q I N A K N N G L F T T V Y D
ttaaacggtgttgctcaaattaatgctaaaaacaatggcttattcactacagtttatgac

K T G K P T K E V Q K T F A V T K E A S
aaaactggtaagccaacgaaagaagttcaaaaaacatttgctgtaacaaaagaagcaagt

L G G N K F Y L V K D Y N S P T L I G W
ttaggtggaaacaaattctacttagttaaagattacaatagtccaactttaattggttgg

V K Q G D V I Y N N A K S P V N V M Q T
gttaaacaaggtgacgttatttataacaatgcaaaatcacctgtaaatgtaatgcaaaca

Y T V K P G T K L Y S V P W G T Y K Q E
tatacagtaaaaccaggcactaaattatattcagtaccttggggcacttataaacaagaa

A G A V S G T G N Q T F K A T K Q Q Q I
gctggtgcagtttctggtacaggtaaccaaacttttaaagcgactaagcaacaacaaatt

D K S I Y L F G T V N G K S G W V S K A
gataaatctatctatttatttggaactgtaaatggtaaatctggttgggtaagtaaagca

Y L A V P A A P K K A V A Q P K T A V K
tatttagctgtacctgctgcacctaaaaaagcagtagcacaaccaaaaacagctgtaaaa

L E H H H H H H
ctcgagcaccaccaccaccaccac
XhoI

Appendix IX. The sequence of the *S. aureus* Atl amidase DNA and protein from pKP9. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence is shown in blue upper case font.

```
   M  A  T  T  T  P  T  T  P  S  K  P  T  T  P  S  K  P  S
gccatggcaacaactacccctactacaccatcaaaaccaacaacaccgtcgaaaccatca
 NcoI
T  G  K  L  T  V  A  A  N  N  G  V  A  Q  I  K  P  T  N  S
actggtaaattaacagttgcagcaaacaatggtgtcgcacaaatcaaaccaacaaatagt

G  L  Y  T  T  V  Y  D  K  T  G  K  A  T  N  E  V  Q  K  T
ggtttatatactactgtttacgacaaaactggtaaagcaactaatgaagttcaaaaaaca

F  A  V  S  K  T  A  T  L  G  N  Q  K  F  Y  L  V  Q  D  Y
tttgctgtatctaaaacagctacattaggtaatcaaaaattctatcttgttcaagattac

N  S  G  N  K  F  G  W  V  K  E  G  D  V  V  Y  N  T  A  K
aattctggtaataaatttggttgggttaaagaaggcgatgtggtttacaacacagctaaa

S  P  V  N  V  N  Q  S  Y  S  I  K  P  G  T  K  L  Y  T  V
tcacctgtaaatgtaaatcaatcatattcaatcaaatctggtacgaaactttatacagta

P  W  G  T  S  K  Q  V  A  G  S  V  S  G  S  G  N  Q  T  F
ccttggggtacatctaaacaagttgctggtagcgtgtctggctctggaaaccaaacattt

K  A  S  K  Q  Q  Q  I  D  K  S  I  Y  L  Y  G  S  V  N  G
aaggcttcaaagcaacaacaaattgataaatcaatttatttatatggctctgtgaatggt

K  S  G  W  V  S  K  A  Y  L  V  D  T  A  K  P  T  P  T  P
aaatctggttgggtaagtaaagcatatttagttgatactgctaaacctacgcctacacca

T  P  K  P  S  T  P  T  T  N  N  K  L  T  V  S  S  L  N  G
atacctaagccatcaacacctacaacaaataataaattaacagtttcatcattaaacggt

V  A  Q  I  N  A  K  N  N  G  L  F  T  T  V  Y  D  K  T  G
gttgctcaaattaatgctaaaaacaatggcttattcactacagtttatgacaaaactggt

K  P  T  K  E  V  Q  K  T  F  A  V  T  K  E  A  S  L  G  G
aagccaacgaaagaagttcaaaaaacatttgctgtaacaaaagaagcaagtttaggtgga

N  K  F  Y  L  V  K  D  Y  N  S  P  T  L  I  G  W  V  K  Q
aacaaattctacttagttaaagattacaatagtccaactttaattggttgggttaaacaa

G  D  V  I  Y  N  N  A  K  S  P  V  N  V  M  Q  T  Y  T  V
ggtgacgttatttataacaatgcaaaatcacctgtaaatgtaatgcaaacatatacagta

K  P  G  T  K  L  Y  S  V  P  W  G  T  Y  K  Q  E  A  G  A
aaaccaggcactaaattatattcagtaccttggggcacttataaacaagaagctggtgca

V  S  G  T  G  N  Q  T  F  K  A  T  K  Q  Q  Q  I  D  K  S
gtttctggtacaggtaaccaaacttttaaagcgactaagcaacaacaaattgataaatct

I  Y  L  F  G  T  V  N  G  K  S  G  W  V  S  K  A  Y  L  A
atctatttatttggaactgtaaatggtaaatctggttgggtaagtaaagcatatttagct

V  P  A  A  P  K  K  A  V  A  Q  P  K  T  A  V  K  L  E  H
gtacctgctgcacctaaaaaagcagtagcacaaccaaaaacagctgtaaaactcgagcac
                                                 NcoI

H  H  H  H  H
caccaccaccaccac
```

