Studies on the biochemistry of the Targeting domain of Lysostaphin

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A thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

ACKNOWLEDEGMENTS

I would like to express my deepest gratitude to my supervisor Professor Richard James without whose help this project would have never been completed. I would also like to thank Dr. Christopher Penfold for his most valuable guidance and advice throughout my project.

Special thanks go to Dr. Dave Walker who had been so patient with me since the very first day in the lab and devoted a great deal of his time to help me through my first experiments. Also, many thanks to Lisa Holland who has been a great lab mate a great flat mate and above all a great friend who was always willing to help and encourage me. This PhD would not have been the same without Dave and Lisa. Furthermore, I would like to thank Mr. Bryan Healy for his valuable technical advice and constant support in the lab. I would never forget the contribution of Dr. Philip Bardelang to my PhD. I cannot thank him enough for being willing to discuss all of my questions and queries; without his help and organization I would have never been able to complete my experimental work so early.

Last and most importantly, I would like to thank my father Mr. Elias Antoniadis and my mother Mrs. Maria Antoniadou for their unconditional love and support. This thesis is cordially devoted to them.

Eleni Antoniadou

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ABBREVIATIONS

Abz	*Ortho*-aminobenzoic acid
bp	DNA base pairs
DMSO	Dimethyl sulfoxide
EDDnp	(2,4-dinitrophenyl)ethylene diamine
EDTA	Ethylene diaminetetraacetic acid
EMRSA	Epidemic methicillin resistant *Staphylococcus aureus*
FRET	Fluorescence resonance energy transfer
Im9	Colicin E9 immunity protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	DNA kilo base pairs
kDa	Kilo Daltons
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin susceptible *Staphylococcus aureus*
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
R-domain	Receptor binding domain of Colicin E9
SDM	Site directed mutagenesis
SDS	Sodium dodeyl sulphate
T-domain	Lysostaphin targeting domain
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol

ABSTRACT

Lysostaphin from *S. simulans* was cloned in expression vector pET21a and expressed and purified in *E .coli*. A spot test and a broth dilution assay indicated that the minimum inhibitory concentration of lysostaphin required to kill EMRSA-16 was 40 nM. Lysostaphin consists of an endopeptidase and a trargeting domain the former of which codes for catalytic activity while the latter is responsible for substrate specificity. In order to find out whether the targeting domain of lysostaphin is an individually functional domain, it was cloned in vector pET21d and expressed in *E. coli*. The purified protein was assayed against EMRSA-16 in the presence of mature lysostaphin and it was found that the targeting domain alone can protect EMRSA-16 cells. This further indicated the potential of the targeting domain of lysostaphin to be used in future domain-swapping studies with other proteins.

Random PCR mutagenesis was used to identify putative active site residues in the C-terminal targeting domain of lysostaphin. One mutation was isolated, where a phenylalanine was replaced by a serine at position 172 of the mature protein. Sequence alignment with other lysostaphin homologues indicated the presence of three more conserved amino acids, two tyrosines at positions 203 and 226 and a tryptophan at position 214. Site-directed mutagenesis was employed to mutate all conserved residues to alanine and F172 to tyrosine to distinguish between important from unimportant sites. All six mutants (F172S, F172A, F172Y, Y203A, Y226A and W214A) were cloned and their proteins expressed and purified in *E. coli*. Their activity was assayed in an agar diffusion assay, a broth dilution assay, turbidimetrically and in a FRET assay. The results indicated that mutants F172S, F172Y, Y203A and Y226A remained bacteriolytic while mutants F172A and W214A had lost most of their activity, suggesting their significance in the activity of lysostaphin. Finally, a reversion experiment carried out with F172A confirmed the importance of phenylalanine at position 172 of the mature protein.

CHAPTER: 1

INTRODUCTION

1.1 Discovery of antibiotics

Since the 1930s the discovery of new antibiotics has been the cornerstone of antibacterial therapy world-wide (Courvalin and Davies, 1999). Antibiotics are known as the wonder drugs of the 20th century because their development made scientists believe that all diseases caused by bacterial infections would soon be conquered and safely forgotten, leaving cancer and viral diseases the only worrying health problems that would have to be dealt with (Salyers and Amabile-Cuevas, 1997). However, the continuous use of antibiotics to treat bacterial infections resulted in an alarming increase in antimicrobial resistance (Morris *et al.*, 1998) and instead of witnessing the elimination of bacterial infections, scientists started to experience their resurgence (Salyers and Amabile-Cuevas, 1997).

The first incidence happened in 1940 and was recorded by Abraham and Chain (Abraham and Chain, 1940) who isolated and characterised an enzyme from *E. coli* that could hydrolyse penicillin (Tenover and Hughes, 1996). Soon after, the rapid appearance of penicillin-resistant infections caused by *Staphylococcus aureus* took place. This is best illustrated by the fact that while in 1941 all *Staphylococcus aureus* strains worldwide were susceptible to penicillin G, by 1944 the same organism had been capable of defending itself by producing a penicillinase (which is nowadays known as β-lactamase) that destroyed penicillin (Neu, 1992). As a result, the effectiveness of penicillin against serious staphylococcal infections was severely reduced (Gold and Moellering, 1996) and raised the first signs of concern. A few years later, penicillin-resistant staphylococci plagued hospitals all over the UK and over the next two decades, 1950s and 1960s, multidrug resistance was reported in Japan, Latin America, Europe and the USA (Levy, 1994).

The situation deteriorated, as the majority of drug-resistant bacteria were no longer confined to organisms from the gastro-intestinal tract, such as *Escherichia coli, Salmonella* and *Shigella*. By the mid 1970s organisms of the respiratory and genito-urinary tract, including *Haemophilus influenzae* and *Neisseria*

gonorrhoeae, had also developed resistance to certain antimicrobials. Their resistance was traced to the acquisition of antibiotic-resistance genes from drug-resistant bacteria of the gastrointestinal tract (Levy, 1994). This evidence suggested that bacteria can obtain resistance genes even from distantly related genera, a finding that was widely doubted when the first cases of antibiotic resistant microorganisms were observed (Levy, 1994, Salyers and Amabile-Cuevas, 1997). What caused great concern among scientists though was the fast rate with which microbes susceptible to certain antibiotics evolved to multi drug-resistant strains (Salyers and Amabile-Cuevas, 1997). This trend was particularly common among certain pathogens that have the capacity to accumulate antibiotic resistance determinants (Struelens, 1998). A notable example is that of *S. aureus* (which emerged again in the 1970s in the form of a multi-resistant strain) and some other Gram-positive bacilli, especially those causing nosocomial outbreaks such as *Enterococcus faecium* (Murray, 1991).

Nevertheless, in the early 1980s fears of outbreaks of untreatable diseases were allayed by the hope that newer broad-spectrum antibacterial agents, including the cephalosporins and the fluoroquinolones would be effective against multiresistant strains. However, resistance to these drugs quickly appeared from several bacterial strains, including *S. aureus*. Other microorganisms, including *Acinetobacter baumannii* and *Enterococcus faecium* later became resistant to all approved antimicrobial agents (Tenover and Hughes, 1996). In the early 1990s it was predicted that penicillin-resistant *S. pneumoniae* strains would become a significant cause of morbidity (Neu, 1992). Indeed by 1998, not only had the number of penicillin resistant pneumococcal isolates increased all over the world, but multidrug resistance of this strain had been also recorded with resistance to chloramphenicol and cephalosporins that dramatically reduced the options available for effective treatment of these infections (Hart and Kariuki, 1998).

Resistance has been a problem for many decades and its effects are being noted on an ever-increasing scale. Multiresistant organisms are diminishing the ability of the clinicians to control the spread of infectious diseases and as a result the need for new antibiotics is growing fast (Tenover and Hughes, 1996). However, since

the mid 1940s bacteria have proved that they have the remarkable ability to overcome every new agent synthesized and therefore the need for new antibiotics will never cease to exist. For example, despite the availability of countless antibiotics in the 1990s, more than 50 penicillins, 70 cephalosporins, 12 tetracyclines, 8 aminoglycosides, 1 monobactam, 3 carbapenems, 9 macrolides, 2 streptogramins and 3 dihydrofolate reductase inhibitors, a patient could die in any hospital in the world as a result of a bacterial infection (Neu, 1992).

What is more, the original predictions regarding the development of antibiotic resistance erred by not realising to what extent antimicrobial agents would be used. Billions of pounds of antibiotics are used every year and this amount is steadily increasing especially in hospitals. As a result of this excessive use and due to environmental selection pressure on microbes, millions of years of bacterial evolution have been compressed into some 40 years (Davies and Webb, 1998). Furthermore, due to the limited knowledge of cellular biochemistry and bacterial genetics available at the time when the first resistant strains appeared, scientists did not expect bacteria to develop and use resistance mechanisms that they had not predicted. More importantly, the various ways of horizontal gene transfer and the biochemical mechanisms that could confer resistance to antibiotics were not known at that time (Davies and Webb, 1998).

1.2. Antibiotic resistance mechanisms

Antibiotic resistant bacteria are able to proliferate and multiply in the presence of certain levels of antibiotics. This is possible due to the fact that some microorganisms are naturally resistant to some antibiotics while others acquire certain resistant genes either by chromosomal mutations or by exchange of genetic material with resistant bacteria. In bacterial genomes and in extra-chromosomal pieces of DNA, there are a vast number of resistance genes that encode various biochemical mechanisms of drug resistance, including efflux pumps, enzymes capable of inactivating antibiotics or changing antibiotic targets. Further to these mechanisms, bacteria can exchange information, critical for their

survival, such as plasmids (containing resistance genes), transposons and integrons. Beneficial mutations may even be selected in the presence of DNA repair mechanisms that could correct them (Shapiro, 1999).

When the first antibiotics were initially introduced it was believed that the frequency of spontaneous mutations of resistance to drugs was very low, in the order of 10^{-8} to 10^{-9} per bacterial generation and therefore it was speculated that the development of bacterial resistance during therapy was not likely to cause serious problems to their use (Davies and Webb, 1998). Nevertheless, this assumption proved to be wrong when it was realised that the nature of the bacteria allows them to acquire antibiotic resistance from the environment and pass it on to other bacteria with remarkable facility and lack of species specificity (Davies, 1994). In addition, due to the extensive use of antibiotics and as a result of the constant exposure of microorganisms to them an environment has been created that provides resistant bacteria with a continual selective advantage (Lancini *et al.*, 1995).

1.2.1 Types of resistance

There are two types of resistance to antibiotics, intrinsic and acquired. The former type of resistance describes those microorganisms that naturally contain a gene that encodes resistance to a certain antibiotic. The latter term refers to bacteria that have acquired resistance to an antibiotic due to expression of a latent gene, a chromosomal mutation or by exchange of genetic material (fig. 1.1) through transformation (the exchange of DNA), transduction (bacteriophage) or conjugation (extrachromosomal DNA) (Neu, 1992). An example of intrinsic resistance is the reduced sensitivity of Gram-negative bacteria to small molecules such as antibiotics. This is due to the fact that, as opposed to Gram-positive bacteria that are surrounded by a single thick, rigid, porous cell wall composed of peptidoglycans, Gram-negative bacteria possess a much thinner peptidoglycan layer, which is however surrounded by a second layer, a hydrophobic outer membrane (Nikaido, 1994; Brody *et al.*, 1994). This layer functions as an effective barrier because its outer leaflet is composed of lipopolysaccharides

(LPS) that contain saturated fatty acids (as opposed to glycerophospholipid, which are found in most other biological membranes and contain unsaturated fatty acids), which enable the tight packing of the hydrocarbon chains, thus making the interior of the LPS leaflet very rigid. Furthermore, an LPS molecule contains six or seven fatty acid chains (as opposed to glycerophospholipid, which contains only two fatty acids). This large number of hydrocarbon chains attached to a single head group decreases significantly the fluidity of the lipid interior (Nikaido *et al.*, 1994).

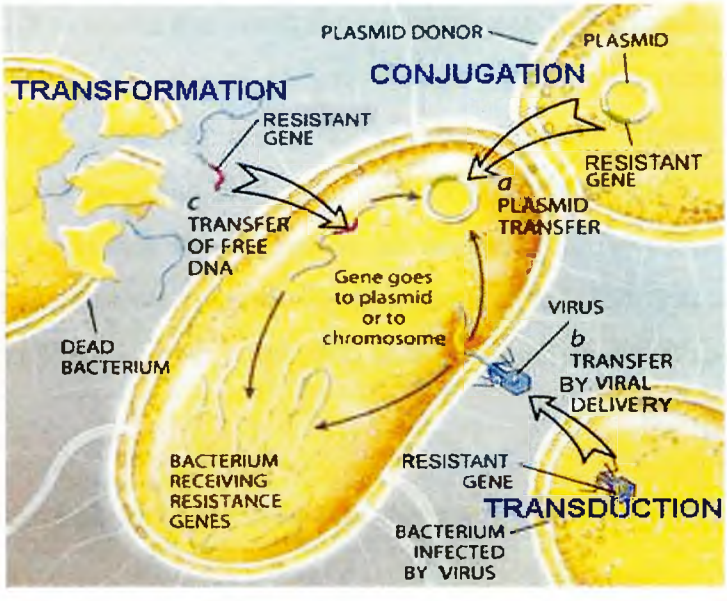

Fig 1.1 The three mechanisms of transfer of genetic material that can lead to acquired antibiotic resistance: Conjugation, Transformation and Transduction (adapted from Levy, 1998).

The basic mechanisms of acquired resistance to antibiotics fall into 5 different categories:

1.2.1.1 Alteration of the target site

For many antibiotics, their mode of action involves inactivation of a specific bacterial enzyme or the ribosome. Through a specific mutation, a large number of resistant bacteria may produce a target protein that cannot bind to the antibiotic. In

some other cases the target protein is not inactivated by the antibiotic because it is not recognised by the latter and the target protein remains fully functional even after the complex with the antibiotic has been formed. This event may be caused by a single point mutation of the genome that results in an important amino acid substitution of the translated protein (Lancini *et al.*, 1995). For instance, the ribosome of staphylococci may become resistant to erythromycin as a result of enzymatic modifications of the rRNA (Davies, 1992). A good example of this is rifampin (Spratt, 1994). On the other hand, in some other cases alteration of the target site is brought about by incorporation of certain genes. For example, if a bacterium acquires a gene that codes for a new enzyme with a much lower affinity for an antibiotic than the target enzyme the antibiotic would normally bind to, resistance to that particular antibiotic will be achieved (Spratt, 1994a). An example of this is penicillin-binding protein 2a (PBP 2a or PBP 2'), which is a drug-resistant target that is encoded by an acquired chromosomal gene called *mecA* that appears to have evolved only as a single event (Ryffel *et al.*, 1990) and has subsequently spread both within and between staphylococcal species (Spratt, 1994a).

1.2.1.2 Reduced uptake of the drug into the cell

As opposed to the cell wall of Gram-positive microorganisms, the cell wall of Gram-negative bacteria has an outer membrane that can act as a molecular filter through certain hydrophilic structures called porins (fig. 1.2). These proteins function as channels for the entrance and exit of hydrophilic low-molecular weight substances. In some cases porins form water-filled channels. Two classes of porins have been identified, specific and non-specific. The former are highly specific because they contain a certain binding site for one or more molecules. On the other hand non-specific porins create a general diffusion channel that allows small substances of any type to cross the membrane into the periplasmic space (Madigan, *et al.*, 1997).

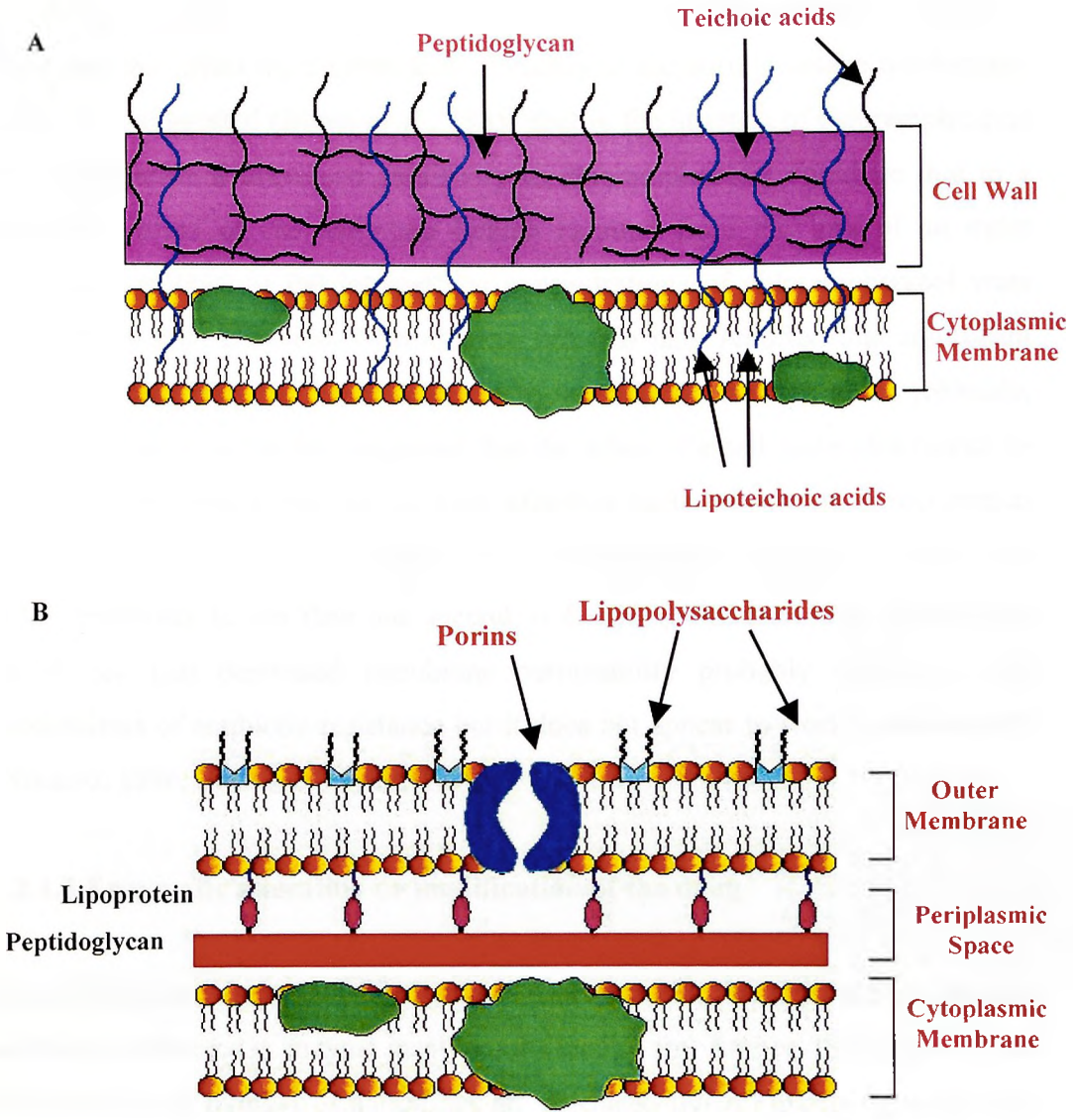

Fig. 1.2 A. Schematic diagram of the structure of a typical Gram-positive bacterial cell envelope, consisting of a cell wall and a cytoplasmic membrane. The cell wall, is composed of lipoteichoic acids located on the surface and embedded in a peptidoglycan layer, linked to the cytoplasmic membrane. Teichoic acids occur on the surface of the cell wall. **B.** The structure of a typical Gram-negative bacterial cell envelope. The cell envelope consists of an outer membrane, a periplasmic space and a cytoplasmic membrane. Here, the peptidoglycan is a very thin layer attached to the outer membrane through lipoproteins. The location of porins and lipopolysaccharides in the outer membrane is indicated by the arrows (adapted from Madigan *et al.*, 1997).

Antibiotic resistance due to reduced drug uptake, can occur as a result of mutations that affect the number and efficiency of the porins' uptake mechanism. It has been suggested (Burns *et al.*, 1985) that in the absence of chloramphenicol acetyltransferase a decreased sensitivity to chloramphenicol could be due to a decreased influx of the antibiotic (which resulted from the loss of an outer membrane protein) as the intracellular concentrations of chloramphenicol were much lower than expected. Nevertheless, Nikaido later reported that significant resistance may only be rarely achieved by permeation barriers alone (Nikaido, 1994). The same author has suggested that the influx of small molecules cannot be completely prevented even by the most effective bacterial barriers. Experimental data show that the half-equilibration time for hydrophilic β-lactams to cross the outer membrane is less than one second in *E. coli* (Nikaido, 1994). It was later established that decreased membrane permeability probably enhances other mechanisms of antibiotic resistance but it does not appear to work independently (Nikaido, 1996; Pratt and Taylor, 1990).

1.2.1.3 Enzymatic alteration or modification of the drug

One of the most important biochemical processes that bacteria use to develop antibiotic resistance is enzyme inactivation (Jacoby and Archer, 1991). Due to the fact that a large number of antibiotics are produced by soil microorganisms it is perhaps not surprising that microorganisms coexisting with antibiotic-producing bacteria might synthesize enzymes that render antibiotics biologically inactive. This mechanism is very important because the genes responsible for the inactivation of the target enzymes can be transferred on plasmids by conjugation and transposition. The abundance of these genes in the pool of common pathogenic bacteria has thus increased significantly with the selective pressure of widespread use. Antibiotics can be inactivated either by enzymatic cleavage or by chemical modification such that they no longer interact with the target site or are no longer taken up by the organism, rendering them inactive (Pratt and Taylor, 1990). Chemical modification can confer clinical resistance to chloramphenicol, penicillins and other β-lactams. Such enzymes are encoded by genes that are mostly transferred by plasmids and they include the β-lactamases that catalyse the

hydrolysis of the β-lactam ring (Brody *et al.*, 1994) and acetyltransferases that inactivate antibiotics by transferring an acetyl group from an acetyl donor to a functional group of the antibiotic (Lancini, 1995). A number of Gram-positive pathogens have an unusual bifunctional aminoglycoside-modifying enzyme that encodes acetyl- and phosphotransferase activities based on protein patterns obtained from two independent resistance genes. This hybrid gene is widely distributed among hospital isolates of staphylococci and can be assumed to have evolved as a fortuitous gene fusion during the process of insertion of the two resistance genes into the cloning site of a transposon (Davies, 1994).

1.2.1.4 Altered amount of target enzyme or receptor

When a drug's activity is based on inhibiting an enzyme that is critical for cell growth, then the cell may be able to produce larger amounts of the enzyme, which will result in sufficient metabolic product for the survival of the organism even in the presence of the drug concentrations that are usually attained during clinical treatment. This may happen in various ways. For example, bacteria may develop resistance to an antibiotic as a result of acquisition of resistance plasmids, and these contain multiple copies of the resistant gene that result in bacteria being capable of producing larger amounts of the target enzyme than usually required for its growth (Pratt and Taylor, 1990). Another mechanism that may result in overproduction of a target enzyme is gene amplification (Berger *et al.*, 1985). Finally, a mutation in a regulatory gene that controls the expression of a gene that encodes the target enzyme could also result in its overproduction. A single mutation in the repressor gene could result in inactivation of its protein or loss of binding of the repressor and consequently in constitutive synthesis of the target enzyme, which could lead to drug resistance (Pratt and Taylor, 1990).

1.2.1.5 Failure to metabolise the drug in its active state

Drugs may be produced in a certain form such that they are inactive (apoenzyme) and need to be introduced into the bacterial cells first and then become converted to the active form (by binding to the coenzyme to produce the holoenzyme). In

these cases, failure of the cell to metabolise the drug into its active form will lead to resistance. This mechanism of resistance usually occurs with drugs that act as antimetabolites (Pratt and Taylor, 1990), that is compounds that 'mimic the structures of normal metabolic constituents such as pyrimidines, folic acid and purines, well enough to inhibit enzymes necessary for folic acid regeneration or for pyrimidine or purine activation for DNA or RNA synthesis in neoplastic cells (Brody *et al.*, 1994).

1.2.2 Methods for transmission of resistance genes

In response to environmental changes, bacteria quickly duplicate and exchange genes that provide them with a selective advantage (Amabile-Cuevas and Chircuel, 1992). The prevalent mechanisms that bacteria use in order to exchange genetic material are via plasmids, transposons and integrons. Plasmids (see section 1.2.2.1) are involved in the exchange of genes by conjugation, through a direct connection formed by a sex pilus (Pratt and Taylor, 1990). Transposons (see section 1.2.2.2) or 'jumping genes' facilitate the movement of a gene between chromosomes and plasmids and between bacteria even of different genera. Integrons (see section 1.2.2.3), constitute a specific subset of transposons. Genes can also be transferred by transduction through infection by a bacteriophage (Lacey, 1975) or by transformation, which is the movement of small pieces of DNA from the environment into the bacterial chromosome. Both mechanisms are considered of major clinical importance, particularly among Gram-positive bacteria (Hakenbeck, 2000).

1.2.2.1 Plasmids

Plasmids that confer resistance to microorganisms are widely distributed in nature and have been identified in virtually all bacteria (Brody *et al.*, 1994). The transmission of resistance to antibiotics, in some cases even to several drugs at the same time, from one bacterium to another is attributed to 'R' or 'r' plasmids. These plasmids are self-replicating double-stranded DNA pieces that occur in circular form and exist in the host microorganism independently from its

chromosome (Williams *et al.*, 1996). The difference between 'R' and 'r' plasmids is that the former are self-transmissable through conjugation whereas the latter are not self-transmissable because they lack the genes for conjugative transfer. Further to this, 'r' plasmids are very common among microorganisms and are very important for Gram-positive bacteria such as staphylococci where they are responsible for most or all of their plasmid-mediated drug resistance (Pratt and Taylor, 1990).

In contrast to 'r', 'R' plasmids may consist of two separate fragments of circular DNA, one of which is responsible for mediating the transfer of both fragments of DNA during conjugation and is called resistance transfer factor (RTF). The other one is responsible for drug resistance and is known as 'r'-determinant. These two DNA fractions may exist independently of each other or may be combined as one plasmid, known as 'R factor' (Pratt and Taylor, 1990). The importance of 'R' plasmids lies on the fact that from an epidemiological point of view, they are transmissible and may be associated with other properties that enable a microorganism to colonise and invade a susceptible host (Brody *et al.*, 1994). Further to this, under certain conditions, the 'r'-determinant components of 'R' factors can be subjected to extensive gene amplification, resulting in an 'R' plasmid that contains multiple tandem copies of 'r' determinants. This may result in resistance of the host microorganism to very high concentrations of antibiotics (Pratt and Taylor, 1990). A plasmid called R751 has been reported to easily cross interspecies barriers (Lewis, 1994) and has been involved in the spread of resistance among several genera within a single hospital (Tompkins *et al.*, 1980).

1.2.2.2 Transposons

Transposons or 'jumping genes' are discrete, movable DNA segments (Lancini *et al.*, 1995). They are characterised by having short terminal sequences at their two ends that range in length from 800 to 1,800 base pairs and are called repeats or insertion sequences (IS). Transposons can integrate into numerous sequences of DNA and can jump from one plasmid to another, from a plasmid to a bacterial chromosome and from a chromosome to a plasmid very easily. It has been

reported that in a large number of bacteria some drug resistance genes have been found in both plasmid and chromosomal location (Pratt and Taylor, 1990). This evidence led to further research, which suggested that it is the abilities of transposition and insertion that make transposons responsible for the reorganisation of the genetic information and as a result for the clustering of many resistance genes in only one plasmid (Lancini *et al.*, 1995). It may be that the mobility of the transposons works in accordance with the site-specific integration system of the plasmids to develop clusters of antibiotic-resistance (Lederberg, 1998).

1.2.2.3 Integrons

Integrons are genetic units, which include genes of a site-specific recombination system capable of capturing and mobilizing genes, contained in mobile elements called gene cassettes (Hall and Collins, 1995). They may be found on R plasmids and occur in both Gram-negative and Gram-positive bacteria (Davies and Webb, 1998). Integrons have a specific structure, which consists of two conserved regions (*intI* region and *sulI* region) flanking a central region, which is known as a gene cassette and contains one or more resistance genes. The 5' end region encodes a site-specific integrase and a strong promoter or promoters that are responsible for expression of the integrated cassettes (Davies, 1994). Most integrons have a *sulI* gene (Vila, 1998), which encodes for resistance to sulfonamides. It is possible that that initial incorporation of that *sulI* gene, during the early use of sulfonamides, preceded the subsequent acquisition of other antibiotic resistance genes. It is also possible that the integron that encodes resistance to sulfonamides has been duplicated and expressed during exposure to antibiotics and has inserted other resistance genes that were also duplicated in response to the stress. These assumptions are enhanced by experiments which concluded that the antibiotic resistant determinants are of diverse origins. This was postulated by nucleotide sequence analysis of multiresistant integrons, which indicated that the inserted resistance gene cassettes differed markedly in codon usage (Davies, 1994).

1.2.2.4 Transduction

Transduction is the transfer of genetic material through a bacteriophage from a donor strain to a recipient one. A phage enters the lytic cycle with newly replicated phage chromosomes packed in its viral capsid. However, a small number of newly replicated phages contain only fragments of the host chromosome, which could include genetic determinants for resistance to an antibiotic. In this case, infection of a bacterium would result in transmission of resistance to the host cell (Lancini *et al.*, 1995). Both plasmids and chromosomal genes may be transferred to bacteria through transduction. It has also been reported that transduction has been found in a wide variety of environments and in at least 60 species of bacteria (Kokjohn, 1989). Even though this mechanism is well studied, it is considered less important in the dissemination of antibiotic resistance genes. This is because phages have limited host ranges and can only infect members of the same or closely related species. Also, the size of the DNA that can be transferred is limited to 50 kb (Davies and Webb, 1998).