Appendix X. The sequence of the *S. aureus* Atl R12 DNA and protein from pKP10. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence of R1 is shown in green, and of R2 in orange upper case font.

```
   M  A  Y  T  V  T  K  P  Q  T  T  Q  T  V  S  K  I  A  Q
gccatggcttatactgttactaaaccacaaacgactcaaacagttagcaagattgctcaa
 NcoI
V  K  P  N  N  T  G  I  R  A  S  V  Y  E  K  T  A  K  N  G
gttaaaccaaacaacactggtattcgtgcttctgtttatgaaaaaacagcgaaaaacggt

A  K  Y  A  D  R  T  F  Y  V  T  K  E  R  A  H  G  N  E  T
gcgaaatatgcagaccgtacgttctatgtaacaaaagagcgtgctcatggtaatgaaacg

Y  V  L  L  N  N  T  S  H  N  I  P  L  G  W  F  N  V  K  D
tatgtattattaaacaatacaagccataacatcccattaggttggttcaatgtaaaagac

L  N  V  Q  N  L  G  K  E  V  K  T  T  Q  K  Y  T  V  N  K
ttaaatgttcaaaacctaggcaaagaagttaaaacgactcaaaaatatactgttaataaa

S  N  N  G  L  S  M  V  P  W  G  T  K  N  Q  V  I  L  T  G
tcaaataacggcttatcaatggttccttggggtactaaaaaccaagtcatttaacaggc

N  N  I  A  Q  G  T  F  N  A  T  K  Q  V  S  V  G  K  D  V
aataacattgctcaaggtacatttaatgcaacgaaacaagtatctgtaggcaaagatgtt

Y  L  Y  G  T  I  N  N  R  T  G  W  V  N  A  K  D  L  T  A
tatttatacggtactattaataaccgcactggttgggtaaatgcaaaagatttaactgca

P  T  A  L  E  H  H  H  H  H  H
ccaactgctctcgagcaccaccaccaccaccac
         XhoI
```

Appendix XI. The sequence of the *S. aureus* Atl GL R3 DNA and protein from pKP11. The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence is shown in blue upper case font.

```
   M  G  W  K  T  N  K  Y  G  T  L  Y  K  S  E  S  A  S  F
catatgggttggaaaacaaacaaatatggcacactatataaatcagagtcagctagcttc
NdeI
T  P  N  T  D  I  I  T  R  T  T  G  P  F  R  S  M  P  Q  S
acacctaatacagatataataacaagaacgactggtccatttagaagcatgccgcagtca

G  V  L  K  A  G  Q  T  I  H  Y  D  E  V  M  K  Q  D  G  H
ggagtcttaaaagcaggtcaaacaattcattatgatgaagtgatgaaacaagacggtcat

V  W  V  G  Y  T  G  N  S  G  Q  R  I  Y  L  P  V  R  T  W
gtttgggtaggttatacaggtaacagtggccaacgtatttacttgcctgtaagaacatgg

N  K  S  T  N  T  L  G  V  L  W  G  T  I  K  T  M  G  A  R
aataaatctactaatactttaggtgttctttggggaactataaagaccatgggagcccgg
                                              NcoI
V  P  V  E  K  M  S  K  G  E  E  L  F  T  G  V  V  P  I  L
gtaccggtagaaaaaatgagtaaaggagaagaacttttcactggagttgtcccaattctt

V  E  L  D  G  D  V  N  G  H  K  F  S  V  S  G  E  G  E  G
gttgaattagatggtgatgttaatgggcacaaattttctgtcagtggagagggtgaaggt

D  A  T  Y  G  K  L  T  L  K  F  I  C  T  T  G  K  L  P  V
gatgcaacatacggaaaacttacccttaaatttatttgcactactggaaaactacctgtt

P  W  P  T  L  V  T  T  F  S  Y  G  V  Q  C  F  S  R  Y  P
ccgtggccaacacttgtcactactttctcttatggtgttcaatgcttttcccgttatccg

D  H  M  K  R  H  D  F  F  K  S  A  M  P  E  G  Y  V  Q  E
gatcacatgaaacggcatgacttttcaagagtgccatgcccgaaggttatgtacaggaa

R  T  I  S  F  K  D  D  G  N  Y  K  T  R  A  E  V  K  F  E
cgcactatatctttcaaagatgacgggaactacaagacgcgtgctgaagtcaagtttgaa

G  D  T  L  V  N  R  I  E  L  K  G  I  D  F  K  E  D  G  N
ggtgataccttgttaatcgtatcgagttaaaaggtattgattttaaagaagatggaaac

I  L  G  H  K  L  E  Y  N  F  N  S  H  N  V  Y  I  T  T  D
attctcggacacaaactagagtacaactttaactcacacaatgtatacatcacgacagac

K  Q  K  N  G  I  K  A  N  F  K  I  R  H  N  I  E  D  G  S
aaacaaaagaatggaatcaaagctaacttcaaaattcgccacaacattgaagatggatcc

V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G  D  G  P  V  L  L
gttcaactagcagaccattatcaacaaaatactccaattggcgatggccctgtccttttta

P  D  N  H  Y  L  S  T  Q  S  A  L  S  K  D  P  N  E  K  R
ccagacaaccattacctgtcgacacaatctgccctttcgaaagatcccaacgaaaagcgt

D  H  M  V  L  L  E  F  V  T  A  A  G  I  T  H  G  M  D  E
gaccacatggtccttcttgagtttgtaactgctgctgggattacacatggcatggatgag

L  Y  K  L  E  H  H  H  H  H  H
ctctacaaactcgagcaccaccaccaccac
         XhoI
```

Appendix XII. The sequence of the GFP tagged lysostaphin targeting domain DNA and protein from pLss-T-GFP (from Philip Bardelang, University of Nottingham). The nucleotide sequence is shown in lower case black font, with restriction sites highlighted in red and the start codon underlined. The amino acid sequence of the lysostaphin T domain is shown in blue upper case font, of GFP is shown in green upper case font, and the linker region in pink upper case font.

Appendix XIII.

The effect of plotting data from the bioluminescence reporter assay as a percentage of the negative control.

As described in chapter five, efforts were made to determine the optimum conditions, and minimise the variability, of the bioluminescence reporter assay. However, during assay runs cells continued to be metabolically active and bioluminescence measurements of buffer control cells were not static. There also remained some inherent variability in the assay format, due to the effect on the host cell metabolism of many factors, including minor changes in temperature, inoculum size, and the exact point in the growth curve at which peptides were added. This variability during and between assays caused difficulties in detecting low levels of activity, and comparing the effects of test proteins in different assay runs.

In order to mitigate this variability, and enable the effective comparison of results from different assay runs, data was plotted as; bioluminescence as a percentage of the negative control. In each assay run the effect on bioluminescence of buffer alone was measured alongside the effects of test proteins. Traces were plotted from the starting bioluminescence, and for each timepoint, the level of bioluminescence of cells to which a test protein had been added was calculated as a percentage of the bioluminescence of buffer control cells. Therefore, the bioluminescence reporter assay plots presented in this study do not show traces of buffer control cells.

To illustrate the effect of presenting data in this format, Figure XIII.1 shows the effect of the lysostaphin targeting domain; Mu50 amidase chimera at 1.8μM, on bioluminescence of *S. aureus* RN6390 (pSB2030). Figure XIII.2 shows the same data presented as a percentage of the negative control. Through plotting the data as a percentage of the negative control it can be shown that the decrease in bioluminescence due to the effect of the chimeric protein is separate from the initial background decrease seen in the buffer control cells. Furthermore, this decrease is maintained as the bioluminescence of the buffer control cells subsequently rises.