1.2.3 The problem of antimicrobial resistance

When antimicrobial resistance first appeared it was thought to be a very slow process. The error rate of DNA repair enzymes for the repair of mistakes made during DNA replication has been shown to vary from 99% to 3%, depending on the nature of the sequence that needs repair (Pennisi, 1998). Also, high-efficiency mechanisms such as proof-reading and repair systems result in DNA replication error rates, which may be in the order of a single mistake per 20 *Escherichia coli* genomes (Shapiro, 1999). Thus, even though mutations are slow to accumulate, changes leading to drug resistance are typically manifest almost immediately (Lederberg, 1998). Several experimental studies on mutations in microorganisms have revealed that there are many biochemical factors that play an important role in mutagenesis, which therefore reject the idea that genetic mutations are a result of chemical fluctuations or incidental damage (Shapiro, 1999). Further studies on antibiotic resistance and pathogenesis determinants suggest that large DNA rearrangements play a much greater evolutionary role than do simpler nucleotide

substitutions. For instance, it has been demonstrated that *E. coli* uses site-specific recombination to accumulate multidrug resistance operons in plasmids and transposons. Therefore, the evolutionary theory is not limited to processes dependent on the slow accumulation of rare, independent mutations (Shapiro, 1999).

It has been suggested that under stressful conditions the cell can initiate its own restructuring (Pennisi, 1998). This involves high rates of mutations, which suggests that when bacteria are in need of genetic changes they search into their environment and do not anticipate these changes to happen naturally (Shapiro, 1999). For example, in 1998, it was found out that the mutation rates in *E. coli* increased when the microbes needed to evolve new capabilities in order to survive changes in their environment (Pennisi, 1998). At the time it seemed that only those genes directly involved with the adaptation changed, and this idea of adaptive or directed evolution was not widely accepted (Pennisi, 1998). Moreover, it has been shown that bacteria have certain natural genetic engineering systems, whose sole function in the organism is to mediate DNA rearrangements (Shapiro, 1999). Scientists have discovered that, certain DNA segments are more likely to be duplicated or transferred to another area of the genome, according to their sequences (Pennisi, 1998). It has also been demonstrated that, when a bacterial population has to survive under stressful conditions, the cells may achieve proliferation due to spontaneous amplifications of a gene (chromosomal or plasmid) coding for an essential function, such as antibiotic resistance (Pratt and Taylor, 1990). However, cascades of antibiotic resistance gene transfer are likely to be provoked even between unrelated microbes due to the intense selective pressure of antibiotic usage (Davies and Webb, 1998). DNA sequencing has revealed similarities between resistance genes found in Gram-positive and Gram-negative bacteria, including plasmid-mediated β-lactamase (Ambler, 1980) and genes for tetracycline resistance (Levy and Miller, 1989). *TetM* in particular, the gene that confers tetracycline resistance is very common and has been found in various microorganisms, including staphylococci, streptococci and enterococci (Roberts *et al.*, 1990).

1.3. *S. aureus* and EMRSA (Epidemic MRSA)

Antibiotics were considered as miracle drugs when they were first discovered however, nowadays multi-drug resistant bacteria that are hard to control have become more and more common (Levy, 1998). *S. aureus* is a particularly worrying problem not only because it is the most common hospital-acquired pathogen causing a variety of diseases such as septicaemia and endocarditis but it is also capable of acquiring a variety of antibiotic resistance genes. This has resulted in the international spread of several epidemic strains of *S. aureus* with multi drug resistance, such as EMRSA-15, EMRSA-16 and more recently EMRSA-17. In this thesis I will now concentrate on methicillin-resistant *S. aureus* (MRSA).

1.3.1 General characteristics

Staphylococci are inherently susceptible to a vast number of antibiotics, excluding those with Gram-negative spectra. However, due to the fact that they develop resistance very easily, both by mutation and DNA transfer, they still remain a widespread problem in hospitals and health care clinics worldwide (Lowy, 1998). Staphylococci belong to a group of microorganisms known as the pyogenic cocci. These are invasive, Gram-positive pathogens that can cause pus-forming infections in humans and other animals. Further to this, staphylococci are non-motile, catalase positive facultative anaerobes that grow in "grapelike" clusters and can be divided into pathogenic and relatively nonpathogenic species on the basis of the synthesis of the enzyme coagulase. Coagulase positive strains of staphylococci are chiefly *S. aureus*, which are sometimes referred to as "golden staph" because they usually produce a yellow carotenoid pigment (Lyon and Skurray, 1987). They may cause a wide variety of infections in the skin and soft tissues, ranging from minor eruptions to infected ulcers, cellulitis and severe impetigo. Further to this, *S. aureus* is very commonly found in surgical and other wounds and in some cases it can even lead to sepsis. It has been reported that the underlying microorganism accounts for 20% of bacteraemias in England and Wales (Reacher *et al.*, 2000). The most common staphylococcal infections occur

in bones and joints due to contamination in orthopaedic surgery. Other common surgical infection sites include prosthetic valve endocarditis, post-neurosurgical meningitis and native valve endocarditis, usually among intravenous narcotic abusers. In rare cases of debilitated patients on ventilators, *S. aureus* may also cause pneumonia (Lowy, 1998). Finally, severe diarrhoea may be caused if food contaminated by certain strains of *S. aureus*, which produce one or more enterotoxins is eaten. Nevertheless, approximately one third of the population carries *S. aureus* innocuously, usually on the moist skin in the nose, axillae and perineum (Livermore, 2000).

Coagulase-negative staphylococci (CNS) include a variety of species, notably *Staphylococcus epidermidis*, *S. haemoliticus* and *S. saprophyticus*. These are considerably less pathogenic than *S. aureus* and normally live on the skin. However, they may become pathogenic by attacking debilitated, hospitalised patients, who have very weak systems and are highly prone to bacteraemia due to the presence of intravascular devices (Livermore, 2000). In general, coagulase negative staphylococci are responsible for about 7-9% of bacteraemias reported in England (Reacher *et al.*, 2000) but are more frequent in cases of prosthetic valve endocarditis and peritonitis while *S. saprophyticus* is usually responsible for urinary tract infections (Livermore, 2000).

1.3.2 Emergence of resistant *S. aureus*

Before the discovery of antibiotics patients with staphylococcal infections had a very poor prognosis. However, when penicillin was developed and introduced into clinical use the situation changed dramatically. Infections such as burns and other serious skin conditions could be treated effectively (Lyon and Skurray, 1987). However, by 1944 the widespread isolation of penicillin resistant *S. aureus* strains was observed (Barber and Rozwadowska-Dowzenko, 1948). The reason for this rapid appearance of penicillin resistant isolates was attributed to the heavy and unnecessary use of the antibiotic. It has been reported that at the time, penicillin powder was dusted on infected wounds and penicillin-containing snuff was available in order to "treat" respiratory infections (Livermore, 2000). Not

surprisingly, in 1948 it was reported that over half of the hospital strains in Britain were resistant to penicillin (Barber and Rozwadowska-Dowzenko, 1948). Not long after these events however, more antibiotics were introduced targeting various aspects of protein synthesis (as opposed to penicillins) such as chloramphenicol, streptomycin, erythromycin and tetracycline (fig. 1.3). Initially, these antibiotics were very effective against staphylococcal infections however resistance was developed to these agents as well. It was then observed that the strains resistant to the above antibiotics were also usually resistant to penicillin because the bacteria produced a β-lactamase (penicillinase). As a result, a large number of *S. aureus* strains had been created with a wide spectrum of resistance and an amazing ability to survive and spread in the hospitals (Lyon and Skurray, 1987).

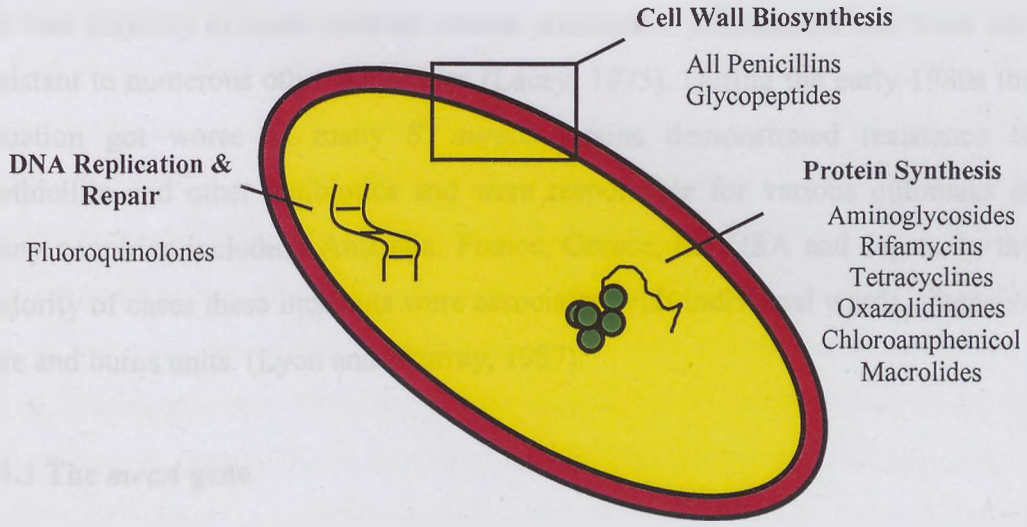

Fig. 1.3 The various sites of action of antibiotics. Cell wall biosynthesis at the stage of cross-linking of peptidoglycan peptide strands by transpeptidases is inhibited by all penicillins (including semi-synthetic penicillins such as methicillin). Protein synthesis is targeted by macrolides, tetracyclines, aminoglycosides, oxazolidinones, chloroamphenicol and rifamycins while fluoroquinolones block DNA replication (adapted from Walsh, 2000). A more detailed description of their mode of action may be seen in Table: 1.1.

In the early 1960s however, new β-lactamase resistant semi synthetic penicillins, such as methicillin, were developed against the multi resistant *S. aureus* strains. These molecules have bulky 6' acyl groups that prevent attack from penicillinase-positive *S. aureus* to the β-lactam ring and therefore allow the antibiotic to kill the cells (Livermore, 2000). Nevertheless, resistance to methicillin appeared soon after the drug was introduced, in 1961 (Jevons, 1961) but these strains were still not a big threat for the overall effectiveness of the antibiotic (Parker and Hewitt, 1970). The problem developed in the late 1960s and early 1970s when strains resistant to the semisynthetic β-lactams appeared in increased frequency in various countries worldwide (Lyon and Skurray, 1987). In Belgium (Klastersky *et al.*, 1971), Poland (Borowski *et al*, 1964) and the United Kingdom (Parker and Hewitt, 1970) these resistant strains (MRSA) increased in frequency from <1% to about 5% in 1980s/1990s (Lyon and Skurray, 1987). It was also then recorded that the vast majority of these resistant strains produced a β-lactamase and were also resistant to numerous other antibiotics (Lacey, 1975). During the early 1980s the situation got worse as many *S. aureus* strains demonstrated resistance to methicillin and other antibiotics and were responsible for various outbreaks in many countries including Australia, France, Greece, the USA and Japan. In the majority of cases these incidents were associated with individual wards, intensive care and burns units. (Lyon and Skurray, 1987).

1.3.3 The *mecA* gene

Clinical isolates of methicillin resistant staphylococci carry a resistance gene called *mecA* that confers resistance to all β-lactams. It is responsible for the production of a penicillin-binding protein (PBP), which is known as PBP2a or PBP2' (Berger-Bachi, 1994). The *mecA* gene is highly conserved among staphylococcal species (Chambers, 1997) and is carried by large pieces of chromosomally inserted DNA, approximately 21-67 kb in size (Hiramatsu *et al.*, 2001) that have no homologues in methicillin-susceptible strains of *S. aureus* (Livermore, 2000). These pieces of DNA are called staphylococcal chromosomal cassette *mec* or 'scc*mec*' and may be found in four different types, that is I, II, III

and IV which vary in the number of genes they carry (besides the *mecA*), their size and organization. In types II and III, the cluster consists of three different genes *mecI-mecRI-mecA*, while in types I and IV much of *mecRI* is deleted (Baba *et al.*, 2002). Finally, the presence of an insertion-sequence-like (IS-like) element (Berger-Bachi, 1994) has been considered to play two different roles. Firstly, it has been suggested to be responsible for the deletion of *mecA* and therefore reversion of MRSA to the methicillin-susceptible phenotype (Georgopapadakou, 1993) and secondly, to act as a trap for additional, unrelated drug-resistance genes, resulting in multi-drug resistance (Berger-Bachi, 1994).

Regulation of the *mecA* gene, is primarily based on two other genes, *mecI* and *mecRI*. *mecI* is responsible for the production of a repressor protein known as MecI while *mecRI* is responsible for the production of a signal-transduction protein, MecRI. In the presence of β-lactam antibiotics, MecRI becomes activated and as a result cleaves MecI repressor protein from the operator region of the *mecA* gene thus initiating production of PBP2' by *mec*A (Hiramatsu *et al.*, 2001). Methicillin resistant staphylococci that carry this fragment have a low-level resistance to methicillin that is slowly inducible and sometimes only expresses borderline resistance (Berger-Bachi, 1994). The *mecA* gene is widely distributed among *S. aureus* strains and coagulase-negative staphylococci and it has therefore been speculated that transfer of the *mecA* gene occurs freely among staphylococcal species (Katayama *et al.*, 2000). The *mecA* gene was acquired by methicillin-resistant staphylococci from an as yet unknown bacterial strain through horizontal gene transfer. However, transduction by bacteriophages also appears to be a likely mechanism (Livermore, 2000). Further to this, it is assumed that evolution of MRSA pre-dated the launch of antistaphylococcal penicillins because MRSA appeared as soon as penicillin was introduced into clinical practice and transfer is not straightforward. In addition to this, it has been suggested that *S. aureus* acquired the methicillin resistance on one occasion and all subsequent MRSA strains have emerged from this one introduction (Livermore, 2000).

1.3.4 Penicillin Binding Proteins (PBPs)

Usually, *S. aureus* strains use four different pencillin-binding proteins whose role is to catalyse the transpeptidation reactions, which are responsible for the production of the glycine residues that form the cross-linking of peptidoglycan. MRSA uses an additional protein called PBP2a or PBP2', which is encoded by the *mecA* gene and has low affinity for β-lactams (Katayama *et al.*, 2000). The size of the protein is approximately 78 kDa. Moreover, it has been reported that the amount of production of PBP2a does not correlate with levels of resistance in MRSA. Expression of the protein may be affected by growth conditions such as pH and temperature (Georgopapadakou, 1993) and the presence of the repressor *mecI* gene (Livermore, 2000). PBP2a is highly resistant to inhibition by all clinically used β-lactams and remains active to maintain cell wall synthesis at normally lethal β-lactam concentrations. Nevertheless, the ability of PBP2a in the formation of the cell wall appears to be restricted to the synthesis of muropeptide dimers, thus leading to a peptidoglycan with a low number of highly crosslinked oligomeric compounds (Berger-Bachi, 1994).

1.3.5 Heteroresistance

The phenomenon of heteroresistance is a typical characteristic of methicillin resistant staphylococci. Approximately 99.9% or more of a staphylococcal cell population (Chambers, 1997) exhibit low level resistance to methicillin. Nevertheless, if the same population becomes exposed to high β-lactam concentrations, a small number of the cells will produce highly resistant subclones, able to survive at methicillin concentrations that exceed 250 mg l^{-1}. The minimum inhibitory concentration, together with the proportion of the highly resistant subpopulation, constitute properties that are strain specific and reproducible under strictly controlled experimental conditions. Further to this, it has been reported that once expressed, high-level resistance to methicillin is stable and the highly resistant subpopulation does not usually revert to the original heterogeneous phenotype. Nevertheless, in some cases, the original low-levels of

resistance may be regained by the subpopulation within approximately 20 generations (Berger-Bachi, 1994).

1.3.6 Fem factors

Six different chromosomal genes have been identified that play a very important role in resistance to methicillin. These are called *fem* (for factor essential for methicillin resistance) or *aux* (auxiliary) factors and are present in both susceptible and resistant strains (Berger-Bachi, 1994). *FemA*, *femB*, *femC* (fig. 1.4), *femD*, *femE* and *femF* have been proved to be involved in specific steps of cell wall biosynthesis. The best identified and most important of the *fem* factors seems to be the *femAB* operon because its inactivation has dramatic effects on the resistance levels of the cells and because it is closely involved in a very specific aspect of cell wall biosynthesis for staphylococci, the addition of glycines to the pentaglycine links in the cell wall (Labischinski *et al.*, 1998).

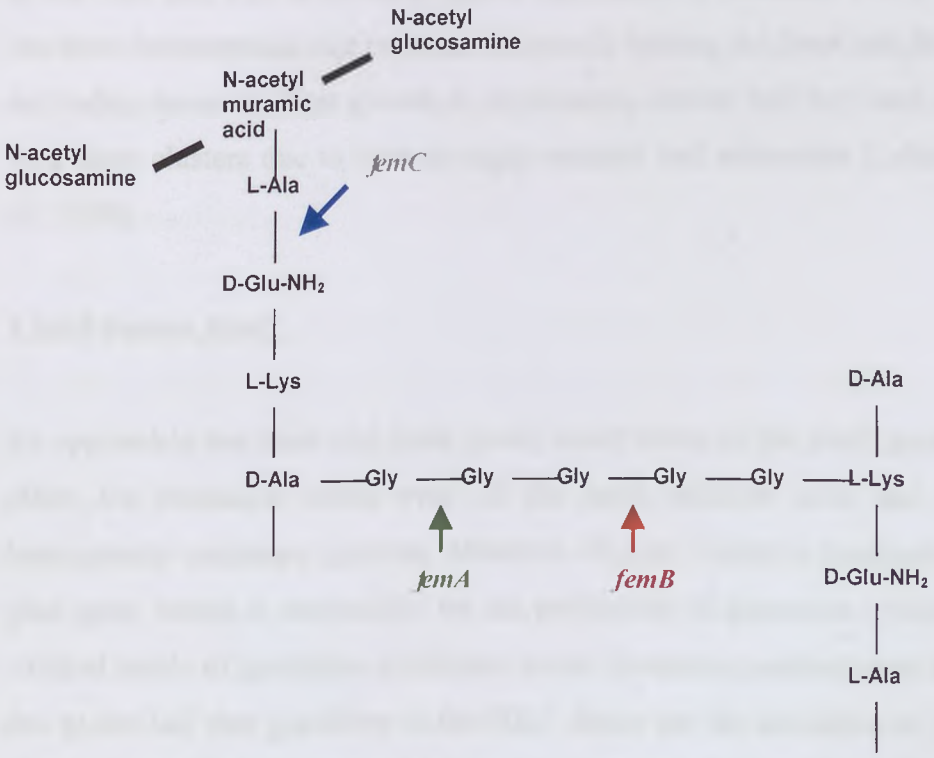

Fig. 1.4 Diagram indicating the basic components of the peptidoglycan backbone and the sites of action of the *femA*, *femB* and *femC* genes.

1.3.6.1 Factors *femA* and *femB*

The *femA* and *femB* factors together form an operon that codes for two similar proteins of 48 and 47 kDa (Berger-Bachi, 1994) and are produced during exponential growth (Labischinski *et al.*, 1998). They are responsible for the formation of the cell wall and in particular the pentaglycine interpeptide bridge that acts as the cross link of the peptidoglycan (Chambers, 1997). This pentaglycine interpeptide bridge is synthesized in an unusual manner, using glycine residues and glycine-adding enzyme(s) that add one glycine at a time (Labischinski *et al.*, 1998). Analysis of the cell wall structure, formed by *femA* and *femB* mutants revealed that the *femA* mutants do not incorporate the second and third glycines into the bridge and that the *femB* mutants produce crosslinks with only three glycines (Ton-That *et al.*, 1998). It has also been shown that disruption of femA and femB genes may result in the MIC (minimum inhibitory concentration) of the cells to fall nearly to susceptible levels while the production of the PBP and PBP2a proteins remain unaffected (Chambers, 1997). Finally, it has been demonstrated that mutants completely lacking the *femA* and *femB* factors are viable, however, their growth is significantly slower and they tend to grow in very large clusters due to their strongly retarded cell separation (Labischinski *et al.*, 1998).

1.3.6.2 Factor *femC*

As opposed to the *femA* and *femB* genes, inactivation of the *femC* gene does not affect the resistance levels even of the most resistant cells and results in homogenous resistance patterns. Mutation of *femC* leads to inactivation of the *glnA* gene, which is responsible for the production of glutamine synthetase. The reduced levels of glutamine synthetase cause glutamine production to halt. Thus, due to the fact that glutamine is the NH_4^+ donor for the amidation of glutamate, which is the third amino acid of the stem peptide, the resulting peptidoglycan cross-linking of the cell wall is reduced (Chambers, 1997). It has been also found that addition of glutamine to the medium relieves the shortage of glutamine,

returning the peptidoglycan composition to normal and therefore restoring resistance to methicillin (Berger-Bachi, 1994).

1.3.6.3 Factors *femD*, *femE* and *femF*

The roles of *femD*, *femE* and *femF* are not fully understood yet. It has been shown that inactivation of the *femF* gene leads to a block in peptidoglycan precursor synthesis at the lysine addition step and as opposed to *femC*, mutation in the *femF* gene results in a heterogenous pattern of resistance. Furthermore, inactivation of *femD* leads to disappearance of unsubstituted disaccharide pentapeptide monomer from the cell wall of MRSA (Chambers, 1997) while the *femE* gene appears to only have a marginal effect on methicillin resistance (Barger-Bachi, 1994).

1.3.7 Treatments used against EMRSA

Because of the multiple antibiotic resistance properties of epidemic MRSA isolates there are few antibiotics that are still effective clinically. Their mode of action as well as their activity spectrum are illustrated in Table: 1.1.

1.3.7.1 Vancomycin

Currently, vancomycin (table: 1.1) is usually the drug of choice for infections caused by MRSA. However, cases of nephrotoxicity and ototoxicity have been documented in patients with MRSA when treated with vancomycin. As a result, pharmacokinetic monitoring of the administered levels is routinely performed and together with the need for parenteral administration renders vancomycin an expensive therapy (Khare and Keady, 2003). Furthermore, in 1997 the first MRSA isolate with reduced susceptibility to vancomycin was reported in Japan (Hiramatsu *et al.*, 1997). According to the British Society for Antimicrobial Chemotherapy, vancomycin-resistant *S. aureus* (VRSA) strains, have a vancomycin minimum inhibitory concentration (MIC) of 8 mg/L. These strains however, have been characterised as vancomycin-intermediate *S. aureus* (VISA) or glycopeptide-intermediate *S. aureus* in the USA, according to the National

Commiteee for Clinical Laboratory standards (CCLS) (Hiramatsu, 2001a). According to Hiramatsu, the principle characteristic of all VRSA strains is the thickened peptidoglycan cell wall as increased amounts of peptidoglycan layers are produced by these resistant strains. Since the identification of the first VRSA strain in Japan, many more incidents with VRSA isolates have been reported worldwide (Korea, Brazil, France, USA, South Africa), making this a global issue (Hiramatsu, 2001a).

1.3.7.2 Linezolid

Linezolid (Table: 1.1) belongs to a new category of antimicrobial agents called oxazolidinones and is highly active against Gram-positive staphylococci, including MRSA. These compounds are not chemically related to currently available agents and their activity is based on selective inhibition of bacterial protein synthesis by binding to the 50S ribosomal subunit (Noskin *et al.*, 1999). The advantages of linezolid include its ability to treat clinical conditions such as bacteraemia, its intravenious as well as oral administration and the low side effects associated with its therapeutic use (Diekema and Jones, 2000). According to experiments carried out by Noskin *et al.*, the potency of linezolid is comparable to that of vancomycin (Noskin *et al.*, 1999) thus providing an equally active alternative choice of treatment for MRSA. Furthermore, successful treatment of vancomycin-resistant enterococci by linezolid could also mean that the drug may be active against VRSA. Nevertheless, resistance to linezolid by a clinical isolate of MRSA has already been recently reported (Tsiodras *et al.*, 2001).

1.3.7.3 Rifampin

Rifampin (Table: 1.1) is a very potent, bactericidal antistaphylococcal agent. Its role in MRSA treatment is controversial and is usually recommended in combination with nafcillin or vancomycin in cases of staphylococcal prosthetic valve endocarditis. However, it has also been reported that vancomycin alone is more effective against MRSA than if combined with rifampin. According to a study, it took longer for patients treated with both of the above agents to recover

than those treated only with vancomycin (Chambers *et al.*, 1997). Due to the fact that high-level resistance to rifampin occurs if the drug is used alone, it is advised to only use it in combination with another antistaphylococcal agent to which the particular strain is still susceptible. Further to this, it has been suggested that rifampin is only used to treat MRSA in cases of prosthetic valve endocarditis and other prosthetic device infections or osteomyelitis (Chambers, 1997).

1.3.7.4 Other agents

Other agents that may be used to treat MRSA infections include teicoplanin and aminoglycosides (Table: 1.1). The former agent is similar to vancomycin but less effective. According to combined results from a clinical study, the average success rate of vancomycin is 76% while the same rate for teicoplanin is only 55% (Chambers *et al.*, 1997). Resistance to teicoplanin has been demonstrated *in vitro* and resistant strains have also been reported during therapy (Chambers *et al.*, 1997). The most active aminoglycoside is gentamicin. These drugs are not used as single agents as resistance rapidly emerges and are thus used with vancomycin. Aminoglycosides are synergistic with vancomycin *in vitro*, however, a large number of MRSA strains produce enzymes that can modify aminoglycosides. In that case aminoglycoside resistance is developed and as a result the synergy is lost (Chambers, 1997). It has been reported that aminoglycosides may also be used in combination with β-lactams, increasing the bacterial killing *in vitro*. This combination therapy led to more rapid clearance of bacteria from the bloodstream, even though the clinical outcome was the same as when a single antimicrobial agent was used (Lowy, 1998). Resistance to gentamicin has also been reported in many cases and therefore susceptibility should be documented before administering the agent (Chambers, 1997).

Antibiotic Class (Examples)	Spectrum	Mode of action
Beta lactams- penicillins & cephalosporins (Penicillin G, Amoxicillin)	Gram-positive, Gram-negative bacteria	Binding to and inhibiting enzymes required for the synthesis of the peptidoglycan wall
Semisynthetic penicillin (Ampicillin, Methicillin)	Gram-positive, Gram-negative bacteria	Inhibition of peptidoglycan synthesis and murein assembly
Aminoglycosides (Streptomycin, Gentamycin)	Gram-positive, Gram-negative bacteria	Bind to the 30S subunit of rRNA & interfere with the formation of initiation complex with mRNA/cause misreading of mRNA leading to the formation of nonsense peptides.
Glycopeptides (Vancomycin, Teicoplanin)	Gram-positive bacteria	Bind to the D-alanines on the precursors of the peptidoglycan cross-bridges preventing their cross-linking.
Rifamycins (Rifampin)	Gram-positive, Gram-negative bacteria	Bind to bacterial RNA polymerase and preventing it from carrying out transcription.
Tetracyclines (Tetracycline)	Gram-positive, Gram-negative bacteria	Bind to the 30S subunit of the bacterial ribosome, preventing the transfer of activated amino acids to the ribosome, halting protein synthesis.
Oxazolidinones (Linezolid)	Gram-positive bacteria	Inhibition of the proper assembly of the complex of fMet-tRNA with the mRNA and the 2 ribosomal subunits.
Chloramphenicol	Gram-positive, Gram-negative bacteria	Bind to 50s-rRNA and inhibit formation of peptide bonds by blocking peptidyl transferase activity.
Fluoroquinolones (Ciproflaxin)	Gram-positive, Gram-negative bacteria	Block the action of the bacterial enzyme that relieves the coils that form in DNA when the helix is being opened for replication/transcription/repair
Macrolides (Erythromycin)	Gram-positive, bacteria	Binding to the 50S subunit of the bacterial ribosome, interfering with the binding of tRNAs thus inhibiting protein synthesis.

Table: 1.1 Indication of the major groups of antibiotics, their spectrum of activity and mode action. Penicillins, semi-synthetic penicillins and glycopeptides target cell wall biosynthesis, while aminoglycosides, rifamycins, tetracyclines, oxazolidinones, chloramphenicol and macrolides, interfere with one or more steps of protein synthesis. Finally, fluoroquinolones inhibit DNA replication by trapping a complex of DNA bound to the enzyme DNA gyrase.

1.4. Development of new antibiotics

1.4.1 Need to develop new antimicrobial agents

The increasingly widespread problem of acquired resistance to the existing antimicrobial agents over the last six decades poses a major threat to global public health and constitutes a growing problem in both nosocomial and community-acquired infections. The underlying problem is exacerbated by demographic factors, such as population growth and urbanization, which generate conditions that facilitate the spread of diseases and the transmission of infections. Another reason for concern is the fact that since the 1980s the development and introduction of new antimicrobial agents has decreased significantly, reflecting the declining commitment to the discovery of antibiotics by the pharmaceutical industry (Chopra *et al.*, 1997). An informal telephone survey has been conducted in the USA about the development of new antibiotics among the heads of departments conducting research on antimicrobial agents in major USA and Japanese pharmaceutical companies. The results indicated that approximately half the companies 'had either reduced or phased out their antibacterial programs five or six years ago, partly because of an erroneous assumption that bacterial diseases were already successfully controlled' (Tomasz, 1994). Even though there are various important guidelines as to how the use of antimicrobials could be reduced in order to prevent the problem of antibiotic resistance from getting worse, it is essential for the pharmaceutical industry to develop agents that will prevent or treat infections caused by resistant pathogens (Chopra *et al.*, 1996).

1.4.2 Discovery of new antibiotics

One solution to the problem of antibiotic resistance would be to develop antimicrobial agents that target novel active sites. This approach is cheaper and probably more successful in avoiding acquired resistance than the development of novel synthetic antibiotics. New strategies include inhibition of biosynthetic pathways, cell division, protein synthesis, secretory function and metabolite transport. Another promising idea for the development of new antibiotics is to

design new agents that would act against expression of bacterial genes or gene products expressed only during infection *in vivo*, as opposed to the 'housekeeping' functions that microbes carry out while growing *in vitro*. An additional advantage of this approach is that it would reduce the chances for selection and spread of resistance genes and thereby prevent the transmission of resistance genes to non-pathogenic strains, because these novel agents would only be targeted against pathogens (Chopra *et al.*, 1996). The research for this kind of agents has not been very successful due to lack of methods for identifying the underlying novel genes and the proteins that they express. However, a number of new techniques have been developed that could be used to identify bacterial genes that express proteins only when infecting a host, thus providing new opportunities to discover an entirely new class of antibiotics. One such method is *in vivo* [gene] expression technology (IVET). It involves usage of an animal as a selective medium for identifying genes that pathogenic bacteria specifically express when infecting host tissues (Mahan *et al.*, 1995). Other methods that have been developed for the discovery of new antibiotics include combinatorial chemistry. This process involves the simultaneous use of large sets of chemically similar reagents (such as amino acids) in binary chemical reactions to produce thousands of products, which are then screened for biological activity. Finally, genome sequencing has helped scientists identify new genes and proteomics have provided information about the structure and function of their protein products. Thus mapped genes could be used as targets for the development of new antibiotics.

However, it is not possible to predict whether and how quickly resistance to these novel antibiotics will be developed. This will probably be affected by factors such as the duration of exposure of the pathogens to the new agent and the ability of bacteria to express drug-resistant targets that retain biological activity (Chopra *et al.*, 1996). It has been also shown that some bacteria, for example *Streptomyces spp.* are natural producers of bacteriocins and as a means of defense they have their own mechanisms of immunity to these compounds. These particular microbes also constitute the origins of the antibiotic resistance determinants found in other bacterial species (Davies, 1994). Therefore it has been postulated that inhibitors of virulence determinants that are basically produced *in vivo* should be

very similar to the antibiotics formed by soil microorganisms such as *Streptomyces*. As a result, selection and prevalence of antibiotic resistance mechanisms targeting virulence mechanisms are not very likely to occur (Chopra *et al.*, 1996).

1.4.3 Problems in developing new antibiotics

The fact that many pathogens have developed multi-resistance to many antibiotics and the lack of new potential target sites make it harder for new agents to be discovered and developed by the pharmaceutical industry. The last major antibiotic class, the oxazolidinones only became available recently and resistance to linezolid (the last remaining therapeutic option against MRSA) has already emerged (Tsiodras *et al.*, 2001). In addition, any new antibiotics introduced throughout the past decade are only improved versions of existing ones (Chopra *et al.*, 1996). Moreover, the current strategy of pharmaceutical companies is to develop antibiotics with commercially good profiles, such as a broad killing spectra. Broad spectrum agents are commercially more advantageous but tend to promote the development of antibiotic resistance by microorganisms (Silver and Bostian, 1993). On the other hand, the discovery of a new class of antibiotics that is based on the identification of targets associated with *in vivo* bacterial infection will produce drugs against more specific targets and would therefore be narrow-spectrum agents. Further to this, it will be some time before as yet undiscovered novel targets for antimicrobial agents can be identified; their safety and efficacy can be evaluated in clinical trials so that they may then enter the market successfully (Chopra *et al.*, 1996).

1.5. Bacteriophages

1.5.1 General characteristics of bacteriophages

Bacteriophages are viruses for which bacteria are the natural hosts (Chanishvili *et al.*, 2001). According to electron microscopy studies in 1942, they were described

as 'sperm-shaped particles consisting of a head and a tail' while studies in 1936 revealed that their chemical composition consists of 50% protein and 50% nucleic acid (Sharp, 2001). Bacteriophages invade bacterial cells and when lytic they disrupt bacterial metabolism and cause their host to lyse. The first step of infection includes absorption of an individual phage by a susceptible bacterium. This event occurs between the phage tail and a certain 'receptor' molecule on the surface of the bacterium. The 'receptor' that the phage tail attaches to is so specific that a phage will not generally interact with unrelated bacteria. The second step of infection occurs when the phage injects its nucleic acid into the bacterium at which point it either replicates itself (lytic cycle) or integrates into the bacterial chromosome (lysogenic cycle). The first mechanism, leads to the formation of mature particles inside the cell, which results in lysis of the bacterium and release of the new daughter phages. On the other hand, during the lysogenic cycle, the phage nucleic acid integrates into the bacterial chromosome and while the bacterium reproduces naturally, the genetic material of the phage is transferred with the bacterial chromosome to the daughter cells. Lysogenic viruses can be also induced after integration of their genetic bacterial into the bacterial chromosome and therefore replication of the virulent phage inside the bacterium is also possible. Thus, the phages selected for clinical use may be either lytic or lysogenic (Chanishvili *et al.*, 2001).

1.5.2 Discovery of bacteriophages

The discovery of bacteriophages dates back to 1896, when Ernest Hankin, a British bacteriologist recorded the presence of marked antibacterial activity against *Vibrio cholerae* in the water of the Ganges river in India and suggested that an unknown substance was responsible for this phenomenon and for limiting the spread of cholera epidemics (Sulakvelidze *et al.*, 2001). Two years later the Russian bacteriologist Gamaleya reported a similar phenomenon and it was not until about 20 years later that the subject was reintroduced by Frederick Twort, an English Bacteriologist, suggesting that the phenomenon could be due to a virus. However, it was the French microbiologist Felix d'Herelle in 1917, who officially discovered bacteriophages at the Institute Pasteur in Paris. He was convinced that

the lytic cycle of bacteriophages could be used in bacterial disease prophylaxis and therapy and from 1920 to 1940 extensive research was carried out on bacteriophage therapy.

Even though bacteriophages appeared to be very promising, especially in treating cholera *in vitro*, they showed less activity in a clinical setting (Alisky *et al.*, 1998). In addition, the early studies were poor and uncontrolled. For instance, reports on the beneficial use of bacteriophages in the treatment of cholera were based on one field trial, which included pouring an undisclosed amount of phage down a drinking well (Barrow and Soothill, 1997) while assessment was based on the number of cases that occurred before and after treatment. Whether the strain was susceptible to that particular phage was not disclosed (Chanishvili *et al*, 2001). Moreover, in certain areas excessive optimism for phage therapy led to vaccination programmes and rigorous sanitation measures being replaced by untested bacteriophage therapy with disastrous results. Further to this, in the 1930s, commercial preparations of phages appeared in the market with exaggerated claims. For example, Enterophagos, was claimed to be effective against infections such as herpes and eczema, which did not even have a bacterial etiology (Barrow and Soothill, 1997 and Chanishvili *et al.*, 2001). As a result of these events the World Health Organisation decided to discontinue research on phage therapy and concentrate on antibiotics, which appeared to be more promising following the success of tetracycline (Barrow, 2001). In addition to this, a number of well-controlled experimental and clinical studies were also carried out on bacteriophage therapy at the time, which demonstrated negative results and effects that were not considered to be significant (Barrow and Soothill, 1997).

Nevertheless, in the Soviet Union major research on bacteriophage therapy was still being carried out at the Eliava institute in Tbilisi, Georgia. The pioneering work at the Institute resulted in the development of phages against infections such as gangrene, in the 1940s (Stone, 2002). Applications of mixtures of phages against anaerobes and *Staphylococcus* and *Streptococcus* phage were used extensively by the Soviet military for treatment of gas gangrene in soldiers during

the World War II. Bacteriophage therapy compared very favourably against other treatments with survival rates among the soldiers of ~80% for phage treatment compared to only ~55% for other methods of treatment (Chanishvili *et al.*, 2001). The period of World War II proved to be very fruitful for the Soviet researchers as they performed new trials and invented new methods for the administration of phages, including applications through tampons and bathing, which were less traumatic for patients. Phage therapy was extensively used against various infections, including *E. coli*, *S. aureus* and *Staphylococcus epidermidis*. In some cases even 100% recovery of patients was achieved (Chanishvili *et al.*, 2001). While work on phages continued in Eastern European countries, in the 1980s research on bacteriophages resumed in the West, initially by Merril, who developed a standard procedure for purifying phage (Ho, 2001) and by Smith and Huggis who demonstrated that phage treatment protected mice from *E. coli* infection and was more effective than four or five antibiotics (Smith and Huggis, 1982). Moreover, Merril and colleagues found that they could prevent mice from eliminating phages by injecting them with mutant bacteriophages able to survive for long periods of time. The mutant bacteriophages also proved to be more potent than their parental strains (Merril *et al.*, 1996). Further to this, clinical trials for a bacteriophage treatment are being carried out against infections caused by *S. aureus* (Koerner, 1998).

1.5.3 Potential of bacteriophage therapy

The long history of research in phage therapy has proved that they possess a large number of advantages over antibiotics that make them a natural alternative to the current search for new antimicrobial agents (Ho, 2001). Firstly, they are highly specific for the target bacteria and would not therefore affect the normal microflora, an important feature that could be valuable in the prophylaxis of enteric infection (Barrow and Soothill, 1997). Due to their high specificity towards bacteria ecological safety is also insured, as they will not accumulate in the environment (Chanishvili *et al.*, 2001). In addition, bacteriophage therapy is very efficient and self-regenerating. A single dose of phage will produce more than a million copies of itself each day (Ho, 2001). Moreover, purified

preparations of bacteriophages do not cause major effects such as allergy and intoxication (Chanishvili *et al.*, 2001). They would also be safe even in cases of administration of the wrong phage particle as the phage would not be able to multiply (Barrow and Soothill, 1997). Also, experiments carried out in the 1980s demonstrated that mutations conferring resistance to *E. coli* occurred less frequently following phage therapy than they did following antibiotic therapy (Stone, 2002).

Bacteriophage therapy for bacterial infections has proved to be a very promising but relatively undiscovered technology so far. It has evolved though into a potentially powerful solution to the worldwide antibiotic resistance problem (Ho, 2001). However, phage therapy can only work under certain environmental conditions, particularly conditions that mimic broth cultures and conditions where phages can spread from cell to cell by direct contact as is the case on the surface of a soft agar plate (Barrow and Soothill, 1997). Another area of debate is the development of resistance to phages by bacteria. However, given the current crisis with antibiotics these concerns do not justify a slowdown in phage research (Koerner, 1998). Besides, phage therapy should not be developed in order to replace antibiotic therapy, because antibiotics have been proved extremely important, successful and necessary weapons against pathogens (Pirisi, 2000).

1.6. Antimicrobial peptides

1.6.1 General characteristics of antimicrobial peptides

It has been widely shown that all types of living organisms, both eukaryotic and prokaryotic, produce a number of substances as part of their defense system, called antimicrobial peptides (Hancock and Lehrer, 1998). Antimicrobial peptides generally refer to all oligo- or polypeptides that have the ability to kill microorganisms or inhibit their growth, including those resulting from cleavage of larger proteins or peptides that are synthesized non-ribosomally (Koczulla and Balls, 2003). They are widely distributed in animals, plants, insects, humans,

bacteria and viruses and constitute one of the most ancient means of host defense (Peschel, 2002). More specifically, all groups of organisms have been shown to produce entire sets of such defence peptides either constitutively or as a survival mechanism against competing bacteria (Sahl *et al.*, 1998). These types of antibacterial agents have recently attracted a lot of attention due to their capability to eliminate pathogens and because bacteria are becoming an increasing threat to human health due to the increase in multiple antibiotic resistance (Nes and Holo, 2000). As a result, antimicrobial peptides are considered important candidates for drug development. During the past half-century alone over 700 antimicrobial peptides have been isolated from organisms of all kingdoms (Koczulla and Balls, 2003). All antimicrobial peptides, obtained either from humans, lower eukaryotes or bacteria, share a number of common properties even though they are different in terms of their activity and structure (Nes and Holo, 2000). They generally consist of between 12 and 50 amino acids (Hancock and Diamond, 2000), their net charge is positive and they are usually hydrophobic (Nes and Holo, 2000). They may be divided into two classes, the non-ribosomally synthesized and the ribosomally synthesized (natural) peptides. The former are largely produced by bacteria while the latter are produced by all species of life, including eukaryotes (Hancock and Chapple, 1999).

1.6.2 Eukaryotic antimicrobial peptides

During their course of life, higher eukaryotic organisms such as humans are exposed to a vast number of bacteria through ingestion, inhalation and contact with infected surfaces (Hancock and Scott, 2000a). In most cases these microorganisms do not cause any harm to the host, however, some times microbes invade humans and become pathogenic (Ganz and Legrel, 1995). Bacteria are capable of multiplying at a particularly fast rate, for example most *S. aureus* strains have a doubling time of under 50 min. A single microorganism is able to produce 5×10^8 cells and therefore a full-blown infection within 24 hours. On the contrary, a primary immune response by the human system can take up to seven days while a secondary response up to three days (Hancock and Diamond, 2000). Therefore, even though the innate immune response can not prevent pathogens

from colonizing and causing infections, it constitutes the first line of defence that the body relies on, to avoid the onset of an infection (Janeway, 1998). The adaptive immune response is characterized by specificity, inducibility and discrimination of self versus non-self. On the other hand, inducible effectors of the innate immune response are relatively non-specific and induced rapidly within minutes to hours. Inducible effectors have conserved molecular patterns of recognition of stimulatory molecules, such as bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) while constitutive effectors such as lysozyme play an additional role in the innate immune response (Hancock and Diamond, 2000).

Even though adaptive immunity is considered to be a mechanism that has only been developed by higher vertebrates, innate host defence is observed among most eukaryotic organisms. In fact, many morphological and functional properties of most effector cells of the innate immune response have been shown to bear a large number of similarities between molluscs, insects and humans (Ganz and Legrel, 1995). These effector cells have been thought to include phagocytic cells such as neutrophils and phagocytes, other leukocytic cells and serum proteins. Nevertheless, it has been recently demonstrated that a group of antimicrobial peptides also play a very significant role in innate host defence (Hancock and Diamond, 2000), even though they constitute an evolutionary and ancient defence mechanism of all species of life with conserved induction pathways in plants, insects and vertebrates (Hoffmann *et al.*, 1999).

Antimicrobial peptides are cationic with a net positive charge of +2 to +7 due to an excess of basic amino acids such as arginine, lysine and histidine (Hancock and Diamond, 2000). They exist in three dimensions, they have a hydrophobic face that consists of non-polar amino acid side-chains and a hydrophilic face of polar and positively charged amino acids, hence the underlying peptides are amphipathic (Hancock, 1997). Furthermore, they are gene-encoded; that is one gene codes for one peptide. The initial post-translational product is generally a propeptide that consists of an N-terminal signal sequence, a pro segment and a C-terminal peptide that becomes active after cleavage. The propeptide is usually anionic and its function includes correct folding of the C-terminus or inhibition of

the activity of the mature peptide. Cleavage of the propeptide may occur during later stages of intracellular processing or after secretion into the extracellular medium (Bals, 2000).

Antimicrobial peptides have a very broad range of activity and target the negatively charged lipid bilayer head group of membranes of Gram-positive and/or Gram-negative bacteria, fungi and enveloped viruses (Zasloff, 2000). Normal human cells are resistant to these peptides, with the exception of melittin from bees, mastoparan from wasps and charybdotoxin from scorpions, which are potent toxins, because they do not contain the correct lipid composition of the target membranes (Hancock and Diamond, 2000). Once bound, the peptide undergoes changes in its secondary and tertiary structure and subsequently several peptide molecules self-assemble into higher-order structures, known as channels. They then form pores, which cause the membrane to become leaky and thus the cell suffers irreversible damage and eventually dies (Zasloff, 2000). Another possible mechanism of activity of antimicrobial peptides involves clustering over the cell membrane, which then collapses due to the formation of holes. This mechanism is called the 'carpet effect' (Hancock and Lehrer, 1998). Finally, disruption of bacterial energy metabolism and interference of biosynthetic pathways have also been proposed as mechanisms for antibacterial activity by antimicrobial peptides (Bevins *et al.*, 1999).

One of the most widely studied groups of antimicrobial peptides produced by eukaryotes are the defensins. They have been shown to constitute the major proteinaceous species in neutrophils, which are the most significant cells involved in immediate defence against bacteria as well as in acute inflammatory reactions (Hancock, 1997). Defensins represent 15% of the total amount of protein in neutrophils. In a number of clinical and laboratory-induced infections and inflammatory states, significantly increased levels of defensins were recorded. More specifically, in patients suffering from pneumonia, the levels of human defensins were increased fourfold. In addition, in pigs infected by *Salmonella*, a threefold rise in the peptide cathelicidin was reported (Hancock and Diamond, 2000). Further experiments carried out with *Drosophila*, involving mutations of

genes responsible for the production of antibacterial peptides, led to reduced survival as a result of bacterial infections (Hancock and Diamond, 2000). The importance of antibacterial peptides in innate host defence may be also demonstrated by the susceptibility of humans that cannot produce (and therefore lack) certain defensins against certain bacterial infections (Hancock and Diamond, 2000).

Apart from their activity against microorganisms, a number of antimicrobial peptides have also been shown to affect the quality and effectiveness of innate immune responses and inflammation. Even though the information available on this area is limited, it is speculated that the role they play is quite important as very high concentrations have been recorded at inflammation sites, such as the plasma of septic individuals and the saliva of patients with oral squamous cell carcinoma. Further to these significant functions, antimicrobial peptides are assumed to play important roles when innate immune mechanisms fail to lead into bacterial clearance and, as a result of chronic inflammation, adaptive immune responses are triggered. For example, antimicrobial peptides can act as chemotaxins for monocytes and in recruitment of T cells through chemotaxis (Hancock and Diamond, 2000).

1.6.3 Bacterial antimicrobial peptides

Production of antimicrobial peptides is very important for microorganisms in their constant fight for survival with other microbes. These compounds are almost always cationic (Nes and Holo, 2000) and produced in large amounts, especially from Gram-positive organisms, which can use non-ribosomal synthesis mechanisms as well as exotic amino acids as building blocks to produce them (Kleinkauf and von Dohren, 1996). On the other hand, Gram-negative bacteria produce ribosomally made antimicrobial peptides and proteins, known as bacteriocins (Sahl *et al.*, 1998). A good example are the colicins produced by *E. coli*. These constitute large domain-structured protein toxins, approximately 30-70 kDa in size, with a narrow spectrum of activity (Guder *et al.*, 2000). On the contrary, Gram-positive microorganisms synthesize shorter peptides that have

many similarities to the peptides produced by the eukaryotic organisms (Sahl *et al.*, 1994).

One of the most widely studied groups of bacteriocins made by Gram-negative organisms are colicins. Their mode of action is either based upon formation of channels in the cytoplasmic membrane, or as nucleases that degrade DNA or RNA. Initially, they bind to specific receptor proteins, located in the outer membrane of the target bacteria. The next step involves unfolding and translocation of the underlying protein through the outer membrane and the periplasmic space to the cytoplasmic membrane. Pore-forming colicins insert into the lipid bilayer forming channels that depolarize the membrane and lyse the cell. Nuclease-type colicins must be transported through the cytoplasmic membrane and kill the cell by cleavage of either DNA or RNA. In terms of structure, colicins consist of an N-terminus domain, which is responsible for the translocation of the protein, a central domain that binds to the outer membrane receptor and a C-terminus which is the cytotoxic domain (James *et al.*, 1996).

Extensive research has been carried out with a specific group of peptides called lantibiotics. In particular, nisin has been very well studied and its killing activity has been reported to occur by disruption of the cytoplasmic membrane by formation of short-lived transmembrane openings, or pores (Guder *et al.*, 2000). The peptide molecules exist unstructured in aqueous solution but adopt an amphiphilic conformation when they interact with the phospholipid head groups of the membranes. They then move through the membrane, remaining surface-bound and carrying the phospholipids across. When accumulation of several peptides at the same site occurs, they insert with their hydrophobic side into the outer leaflet of the bilayer causing the formation of transient pores (Sahl *et al.*, 1998). These antimicrobial peptides are synthesized as pre-propeptides and their general structure appears to consist of a C-terminal propeptide domain, which after a series of post-translational modifications is cleaved from the N-terminal domain that leads to the formation of the mature and active antimicrobial molecule (Jack *et al.*, 1995).

1.6.4 Potential of antimicrobial peptides

Eukaryotic antimicrobial peptides of the innate immune system of higher organisms are very important in preventing infections by bacteria. Similarly, prokaryotic antimicrobial peptides produced by microorganisms play a major role in their fight for survival against competing bacteria. Considering this and the current crisis of universal antibiotic resistances, it is clear that antimicrobial peptides could be used to our advantage and exploited as a new class of antibiotics. In general, the underlying compounds have many advantages, for example they kill bacteria rapidly, some of them have very a broad spectrum of activity, they remain unaffected by classical antibiotic resistance variants and can work in synergy with classical antibiotics (Hancock and Scott, 2000a). Nevertheless, a few issues relating to their safety still remain to be solved. For instance, even though *in vitro* antimicrobial peptides appear to have admirable properties their stability *in vivo* has not been fully demonstrated. Another major concern are unknown potential toxicities (Hancock, 1999a). Certain cationic peptides are particularly toxic to humans, for example, bee venom milletin and mastoparan from wasps (Hancock and Diamond, 2000). Further to this, large amounts of these peptides are not produced naturally and therefore they will have to be synthesized chemically, which is an expensive process (Hancock, 1999a). Another potential problem with these peptides is associated with their high molecular mass compared to most antibiotics; therefore they will have to be produced by recombinant technology in order to keep the prices low (Hancock and Scott, 2000a). These are critical considerations that major pharmaceutical companies have taken into account and constitute some of the reasons why they have not investigated the potential of antimicrobial peptides to a greater extent as yet (Hancock, 1999a). There are however, promising clinical trials underway, including human phase I-III studies on peptides such as magainin and iseganan (Bals, 2000). Finally, the bacterial cationic antimicrobial peptide nisin should also be mentioned, as it has been licensed as a food preservative in over 50 countries with great success (Hancock and Lehrer, 1998), implying the potentially significant role of antimicrobial peptides as a starting point for the development of new antibiotics (Bals, 2000).

1.7. Lysostaphin

1.7.1 History of lysostaphin

The first natural antibiotic substance was documented in 1899 and was an enzyme called pyocyanase, produced by *Pseudomonas aeruginosa*. Even though the agent proved to be toxic and unstable and thus could not be used as an antibiotic, research on bacteriolytic agents continued and in 1922 Fleming isolated a bacteriolytic enzyme from egg white, which was called lysozyme and could lyse the cells of several Gram-positive bacteria. Since then, many other bacteriolytic agents have been reported, produced by various organisms. Extensive research, performed on the bacterial cell wall led to the identification of enzymes that disrupt the cell wall at specific sites. In 1964, a unique antistaphylococcal agent, called lysostaphin was discovered (Schindler and Schuhardt, 1964). Schindler and Schuhardt cross-streaked various *S. aureus* strains with a staphylococcal strain from another species, which was at the time called K-6-W1. It was observed that growth of *S. aureus* colonies adjacent to K-6-W1 were inhibited after 16 hours of growth and that further incubation for 40 h resulted in clearance of *S. aureus* adjacent to the K-6-W1 streak (Schindler and Schuhardt, 1964). At the time of its discovery, lysostaphin appeared to be a very promising agent as it had a number of significant properties, including selectivity, rapidity of action and stability. It was therefore considered as a potentially efficient agent to offer therapeutical use against *S. aureus* (Zygmunt and Tavormina, 1972).

1.7.2 General properties

Lysostaphin is an extracellular enzyme (Heinrich *et al.*, 1987), which was discovered by Schindler and Schuhardt (1964). It is produced by *Staphylococcus simulans* biovar *staphylolyticus* and lyses *S. aureus* cells (Heath *et al.*, 1987). The polypeptide is a monomer with a molecular weight of ~42 kDa and the translated protein is encoded by the lysostaphin gene, which is 1.5 kb in size (Recsei *et al.*, 1987). Lysostaphin has an isoelectric point of 9.5 (Heinrich *et al.*, 1987), optimum pH of 7.5 and contains one molecule of zinc per mole of protein (Browder *et al.*,

1965 and Trayer and Buckley, 1970). Lysostaphin can be produced by *S. simulans* in liquid culture by fermentation (Zygmunt and Tavormina, 1972). The gene has been successfully cloned in *E. coli* and *Bacillus* species (Bramley and Foster, 1990) and lysostaphin has been expressed and purified (Recsei *et al.*, 1987). Lyophilised preparations of lysostaphin have been shown to exhibit indefinite stability when stored at –20°C in the dry state. The enzyme is also very stable at room temperature when stored in the dark and kept dry (Zygmunt and Tavormina, 1972). Finally, one unit of lysostaphin has been defined as the amount of protein required to reduce the turbidity of a standard suspension of *S. aureus* after a 10-min incubation at 50°C by 50% (Schindler and Schuhardt, 1964).

1.7.3 General structure and mode of action of lysostaphin

The cell wall lytic activity of lysostaphin is due to its ability to cleave the pentaglycine cross-links that are characteristically found in the staphylococcal cell wall and is therefore active against many known staphylococcal species (Zygmunt and Tavormina, 1972) but is inactive against species of all other genera (Recsei *et al.*, 1987). Lysostaphin is primarily produced as a pre-pro-enzyme (fig. 1.5), which is exported from the bacterial cytoplasm by an amino-terminal signal peptide. Subsequently, a leader peptidase cleaves the signal peptide, leaving the pro-enzyme to be released into the culture medium. Pro-lysostaphin consists of 15 tandem repeats of a 13-residue peptide, located at the N-terminal end of the gene. Finally, once prolysostaphin is secreted into the culture medium of *S. simulans* a cysteine protease removes the 15 tandem repeats of lysostaphin thus releasing the mature form of the protein (Baba and Schneewind, 1998). After secretion of mature lysostaphin by its producing organism, the polypeptide is directed to the *S. aureus* cell walls that harbor the appropriate receptor by its targeting domain located in the carboxy-terminal end of the protein. Immunity of *S. simulans* to its secreted polypeptide requires an immunity factor, which is encoded by the *lif* gene. This gene modifies the peptidoglycan of *S. simulans* as soon as lysostaphin is exported into the medium thus preventing cleavage of its own cell wall by the mature protein and thereby conferring effective natural resistance to the active peptide (Thumm and Gotz, 1997).

1	MKKTKNNYYT	RPLAIGLSTF	ALASIVYGGI	QNETHASEKS	NMDVSKKVAE	VETSKAPVEN	60
61	TAEVETSKAP	VENTAEVETS	KAPVENTAEV	ETSKAPVENT	AEVETSKAPV	ENTAEVETSK	120
121	APVENTAEVE	TSKAPVENTA	EVETSKAPVE	NTAEVETSKA	PVENTAEVET	SKAPVENTAE	180
181	VETSKAPVEN	TAEVETSKAP	VENTAEVETS	KAPVENTAEV	ETSKAPVENT	AEVETSKALV	240
241	QNRTALRAAT	HEHSAQWLNN	YKKGYGYGPY	PLGINGGMHY	GVDFFMNIGT	PVKAISSGKI	300
301	VEAGWSNYGG	GNQIGLIEND	GVHRQWYMHL	SKYNVKVGDY	VKAGQIIGWS	GSTGYSTAPH	360
361	LHFQRMVNSF	SNSTAQDPMP	FLKSAGYGKA	GGTVTPTPNT	GWKTNKYGTL	YKSESASFTP	420
421	NTDIITRTTG	PFRSMPQSGV	LKAGQTIHYD	EVMKQDGHVW	VGYTGNSGQR	IYLPVRTWNK	480
481	STNTLGVLWG	TIK					493

Fig. 1.5 Diagram indicating the organisation of the lysostaphin gene. Amino acids positions: 1-36 comprise the signal sequence (indicated in brown), 37-493 comprise the proenzyme (indicated in purple) and 248-493 comprise mature lysostaphin. The N-terminal catalytic domain of the mature protein is shown in red, the linker region in green and the C-terminal targeting domain in blue.

1.7.4 Activity of lysostaphin against various bacterial strains

As soon as lysostaphin was discovered in 1964 many experiments were carried out with *S. aureus* strains to demonstrate its efficacy *in vitro*. Cropp and Harrison (1964) were the first scientists to perform a study on the *in vitro* effect of the underlying protein against clinical isolates of *S. aureus*. The results of the experiments were very encouraging as lysis occurred with all of the 252 strains that were tested (Cropp and Harrison, 1964). In 1966, further experiments were carried out to compare the activity of lysostaphin against *S. aureus* with that of bacitracin and neomycin, two compounds that were widely used at the time as topical antibacterial agents. The results revealed the superiority of lysostaphin over bacitracin and neomycin in inhibiting the growth of staphylococcal strains (Zygmunt *et al.*, 1966). In 1967 more experiments were performed in order to investigate the lytic action of lysostaphin on *S. aureus* strains (Zygmunt *et al.*, 1967) and the results supported the conclusions drawn from previous experiments. By 1989, MRSA strains had become a very common problem worldwide and due

to the limited availability of antibiotics active against MRSA, Huber and Huber decided to test lysostaphin against a large number of MRSA strains over a period of 4 years, from 1983-1987. The results of the study indicated that lysostaphin was as effective against MRSA as it was against methicillin susceptible *S. aureus*, (MSSA) therefore indicating its potential as an antimicrobial agent (Huber and Huber, 1989).