Through removing the necessity to plot the buffer control, it is also possible to expand the y axis over the range required for the effect of the test proteins. This is useful as, if plotting bioluminescence itself, often the decrease in CPS is relatively small compared to the background increase seen in buffer control cells. This hampers the differentiation between similar levels of activity if the buffer control trace is plotted. Therefore, by plotting bioluminescence as a percentage of the negative control, variance between assays can be reduced, and small changes in activity can be measured.

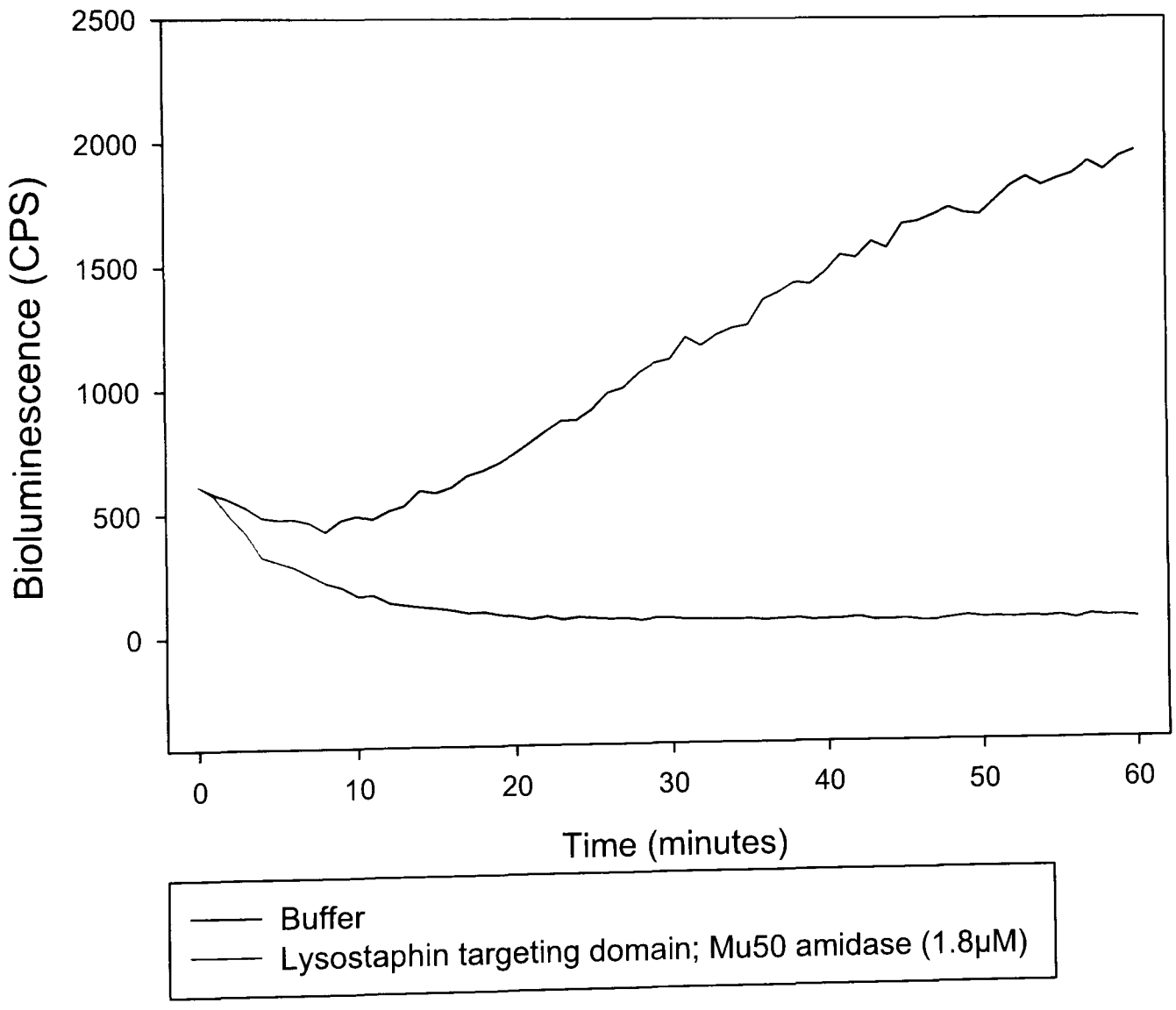

Figure XIII.1 Effect of LssT:Mu50 ami in bioluminescence reporter assay, shown in counts per second