In vitro experiments against staphylococcal strains have also been performed with lysostaphin combined with other antibacterial agents. In particular, Cisani and co-workers investigated the possibility of enhancing the anti-staphylococcal efficacy of lysostaphin in combination with lysozyme. Lysozyme is a well-characterised enzyme, highly active on micrococci but only slightly active on staphylococci. A large number of staphylococcus strains (235) were used for this experiment and the results showed that when combined with lysozyme, the activity of lysostaphin can be enhanced by 16- to 200-fold depending on the staphylococcus strain (Cisani *et al.*, 1982). Recently, lysostaphin has also been combined with other antibiotics to treat MRSA strains. In 1993, combinations of lysostaphin with a wide range of antibiotics, including β-lactams (such as benzylpenicillin, methicillin and cephalosporin B), bacitracin, polymyxin B, vancomycin, gentamicin, tetracycline and erythromycin were tested against 5 different MRSA strains (Polak *et al.*, 1993). During this study it was observed that when combined with all of the β-lactams, bacitracin, polymyxin B and lysostaphin exhibited rapid synergistic bactericidal activity against all 5 MRSA strains. Finally, when combined with either vancomycin, gentamicin or erythromycin, the activity of lysostaphin was significantly enhanced. These results proved once again that synergistic combinations with lysostaphin and other antibiotics may prove to be very efficient in controlling MRSA infections (Polak *et al.*, 1993).

1.7.5 Activity of lysostaphin in animal models

Schuhardt and Schindler (1964) carried out the first *in vivo* experiments using lysostaphin as a therapeutic agent. Mice infected with *S. aureus* were treated with lysostaphin and the authors recorded very positive results, including survival rates

of 100% compared to untreated controls. Therefore, further studies followed by Cropp and Harrison (1964) who investigated the therapeutic activity of lysostaphin in mice and rabbits, the former suffering from peritonitis and leg oedema produced by localized infection while the latter had dermal ear infections. Their results were also very successful and in agreement with those recorded by Schuhardt and Schindler (1964). An additional observation derived by this study was that lysostaphin was a more effective antistaphylococcal agent than vancomycin, cloxacillin, methicillin and penicillin G for mouse leg edema (Harrison and Cropp, 1966). Further studies were performed on mice injected with extremely lethal intraperitonal staphylococcal injections. The infected animals were treated with lysostaphin and penicillin and 72 h after administration of the injection, it was observed that only 53% of the penicillin-treated mice were still alive. On the other hand, 100% survival rates were achieved for the lysostaphin-treated group, compared to only 6% viability of the control treatment (Zygmunt and Tavormina, 1972).

1.7.6 Activity of lysostaphin on humans

The first experiments with lysostaphin on humans were carried out by Martin and White in 1967 in order to evaluate the efficacy and toxicity of the underlying protein when used topically in humans before proceeding with systemic use. Nasal carriers of staphylococci were treated with topically applied lysostaphin which reduced staphylococcal carriage dramatically without interfering significantly with the commensal nasal flora (Martin and White, 1967). A subsequent study was conducted on a group of infants and children, who were carriers of nasal staphylococci. The purpose of the study was to investigate the toxicity of topical lysostaphin and to determine whether an immune response to topically administered lysostaphin occurred in a pediatric population (Harris *et al.*, 1967). The results showed that the lysostaphin spray effectively eradicated *S. aureus* from the noses of infants and children. Further to this, no clinical side effects were recorded and no significant difference between pre- and post-therapy blood and urine samples was observed even though antibody formation was induced (Harris *et al.*, 1967).

In 1974, lysostaphin was used on a young soldier suffering from acute myelocytic leukemia, a severe staphylococcal pneumonia and other systemic infections caused by MRSA. The patient had not been responding for over three weeks to methicillin, cephalothin and vancomycin and although the patient died 3 days after the administration of lysostaphin due to heart failure, there was some evidence that lysostaphin had cleared most of the MRSA from the patient's body (Stark *et al.*, 1974).

Many more experiments have also been carried out recently with lysostaphin *in vivo*. In 1990, the positive effect of lysostaphin on *S. aureus* infections of the mouse mammary gland was demonstrated with survival rates of more than 99% (Bramley and Foster, 1990). Lysostaphin was more effective than vancomycin in the treatment of experimental MRSA aortic valve endocarditis in rabbits, with no visible side effects even after 9 weeks of treatment (Climo *et al.*, 1998). Lysostaphin has also been shown to be effective against *S. aureus* strains with reduced susceptibility to vancomycin (Patron *et al.*, 1999) and against MRSA keratitis in rabbits (Dajcs *et al.*, 2000). Finally, experiments have been carried out with transgenic mice that express lysostaphin in mammary glands for protection against staphylococcal infections, such as mastitis in cows. The transgenic mice were completely resistant to the pathogen and their milk protein content was similar to that of non-transgenic mice, providing the potential to use lysostaphin and genetic engineering to prevent the devastating losses incurred by the agricultural industry to *S. aureus* infections in cattle (Kerr *et al.*, 2001).

1.7.7 Lysostaphin- The potential for therapeutic use

Antibiotic resistance has nowadays become a major problem worldwide and its effects are being noted on an ever-increasing scale. More precisely, MRSA constitutes one of the most threatening pathogens in hospitals and non-hospital settings. In particular, two epidemic MRSA strains (EMRSA) types 15 and 16 have been identified in hospitals in the UK posing a major threat due to their multiple antibiotic resistance. EMRSA 15 has developed resistance to

ciprofloxacin and erythromycin, while EMRSA 16 is also resistant to aminoglycosides. Unfortunately, in 2000, a new more potent strain of EMRSA was identified in UK hospitals. This strain has been designated EMRSA 17 and is resistant to ciprofloxacin, erythromycin, aminoglycosides, fusidic acid, rifampin, tetracycline and occasionally to high levels of mupirocin. It has also been shown to have borderline resistance to teicoplanin (Aucken *et al*, 2002).

Only a very limited number of drugs are nowadays still effective against MRSA infections and as a result the most widely trusted, vancomycin is routinely prescribed for all MRSA infections and even MSSA infections before diagnosis is complete (Enright *et al.*, 2002). Due to the lack of new antistaphylococcal agents, the overuse of vancomycin to treat MRSA infections and the several strategies that microorganisms adopt in order to overcome antibiotic susceptibility, it was inevitable for vancomycin resistance to eventually develop (Climo *et al.*, 1998). Furthermore, many staphylococci have developed resistance to more than one antibiotic. Thus, the potential for vancomycin failure rates and the lack of alternative therapeutic agents have prompted a desperate search for new antistaphylococcal agents with activity against MRSA.

Even though lysostaphin was discovered in 1964, its potential had not been extensively investigated due to the availability of many other antibiotics and because the underlying problem of antibiotic resistance was not at the time considered. Lysostaphin constitutes a highly specific and effective antimicrobial agent against MRSA and *S. aureus* strains with reduced susceptibility. Moreover, even though there is not much data on the immunogenicity of lysostaphin in human subjects due to limited studies investigating its topical use, there has been very little evidence of sensitization or antibody formation among patients treated with topical lysostaphin in attempts to eradicate nasal staphylococcal carriage (Climo *et al*, 1998).

There has also been very little research into the basic biology of lysostaphin. There is currently no information on the nature of the interaction between the targeting domain of lysostaphin and the cell surface receptor, the identity of the

receptor, nor on the interaction of the pentaglycine crossbridge with the endopeptidase domain and its subsequent cleavage. Thus, the present study was undertaken in order to investigate how the primary structure of the targeting domain relates to its function of binding to *S. aureus* cell walls. Site-directed and random mutagenesis were used to engineer inactive mutations in the targeting domain of lysostaphin so as to identify residues essential for binding to the *S. aureus* cell wall. This would then provide crucial information on the type of the ligand-receptor interaction before and after the 3-D structure of lysostaphin becomes available.

CHAPTER: 2

MATERIALS AND METHODS

2.1 Bacterial strains and media

E.coli Strain	Relevant genotypes	Source
JM83	Δ (*lac-proAB*), ϕ80d*lacZ*ΔM15	Lab strain
DH5α	*lacZ*ΔM15	Lab strain
ER2566	*lacZ* :: T7	New England Biolabs
B834(DE3)	λ (DE3)	Novagen
BL21(DE3)	λ (DE3)	Studier and Moffat, 1986

Bacterial cultures were grown in LB broth or on plates of LB agar (Miller, 1972). Ampicillin (100 μg ml^{-1}), and chloramphenicol (35 μg ml^{-1}) were added as selective agents where appropriate. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a final concentration of 1 mM to induce expression of T7 RNA polymerase in λ (DE3) lysogenic cultures.

2.2 Long term storage of bacterial clones

One colony was transferred to a 5 ml culture followed by incubation at 37°C for 24 h. One millilitre of the culture was centrifuged and the supernatant was removed. The pellet was resuspended in 1 ml of 20% (v/v) glycerol and the resuspended cells were kept at –20°C.

2.3 Plasmids

pUC18 (Vieira and Messing, 1983 and Yanisch-Perron *et al.*, 1985), supplied by Pharmacia Biotech, was used for cloning blunt-ended PCR products.

pET21a and pET21d (Studier and Moffat, 1986), supplied by Novagen, were used for the expression of target genes under the control of strong bacteriophage T7 transcription and translation signals (fig. 2.1). These vectors include a polyhistidine tag sequence that can be expressed at the carboxyl terminus of the target protein. This allows the target protein to be purified rapidly by metal chelation chromatography.

pLysS (Dunn and Studier, 1983) encodes the production of low levels of T7 lysozyme, an inhibitor of T7 RNA polymerase.

pCS4 (Garinot-Schneider, 1996) is a pET21a-derived plasmid and encodes ColE9 and a His-tagged Im9.

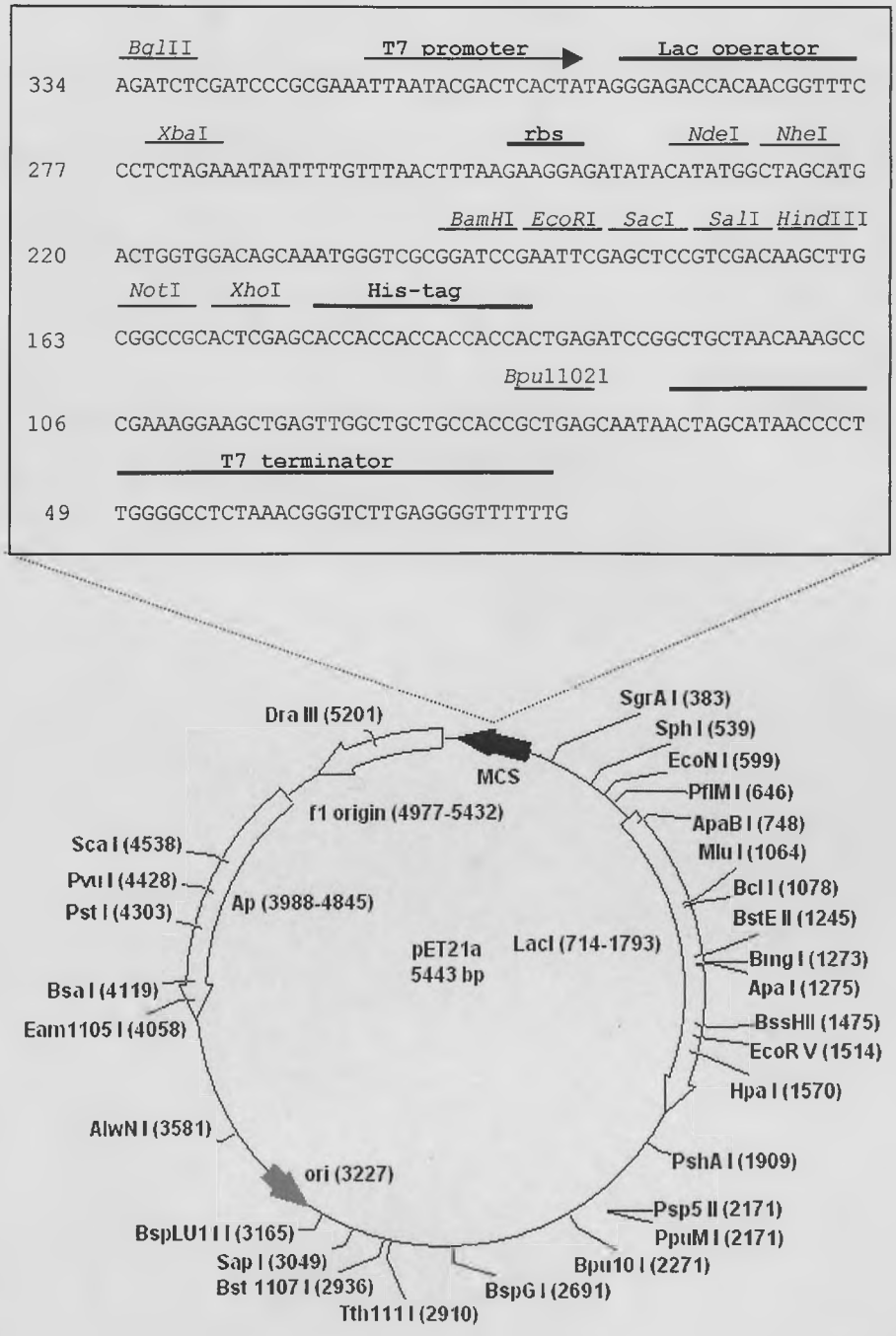

Fig. 2.1 Map of pET21a. The multiple cloning site and expression region is shown in the expanded region. Plasmid pET21d differs from pET21a in that it has an *Nco*I site substituted for the *Nde*I site at position 238 resulting in a net loss of 1 bp.

pEA1-pEA8 (Table 2.1) are the plasmids containing the mature lysostaphin gene, *S. aureus* mature *lytM* and *E. coli lytM* and mature *lytM* fragments in vectors, pET21a, pET21d and pCS4.

Plasmid	Relevant characteristics
pEA1	Ap^R, 0.88 kb mature *S. aureus lytM* gene with a C-terminal His-tag cloned in the *NdeI XhoI* sites of pET21a
pEA2	Ap^R, 1.2 kb *E. coli lytM* gene with a C-terminal His-tag cloned in the *NdeI*/*Xho*I sites of pET21a
pEA3	Ap^R, 0.75 kb mature lysostaphin gene with a C-terminal His-tag cloned in the *NdeI*/*Xho*I sites of pET21a
pEA4	Ap^R, 1.5 kb *E. coli lytM* gene with a C-terminal His-tag cloned in the *NdeI*/*Xho*I sites of pET21a
pEA5	Ap^R, 1.5 kb *E. coli lytM* gene with a C-terminal His-tag cloned in the *NcoI*/*Xho*I sites of pET21d
pEA6	Ap^R, 1.5 kb *E. coli lytM* gene cloned in the *NcoI*/*Xho*I sites of pCS4
pEA7	Ap^R, Targeting domain of lysostaphin with a C-terminal His-tag cloned in the *NcoI*/*Xho*I sites of pET21d
pEA8	Ap^R, Targeting domain of lysostaphin cloned "blunt-ended" in pUC18

Table 2.1 Listing of plasmids pEA1-pEA8, in vectors pET21a, pET21d and pCS4.

pEA9-pEA15 (Table 2.2) are the plasmids containing the various lysostaphin (Lss) targeting domain (T-domain) mutants in pET21a.

Plasmid	Relevant characteristics
pEA9	Ap^R, Lss T-domain mutant (F172S) with a C-terminal His-tag cloned in the *Nde*I/*Xho*I sites of pET21a
pEA10	Ap^R, Lss T-domain mutant (F172A) with a C-terminal His-tag cloned in the *Nde*I/*Xho*I sites of pET21a
pEA11	Ap^R, Lss T-domain mutant (F172Y) with a C-terminal His-tag cloned in the *Nde*I/*Xho*I sites of pET21a
pEA13	Ap^R, Lss T-domain mutant (W214A) with a C-terminal His-tag cloned in the *Nde*I/*Xho*I sites of pET21a
pEA14	Ap^R, Lss T-domain mutant (Y203A) with a C-terminal His-tag cloned in the *Nde*I/*Xho*I sites of pET21a
pEA15	Ap^R, Lss T-domain mutant (Y226A) with a C-terminal His-tag cloned in the *Nde*I/*Xho*I sites of pET21a

Table 2.2 Listing of plasmids pEA9, pEA10, pEA11, pEA13, pEA14 and pEA15, in pET21a, carrying the various lysostaphin T-domain mutants.

2.4 Plasmid restriction, ligation, transformation and electrophoresis

Endonuclease digestion of plasmid DNA, ligation of dsDNA fragments, transformation of competent *E. coli* cells and agarose gel electrophoresis were carried out as described by Sambrook *et al* (1989).

2.5 Electroporation

In addition to the use of chemically competent cells, DNA uptake by bacterial cells was also accomplished by electroporation. Electrocompetent B834(DE3) cells were used for electroporation with the Gene Pulser (Bio-Rad). The voltage was set to 2-50 kV and time was set to 3.5-5.5 msec. The mini-prep plasmid sample was initially diluted 1 in 20 while clean and dry cuvettes were kept on ice. Two microlitres of plasmid DNA were added to ice cold electrocompetent cells and incubated for 1 min keeping the cells on ice. Forty microlitres of the mix were added to a cuvette, which was placed into the chamber attached to the gene pulser. Pulsing followed until beeps were heard, the time displayed should be between 5 and 5.6 msec.

2.6 Isolation of plasmid DNA

Small-scale preparations of plasmid DNA were prepared by the alkaline extraction method (Birnboim and Doly, 1979). Midi Qiagen plasmid isolation kits (Qiagen) were used to prepare high yields of pure plasmid DNA for automated sequencing reactions.

2.7 Extraction of DNA from agarose gels

DNA was recovered from agarose gels using the QIAEX II Gel extraction Kit according to the manufacturer's instructions (Qiagen).

2.8 Polymerase Chain Reactions (PCR)

PCR was carried out using a Primus MWG thermal cycler with either the Taq Expand High Fidelity PCR system or Pwo Taq polymerase (both supplied by Roche). Reactions were allowed to proceed through thirty cycles of 94°C for 30 seconds, 54°C for 1 min and 72°C for 1 min.

2.9 Site-directed mutagenesis (SDM)

Site directed mutants were made using a two stage PCR method (Sarkar and Sommer, 1990). In the first stage, a mutagenic primer (designed to give at least 10 complementary bases either side of the introduced mutation) was used in conjunction with a suitable flanking primer having complementarity to the opposite strand. The first stage PCR product was purified from an agarose gel using the Qiaex II kit (see section 2.7) and used as a "mega-primer" in a second stage PCR with another suitable flanking primer. When Taq polymerase is used in this Site-directed mutagenesis method, extra care must be taken when designing the mutagenic primer to allow for the non-template dependent addition of a single adenosine residue to the 3' end of extended chains.

2.10 Random PCR mutagenesis

Random PCR mutagenesis was developed from the method of Spee *et al.* (1993). The technique relies on the fact that (i) one of the four dNTPs is present in limiting amounts (10 fold less than normal) and replaced with a high concentration of dITP in each of four separate reaction mixes, (ii) when the pool of the limiting dNTP has been exhausted misincorporation of dITP occurs, (iii) dITP will allow the misincorporation of any one of the remaining three natural nucleotides as a complementary base during the next PCR cycle, resulting in a mutation at that position.

Five PCR reactions were set up: one used as the control with the normal complement of nucleotides, and the other four with one of the mutagenic mixes (*) shown in table 2.2. The reactions were run as in section 2.8. Successful random PCR mutagenesis reactions should produce weaker bands for the mutagenic mixes on the gel compared to the bands representing the controls because incorporation of dITPs is not as efficient as that of dNTPs. The successful reactions were then mixed and purified using the Qiaex II kit (see section 2.7).

Mutagenic mix	dA	dC	dG	dT	dI	H_2O
A*	0.4µl	4 µl	4 µl	4 µl	4 µl	183.6 µl
C*	4 µl	0.4µl	4 µl	4 µl	4 µl	183.6 µl
G*	4 µl	4 µl	0.4µl	4 µl	4 µl	183.6 µl
T*	4 µl	4 µl	4 µl	0.4µl	4 µl	183.6 µl
Control dNTP	4 µl	4 µl	4 µl	4 µl	0.4µl	184 µl

Table: 2.2 Description of the mutagenic dNTP mixes (*) used in the PCR reactions. dNTP mixes were diluted 1 in 10 in the final PCR reaction with the final concentration of each nucleotide at 200 µM. In each of the mutagenic dNTP mixes, the nucleotide was used at a final concentration of 20 µM while the dITP was used at 200 µM.

2.11 DNA sequencing

DNA sequencing reactions were carried out using the BigDye Terminator Kit (PE Applied Biosystems) according to the manufacturer's instructions. The reaction involved 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequencing reactions were run in-house on an ABI Prism sequencer in the Division of Immunology, School of Clinical Laboratory Sciences, Universiy of Nottingham.

2.12 Induction and expression of lysostaphin, lysostaphin mutants and the targeting domain of lysostaphin

A single colony of the clone of interest was added to a 5 ml LB broth containing 100 μg ml^{-1} of ampicillin and grown overnight at 37°C. Fifty microlitres of the overnight broth were transferred to 5 ml of fresh LB broth and incubated with aeration at 37°C until it reached an OD_{600} of 0.5. In order to induce expression of the protein, 2.5 ml of the culture were transferred to a clean sterilin tube containing 25 μl of 100 mM IPTG. The remaining culture was untreated and used as a control. Both tubes were incubated for 2 h at 37°C. One mollilitre of each culture were transferred to 1.5 ml microfuge tubes and centrifuged for 1 min to pellet the cells. The supernatant was discarded and the pellet was resuspended in 50 μl of 2x SDS loading buffer, heated at 90°C for 5 min and run on a SDS-PAGE gel alongside a protein ladder. The SDS-PAGE gel was stained with Coomassie brilliant blue and then destained overnight.

2.13 Protein purification of lysostaphin, lysostaphin mutants and the targeting domain of lysostaphin

After induction of recombinant lysostaphin wild–type and mutant protein, the expressed cells were centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the cell pellet was carefully re-suspended in 45 ml ice cold NiC buffer containing 300 μl of 100 mM PMSF. The sample was sonicated on ice for 20 s bursts on and 30 s off, over a 40 min period and then centrifuged for 30 min at 15000 rpm. The supernatant, containing soluble proteins, was removed, centrifuged a second time under the same conditions to remove any traces of cell debris, and subjected to Ni^{2+}-NTA chelate chromatography using a BiologicLP automated protein purification system (BioRad).

A 5 ml HiTRAP column was charged with 50 mM of Ni^{2+}, and then equilibrated with 10 ml of low imidazole buffer. The prepared cell free extract was loaded

onto the column at a flow rate of 1 ml min^{-1}. The column was washed with 20 ml of low imidazole buffer to remove weakly interacting proteins, and the His-tagged protein was eluted in 2 ml fractions using a 5%-85% imidazole gradient in a total volume of 50 ml. Fractions containing recombinant protein were analysed by SDS-PAGE, and purified lysostaphin was pooled and dialysed against 25 mM sodium phosphate pH 7.0, 500 mM NaCl, 5% (v/v) glycerol. The A_{280} measurements were recorded from a Shimatzu UV-160 spectroscope, and protein concentrations were calculated using the Beer-Lambert Law:

$A_{280} = \text{Conc} \times \varepsilon\lambda_{280} \times l$

The Extinction Coefficient ($\varepsilon\lambda_{280}$) for mature lysostaphin is 0.4255 mg ml^{-1} and for the targeting domain is: 0.3966 mg ml^{-1}. l is the path length of the cuvette (in cm).

The extinction coefficient value of lysostaphin was calculated using the following equation:

$$\varepsilon = \frac{(\text{No of tyrosine residues} \times 1440) + (\text{No of tryptophan residues} \times 5050)}{\text{Molecular mass of protein}}$$

Composition of buffers:

Buffer A: 25 mM NaH_2PO_4, pH 7.0, 0.5 M NaCl, 5% (v/v) glycerol.

Buffer B: 25 mM NaH_2PO_4, pH 7.0, 0.5 M NaCl, 5% (v/v) glycerol, 1 M Imidazole.

Buffer C: Deionised water.

Buffer D: 50 mM $NiSO_4$

Buffer E: Sample in 35 ml of NiC buffer (plus NaCl to 0.5 M, 0.1% (v/v) Triton-X100.

Buffer NiC: 20 mM Imidazole pH 7.0, 50 mM NaCl, 10% (v/v) glycerol.

2.14 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a mini Atto gel system. 10-16 % resolving and 6 % stacking polyacrylamide gels were prepared and run according to Sambrook *et al.* (1989).

Gels were stained using Coomassie blue stain (0.2 % coomassie blue R-250, 45% methanol, 10% acetic acid) and destained (10% methanol, 10% acetic acid).

2.15 Stab test

A colony of ER2566 cells expressing the protein that needed to be tested was stabbed onto an LB agar plate and grown overnight at 37°C. The plate was then exposed to chloroform for approximately 20 min to lyse the cells and release the protein. Four millitres of molten 0.7% (w/v) non-nutrient agar, inoculated with 50 µl of an overnight culture of EMRSA-16 was poured over the surface of the LB agar plate and allowed to set. The plate was then incubated at 37°C and inspected after 24 h. If the stabbed colony produced an active lysostaphin protein there was a zone of inhibition of bacterial growth around the stab.

2.16 Spot test

Four millilitres of molten 0.7% (w/v) non-nutrient agar, inoculated with 50 µl of an overnight culture of test bacteria (EMRSA-16) was poured over the surface of an LB agar plate and allowed to set. Three microlitres of dilutions of purified bacteriocin was spoted onto the non-nutrient agar surface and allowed to dry. The plate was incubated at 37°C overnight. If the test culture produced an active bacteriocin there was a zone of inhibition of bacterial growth around the spot.

2.17 Turbidity assay for lysostaphin activity

Turbidity tests were carried out using Shimatzu UV-160. EMRSA-16 cells were cultured in LB until an OD_{600} of 0.8 and then harvested by centrifugation. The cell pellet was washed twice with 25 ml of cold 10 mM Tris pH 7.0 and then twice with 25 ml deioinised water. The final pellet was resuspended in 5 ml deionised water, frozen at –80°C for 2 h and then lyophilized. The lyophilized EMRSA-16 was resuspended in 5% (v/v) glycerol, 0.5 M NaCl and 25 mM sodium-phosphate pH 7.0 to an OD_{600} of 0.8-1.2. The turbidity of the cells was monitored for 5 min at an OD_{600}, and then challenged with 1 μM of lysostaphin. A change in the OD_{600} was monitored for a further 15 min after the addition of lysostaphin.

2.18 Liquid assay for lysostaphin activity

A 100 ml LB broth was inoculated with 500 μl of an overnight culture of EMRSA-16 and grown at 37°C to an OD_{600} of 0.1. Ten millilitres aliquots were transferred to sterile 250 ml conical flasks containing a specific concentration of lysostaphin protein. The flasks were incubated at 37°C for at least 5 h and bacterial growth was monitored by OD_{600} measurements every 30 min. The results were used to identify the minimum inhibitory concentration of purified protein required to kill EMRSA-16.

2.19 Fluorescence Resonance Energy Transfer (FRET) assay

The FRET assay was conducted using a Perkin Elmer LS50b fluorimeter (running software; Luminescence Spectroscopy: FL Winlab).

A pentaglycine peptide substrate was chemically synthesised in the School of Chemical Sciences by Rachel Warfield that incorporated a donor fluorophor (*ortho*-aminobenzoic acid (Abz)) at the N-terminal glycine and an acceptor or quenching fluorophor (2, 4-dinitrophenyl)ethylene diamine (EDDnp)) at the C-terminal glycine residue. The Abz-Gly5-EDDnp substrate was highly insoluble and had to be dissolved in DMSO.

Assays were performed using 10 μM Abz-Gly5-EDDnp substrate with 1 μM lysostaphin and lysostaphin mutants in 50 mM sodium-phosphate pH. 7.0, 150 mM NaCl, 2% DMSO using a 3 ml quartz cuvette at 37°C. Fluorescence measurements were taken every 15 min for 4 h with excitation and emission wavelengths of 325 nm and 360-510 nm (λmax of 420 nm) respectively, Ex and Em slit widths of 5 and 10 nm respectively, and a scan speed of 100 nm min^{-1}. Using these settings, background fluorescence generated from Abz-Gly5-EDDnp, the W and Y residues of the proteins, and the buffer was kept to a minimum. Cleavage was measured as an increase in the emitted fluorescence at the λmax over time.

CHAPTER: 3

CLONING, EXPRESSION, PURIFICATION AND ASSAY OF LYSOSTAPHIN AND HOMOLOGUES

3.1 Introduction

The bacterial cell wall of Gram-positive microorganisms is host to a wide variety of molecules and serves a wide range of functions that are critical for the survival of the cell. Even though the primary role of the cell wall is to provide a rigid exoskeleton for protection against mechanical and osmotic lysis it also functions as an essential attachment site for proteins that interact with the bacterial environment. The range of activities that proteins can perform once anchored to the cell wall may be incredibly diverse. For example, autolysins (such as Atl from *S. aureus*) become attached to the targeted bacterial division sites in order to carry out peptidoglycan synthesis and turnover during cell growth and division. In other cases, Gram-positive bacteria that produce cell wall-targeted endopeptidases (such as lysostaphin from *S. simulans*) attach to the cell surface of their competitors (*S. aureus*) in order to cleave the peptidoglycan and lyse the cells. Investigation of the mechanisms employed by these cell wall-targeted proteins could reveal critical information that could be used for the development of antimicrobials that control bacterial division or inhibit the growth of target cells.