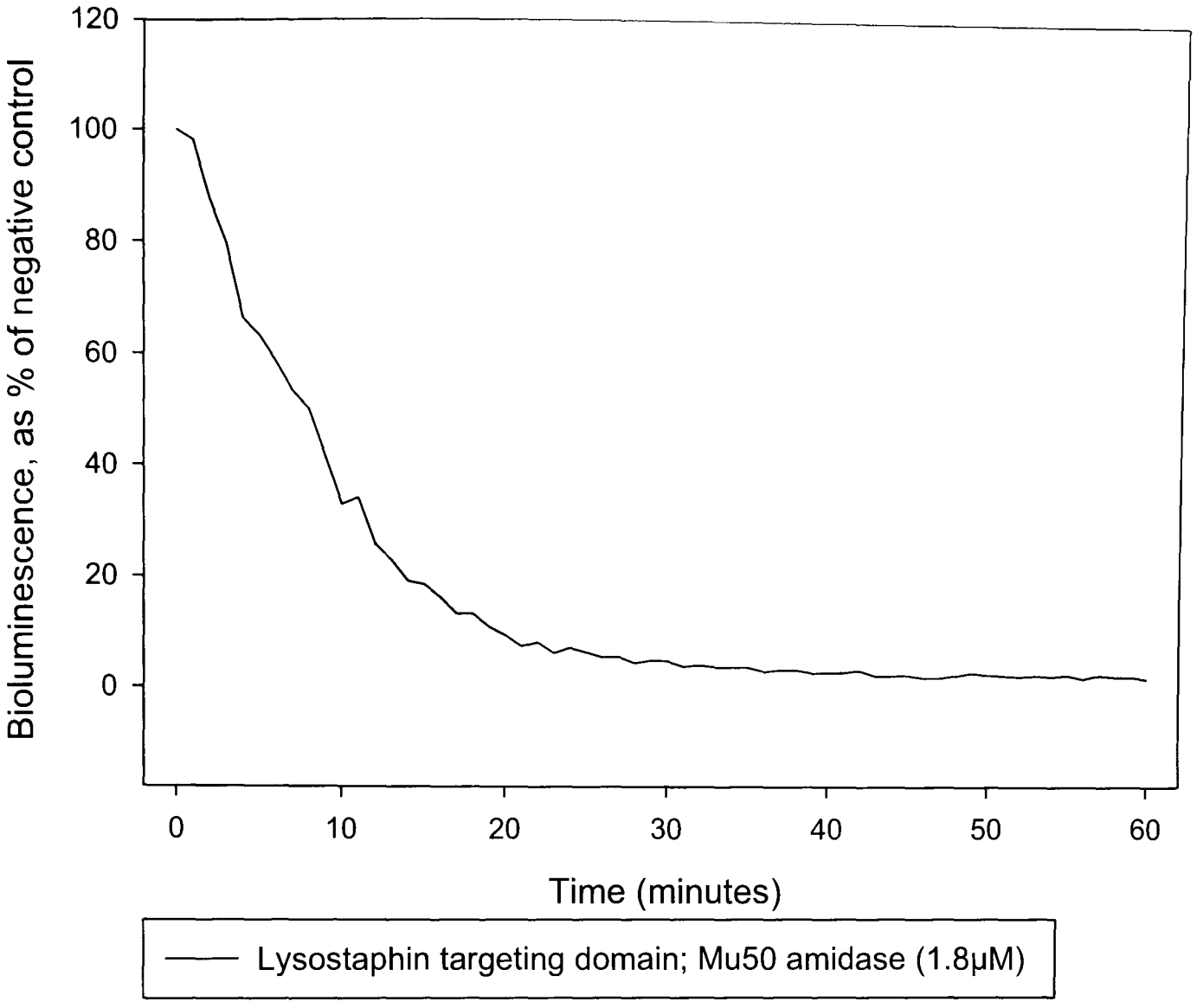

Figure XIII.2 Effect of LssT:Mu50 ami in bioluminescence reporter assay, shown as percentage of negative control.