3.1.1 Cell wall structure of *S. aureus.*

Staphylococcal cells consist of three separate compartments, the cytosol, a cytoplasmic membrane and the surrounding cell wall. The cell wall of staphylococci has all the characteristic features of Gram-positive bacterial cell walls and constitutes a relatively thick homogeneous structure, of about 20 to 40 nm. Its major component is a peptidoglycan macromolecule with several molecules attached, such as carbohydrates and teichoic acids (Navarre *et al.*, 1999). This heteropolymer consists of a disaccharide backbone of alternating β-1-4-*N*-acetylglucosamines and *N*-acetylmuramic acids (MurNAc-GlcNAc) repeats, on average of about 10 disaccharides in length (Sidow *et al.*, 1990). Tetrapeptides that consist of L-alanine, D-glutamine, L-lysine, and D-alanine are attached as side chains to the *N*-acetylmuramic acid. The vast majority (about 90%) of these stem peptides are cross-linked to the stem peptides of an adjacent glycan chain by a

pentaglycine peptide (Labischinski, 1992). This pentaglycine is a characteristic feature of the staphylococcal peptidoglycan and connects the amino group of the L-lysine of one stem peptide to the D-alanine of the adjacent stem peptide. The tetrapeptides carry an additional D-alanine that is cleaved during the cross-linking reaction (fig. 3.1).

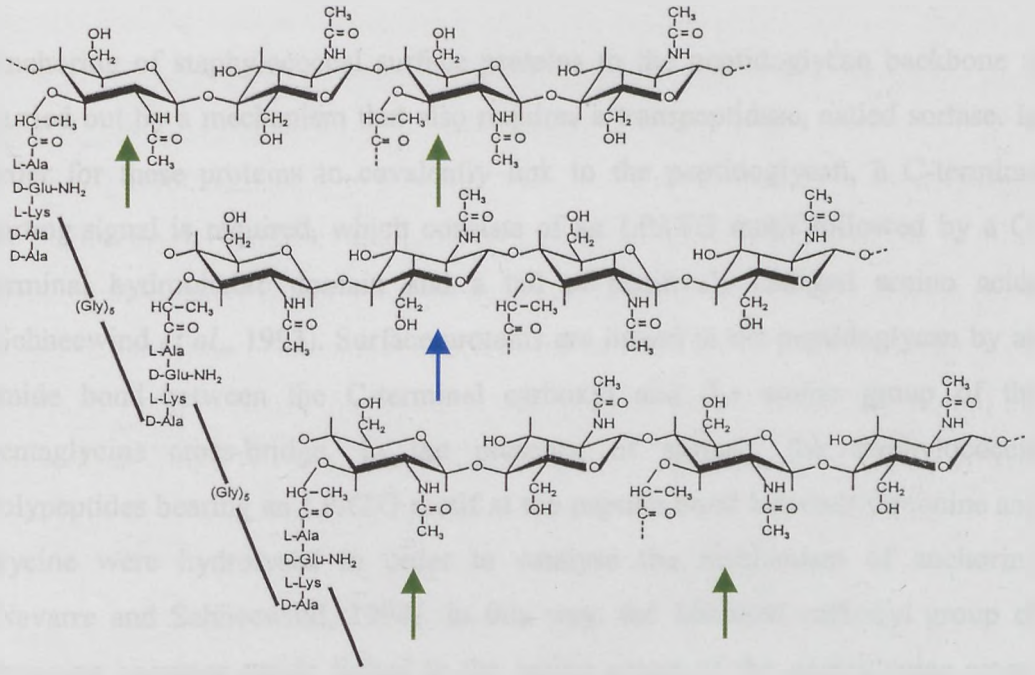

Fig. 3.1 Schematic diagram of the structural components of peptidoglycan. Three glycan strands, consisting of alternating *N*-acetylmuramic acid (indicated by a green arrow) and *N*-acetylglucosamine (indicated by a blue arrow) are indicated. The tetrapeptides, branching from *N*-acetylmuramic acid, are interconnected by pentaglycine cross bridges following an L-lys/D-ala transpeptidation reaction (Giesbrecht *et al.*, 1998).

S. aureus uses four different penicillin binding proteins (PBPs) in order to catalyse the process of cell wall cross-linking reactions between two peptidoglycan chains, which are called transpeptidation reactions (Giesbrecht *et al.*, 1998). There is lack of information on the exact functions of the four staphylococcal PBPs. However, it has been reported that the function of PBP1 is the most important for the survival of staphylococci exposed to beta-lactams since they can survive with only this PBP as the functional enzyme (Beise *et al.*, 1988).

On the other hand, PBP4 seems to be responsible for secondary cross-linking, because PBP4-defective *S. aureus* mutants exhibited a low cross-linking rate (Henze *et al.*, 1993). Finally, PBP2a, the reason for methicillin resistance in staphylococci, appears to be responsible for the formation of muropeptide dimers leading to the synthesis of peptidoglycan with reduced highly crosslinked oligomeric compounds (Berger-Bachi, 1994).

Anchoring of staphylococcal surface proteins to the peptidoglycan backbone is carried out by a mechanism that also requires a transpeptidase, called sortase. In order for these proteins to covalently link to the peptidoglycan, a C-terminal sorting signal is required, which consists of an LP*X*TG motif followed by a C-terminal hydrophobic domain and a tail of positively charged amino acids (Schneewind *et al.*, 1993). Surface proteins are linked to the peptidoglycan by an amide bond between the C-terminal carboxyl and the amino group of the pentaglycine cross-bridge. In the presence of sortase, the staphylococcal polypeptides bearing an LP*X*TG motif at the peptide bond between threonine and glycine were hydrolysed in order to catalyse the mechanism of anchoring (Navarre and Schneewind, 1994). In this way, the liberated carboxyl group of threonine becomes amide linked to the amino group of the pentaglycine cross-bridge, thus linking the C-terminal end of the polypeptide chain to the bacterial cell wall (Ton-That *et al.*, 1999). The LP*X*TG motif is conserved in more than 100 surface proteins of Gram-positive pathogens, suggesting that anchoring of these polypeptides occurs by a universal mechanism (Ton-That *et al.*, 1999a).

3.1.2 Murein hydrolases of staphylococci

The various components of the peptidoglycan backbone constitute targets for the polypeptides released into the surrounding medium of staphylococci. In some cases, these molecules bind to various sites of the peptidoglycan and play an important role in the synthesis and turnover of the peptidoglycan exoskeleton for daughter cells as well as the survival of staphylococci over competing microorganisms. These molecules are called murein hydrolases and may be classified according to their enzymatic activities on the various peptidoglycan

cleavage sites (fig 3.2). *N*-acetylmuramidases (muramidase) and *N*-acetylglucosaminases (glucosaminidase) cleave the MurNAc(β1-4)GlcNAc and GlcNAc(β1-4)MurNAc respectively. Moreover, *N*-acetylmuramyl-*L*-alanine amidase (amidases) hydrolyze the amide bond between the D-lactyl group of MurANc and the amino group of L-Ala. Furthermore, there are glycyl-glycine endopeptidases, such as lysostaphin, which can cleave the pentaglycine crossbridge of the staphylococcal peptidoglycan backbone (Ghuysen *et al.*, 1968). Finally, the presence of murein hydrolase LytA from phage ϕ11 breaks the bond between the D-Ala and the pentaglycine (Navarre *et al.*, 1999).

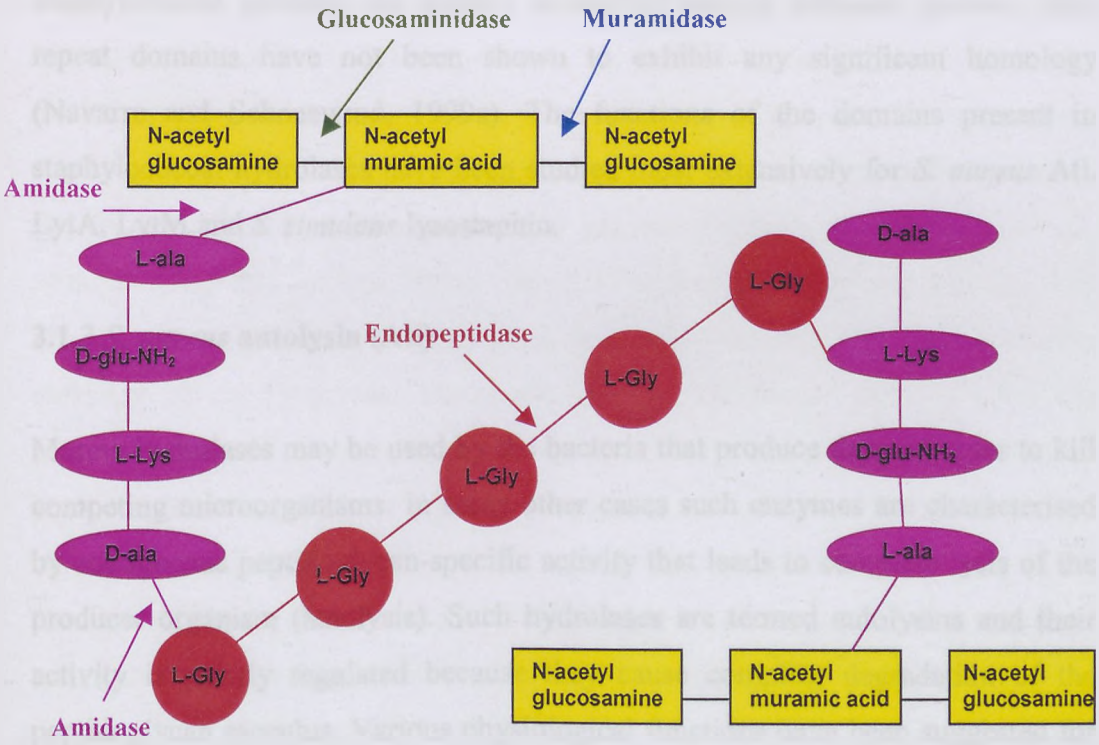

Fig. 3.2 Sites of hydrolysis of the various peptidoglycan hydrolases found in *S. aureus*. The sites of hydrolysis for amidases are indicated in red, for muramidases in blue for endopeptidases in brown and for glucosaminidases in green.

According to Navarre and Schneewind (1999a) most of these hydrolases display a certain domain structure. They are characterised by the presence of a signal peptide at the N-terminal end of the protein followed by a second domain that is responsible for the enzymatic activity and in some cases by a C-terminal targeting domain that provides target specificity to the enzyme. Further to this, some hydrolases have repeat domains that flank either the N- or the C- teminal end of the protein (Navarre and Schneewind, 1999a). For example, *S. simulans* lysostaphin contains 15 tandem repeats of 13 amino acids at its N-terminal side. *S. aureus* autolysin contains 3 repeat domains (R1, R2 and R3), two of which are located at the C-terminal end of amidase and one at the N-terminal end of glucosaminidase (fig. 3.3). Even though the enzymatic domains of various staphylococcal proteins are usually conserved among different species, their repeat domains have not been shown to exhibit any significant homology (Navarre and Schneewind, 1999a). The functions of the domains present in staphylococcal hydrolases have been studied most extensively for *S. aureus* Atl, LytA, LytM and *S. simulans* lysostaphin.

3.1.3 *S. aureus* autolysin (Atl)

Murein hydrolases may be used by the bacteria that produce them in order to kill competing microorganisms. In some other cases such enzymes are characterised by endogenous peptidoglycan-specific activity that leads to complete lysis of the producer organism (autolysis). Such hydrolases are termed autolysins and their activity is strictly regulated because they cause complete degradation of the peptidoglycan sacculus. Various physiological functions have been suggested for these autolysins including their roles in cell separation, wall growth, wall turnover, sporulation, formation of flagella and transformation (Yamada *et al.*, 1996). *S. aureus* produces two major peptidoglycan hydrolases, a 51 kDa glucosaminidase (endo-β-N-acetylglucosaminidase) and a 62 kDa amidase (N-acetylmuramyl-L-alanine) (Sugai *et al.*, 1995).

3.1.3.1 Cloning and sequence analysis of *S. aureus atl*

Cloning and expression of the *S. aureus* glucosaminidase was first reported by Biavasco *et al.* (1988). Oshida and colleagues later (1995) reported cloning of both amidase and glucosaminidase. Sequence analysis carried out by the same authors (1995) revealed that both hydrolases are 1256 amino acids long with a molecular mass of 137,381 Da and encoded by a gene called *atl*. The *atl* operon has four open reading frames (ORF) however both hydrolases are encoded by a single ORF (4th ORF) (Oshida *et al.*, 1995). A putative signal sequence has been also located at the N-terminal end of the Atl protein within the first 29 amino acids. Finally, three repeated sequences (R1, R2 and R3) were identified, each of between 140 and 164 residues long (Foster, 1995). Alignment of R1 with R2 indicated 76% similarity between the two sequences whereas alignment of repeats R1-R3, and R2-R3 indicated much lower identity, 34% and 35% respectively (Oshida *et al.*, 1995). The three repeat domains, are positioned at the center of pro-Atl such that mature amidase and glucosaminidase retain either two C-terminal (R1, R2) or one N-terminal (R3) repeat domains, respectively. The bifunctional Atl protein is synthesized as a preproenzyme (fig. 3.3) and after cleavage of its N-terminal signal peptide the enzyme is exported into the extracellular medium in the form of a proprotein. Further cleavage of pro-Atl at positions 198 and 775, releases a propeptide of unknown function, mature amidase with C-terminal R1 and R2 repeats and glucosaminidase with an N-terminal R3 repeat (Navare *et al.*, 1999a). It has been proposed that the *atl* gene was developed through a fusion of the amidase and glucosaminidase genes and that the DNA sequences of the three repeats were formed by duplication of an ancestral DNA segment. The high similarity between R1 and R2 indicates that they were formed by two rounds of duplication (Oshida *et al.*, 1995).

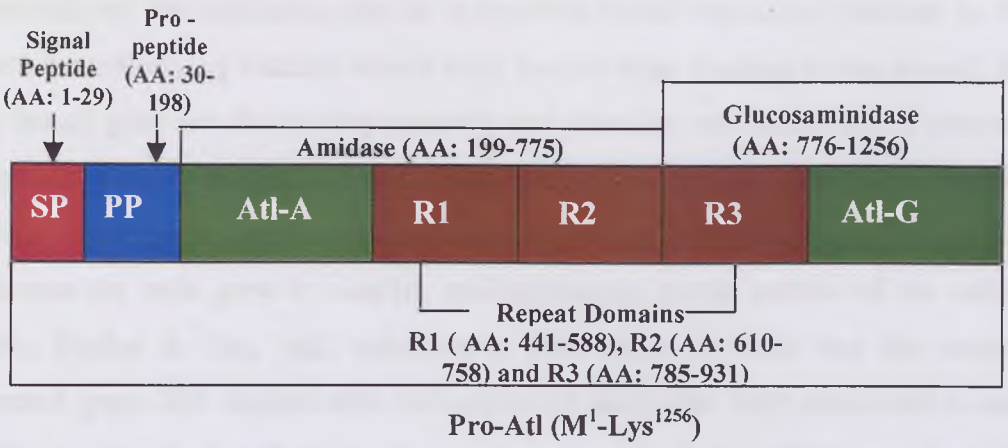

Fig. 3.3 Diagram indicating the domain structure of the *S. aureus* Atl. The protein consists of a signal peptide (SP) sequence (see section 3.1.3.4), a propeptide (PP) of unknown function and mature amidase (harbouring 2 repeat domains, R1 and R2) and glucosaminidase (with 1 repeat domain, R3).

3.1.3.2 Enzymatic activity of *S. aureus* Atl

The amidase and glucosaminidase gene products of *S. aureus atl* have been found to form a ring structure on the cell surface at the septal region of the equatorial surface ring, for the upcoming cell division site. According to scanning electron microscopy, after completion of septum formation and processing of the *atl* gene at the septal site, the mature forms of amidase and glucosaminidase are released and start to digest the peripheral peptidoglycan that connects the two daughter cells. Completion of the cell wall digestion produces two daughter cells that have already started to produce the new *atl* gene products at the site of new septum formation (Yamada *et al.*, 1996). As a result it has been postulated that *atl* is involved in cell separation.

3.1.3.3 Isolation and characterisation of Atl mutants

Oshida and Tomasz (1992), Foster (1995) as well as Takahashi and coworkers (2002) have isolated and characterised *atl* mutants. However, it was observed that these mutants did not form giant clusters when grown in broth culture. This result

contradicted the conclusion that *atl* is involved in cell separation, because in that case the underlying mutants should have formed large clusters during growth due to the *atl* gene not functioning properly and therefore not being able to properly separate the two new cells. Experiments carried out by Takahashi and colleagues (2002) with an *atl* knockout mutant indicated that *atl* is involved in cell saparation because the cells grew in clusters and sedimented at the bottom of the culture tube. Further to this, light microscopic observation revealed that the mutants formed grape-like clusters with well-separated cocci that were connected to each other by marginal cell walls. It was thus postulated that there may be other peptidoglycan hydrolase(s) involved in cell separation that can compensate for the deficit of *atl* function. Furthermore, deletion of the *atl* gene resulted in alteration of the cell surface into rough appearance that may have been caused by the presence of undigested protein targets of the Atl protein. This also indicated that the primary role of the *atl* gene may be the metabolic turnover of peptidoglycan through trimming off the outer surface peptidoglycan. Thus, it may be suggested that the rough surface produced by the peptidoglycan present in the *atl* mutant is structurally or chemically different to that forming the standard sacculus (Takahashi *et al.*, 2002).

3.1.3.4 The targeting mechanism of Atl

In terms of structure, the peptidoglycan backbone of most Gram-positive microorganisms is very similar if not identical. Once exported from the cytosol and secreted into the extracellular medium with mixed bacterial populations, murein hydrolases need to distinguish between the surface receptors of different microbial species in order to bind to the desired target. Thus, targeting of muralytic enzymes cannot be achieved by simple enzyme-substrate interactions but requires specific sequence elements to direct them to the corresponding surface receptors on the peptidoglycan of the target species (Baba and Schneewind, 1998). As a result, it may be assumed that the targeting mechanisms of murein hydrolases employ species-specific receptors for either physiological cell-wall turnover or the bacteriolytic killing of competing microorganisms (Baba and Schneewind, 1998a). For example, the six repeat domains at the C-terminal

end of *Streptococcus pneumoniae* LytA function to target this muralytic enzyme to the bacterial surface via binding to choline within the pneumococcal teichoic acid moieties (Sánchez-Puelles *et al.*, 1990). Experiments with *S. aureus* pro-Atl (Baba and Schneewind, 1998) revealed that the three repeat domains of Atl were each sufficient to direct both amidase and glucosaminidase to bind to the equatorial surface ring, either when secreted by staphylococci or when added externally to the cells. Finally, the same authors suggested the existence of a specific receptor at the equatorial surface ring due to the presence of an abundance of repeat domain-binding sites per colony forming unit (10^8).

3.1.4 Staphylococcal phage-encoded LytA

Bacteriophage ϕ11 was first identified as a prophage of *S. aureus* in 1967 (Novick, 1967) and is now one of the most genetically characterised of all staphylococcal phages. ϕ11 is a temperate, generalised transducing phage whose DNA is a linear, double-stranded molecule of approximately 46 kb in size. Its insertion into the *S. aureus* chromosome is a site-specific recombination event that occurs between an attachment site on the phage genome called *attP* and another location called *attB* on the *S. aureus* DNA. After infection of a cell by the phage and integration of viral DNA into that of the host, replication of the viruses occurs and lysis of the cell is induced. This event is achieved by certain phage-encoded proteins that are able to hydrolyse the covalent bonds in the bacterial cell wall, which may result in release of the progeny phage. Such phage-encoded proteins are called endolysins or lysins and a typical example is LytA from *S. aureus* NCTC 8325 lysogenic for bacteriophage ϕ11 (Jayaswal *et al.*, 1990 and Wang *et al.*, 1991).

3.1.4.1 Sequence analysis and structure of the *lytA* gene.

The *lytA* gene has been cloned and sequenced by Jayaswal and colleagues (1990). Sequence analysis revealed that the size of the gene corresponds to a 2.0 kb DNA fragment while a nucleotide sequence of ~1.2 kb was identified in the upstream region of *lytA*, containing three ORFs. The third ORF was found to overlap with

lytA by 18 nucleotides (Weerakoon *et al.*, 1995). The 481 amino acid putative LytA protein has an M_r of ~ 54,000 Da in its proform while after enzymatic processing it is converted into a mature amidase of M_r ~ 23,000 Da (Wang *et al.*, 1992). Furthermore, unlike other murein hydrolases, it has been reported that LytA is devoid of an N-terminal signal peptide (Navarre and Schneewind, 1999a). The enzyme consists of two enzymatic domains (fig 3.4), one at the N-terminal end of the protein that is responsible for endopeptidase activity (LytA-DL) and is 180 aa long and one between amino acids 181 and 370 that codes for amidase activity (LytA-A). Finally, the presence of a C-terminal cell wall targeting domain has been reported. It is 111 amino acids long and displays striking homology to the cell wall targeting domain of lysostaphin (Navarre *et al.*, 1999).

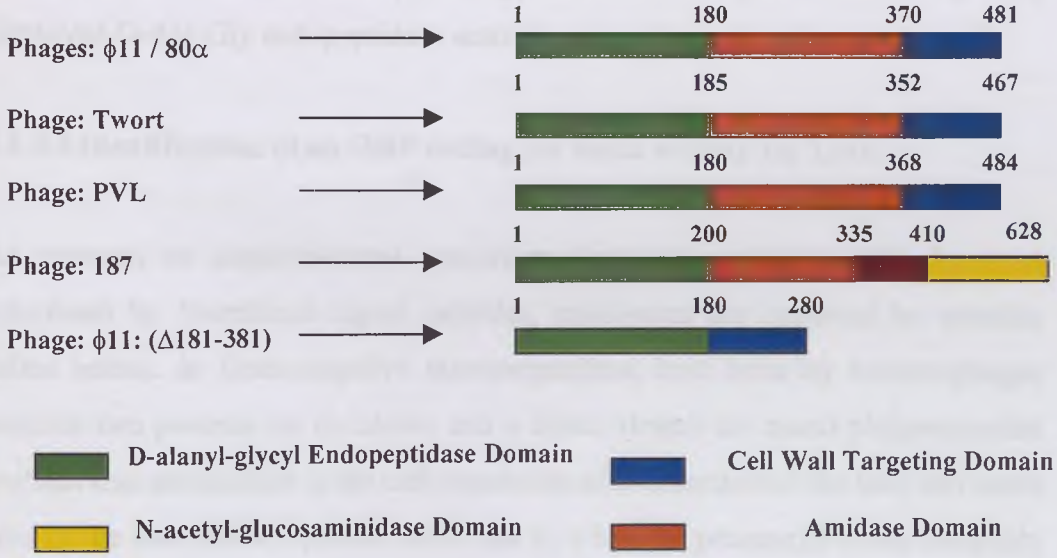

Fig. 3.4 Diagram indicating the structural similarities in domain organisation of the various murein hydrolases of staphylococcal bacteriophages (Navarre *et al.*, 1999).

3.1.4.2. Enzymatic activity of the two N-terminal domains of LytA

The amidase activity of the *lytA* gene was first reported by Jayaswal and colleagues (1990). Further studies performed by Navarre and colleagues (1999), revealed that the LytA protein consists of an N-terminal domain that displays striking similarity to endolysins of other phages that infect staphylococci, such as

ϕ187, 80α, Twort and ϕPVL (fig. 3.4). Furthermore, a central domain was also identified and found to exhibit sequence similarity with other *N*-acetylmuramyl-L-alanine amidases such as Atl from *S. aureus*. In order to investigate the activity of these domains further, a deletion mutant lacking the amidase homologous sequence ϕ11 (Δ181-381) was isolated and used with the wild type ϕ11 hydrolase to cleave the peptidoglycan backbone. The cleavage products were purified and revealed a NH_2-L-Ala-D-iGln-L-Lys-(NH_2-Gly_5)-D-Ala-COOH muropeptide that resulted from the wild type LytA activity and a MurNAc-(L-Ala-D-iGln-L-Lys-(NH_2-Gly_5)-D-Ala-COOH)-(β1-4)-GlcNAc muropeptide that was the cleavage product of the LytA mutant (lacking the amidase homologous sequence). These results, suggested that LytA has N-acetylmuramyl-L-Ala amidase as well as D-Ala-Gly endopeptidase activity, as opposed to the LytA mutant that only displayed D-Ala-Gly endopeptidase activity.

3.1.4.3 Identification of an ORF coding for holin activity for LytA.

As opposed to staphylococcal autolysins that are secreted to the bacterial cytoplasm by N-terminal signal peptides, endolysins are exported by proteins called holins. In Gram-negative microorganisms, host lysis by bacteriophages requires two proteins an endolysin and a holin. Holins are small phage-encoded proteins that accumulate in the cell membrane after infection of the host and cause lysis of the host cell at a precise time, that is, when the process of virion assembly has generated an appropriate number of progeny. Thus, holins are the sole direct determinants of the length of the infective cycle and the yield of progeny phage (Young *et al.*, 2000). In the case of LytA, the third ORF identified immediately upstream of the *lytA* nucleotide sequence, has been assumed to play this role due to its significant sequence homology to the holins of the bacteriophages of Gram-negative bacteria (Borchardt *et al.*, 1993). According to experiments carried out by Weerakoon and colleagues (1995) in order to specify the role of the third ORF identified adjacent to the *lytA* gene, the ORF3 product is involved in the process of cell lysis and phage release. Mutants containing the disrupted ORF3 were isolated and it was observed that lysogens containing the disrupted ORF had a very low or no phage titer, as opposed to lysogenised strains containing the intact

ORF, which showed lysis and high phage titers. This suggested that the plasmid containing the mutated ORF behaved as a suicide vector indicating that in the case of ϕ11 infecting *S. aureus*, phage-induced lysis by LytA requires a second protein that is a holin-like enzyme, encoded by ORF3 that helps the endolysin to attack the peptidoglycan and thus cause cell death. A similar mechanism of cell lysis has been reported with the Twort and 80α bacteriophages that also infect *S. aureus*. Therefore, it has been suggested that in Gram-positive bacteria too, phage encoded murein hydrolases are released from the cytoplasm only after the holin-induced disruption of the cytoplasmic membrane (Navarre and Schneewind, 1999a).

3.1.4.4 Identification of the CHAP domain in endolysins and autolysins

The N-terminal amidase domain of phage-encoded LytA from bacteriophage ϕ11 has been used in BLAST searches in an attempt to investigate the presence of potentially uncharacterised domains of several other endolysins and bacterial autolysins (Bateman and Rawlings, 2003). In this way, new components of peptidoglycan metabolism may be revealed that could constitute potential antimicrobial targets for the development of antibiotics. The results of the aligned sequences indicated the presence of a domain, called the CHAP (cysteine, histidine-dependent amindohydrolases/peptidases) domain. This new superfamily of amidohydrolases are characterised by conserved cysteine and histidine residues (fig. 3.5), which appear to be essential for amidase activity, as well as aspartic acid residues. Another characteristic feature of these proteins is the presence of a complex domain organization that results in the formation of bifunctional enzymes and thus makes the exact location of the peptidoglycan hydrolase activity unclear (Rigden *et al.*, 2003). Nevertheless, amidase activity has been mapped to the CHAP domain in several other endolysins including LytA (Wang *et al.*, 1991). Another example, is that of endolysin from the *S. aureus* bacteriophage Twort, whose N-terminal 172-amino acid fragment was shown to possess an *N*-acetylmuramoyl L-alanine amidase activity, whereas the C terminus of the protein was similar to lysostaphin (Loessner *et al.*, 1998). The large number of such multifunctional hydrolases belonging to the CHAP family, suggests that

these proteins may act in a cooperative manner in order to cleave specialised substrates, which could constitute new targets for the development of novel antibiotics.

```
LytA          LKTSEGKQFNVDLWYGFQCFDYANAGWKVLFGLLLKGLGAKDI
Phage 187     AINLIGSGVDVDGYYGRQCWDLPNYIFNRYWNFKTPGNARDMA
Phage Twort   IKSKVNTGTDFDGLYGYQCMDLAVDYIYHVTDGKIRMWGNAKD

LytA          -17-DFLAQPGDMVVFG-5-GYGHVAWVIEAT
Phage 187     -15-DFVPKPGDIAVWT-7-TWGHTGIVVGPS
Phage Twort   -15-AFRPKYGDVVVWT-5-TYGHIAIVTNPD
```

Fig. 3.5 Sequence alignment indicating the conserved core of the CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domains of staphylococcal phage endolysins Twort and 187 as well as LytA from bacteriophage ϕ11. Predicted catalytic cysteine and histidine residues are indicated in red. Hydrophobic and small conserved aa are shown in green and blue respectively, while acidic residues are indicated in orange. The numbers between the aligned blocks indicate the lengths of variable inserts in the respective protein sequences (Rigden *et al.*, 2003).

3.1.5 Organisation of lysostaphin

The lysostaphin endopeptidase gene has been located on the largest of the five plasmids in *S. simulans* biovar *staphylolyticus*, called pACK1 (Heath *et al.*, 1987). Lysostaphin has been cloned and sequenced (Recsei *et al.*, 1987; Heinrich *et al.*, 1987; Williamson *et al.*, 1994) while analysis of the sequence of the lysostaphin gene and the sequencing of the amino-terminus of purified prolysostaphin and of mature lysostaphin have revealed that lysostaphin is organised as a preproenzyme and consists of 493 amino acids (fig. 3.6). It contains a signal sequence, which consists of 36 amino acids, a proenzyme of 211 amino acids and the mature enzyme of 246 amino acids. 195 amino acids of the proenzyme are organised in 15 tandem repeats of 13 amino acids long, which start at amino acid position 49 and finish at position 243. Finally, the mature enzyme consists of an endopeptidase domain (113 amino acids), a linker region (17 amino acids) and a

targeting domain (93 amino acids) (Thumm and Gotz, 1997). In the form of a preproenzyme, lysostaphin has a molecular mass of 42 kDa and 27 kDa when it is released as a mature protein.

```
1    ATGAAAAAAACTAAAAATAATTATTATACTCGTCCTCTTGCTATTGGTCTTTCTACTTTT
1     M  K  K  T  K  N  N  Y  Y  T  R  P  L  A  I  G  L  S  T  F
61   GCTCTTGCTTCTATTGTTTATGGTGGTATTCAAAATGAAACTCATGCTTCTGAAAAATCT
21    A  L  A  S  I  V  Y  G  G  I  Q  N  E  T  H  A  S  E  K  S
121  AATATGGATGTTTCTAAAAAAGTTGCTGAAGTTGAAACTTCTAAAGCTCCTGTTGAAAAT
41    N  M  D  V  S  K  K  V  A  E  V  E  T  S  K  A  P  V  E  N
181  ACTGCTGAAGTTGAAACTTCTAAAGCTCCTGTTGAAAATACTGCTGAAGTTGAAACTTCT
61    T  A  E  V  E  T  S  K  A  P  V  E  N  T  A  E  V  E  T  S
241  AAAGCTCCTGTTGAAAATACTGCTGAAGTTGAAACTTCTAAAGCTCCTGTTGAAAATACT
81    K  A  P  V  E  N  T  A  E  V  E  T  S  K  A  P  V  E  N  T
301  GCTGAAGTTGAAACTTCTAAAGCTCCTGTTGAAAATACTGCTGAAGTTGAAACTTCTAAA
101   A  E  V  E  T  S  K  A  P  V  E  N  T  A  E  V  E  T  S  K
361  GCTCCTGTTGAAAATACTGCTGAAGTTGAAACTTCTAAAGCTCCTGTTGAAAATACTGCT
121   A  P  V  E  N  T  A  E  V  E  T  S  K  A  P  V  E  N  T  A
421  GAAGTTGAAACTTCTAAAGCTCCTGTTGAAAATACTGCTGAAGTTGAAACTTCTAAAGCT
141   E  V  E  T  S  K  A  P  V  E  N  T  A  E  V  E  T  S  K  A
481  CCTGTTGAAAATACTGCTGAAGTTGAAACTTCTAAAGCTCCTGTTGAAAATACTGCTGAA
161   P  V  E  N  T  A  E  V  E  T  S  K  A  P  V  E  N  T  A  E
541  GTTGAAACTTCTAAAGCTCCTGTTGAAAATACTGCTGAAGTTGAAACTTCTAAAGCTCCT
181   V  E  T  S  K  A  P  V  E  N  T  A  E  V  E  T  S  K  A  P
601  GTTGAAAATACTGCTGAAGTTGAAACTTCTAAAGCTCCTGTTGAAAATACTGCTGAAGTT
201   V  E  N  T  A  E  V  E  T  S  K  A  P  V  E  N  T  A  E  V
661  GAAACTTCTAAAGCTCCTGTTGAAAATACTGCTGAAGTTGAAACTTCTAAAGCTCTTGTT
221   E  T  S  K  A  P  V  E  N  T  A  E  V  E  T  S  K  A  L  V
721  CAAAATCGTACTGCTCTTCGTGCTGCTACTCATGAACATTCTGCTCAATGGCTTAATAAT
241   Q  N  R  T  A  L  R  A  A  T  H  E  H  S  A  Q  W  L  N  N
781  TATAAAAAAGGTTATGGTTATGGTCCTTATCCTCTTGGTATTAATGGTGGTATGCATTAT
261   Y  K  K  G  Y  G  Y  G  P  Y  P  L  G  I  N  G  G  M  H  Y
841  GGTGTTGATTTTTTTATGAATATTGGTACTCCTGTTAAAGCTATTTCTTCTGGTAAAATT
281   G  V  D  F  F  M  N  I  G  T  P  V  K  A  I  S  S  G  K  I
901  GTTGAAGCTGGTTGGTCTAATTATGGTGGTGGTAATCAAATTGGTCTTATTGAAAATGAT
301   V  E  A  G  W  S  N  Y  G  G  G  N  Q  I  G  L  I  E  N  D
961  GGTGTTCATCGTCAATGGTATATGCATCTTTCTAAATATAATGTTAAAGTTGGTGATTAT
321   G  V  H  R  Q  W  Y  M  H  L  S  K  Y  N  V  K  V  G  D  Y
1021 GTTAAAGCTGGTCAAATTATTGGTTGGTCTGGTTCTACTGGTTATTCTACTGCTCCTCAT
341   V  K  A  G  Q  I  I  G  W  S  G  S  T  G  Y  S  T  A  P  H
1081 CTTCATTTTCAACGTATGGTTAATTCTTTTTCTAATTCTACTGCTCAAGATCCTATGCCT
361   L  H  F  Q  R  M  V  N  S  F  S  N  S  T  A  Q  D  P  M  P
1141 TTTCTTAAATCTGCTGGTTATGGTAAAGCTGGTGGTACTGTTACTCCTACTCCTAATACT
381   F  L  K  S  A  G  Y  G  K  A  G  G  T  V  T  P  T  P  N  T
```

1201 GGTTGGAAAACTAATAAATATGGTACTCTTTATAAATCTGAATCTGCTTCTTTTACTCCT

401 G W K T N K Y G T L Y K S E S A S F T P

1261 AATACTGATATTATTACTCGTACTACTGGTCCTTTTCGTTCTATGCCTCAATCTGGTGTT

421 N T D I I T R T T G P F R S M P Q S G V

1321 CTTAAAGCTGGTCAAACTATTCATTATGATGAAGTTATGAAACAAGATGGTCATGTTTGG

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

1381 GTTGGTTATACTGGTAATTCTGGTCAACGTATTTATCTTCCTGTTCGTACTTGGAATAAA

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

1441 TCTACTAATACTCTTGGTGTTCTTTGGGGTACTATTAAA

481 S T N T L G V L W G T I K

Fig. 3.6 Indication of the *S. simulans* lysostaphin nucleotide and deduced amino acid sequence indicated in red and blue respectively.

3.1.5.1 The Lysostaphin Immunity Factor (*Lif*)

The lysostaphin immunity factor is plasmid-encoded in *S. simulans* biovar *staphylolyticus* and also located on the plasmid pACK1 (Heath *et al.*, 1987). The gene is a 413 codon open reading frame, located adjacent to that of lysostaphin but in the reverse orientation. There is a non-coding region of 208 base pairs (bp) between the start codons of lysostaphin and *lif* (Thumm and Gotz, 1997). Various *S. aureus* strains with reduced susceptibility to lysostaphin have been shown to contain an increased number of serine residues and fewer glycines in their pentaglycin (Maidhof *et al.*, 1991). According to experiments by Thumm and Gotz (1997), lysostaphin immunity factor primarily confers immunity to the producing organism by replacing glycine residues in the staphylococcal peptidoglycan with serine residues (fig. 3.7) (Thumm and Gotz, 1997). Further experiments by Tschierske *et al.*, (1997) have showed that *lif* functions as a *femB* analogue. This was indicated when the peptidoglycan composition of *femB* null mutants was analysed and it was observed that their serine content was increased fourfold (once *lif* was expressed) compared to *femA* and *femAB* null mutants. It was therefore postulated that *lif* is responsible for the incorporation of serines in a way similar to that of *femB* in glycine incorporation. However, in contrast to this postulation, experiments carried out by Ehlert *et al.*, (2000) proved that the

activity of the *lif* gene depends upon the presence of *femA* and/or *femB* and that Lif alone cannot catalyse the incorporation of serines in the interpeptide cross bridge. This conclusion was drawn when *femAB* mutants expressing *lif* were unable to extend their shortened cross bridges by the addition of serine residues. Further experiments carried out by the same author showed that *Lif* is responsible for the addition of serine residues to the interpeptide side chain only at positions 3 and 5.

Increased resistance to lysostaphin has been also observed by organisms such as *S. epidermidis*, which have substituted their glycine residues with alanine or serine residues. These observations also indicate the inability of mature lysostaphin to hydrolyse glycyl-serine and seryl-glycine peptide bonds (Robinson *et al.*, 1979).

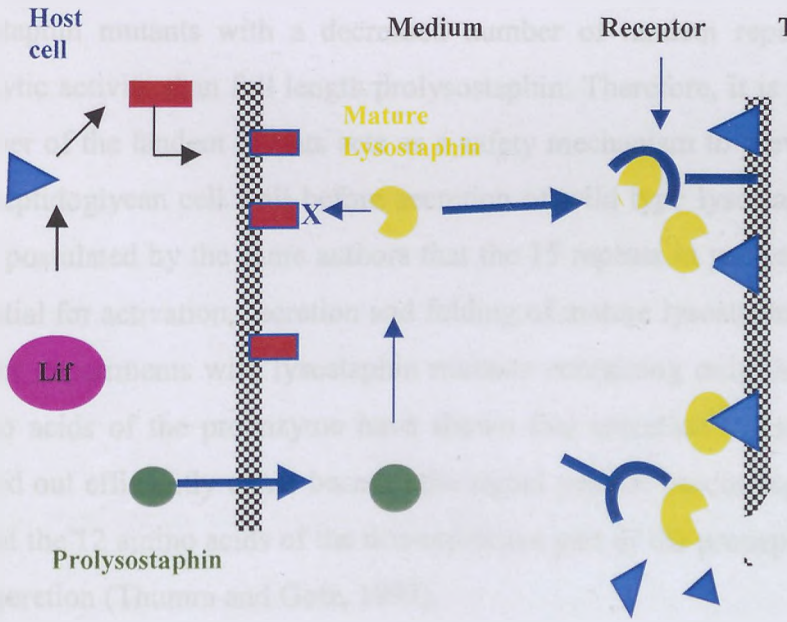

Fig. 3.7 *Staphylococcus simulans* is not susceptible to lysostaphin due to the *lif* gene indicated in pink. The immunity factor protects its host by replacing some glycine residues of the pentaglycine with serines, resulting in a modified cell wall (red) that mature lysostaphin cannot hydrolyse (adapted from Baba and Schneewind, 1998).

3.1.5.2 The signal peptide of lysostaphin

The signal peptide of lysostaphin has a 6 amino acid long hydrophilic region at the amino-terminal of the sequence and a 27 amino acid long hydrophobic region from position 9 to 36 (Heinrich *et al.*, 1987). When *S. simulans* biovar *staphylolyticus* requires the production of mature lysostaphin, preprolysostaphin is initiated into the secretory pathway by the amino-terminal signal peptide and cleavage of the hydrophobic signal sequence occurs intracellularly by leader peptidase (Baba and Schneewind, 1996).

3.1.5.3 The proenzyme form of lysostaphin

The remaining propeptide of lysostaphin has been shown to play a role in the degree of activity of lysostaphin. Thumm and Gotz (1997) have shown that lysostaphin mutants with a decreased number of tandem repeats have higher autolytic activity than full length prolysostaphin. Therefore, it is possible that the number of the tandem repeats acts as a safety mechanism to prevent autolysis of the peptidoglycan cell wall before secretion of wild type lysostaphin. It has also been postulated by the same authors that the 15 repeats in prolysostaphin are not essential for activation, secretion and folding of mature lysostaphin. Nevertheless, further experiments with lysostaphin mutants containing only the first 3, 4 or 5 amino acids of the proenzyme have shown that secretion of lysostaphin is not carried out efficiently either because the signal peptide processing site is affected or that the 12 amino acids of the non-repetitive part of the propeptide are required for secretion (Thumm and Gotz, 1997).

Experiments have also been carried out with *S. carnosus* in order to identify a role for the 15 tandem repeats of the prolysostaphin molecule (Thumm and Gotz, 1997). The growth of *S. carnosus* expressing the lysostaphin gene without the 15 tandem repeats was reduced when compared to similar cultures of *S. carnosus* expressing lysostaphin with all 15 tandem repeats present. It was therefore concluded that the *lif* gene cannot fully protect a lysostaphin-producing organism

against the more potent form of lysostaphin that lacks the 15 tandem repeats (Thumm and Gotz., 1997).

3.1.5.4 Processing of prolysostaphin to the mature lysostaphin

In the absence of extracellular proteases prolysostaphin cannot be converted into its mature form. Experiments have showed that in order for *S. simulans* biovar *staphylolyticus* to process prolysostaphin into the mature enzyme the presence of a cysteine protease is required in the supernatant, which is chromosomally encoded. This was concluded after incubation of purified prolysostaphin together with cysteine protease that led to the formation of mature lysostaphin. The processing occurs stepwise by the cysteine protease molecule and the resulting mature lysostaphin has 4.5-fold higher staphylolytic activity than that of prolysostaphin (Thumm and Gotz, 1997).

3.1.6 LytM – A *Staphylococcus aureus* autolysin

According to BLAST searches from the NCBI Entrez protein databases, significant homology has been observed (51%) between the amino acid sequence of lysostaphin and that of a unique autolytic gene of *Staphylococcus aureus* called *lytM* (fig.3.8).

```
LytM213 VDYAMPEUSP VYSLTDGFVV QAGWSNYGGG HQVTIKEANS NNYQWYMHNH RL 264
        .. .    . .     . .  ......... ..    .       .....
        ... .   .. . ... . .. ......... ... . . .   . ..... . .
Lyso282 VDFFMNIGTP VKAISSGKIV EAGWSNYGGG NQIGLIENDG VHRQNYMHLS KY 333

LytM265 TVSAGDKVKA GDQIAYSGST GHSTAPHVHF QRHSGGIGHQ YAVDPTSYLQ S 315
          .  .. ... .  .  .... . ..... .. ...     .   . ..   .  .
          .  .. ... .  . ..... . ......... ...     .   . ..  ... .
Lyso334 NVKVGDYVKA GQIIGWSGST GYSTAPHLHF QRMVNSFSNS TAQDPMPFLK S 384
```

Fig. 3.8 Alignment of the homologous regions of the LytM (C-terminal region) and lysostaphin genes (N-terminal region). Identical amino acids are represented with two dots while similar amino acids are indicated with one dot.

The N-terminal region of the mature lysostaphin sequence was found to be very similar to the C-terminal region of LytM. This equates to the endopeptidase domain of lysostaphin and indicates that LytM also has a catalytic activity (Ramadurai and Jayaswal, 1997). The *lytM* gene contains 0.9 mol of zinc per *lytM* molecule (Ramadurai *et al.*, 1999). This suggests that LytM may belong to a novel class of zinc metalloproteases, similar to lysostaphin which has 1 zinc atom per lysostaphin molecule (Trayer and Buckley, 1970). These similarities between the two genes indicate that LytM may have similar properties to lysostaphin and therefore could be used as an antimicrobial peptide. Furthermore, studies on the LytM protein may provide significant information on our understanding of the biological activity of lysostaphin.

LytM is the first reported chromosomally encoded glycylglycine endopeptidase in *S. aureus* (Ramadurai and Jayaswal, 1997). The gene has been cloned and sequenced by Ramadurai and Jayaswal (1997) and was found to be located within a 1.2 kb fragment. Two overlapping open reading frames of 966 bp and 948 bp have been located. It is however, the start codon of the second open reading frame that constitutes the start site for translation of the gene, because an appropriate ribosome binding site occurs upstream of this start codon. A signal peptidase cleavage site has been located at amino acid position 26 while the signal sequence has been proposed to be present within these 26 amino acids (fig. 3.9).

```
1   ATGAAAAAATTAACAGCAGCAGCGATTGCAACGATGGGCTTCGCTACATTTACAATGGCG
1    M  K  K  L  T  A  A  A  I  A  T  M  G  F  A  T  F  T  M  A
61  CATCAAGCAGATGCAGCAGAAACGACAAACACCCAACAAGCACATACACTAATGTCAACA
21   H  Q  A  D  S  A  E  T  T  N  T  Q  Q  A  H  T  Q  M  S  T
121 CAATCACAAGACGTATCTTATGGTACTTATTACAATTGATTCTAATGGGGATTATCATCA
41   Q  S  Q  D  V  S  Y  G  T  Y  Y  T  I  D  S  N  G  D  Y  H
181 CACACCTGATGGTAACTGGAATCAAGCAATGTTTGATAATAAAGAATATAGCTATACATT
61   H  T  P  D  G  N  W  N  Q  A  M  F  D  N  K  E  Y  S  Y  T
241 CGTAGATGCTCAAGGACATACGCATTATTTTTATAACTGTTATCCAAAAAATGCAAATGC
81   F  V  D  A  Q  G  H  T  H  Y  F  Y  N  C  Y  P  K  N  A  N
301 CAATGGAAGCGGCCAAACATATGTGAATCCAGCAACAGCAGGAGATAACAATGACTACAC
101  A  N  G  S  G  Q  T  Y  V  N  P  A  T  A  G  D  N  N  D  Y
361 AGCGAGTCAAAGCCAACAGCATATTAATCAATATGGTTATCAATCAAATGTAGGTCCAGA
121  T  A  S  Q  S  Q  Q  H  I  N  Q  Y  G  Y  Q  S  N  V  G  P
421 CGCGAGCTATTATTCACATAGTAACAACAACCAAGCGTATAACAGCCATGATGGTAATGG
141  D  A  S  Y  Y  S  H  S  N  N  N  Q  A  Y  N  S  H  D  G  N
481 AAAGGTCAATTATCCTAATGGCACATCTAATCAAAATGGTGGATCAGCAAGTAAAGCGAC
161  G  K  V  N  Y  P  N  G  T  S  N  Q  N  G  G  S  A  S  K  A
541 AGCTAGTGGTCATGCGAAAGACGCAAGCTGGTTAACAAGTCGTAAACAACTACAACCATA
181  T  R  S  G  H  A  K  D  A  S  W  L  T  S  R  K  Q  L  Q  P
601 TGGACAATATCACGGTGGTGGTGCGCATTACGGTGTCGACTATGCAATGCCTGAAAATTC
201  Y  G  Q  Y  H  G  G  G  A  H  Y  G  V  D  Y  A  M  P  E  N
661 ACCAGTTTACTCATTAACTGATGGTACAGTAGTACAAGCAGGTTGGAGTAACTATGGTGG
221  S  P  V  Y  S  L  T  D  G  T  V  V  Q  A  G  W  S  N  Y  G
721 CGGCAATCAAGTAACTATTAAAGAAGCGAACAGTAATAACTACCAATGGTATATGCATAA
241  G  G  N  Q  V  T  I  K  E  A  N  S  N  N  Y  Q  W  Y  M  H
781 TAATCGTTTAACTGTTTCAGCTGGTGATAAAGTCAAAGCTGGTGACCAAATTGCATATTC
261  N  N  R  L  T  V  S  A  G  D  K  V  K  A  G  D  Q  I  A  Y
841 AGGTAGTACGGGTAATTCAACAGCGCCTCACGTACACTTCCAACGTATGTCTGGTGGCAT
281  S  G  S  T  G  N  S  T  A  P  H  V  H  F  Q  R  M  S  G  G
901 CGGTAATCAATATGCAGTAGACCCAACGTCATACTTGCAAAGTAGATA
301  I  G  N  Q  Y  A  V  D  P  T  S  Y  L  Q  S  R
```

Fig. 3.9 Indication of the *S. aureus lytM* nucleotide and deduced amino acid sequence indicated in blue and red respectively.

The protein encoded by *lytM* consists of 316 amino acid residues, has a molecular mass of 34.4 kDa and a pI of 6.3. After removal of the signal peptide the protein's

molecular mass has been estimated 32 kDa with a pI of 6.02. LytM is mainly hydrophilic with the exception of its signal peptide sequence that is hydrophobic (Ramadurai and Jayaswal, 1997). Similarly to other autolysins, LytM remains active within a pH range 5-8.

3.1.7 An *E. coli* autolysin - LytM

E. coli lytM is another gene with significant homology to lysostaphin (fig. 3.10), the size of which is 1257 bp. The protein is 419 amino acids long, shown in fig. 3.11 and has a molecular mass of 45 kDa. There is a signal sequence located at the N-terminal of the protein, the length of which is 16 amino acids. A targeting domain has not been identified so far, which suggests that the gene could be used for the production of chimeras that will use the killing activity of the LytM protein and the targeting domain of another protein to direct the fused product to a desired target depending on the targeting domain used.

```
Lys  -MKKTKNNYYTRPLAIGLSTFALASIVYGGIQNETHASEKSNMDVSKKVAEVETSKAPVE 59
LytM MLGSLTVLTLAVAVWRPYVYHRDATPIVKTIELEQNEIRSLLPEASEPIDQAAQEDEAIP 60

Lys  N---TAEVETSKAPVENTAEVETSKARVENTAEVETSKAPVENTAEVETSKAPVENTAEV 116
LytM QDELDDKIAGEAGVHEYVVSTGDTLSSILNQYGIDMGDITQLAAADKELRNLKIGQQLSW 120

Lys  ETSKAPVENTAEVETSKAPVENTAEVETSKAPVENTAEVETSKAPVENTAEVETSKAPVE 176
LytM TLTADGELQRLTWEVSRRETRTYDRTAANGFKMTSEMQQGEWVNNLLKGTVGGSFVASAR 180

Lys  NTAEVETSKAPVENTAEVETSKAPVENTAEVETSKAPVENTAEVETS-------KAPVEN 229
LytM NAGLTSAEVSAVIKAMQWQMDFRKLKKGDEFAVLMSREMLDGKREQSQLLGVRLRSEGKD 240

Lys  TAEVETSKALVQNRTALRAATHEHSAQWLNNYKKGYGYGP---YPLGINGGMHYGVDFFM 286
LytM YYAIRAEDGKFYDRNGTGLAKGFLRFPTAKQFRISSNFNPRRTNPVTGRVAPHRGVDFAM 300

Lys  NIGTPVKAISSGKIVEAGWSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDYVKAGQI 346
LytM PQGTPVLSVGDGEVVVAKRS--GAAGYYVAIRHGRSYTTRYMHLRKILVKPGQKVKRGDR 358

Lys  IGWSGSTGYSTAPHLHFQRMVNSFSNSTAQDPMPFLKSAGYGKAGGTVTPTPNTGWKTNK 406
LytM IALSGNTGRSTGPHLHYEVWIN----QQAVNPLTAKLPRTEGLTG---------------- 399

Lys  YGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIHYDEVMKQDGHVWVGYTGN 466
LytM ------SDRREFLAQAKEIVP------------------QLRFD---------------- 419
```

Fig. 3.10 Homology of the primary amino acid sequence of Lysostaphin (Lys) from *S. simulans* and *E. coli* LytM (LytM). Identical residues (*), conserved (:) and semi-conserved sustitutions (.) are indicated. The hyphens indicate gaps introduced to maximise homology.

```
1   MLGSLTVLTL AVAVWRPYVY HRDATPIVKT IELEQNEIRS LLPEASEPID QAAQEDEAIP   60
61  QDELDDKIAG EAGVHEYVVS TGDTLSSILN QYGIDMGDIT QLAAADKELR NLKIGQQLSW  120
121 TLTADGELQR LTWEVSRRET RTYDRTAANG FKMTSEMQQG EWVNNLLKGT VGGSFVASAR  180
181 NAGLTSAEVS AVIKAMQWQM DFRKLKKGDE FAVLMSREML DGKREQSQLL GVRLRSEGKD  240
241 YYAIRAEDGK FYDRNGTGLA KGFLRFPTAK QFRISSNFNP RRTNPVTGRV APHRGVDFAM  300
301 PQGTPVLSVG DGEVVVAKRS GAAGYYVAIR HGRSYTTRYM HLRKILVKPG QKVKRGDRIA  360
361 LSGNTGRSTG PHLHYEVWIN QQAVNPLTAK LPRTEGLTGS DRREFLAQAK EIVPQLRFD   419
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Fig. 3.11 Indication of the *E. coli* LytM amino acid sequence in blue and its signal sequence at the N-terminus of the protein in green.

3.1.8 The pET21a vector system used for cloning of *S. simulans* lysostaphin, *S. aureus lytM* and *E. coli lytM*.

The vector system that has been used in this study is the pET System (by Novagen), a very powerful and efficient approach for the production of recombinant proteins in *E. coli*. It is based on the T7 promoter-driven system that was originally developed by Studier and Moffat (1986). The pET21a and pET21d plasmids are under the control of the strong bacteriophage T7 transcription and translation signals while expression is induced by providing a source of T7 RNA polymerase in the host cell. One of the main advantages of this vector system is that T7 RNA polymerase has a very high selection and activity rate. In this way all of the cell's resources are used to target gene expression; as a result the desired protein may comprise more than 50% of the total cell protein only after a few hours of induction.

Another advantage of pET21a and pET21d vectors is that they carry the His-Tag sequence. This is a stretch of 6 consecutive histidine residues that can be expressed at either the N-terminus or the C-terminus of the target protein. Divalent cations (Ni^{2+}) are immobilised onto a His-Bind metal chelation resin to which the His-Tag sequence binds. Wash buffers are then used to remove any unbound proteins and recovery of the target protein follows by elution with imidazole, which binds to the resin and thus releasing the his-tagged protein from the column. The columns required for the purification of a protein can be regenerated and reused making the process convenient and a very economical means of protein purification without the need to develop new protocols for each protein. Furthermore, pET21a and pET21d vectors allow purification under gentle and native conditions for maintaining activity of soluble proteins and under denaturing conditions that may be required for solubilisation of inclusion bodies.

3.1.9 Aims

The lysostaphin molecule appears to be a promising solution to the problem of antibiotic resistance to many existing staphylococcal species including the

nosocomial pathogen EMRSA-16, which has developed resistance to all existing antibiotics. However, little information is currently available on its mode of action against its target bacteria. Hence it was decided to initially clone the lysostaphin gene and purify the polypeptide in order to investigate its mode of action. Furthermore, the significant homology that has been observed between the catalytic domain of lysostaphin and the C-terminal domain of *S. aureus* LytM indicate that it may be possible to use these proteins in order to successfully construct chimeras against EMRSA-16, as alternatives to lysostaphin. Such fusion proteins could be very important because they could be used in order to prevent natural resistance to lysostaphin from developing. In addition, the high homology between lysostaphin and *E. coli* LytM suggests that the later may also be an autolysin. Also, the similar domain structure between *S. aureus* LytM and *E. coli* LytM indicate that the later protein could also be used for the construction of chimeras targeted at Gram negative bacteria.

3.2 RESULTS

3.2.1 Cloning, expression, purification and assay of mature lysostaphin

Lysostaphin was amplified from the genomic DNA of *Staphylococcus simulans* biovar *staphylolyticus* (as described in section 2.8) using reverse primer DW41 (G CTC GAG CTT TAT AGT TCC CCA AAG) and forward primer RJ84 (G CAT ATG GCT GCA ACA CAT GAA CAT TC). Primer DW41 was used to introduce an *Xho*I site in place of the stop codon of mature lysostaphin while RJ84 was used to introduce an *Nde*I site and thus a methionine codon, immediately upstream and in frame with the first alanine codon (Ala1) of the mature lysostaphin encoding fragment of the lysostaphin gene. The resulting 750-bp PCR product was gel purified and digested with *NdeI* and *XhoI*. The restricted mature lysostaphin fragment was then ligated into expression vector pET21a also restricted with *Nde*I and *Xho*I and transformed initially into competent *E. coli* JM83 cells. Recombinant plasmid pEA3 containing the lysostaphin gene was sequenced to check for the presence of the desired gene and fidelity of the PCR.

Plasmid pEA3 was transformed into *E. coli* B834 [DE3] cells for the overexpression of mature lysostaphin with IPTG. However, after addition of IPTG it was observed that the rate of growth of the *E. coli* cells harboring lysostaphin decreased, suggesting that the cells died while trying to express mature lysostaphin due to instability or potential toxicity of the protein. It was therefore decided to attempt expression with a different *E. coli* strain called BL21 (DE3). Similar to *E. coli* B834 [DE3], BL21 (DE3) cells can be also used with protein expression vectors that are under the control of the T7 promoter such as pET21a that lysostaphin is cloned into. BL21(DE3) are also lysogenic for lambda-DE3 and contain the T7 bacteriophage gene 1 that codes for T7 RNA polymerase under the control of the lacUV5 promoter. Nevertheless, BL21(DE3)pLysS also carries the pLysS plasmid, which contains the gene that codes for T7 lysozyme (Dunn and Studier, 1983). T7 lysozyme lowers the background expression level of target genes under the control of the T7 promoter and still allows considerable

expression following induction with IPTG, thus providing a more stable expression system that can be easily tolerated by *E. coli*. In this way, expression of mature lysostaphin was carried out successfully. The mature lysostaphin polypeptide containing an N-terminal methionine and a C-terminal polyhistidine tag was purified from the IPTG culture using metal chelate chromatography. Fractions 10 (fig. 3.12A) and 12-17 (fig. 3.12B) were pooled together and dialysed overnight.

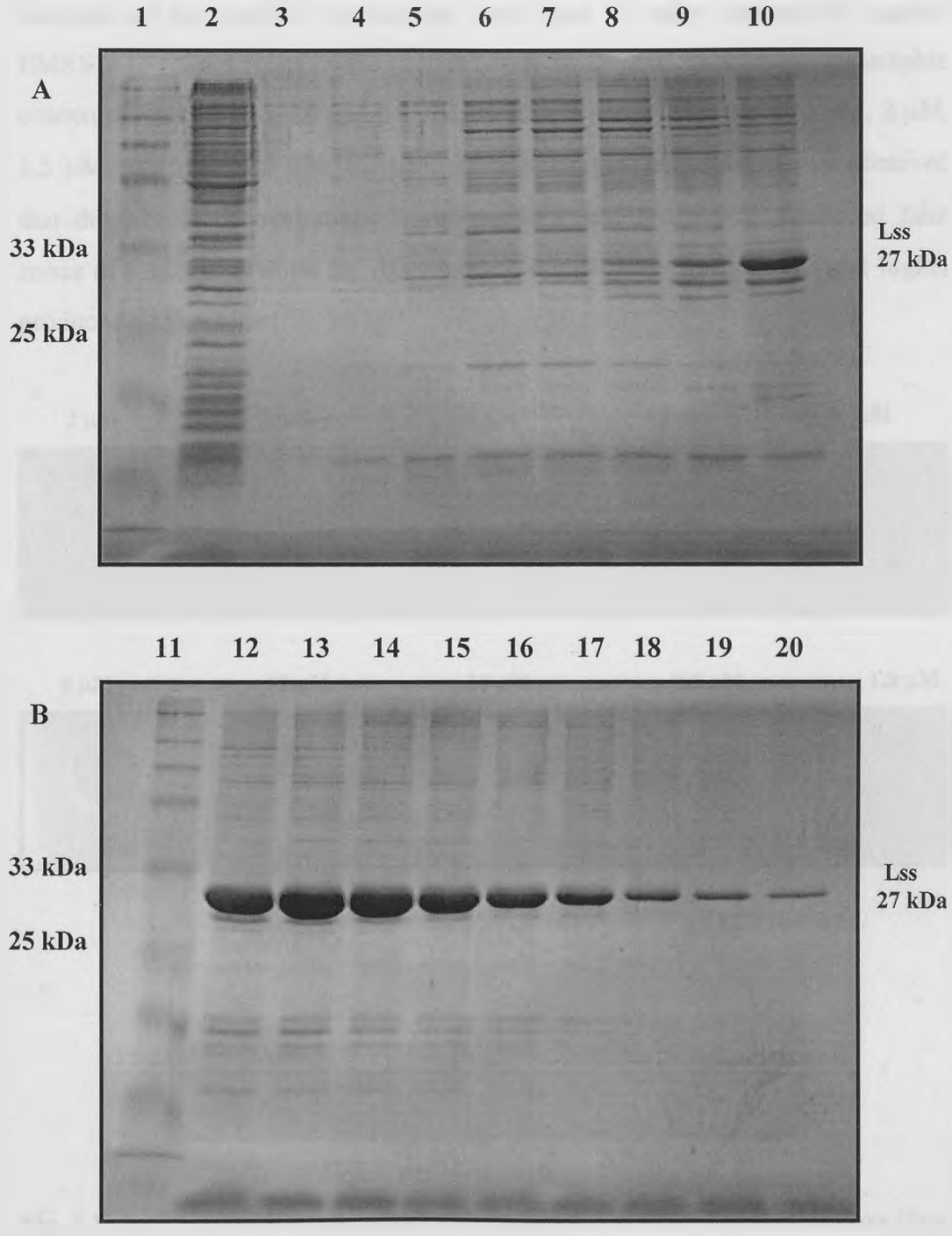

Fig. 3.12A, B 16% acrylamide SDS-PAGE gel indicating lysostaphin fractions released from the nickel column in lanes: 3-10 and 12-20. The samples range from very concentrated (~10 mg/ml) to less concentrated ones. Protein markers (83 kDa, 62 kDa, 48 kDa, 33 kDa, 25 kDa, 17 kDa and 7 kDa) are indicated in lanes: 1 and 11. A flowthrough fraction containing material unbound to the column is indicated in lane: 2. Fractions 10 and 12-17 were pooled together and dialysed overnight.

Samples of the purified lysostaphin were used to assay its activity against EMRSA-16. Spot tests were carried out with the following lysostaphin concentrations: 32 μM, 24 μM, 16 μM, 12 μM, 8 μM, 6 μM, 4 μM, 3 μM, 2 μM, 1.5 μM, 1.0 μM, 0.75 μM, 0.5 μM. After overnight incubation it was observed that drops with concentrations lower than 1.5 μM lysostaphin produced faint zones of inhibition, while the drops containing 3 μM of lysostaphin and higher produced a clear zone (fig. 3.13).

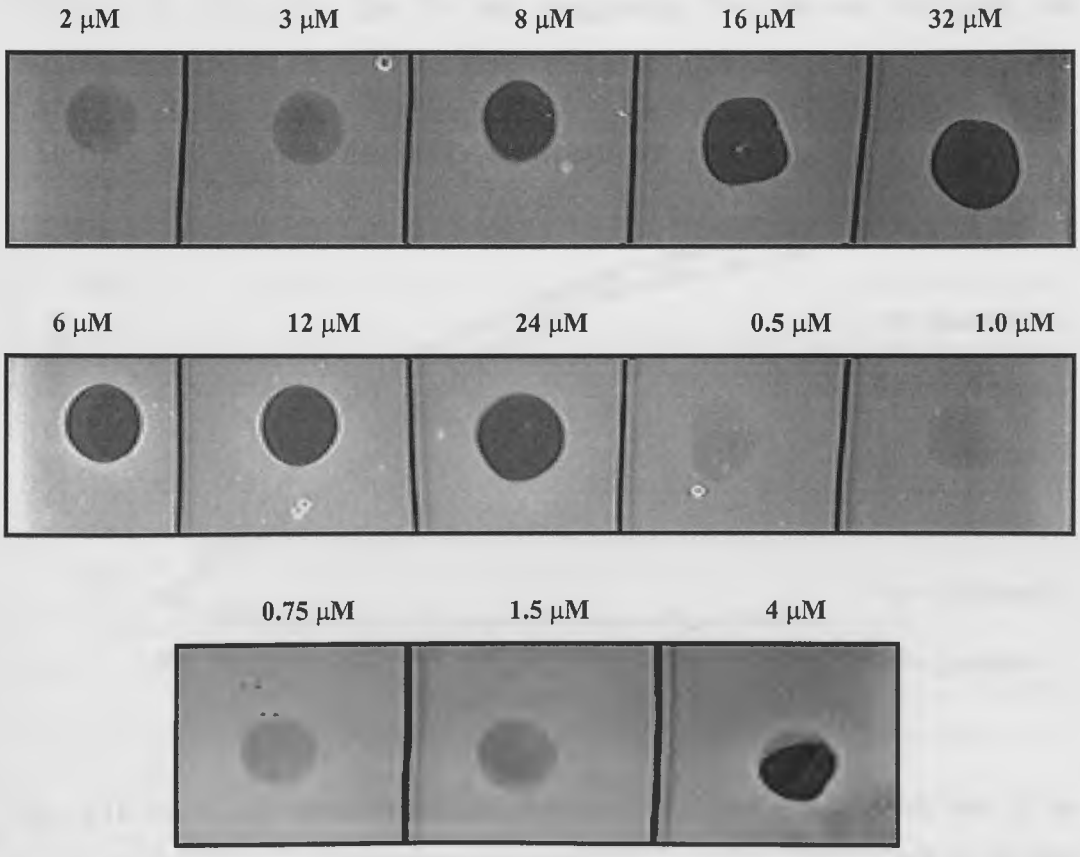

Fig. 3.13 Spot test that was carried out with lysostaphin at different concentrations (from 0.5 μM to 32 μM) to determine the minimum concentration required to kill EMRSA-16. Clear zones of inhibition may be observed at concentrations of approximately 3 μM or higher.

In order to obtain a more accurate value for the minimum concentration of lysostaphin required to kill EMRSA-16, the purified protein was assayed with a

broth dilution assay. This assay is more sensitive and effective than the spot test because the test protein comes into direct contact with the microorganism. Therefore, it was assumed that lysostaphin may be in fact effective against EMRSA-16 even at concentrations lower than 0.75μM, at which faint zones of inhibition were observed in the spot test. Thus, it was decided to use the following concentrations of purified protein against EMRSA-16: 60 nM, 50 nM, 40 nM, 30 nM, 20 nM, 10 nM and 0 nM (represents the control). According to the results (fig. 3.14) concentrations of 40 nM, 50 nM and 60 nM inhibited the growth of EMRSA-16 within the first 90 min, suggesting that the cut off point for lysostaphin activity is at 40 nM at these cell concentrations.

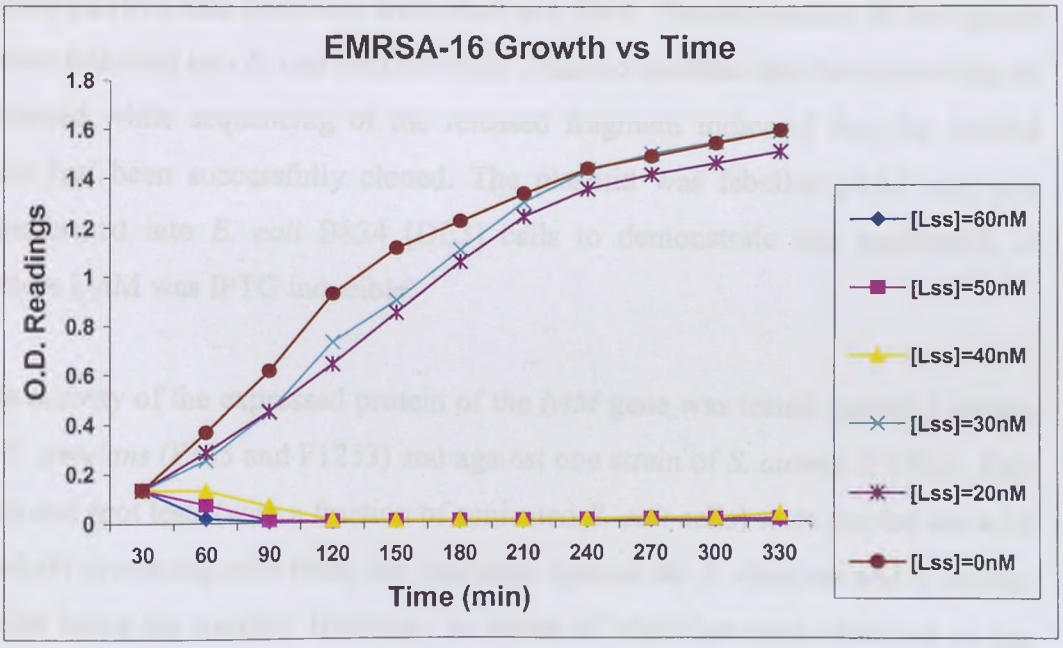

Fig. 3.14 Graph indicating EMRSA-16 growth over a time period of 300 min in the presence of different concentrations of lysostaphin, ranging from 0 nM to 60 nM. Samples from each broth containing EMRSA-16 and a certain concentration of lysostaphin were taken every 30 min and their OD_{600} readings (Table: 3.1) were recorded and used to produce the plot.

The fact that at 40nM lysostaphin can completely inhibit MRSA-16 growth, whereas at concentrations of 30nM or less EMRSA-16 growth does not seem to be affected implies that there may be a certain number of key receptors on *S. aureus* that need to be occupied for the cell population to survive.

3.2.2 Cloning, expression and assay of *S. aureus* LytM

Plasmid pRSETa, carrying the *lytM* gene (Ramadurai and Jayaswal, 1997) was used as a template in a PCR reaction using reverse primer RJ78 (G CTC GAG TTA TCT ACT TTG CAA GTA TGA) and forward primer RJ81 (CCC ATG GCA GAA ACG ACA AAC ACC). Primer RJ78 was used to introduce an *Xho*I restriction site after the stop codon of *lytM* while RJ81 was used to introduce an *Nco*I site after the signal sequence of the *lytM* gene. The successful PCR reaction produced a fragment 879 bp long, which was gel purified and then restricted with *Nco*I and *Xho*I. The restricted *lytM* fragment was then ligated into expression vector pET21d also restricted with *Nco*I and *Xho*I. Transformation of the ligated vector followed into *E. coli* ER2566 cells. Plasmid isolation and restriction digests followed while sequencing of the released fragment indicated that the desired gene had been successfully cloned. The plasmid was labelled pEA1 and was transformed into *E. coli* B834 [DE3] cells to demonstrate that expression of mature LytM was IPTG inducible.

The activity of the expressed protein of the *lytM* gene was tested against 2 strains of *S. simulans* (F335 and F1253) and against one strain of *S. aureus* (F1563). Stab tests and spot tests (with a fraction of sonicated *E. coli* cells) were carried out with the *lytM* producing cells being the inoculum against the *S. simulans* and *S. aureus* strains being the overlay. However, no zones of inhibition were observed on the plates, indicating that the protein was inactive against these targets. This most likely suggests that the LytM protein cannot target itself to the receptor on the bacterial cell wall as it does not have a defined targeting domain. Another possibility may be that the protein was inactive due to instability or a problem with expression, however these suggestions do not seem very likely because expression of the mature protein had been shown to be IPTG inducible.

3.2.3 Cloning, expression, purification and assay of *E .coli* LytM

The *lytM* gene was isolated from a genomic prep of *E. coli*. The genomic DNA was PCR amplified using reverse primer RJ83 (G CTC GAG TTA ATC AAA CCG TAG CTG) and forward primer RJ82 (G CAT ATG TTG GGG TCG CTC ACC). Primer RJ83 was used to introduce an *Xho*I site after the stop codon of *lytM* while RJ82 was used to introduce an *Nde*I site at the start codon of *lytM*. The resulting PCR product released a 1200 bp fragment, which was gel purified and then restricted with *Nde*I only, because after examination of the *lytM* sequence it was observed that an additional *Xho*I site occurs in the gene. The fragment was therefore cloned "blunt-ended" and then ligated into expression vector pET21a. The vector had been previously restricted with *XhoI* then treated with dNTPs and T4 DNA polymerase to fill the *Xho*I end of the plasmid and finally restricted with *Nde*I (fig. 3.15). Sequencing was also carried out to confirm that the desired gene had been successfully cloned.

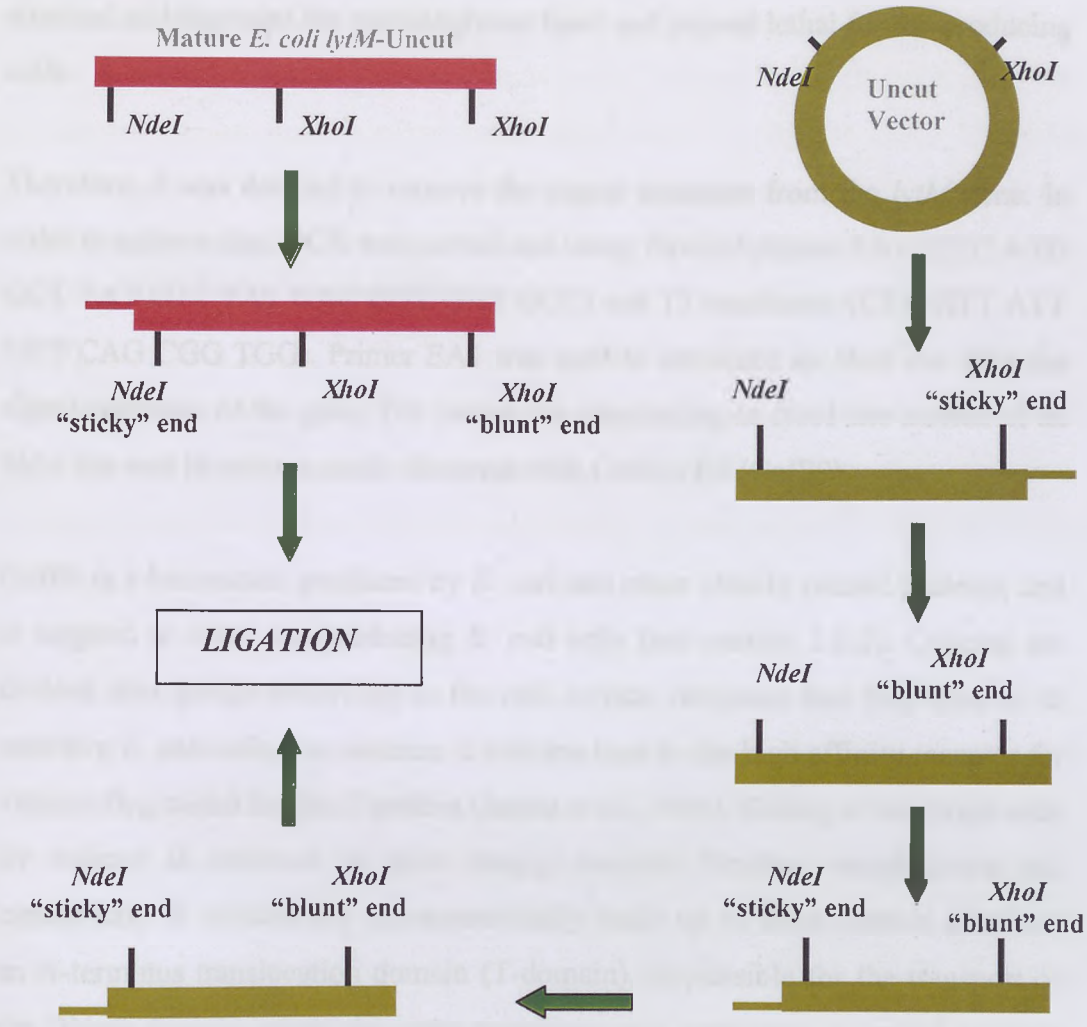

Fig. 3.15 Diagram indicating blunt-ended cloning of *E. coli lytM*. The gene was cut with *NdeI* only. The vector was initially cut with *XhoI* while T4 DNA polymerase was used to add dNTPs to fill the "sticky" end produced. The vector was finally cut with *NdeI* to achieve ligation with the *lytM* gene.

The plasmid was labelled pEA2 and was transformed into *E. coli* B834 [DE3] cells to demonstrate that expression of the *lytM* gene was IPTG inducible. Expression of *lytM* was attempted, however the cell density decreased over time indicating that overexpression of the *E. coli lytM* gene is toxic to the host cells. It was then assumed that the reason the expressing cells did not grow was the same as with the *E. coli* cells when Ramadurai and Jayaswal (1997) failed to express the *S. aureus* LytM protein. Therefore, it was postulated that the presence of a

signal sequence in the *E. coli* lytM targeted the protein to the periplasm, where it attached and degraded the peptidoglycan layer and proved lethal for the producing cells.

Therefore, it was decided to remove the signal sequence from the *lytM* gene. In order to achieve that, PCR was carried out using forward primer EA1 (CCC ATG GCT TAT GTT TAT CAC CGT GAT GCC) and T7 terminator (CTA GTT ATT GCT CAG CGG TGG). Primer EA1 was used to introduce an *Nco*I site after the signal sequence of the gene. The reason for introducing an *Nco*I site instead of an *NdeI* site was in order to make chimeras with Colicin E9 (ColE9).

ColE9 is a bacteriocin produced by *E. coli* and other closely related bacteria, and is targeted at other non-producing *E. coli* cells (see section 1.6.2). Colicins are divided into groups according to the cell surface receptors that they bind to in sensitive *E. coli* cells, for instance, E colicins bind to the high affinity receptor for vitamin B_{12}, called the BtuB protein (James *et al.*, 1996). Killing of the target cells by colicins is achieved in three stages: receptor binding, translocation and cytotoxicity. E colicins are characteristically made up of three distinct domains, an N-terminus translocation domain (T-domain), responsible for the transport of the DNase domain across the outer membrane and periplasm, a central receptor binding domain (R-domain), which is responsible for BtuB receptor-binding activity and a C-terminal DNase domain that codes for the protein's cytotoxic activity (fig. 3.16). Their mode of action is either based upon formation of ion channels that result in depolarization of the cytoplasmic membrane as is the case with colicin E1, or as endonucleases that degrade DNA through a 32 aa structure known as H-N-H motif (for example colicins E2, E7, E8 and E9) or a single phosphodiester bond in 16S ribosomal RNA hence inhibiting translation (such as colicins E3, E4, E5 and E6) (James *et al.*, 2002). Protection from colicins by the producing organism is achieved by the synthesis of immunity (Im) proteins (one of the fastest folding protein in nature, (Kleanthous *et al.*, 1999)) that bind to the DNase domain and therefore prevents its activity. Once bound to the BtuB receptors, group A colicins (such as E colicins and cloacin DF13) must be translocated across the outer membrane and periplasmic space while in the case of

group B colicins (such as colicin B) whose targets are located in the cytoplasm the inner membrane must be also penetrated. However, the molecular mechanism by which a colicin nuclease domain is transported across the outer membrane, the periplasmic space and the cytoplasmic membrane, in order to reach its DNA target in the cytoplasm, is currently unknown (James *et al.*, 2002). It has been reported that certain periplasmic components of the translocation system are responsible for maintaining cell envelope structure and integrity (Bernadac *et al.*, 1998). Therefore, a detailed understanding of this system could be very useful in the design of novel antibiotics for Gram negative pathogens. Furthermore, the characteristic structure of colicins, consisting of individually functional domains make colicins ideal for domain swapping studies and the construction of chimeric proteins.

Colicin E9 is encoded by a plasmid called pCS4, which has an *Nco*I and an *Xho*I site (fig. 3.16). After successfully introducing the *Nco*I restriction site in *lytM* (pEA5) it was decided to make a chimera between ColE9 and *lytM*, by replacing the DNase and Immunity Protein domains of ColE9 with the *lytM* gene, assuming that the ColE9 T+R domains present would be able to direct the LytM protein to the target *E. coli* cell wall and the killing activity of LytM would kill the cells. Thus, the successful PCR product was gel purified and restricted with *Nco*I and *Xho*I. The restricted mature *lytM* fragment was then ligated into expression vector pET21d also restricted with *Nco*I and *Xho*I. The plasmid was labelled pEA6 and was transformed into *E. coli* B834 [DE3] cells to demonstrate that expression of mature LytM was IPTG inducible and not lethal. The protein was expressed and successful purification of the chimeric protein followed using metal chelate chromatography, producing a ~45,000 Da band on the protein gel.

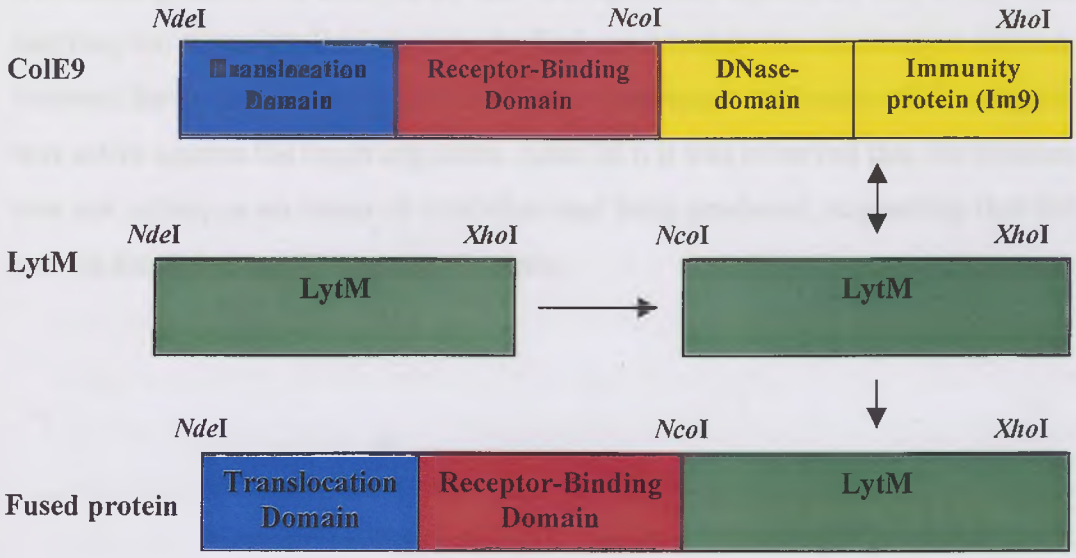

Fig. 3.16 Schematic diagram indicating the construction of the ColE9-LytM fused protein, where the DNase and Immunity protein domains of ColE9 is replaced by LytM. In pCS4, *Nco*I and *Xho*I restriction sites are positioned at the start and end of the DNase domain of ColE9 respectively. Therefore, by replacing the *Nde*I site with *Nco*I at the start of LytM, it was possible to clone LytM into pCS4, at the desired position.

In order to be able to study the *lytM* gene it was then considered essential to remove the internally located *Xho*I site. Site Directed Mutagenesis was thus carried out with forward primer DW50 (G GTG TTA ATG TCT CGG GAA ATG CTT GAT GG) and T7 terminator (CTA GTT ATT GCT CAG CGG TGG). DW50 was specifically designed to remove the internal *Xho*I site from the *E. coli lytM* gene. The first stage of the PCR reaction produced the megaprimer fragment, which was ~750 bp long. In the second stage PCR the product of the first reaction was used as the forward primer and T7 terminator was used as the reverse primer. The reaction produced 2 bands one of which corresponded to the desired fragment. The fragment was gel purified and restricted with *Nde*I and *Xho*I. The restricted mature *lytM* gene was then ligated into expression vector pET21a also restricted with *Nde*I and *Xho*I. Transformation of the ligated vector followed into *E. coli* JM83 cells. Plasmid isolation indicated that the desired fragment had been successfully cloned. The plasmid was labelled pEA4.

The fused protein was assayed by stab and spot tests against *E. coli* JM83 cells carrying the plasmid pEA6 in order to find out whether the constructed chimera between the T+R domains of ColE9 and the endopeptidase domain of *E. coli lytM* was active against the target organism. After 24 h it was observed that the chimera was not active, as no zones of inhibition had been produced, suggesting that the protein had failed to kill the target bacteria.

3.3 DISCUSSION

3.3.1 Lysostaphin – Cloning, efficient purification and assay results

During their course of life all types of microorganisms produce antibiotic substances that cause the selective destruction of their competitors. Even though a large amount of information is known for some of these molecules, important details concerning their mode of action are still missing. Lysostaphin is one of these molecules with great therapeutic potential and various laboratory applications. It is therefore very important to study the protein in much more detail so as to understand its structure and how this relates to the function of the polypeptide. In order to achieve this it was essential to initially clone and express the mature lysostaphin gene. Subsequent purification of the protein would then allow assay of the mature enzyme to determine its minimum inhibitory concentration required to kill antibiotic-resistant strains of *S. aureus*.

The lysostaphin endopeptidase gene has been located on the largest of the five plasmids in *S. simulans* biovar *staphylolyticus*, called pACK1 (Heath *et al.*, 1987). Cloning, sequence and expression of lysostaphin has been reported in the literature in both bacterial (Recsei *et al.*, 1987; Heinrich *et al.*, 1987, Chen, 1996) and eukaryotic expression systems (Williamson *et al.*, 1994). Both Recsei and collegues (1987) and Heinrich and collegues (1987) cloned the prepropeptide form of the gene and its flanking sequences. The cloned product corresponded to a 1.5 kb fragment, which when expressed produced the prepropeptide form of the enzyme with a molecular weight of 42,000 Da. In both cases, cloning and expression of preprolysostaphin was achieved through a very involved process with respect to both time and materials requirements. Here, genomic DNA was isolated from *S. simulans* biovar *staphylolyticus* and used as the template in a PCR reaction in order to amplify the desired mature lsysostaphin fragment. Cloning and expression of mature lysostaphin followed using pET vectors.

pET vectors also constitute a very efficient approach for the production of recombinant proteins in *E. coli* because they are based on the T7 promoter-driven

system (Studier and Moffat, 1986). Furthermore, pET vectors have the distinctive advantage of introducing the His-tag sequence, which makes purification of proteins a very straightforward process. The His-tag is a stretch of 6 consecutive histidine residues that can be expressed at either the N-terminal end or the C-terminal end of the target protein. Here, the mature lysostaphin was cloned in pET21a so that the His-tag is attached to its C-terminal end. The *E. coli* cells expressing lysostaphin were lysed by sonication and the protein was purified using metal chelate chromatography. This method is very rapid and convenient because the his-tagged protein binds to the nickel particles in a matter of minutes while the unbound molecules may be subsequently washed away and the target protein is eventually recovered by elution with imidazole.

Utilisation of overproducing pET vectors has been reported in the past for cloning and purification of lysostaphin (Chen, 1996). The purification process described was very similar to the one used in this study. Thus, purification was based on a single-step affinity method while expression of the protein was attempted through a pET vector system. Cloning, expression and purification of mature lysostaphin has been also reported by Szweda and colleagues (Szweda *et al.*, 2001). In this case, purification of the protein was also carried out in a single chromatography step using however, the Intein-Chitin-Binding Domain (CBD). This is another quick method for protein purification that produced in excess of 6 mg of lysostaphin from 1 litre of induced culture that was >98% pure. However, in this study, purification of lysostaphin yielded a significantly larger amount of product that is ~ 120 mg from only 500 ml of induced culture and with only insignificant background present. By comparison of the SDS-PAGE gels indicated in fig. 3.12A, B with that obtained by Szweda *et al.* (2001) it is evident that the protein obtained from this study was 90%-95% pure.

In order to obtain the minimum inhibitory concentration (MIC) required to kill EMRSA-16, an agar diffusion assay was carried out using the purified protein against EMRSA-16 at a wide range of concentrations, varying from 32 μM to 0.5 μM (fig. 3.13). Because of the nature of the assay, it would be impossible to get a very accurate and reliable value for the desired MIC, however, it would provide

us with an approximate value that could be then used as a reference in order to subsequently test mature lysostaphin at a narrower range of concentrations with a liquid broth assay. This could be due to the fact that in the agar diffusion test, the size of the microbial inhibition zone depends upon the solubility and diffusibility of the test substance and, therefore, may not express its full effective potential. However, the liquid broth assay is correlated to substance effectiveness and its direct contact with the microorganism (Estrela *et al.*, 2003). The results of the liquid broth assay indicated that only at concentrations of 3 μM or higher was a clear zone of inhibition produced. At concentrations lower than 3 μM faint zones were observed at the protein spots still indicating signs of activity that can inhibit growth of EMRSA-16. Thus, it was decided to use the purified lysostaphin at concentrations between 0 and 60 nM in a liquid broth dilution assay in order to get a more precise MIC. The results illustrated in fig. 3.14, showed that the minimum concentration required to kill EMRSA-16 corresponds to a concentration of 40 nM / 0.04 μM.

3.3.2 Lysostaphin homologues – Cloning, expression and construction of chimeras

Autolysins have been defined as bacteriolytic enzymes that hydrolyse the cell-wall peptidoglycan of the organisms that produce them (Smith *et al.*, 2000). The possibility that peptidoglycan hydrolases are involved in the selective removal of peptidoglycan has led to proposals that they are involved in several cellular processes including cell wall growth, cell separation, peptidoglycan maturation, cell wall turnover, cell division, lysis caused by cell wall-acting antibiotics, competence for genetic transformation, flagellum formation, sporulation and bacterial pathogenicity (Ramadurai and Jayaswal, 1997). Autolysins of *S. aureus* have not been studied extensively and as a result their role in the processes mentioned above has not yet been established. It is known that *S. aureus* has three different autolytic enzymes, an *N*-acetylglucosaminidase, an *N*-acetylmuramidase, and an endopeptidase (Giesbrecht *et al.*, 1998). Only the role for the *S. aureus* ATL autolysin has been investigated and found to encode glucosaminidase and

amidase domains and be involved in cell separation after cell division (Sakuo *et al.*, 1996).

The *lytM* gene constitutes one of the recently studied *S. aureus* peptidoglycan hydrolases and due to its high homology to lysostaphin, it may constitute a promising agent for the development of antibiotics. Cloning of the gene and expression of the protein have been attempted in the past by Ramadurai and Jayaswal (1997). The gene was cloned into the expression vector pRSETa and transformed into *E. coli* BLR(DE3)pLysS cells. However, expression failed to occur as the gene appeared to be toxic to the *E. coli* cells. It was observed that the growth rate of the host cells was significantly lower than that of the control cells (carrying the vector alone). In this study, the *lytM* gene was cloned into vector pET21d and expression was carried out successfully in *E. coli* B834 [DE3] cells. The reason for this is because the *lytM* gene was cloned without the signal sequence. Since the signal sequence is responsible for exporting the LytM protein across the cytoplasmic membrane of *S. aureus*, the LytM expressed in *E. coli* could not be exported to the periplasmic space where it could not attack the peptidoglycan of the bacterial cell envelope and thus kill the cell.

The protein expressed by the cloned *lytM* gene was tested for activity against 2 strains of *S. simulans* (F335 and F1253) and against a *S. aureus* strain (F1563). However, no zones of inhibition were observed in the stab and spot tests, possibly because the protein could not direct itself against the targeted microorganisms. This suggested that the LytM protein may require a targeting domain to provide target specificity for *S. aureus* or *S. simulans* in order to be able to kill these microorganisms. *LytM* codes for a protein that harbours an N-terminal signal peptide followed by an uncharacterised domain. The C-terminal domain of the protein displays striking homology to the endopeptidase domain of lysostaphin and is assumed to also function as a glycyl-glycine endopeptidase (Ramadurai and Jayaswal, 1997). Based on this information, the problem encountered above could be solved by the construction of a chimera. More precisely, fusion of the targeting domain of lysostaphin and the *lytM* gene (in place of the lysostaphin endopeptidase domain) could direct *lytM* to *S. aureus* as a result of the lysostaphin

targeting domain and act against *S. aureus* as a result of the potential bactericidal killing activity of the *lytM* gene.

LytM from *E. coli* is another gene that exhibits significant homology to lysostaphin and is assumed to be an autolysin. Like the LytM protein expressed from *S. aureus*, LytM from *E. coli* lacks a C-terminal targeting domain and has a signal peptide located at the N-terminal end of the protein. There has not been reported any experimental work about this gene in the literature to date. As a result, there is not any information available concerning its mode of action. However, due to the fact that its structure and distribution of domains is very similar to that of *lytM* from *S. aureus*, and because of its homology to lysostaphin it could be assumed that its mode of action is similar to that of LytM from *S. aureus* and lysostaphin from *S. simulans* but its target specificity is restricted to *E. coli* cells. Based on these conclusions we decided to construct a chimera between *E. coli lytM* and a bacteriocin called ColE9 (fig. 3.16), also targeted at and produced by *E. coli* cells. This chimera was constructed in order to find out if the T+R domains of ColE9 could deliver LytM from the outside of the cell through the periplasm and to the peptidoglycan for cell wall cleavage. Colicin E9 causes cell death by binding to specific outer membrane receptor proteins, translocating through the cell envelope and cleaving the DNA.

Chimeras have been previously constructed with ColE9. Penfold and coworkers (2000) successfully fused the DNAase domain of ColE9 with the translocation and receptor binding domains of another bacteriocin from Enterococcus spp, called cloacin DF13. Another chimera was also successfully constructed between the R and DNAase domains of ColE9 and the T-domain of cloacin DF13. Both of the resulting fusion proteins were fully functional, and had retained both their activity and specificity against the complementary indicator strains. Here, it was decided to construct a chimera between the T+R domains of ColE9 and the endopeptidase domain of *E. coli* LytM to see whether the T+R domains of ColE9 could deliver LytM from the outside of the cell through the periplasm and to the peptidoglycan for cell wall cleavage. The stab and spot tests that were carried out to test the efficiency of the chimera against *E. coli* JM83 indicated no zones of

inhibition. One reason for this could be that the chimera did not bind to the cell receptors or was not translocated across the outer membrane and therefore failed to reach its target. Alternatively, if the protein did manage to reach the target membrane it could be that after fusion with the translocation and binding domain the *E. coli* LytM endopeptidase domain was not in the correct conformation for cell wall hydrolysis.

These possibilities could be investigated by carrying out a protection and a FRET (Fluorescence Resonance Energy Transfer) assay (see chapter: 6). The former test, carried out with wild type colicin, would primarily reveal if the chimera can bind to the cells. If the *E. coli* cells survived the wild type colicin, this would then mean that the fusion protein managed to bind to the cell receptors, preventing wild type colicin from binding and killing the cells. On the other hand if the *E. coli* cells did not survive the wild type colicin, this would suggest that the chimera was unable to bind to the cell receptors. Any loss or reduction of activity may also be investigated by performing a real-time assay. The FRET assay would indicate if there is any activity, by measuring fluorescence production of the chimera against an appropriate substrate, bound to a suitable donor and quencher, which would fluoresce in the presence of substrate hydrolysis.

Such a substrate could be the stem peptide (L-ala – D-glu – DAP – D-ala – D-ala) attached to the NAM-NAG of the *E. coli* peptidoglycan backbone. Rachel Warfield (Schoool of Chemistry, University of Nottingham) has been working on a similar peptide (L-ala – D-glu – L-lys – D-ala – D-ala). The peptide sequence has been attached to a donor and an acceptor, EDANS (4-((4-(dimethylamino)phenyl)azo)benzoic acid) and DABCYL (5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid) respectively. The method of solid phase peptide synthesis was employed to assemble the peptide chain. The donor EDANS was covalently attached to an insoluble polystyrene resin, making it relatively simple and quick to wash away excess reagents and by-products. The C-terminal of the first aa (in this case L-ala) was then added to the N-terminal of EDANS using coupling reagents. The N-terminal of the aa as well as any functional side chain groups were protected with Fmoc protecting groups so that

during the coupling stage they could not react with the N-terminal of the aa at the end of the growing polypeptide chain. Upon addition of the next aa the Fmoc group was removed after treatment with a certain base called piperidine, to leave the N-terminal free for the next aa. The process was repeated until the donor group, DABCYL was added. Removal of the resin and the remaining protecting groups was carried out with trifluroacetic acid (that can cleave the bond between EDANS and the resin) thus leaving the desired polypeptide only attached to EDANS and DABCYL, which can then be purified by HPLC.

Comparison of the amount of fluorescence produced by the chimera to that produced by the wild type colicin against the same synthetic polypeptide substrate would provide information on the levels of activity of the fusion protein. In the case of no fluorescence production and provided that the chimera protected *E. coli* cells during the protection assay, the absence of any fluorescence would indicate that the protein can either not be translocated or after translocation the *E. coli* LytM endopeptidase domain could not function properly. Finally, another possibility would be the production of a small amount of fluorescence, which would suggest that the chimera can in fact be translocated but cannot act efficiently against the substrate, which would explain the absence of zones in the spot and stab tests.

Despite the increased research on the properties and potential of lysostaphin as an antibiotic there is still some critical information missing that is essential in order to fully comprehend its mode of action. For example, apart from Baba and Schneewind (1996) and Thumm and Gotz (1997) no other detailed group studies have been reported in the literature on the lysostaphin targeting domain. It was therefore considered essential to investigate further the activity and biochemistry of the C-terminal targeting domain in an attempt to understand the mechanism behind the target specificity of lysostaphin.

CHAPTER: 4

CLONING, EXPRESSION, PURIFICATION AND ASSAY OF THE C-TERMINAL TARGETING DOMAIN OF LYSOSTAPHIN

4.1 INTRODUCTION

Antimicrobial peptides isolated from Gram-positive microorganisms are generally narrow spectrum enzymes and therefore inhibit the growth of bacteria closely related to the producing species, such as lysostaphin from *S. simulans* and zoocin A from *Streptococcus zooepidemicus*. However, bacteriolytic agents with a broader spectrum of activity have also been identified. For example, enterolysin A has been reported to inhibit the growth of a wide range of Gram-positive bacteria including bacteria from more diverse genera than the producing organism. Nevertheless, in both narrow and wide spectrum agents, sequences of TP-rich linker regions have been identified that usually occur between an N-terminal catalytic domain and a C-terminal targeting module (Nilsen *et al.*, 2003). The separation of both catalytic and targeting domains suggests that these proteins may belong to a family of enzymes that consist of individual functional modules. This is a very important feature because it renders these enzymes ideal for domain-swapping studies for the production of antibiotics with improved catalytic activities and/or more broad range specificities (Schuch *et al.*, 2002). Lysostaphin is an enzyme with striking homology to other antibacterial proteins (such as internalin B, enterolysin A and zoocin A) (Simmonds, *et al.*, 1997, Nilsen *et al.*, 2003, Lai *et al.*, 2002) and an individually functional targeting module that can be used with catalytic domains from similar murein hydrolases such as *S. aureus* LytA for the production of chimeras.

4.1.1 InlB by *Listeria monocytogenes* has a domain architecture similar to lysostaphin

Listeria monocytogenes is a Gram-positive bacterium responsible for severe food-borne infections in humans and animals. The microorganism produces several surface proteins that play a critical role in the infectious process. Such proteins include internalins A (InlA) and B (InlB), both of which are necessary and sufficient for entry into various cell lines, each with its own specificity (Jonquieres *et al.*, 1999). InlA is required for promoting invasion of enterocytes while InlB is more important for infection of other tissues including hepatocytes,

endothelial and epithelial cell lines (Marino *et al.*, 2002). As opposed to InlB, the molecular basis of surface attachment of InlA has been well characterised (Braun *et al.*, 1997). It covalently binds to the peptidoglycan via a C-terminal signal consisting of an "LPXTG" motif, followed by a hydrophobic region and a few positively charged amino acids. The presence of similar C-terminal domains as well as the anchoring process that occurs after cleavage of the T-G bond (Navarre and Schneewind, 1994) have been also described for the staphylococcal surface proteins (see section 3.1.1). Similar to lysostaphin, both InlA and InlB are characterised by the presence of individual functional modules, including an N-terminal enzymatic region and a C-terminal region responsible for directing the protein to its appropriate receptors. Experiments based on a domain "swapping strategy" have been performed to reveal the mechanism behind anchoring of InlB (Braun *et al.*, 1997). The results suggested that the 232 amino acid C-terminal module was necessary and sufficient for surface recognition of the target cells, since mutant proteins lacking this region were found in the extracellular medium. Furthermore, InlB is characterised by the presence of three modules in its C-terminal domain, consisting of approximately 80 amino acids each, that start with the dipeptide GW and have thus been named "GW motifs" (see section 4.1.4) (Braun *et al.*, 1997). A database search performed to identify homologues of the C-terminal domains of InlB indicated significant similarities with the C-terminal domains of lysostaphin that contains one such "GW motif", and Ami, a newly identified surface protein of *L. monocytogenes*, which contains eight such "GW" modules (fig. 4.1). These results suggested that Listeria and Staphylococci may have evolved a common motif of about 80-90 amino acids long, beginning with the sequence GW. This motif is assumed to affect the targeting of proteins to the appropriate cell wall receptors (Braun *et al.*, 1997).

```
Ami   346 GWVDSKALNTF-YTPSME--KTITGTRYVLPSKQT--...PVE...PLSKFNGQALTLQREAT...LWY...L
Ami   423 GWVKAVNLTTTKYDL-IEYDK--AITAYSRV-KTAAG PNK...ALSTYSGKNMRIIREAK...IWY...I
Ami   507 GWVDTKALNTF-YTPSME--KNLTATRYVAPG-QET-...PVA...PLSKFAGQTLTVQREAT...LWY...L
Ami   584 GWTKASTLTATQYDK-LEYDK--AITAYSRV-KTATG...PYR...PLSSYAGKNLRIIREAK...IWY...I
Ami   667 GWVDSKALNTF-YTPSME--KTITGTRYVLPSKQT--...PVE...PLSKFNGQALTLQREAT...LWY...L
Ami   744 GWVKAANLTTTKYDT-LSYDK--AITAYSRV-KTATG...PNK...ALSTYSGKNMRILREAK...IWY...I
Ami   828 GWVETKALNTF-YTPSME--KNLTATRYVLTSK--KN...PVV...PLSKFSGKTLTVQREAT...LWY...L
Ami   905 GWTKAANLSAKKQ

InlB  399                          LTRYVKYIRGN--...PRE...TLASHRCKALTVDREAR...LWY...I
InlB  455 GWTKAENLSLDRYDK-MEYDK--GVTAYARVRNAS-G...PYN...KLSVYQGKNMRILREAK...TWY...I
InlB  538 GWVDTRALNTF-YKQSME--KPTRLTRYVSANKA--G...PVA...TLAKYKNQKLIVDCQAT...LWY...I
InlB  619 GWTKAANLRAQK

Lys   297 GW-KTNKYGT-LYK-S-ESAS---FTPNTDIITRTTG...PQS...QTIHYDEVMKQDGHVWV...NSG...T
Lys   374 -WNKSTNTLGVLWGTIK
```

Fig. 4.1 Amino acid sequences indicating the presence of "GW-motifs" in Ami, InlB and lysostaphin (Lys). Eight such motifs may be observed in Ami, three in InlB and one in the C-terminal targeting domain of lysostaphin (shown in blue, bold type). All motifs are approximately 80-90 amino acids long. The presence of homologous residues between Ami and InlB and Ami and lysostaphin are indicated in blue. The hyphens indicate gaps introduced to maximise homology.

4.1.2 The presence of a TP-rich region in Enterolysin A indicates similar domain structure to lysostaphin

Enterococcus faecalis is an important pathogen in community-acquired and hospital-acquired infections causing bacteremia, infective endocarditis and urinary tract infections (Moellering *et al.*, 1992). An antimicrobial protein has been identified that is produced by strain *E. faecium* LRG 2333, designated enterolysin A and is considered a very important novel antimicrobial agent. In contrast to lysostaphin, enterolysin A is active against a wide range of Gram-positive species including enterococci, pediococci, lactococci, and lactobacilli, but interestingly not staphylococci nor any other of the Gram-negative bacteria tested (Hickey *et al.*, 2003). It is a 327 amino acid protein that consists of two distinct domains, an N-terminal enzymatic domain and a C-terminal region coding for substrate

specificity, separated by a short threonine-proline (TP)-rich putative linker sequence (fig. 4.2). This sequence is also present between the N-terminal catalytic domain and the C-terminal targeting domain of other antimicrobial peptides such as lysostaphin and zoocin A. Homology searches (fig. 4.3) have indicated that the N-terminal catalytic domain of enterolysin A exhibits significant homology with lysostaphin, LytM and Ale-1 (a glycylglycine endopeptidase produced by *Staphylococcus capitis* EPK1 with significant homology to lysostaphin) (Nilsen *et al.*, 2003). There are not any common features in the interpeptide bridges of the peptidoglycan chains of the bacteria that are sensitive to enterolysin A. Nevertheless, the following stem peptide sequence has been identified in their peptidoglycan: L-Ala-D-Glu-L-Lys-D-Ala, which is also present in microorganisms that are inhibited by Millericin B, a murein hydrolase from *Streptococcus milleri* NMSCC 061 (Beukes *et al.*, 2000). This indicates that it is possible for enterolysin A to exert its activity in a similar way as millericin B provided that the protein acts on the stem peptide (Nilsen *et al.*, 2003).

```
1    MKNILLSILG VLSIVVSLAF SSYSVNAASN EWSWPLGKRY AGRYEEGQQF    50
51   GNTAFNRGGT YFHDGFDFGS AIYGNGSVYA VHDGKILYAG WDPVGGSLGA   100
101  FIVLQAGNTN VIYQEFSRNV GDIKVSTGQT VKKGQLIGKF TSSHLHLGMT   150
151  KKEWRSAHSS WNKDDGTWFN PIPILQGGST PTPPNPGPKN FTTNVRYGLR   200
201  VLGGSWLPEV TNFNTNDGFA GYPNRQHDML YIKVDKGQMK YRVHTAQSGW   250
251  LPWVSKGDKS DTVNGAAGMP GQAIDGVQLN YITPKGEKLS QAYYRSQTTK   300
301  RSGWLKVSAD NGSIPGLDSY AGIFGEPLDR LQIGISQSNP F           341
```

Fig. 4.2 Indication of the aa sequence of Enterolysin A. The signal sequence is highlighted in blue, the N-terminal catalytic and C-terminal targeting domains are shown in red and green respectively. Finally, the TP-rich linker sequence (residues 180-193) is indicated in brown.

```
EnlA   45 EGQQFGNTAFNRGGTYFHDGFDFGSAIYGNGSVYAVHDGKILYAG---
ZooA   32 TGNITTGFNGYPG------HVGVDYAVPVGTPVRAVANGTVKFAGNGA
Lys    13 NYKKGYGYGPYPLGINGGIHYGVDFFMNIGTPVKAISSGKIVEAG---
LytM  200 SRKQLQPYGQYHG---GGAHYGVDYAMPENSPVYSLTDGTVVQAG---
ALE1  132 NYKKGYGYGPYPLGINGGNHYGVDFFMNVGTPVRAISDGKIVEAG---

EnlA   90 ----WDPVGGGSLGAFIVLQAGNTNVIYQEFSRNVGDIKVSTGQTVKK
ZooA   75 NHPWVLWVAGNCVLIQHADG-MHTGYAHLSKISVSTDSTVKQGQIIGY
Lys    58 ---WSNYGGGNQIGLIENDGVHRQWYMHLSKYNVKVGDYVKAGQIIGW
LytM  246 ---WSNYGGGNQVTIKEANSNNYQWYMHNNRLTVSAGDKVKAGDQIAY
ALE1  177 ---WTNYGGGNEIGLVENDGVHRQWYMHLSKFNVKVGDRVKAGQIIGW

EnlA  134 GQLIGKFTSSHLHLGMTKKEWRSAHSSWNKDDGTWFNPIPILQGGSTP
ZooA  123 TGATGQVTGPHLHFEMLPANPNWQNGFSGRIDPTGYIANAPVFNGTTP
Lys   103 SGSTGYSTAPHLHFQRMVN----SFSNSTAQDPMPFLKSAGYGKAGG-
LytM  291 SGSTGNSTAPHVHFQRMSG----GIGNQYAVDPTSYLQSR--------
ALE1  222 SGSTGYSTAPHLHFQRMTN----SFSNNTAQDPMPFLKSAGYGSNS--

EnlA  182 TPPNPGPKNFTTNVRYGLPVLGGSWLPEVTNFNNTNDGFAGYPNRQHD
ZooA  171 TEPTTPTTNLKIYKVDDLQKINGIWQVRNNILVPTDFTWV--------
Lys   150 TVTPTPNTGWKTNKYGTLYKSESASFTPN--------------------
LytM      ----------------------------------------------
ALE1  264 -TSSSNNNGYKTNKYGTLYKSESASFTAN--------------------

EnlA  230 LYIKVDKGQMKYRVHTAQSGWLPWVSKGDKSDTVNGAAGMPGQAIDGV
ZooA  211 -----------DNGIAADDVIEVTSNGTRTSDQVLQKGGYFVINPNNV
Lys   179 ----------------TDIITRTTGPFRSMPQSGVLKAGQTIHYDEVM
LytM      ----------------------------------------------
ALE1  292 ----------------TDIITRTTGPFRSMPQSGVLRKGLTIKYDEVM

EnlA  278 QLNYITPKGEKLSQAYYRSQTTKRSGWLKVSADNGSIPGLDSYAGIFG
ZooA  248 KSVGTPMKGSGGLSWAQVNFTTGGNVWLNTTSKDNLLYGK--------
Lys   211 KQDGHVWVGYNTNSGQRIYLPVRTWNKSTNTLGVLWGTIK--------
LytM      ----------------------------------------------
ALE1  323 KQDGHVWVGYNTNSGKRVYLPVRTWNESTGELGPLWGTIK--------
```

Fig. 4.3 Sequence alignment of the N-terminal region of enterolysin A (EnlA) with zoocinA (ZooA), mature lysostaphin (Lys), LytM and ALE-1, indicating the presence of several conserved residues, which are highlighted in black. The hyphens indicate gaps introduced to maximise homology.

4.1.3 Zoocin A is a lysostaphin-like bacteriolytic agent

The Lancefield group C streptococci that includes *Streptococcus zooepidemicus*, *S. dysgalactiae*, *S. equi* and *S. equisimilis* constitute commensal and occasional pathogens for a wide variety of animal hosts including humans. This group of streptococcal species is known to produce bacteriocin-type substances that can

inhibit the growth of a range of microorganisms. In particular, *S. zooepidemicus* 4881 is of special interest because of its ability to produce a high molecular mass, heat-labile bacteriolytic agent against all streptococcal species including all *S. pyogenes*, *S. mutans* and all *S. zooepidemicus* strains apart from the producing 4881 strain, but not *S. rattus*. This protein is called zoocin A and has been characterised as a lysostaphin-like agent because the two proteins share a great deal of similarities (Simmonds *et al.*, 1996). Analysis of the gene sequence of *zoo A* has revealed that the respective protein exhibits a typical "mix and match" of domain-type structures that is found in many Gram-positive bacteria including lysostaphin and many other murein hydrolases, such as LytA and enterolysin A (Simmonds *et al.*, 1997). Furthermore, Simmonds and co-workers (1997) confirmed the presence of a domain structure similar to that of lysostaphin, including a leader sequence that comprises the signal peptide, an N-terminal region, a short TP-rich linker sequence and a C-terminal domain. Moreover, the same authors have reported that the N-terminal region exhibits striking homology to the catalytic domain of lysostaphin and codes for enzymatic activity (fig. 4.4).

```
ZooA 20 PGHVGVDYAVPVGTPVRAVANFTVKFAGNGANHPWMLWMAGNCVLIQH
Lys  30 GMHYGVDFFMNIGTPVKAISSGKIVEAGWSNYGG-----GNQIGLIEN

ZooA 69 ADGMHTGYAHLSKISVSTDSTVKQGQIIGYTGATGQVTGPHLHFEMLP
Lys  75 DGVHRQWYMHLSKYNVKVGDYVKAGQIIGWSGSTGYSTAPHLHFQRMV
```

Fig. 4.4 Sequence alignement indicating significant homology between the zoocin A (ZooA) and lysostaphin (Lys) N-terminal catalytic domains from positions 20-116 and 30-126 respectively. The identical residues between the two protein sequences are highlighted in red. The hyphens indicate gaps introduced to maximise homology.

Studies of the mode of action of zoocin A have demonstrated that the protein is capable of cleaving a hexaglycine substrate with equal efficiency to that of lysostaphin and thus can function as an endopeptidase, causing cell lysis by attacking the cell wall (Simmonds *et al.*, 1996). However, further experiments have been carried out with zoocin A to investigate its ability to cleave hexaglycine

(P. Bardelang, unpublished data). His-tagged zoocin A has been purified and used against a pentaglycine substrate in a FRET assay (for details on the assay see chapter: 6), however no evidence was shown for any cleavage ability even though the protein was active against *S. pyogenes* on an agar diffusion assay, as expected. The linker TP-rich protein that has been identified between the N-terminal and C-terminal modules, has also been reported for many other hydrolases, including lysostaphin and endolysin A. Both the particularly strong homology between the catalytic domains of lysostaphin and zoocin A and the presence of the TP-rich linker regions suggest the ability of each domain of the latter protein to function individually and that these regions may not have therefore evolved independently. Interestingly though, lysostaphin is a plasmid-located gene (Heath *et al.*, 1987) whereas *zooA* is chromosomally located. This suggests that there may have been exchange of genetic material between the two bacteria and that the latter gene may have evolved from the transfer of plasmid-located lysostaphin from *S. simulans* to *S. zooepidemicus* (Simmonds *et al.*, 1997). Similar observations have been described with Pyocin AP41 from *Pseudomonas aeruginosa* and colicin genes from *E. coli* (Sano and Kageyama., 1993). Even though no homology has been found between the lysostaphin and zoocin A C-terminal domains, it has been reported likely that this region plays the same role in target recognition probably by binding to a different component of the cell wall (Lai *et al.*, 2002).

4.1.4 Proline-rich regions called SH-3 domains may confer target specificity

The SH-3 family of domains (Src Homology 3) are generally non-catalytic modules that bind with moderate affinity and selectivity to proline-rich ligands and play a very important role in a wide variety of biological processes such as intracellular signalling (Mayer, 2001). SH-3 domains were initially located in eukaryotic organisms and viruses, but several homologues have now been identified in many prokaryotic microoganisms in which they might have two possible functions. Firstly, for promoting the survival of the pathogen within the invaded cell by modulating pathways controlled by SH-3 domains and secondly for promoting invasion by binding to receptors on the target cells (Whisstock and Lesk, 1999). Target specificity of SH-3 domains has been identified in many

prokaryotic murein hydrolases with proline-rich regions including lysostaphin (Ponting *et al.*, 1999) and Ale-1 from *S. capitis* (Reinscheid *et al.*, 2002). Interestingly, the "GW motifs" of *L. monocytogenes* InlB (fig. 4.1) resemble the SH-3 domains. However, even though they are non-catalytic and responsible for target specificity they have been reported unlikely to act as functional mimics of SH-3 domains since their proline-binding sites are blocked or destroyed (Marino *et al.*, 2002). Finally, it has been suggested that the presence of SH-3 domains have resulted from an early horizontal gene transfer between eukaryotes and bacteria, but the direction of transfer is currently unclear (Ponting *et al.*, 1999).

4.1.5 The 92 amino acids C-terminal domain of lysostaphin confers target specificity to the protein

Factors responsible for the export, processing and subsequent cellular targeting of lysostaphin to *S. aureus* cells were initially investigated by Baba and Schneewind (1996) by expressing lysostaphin in both the producing and the target cell organisms. It was observed that when expressed in *S. simulans*, preprolysostaphin was converted into its proform within 2 min of its synthesis while cleavage to the mature form required over 60 min and occurred exclusively during stationary phase. On the other hand, when expressed by *S. aureus*, preprolysostaphin was cleaved to its mature form in less than 30 sec. However, the protein expressed in *S. aureus* remained cell associated, trapped within the cell wall and degraded very quickly. In order to determine the location of the T-domain function of the lysostaphin molecule, a 92 residue C-terminal region was deleted from the bacteriocin and the experiment was repeated with the deleted protein. The results showed that the deleted lysostaphin was successfully secreted into the culture medium of *S. aureus*, suggesting that it is the C-terminal domain that is necessary for targeting of lysostaphin to the envelope of *S. aureus* cells. Due to the fact that *S. aureus* and *S. simulans* share the same peptidoglycan structure in their cell walls, it was assumed that the targeting domain of lysostaphin may identify a non-peptidoglycan component of the *S. aureus* cell wall.

4.1.6 The presence of the C-terminal targeting domain of lysostaphin is essential for the specific killing of *S. aureus* cells.

Baba and Schneewind, (1996) developed an assay in order to measure the bacteriolytic activity and target cell specificity of lysostaphin. According to the assay the target cell specificity of mature lysostaphin was the number of surviving *S. simulans* cells (ie host cells) divided by the number of surviving *S. aureus* cells (ie target cells). The assay produced a value of 5.3×10^4. The bacteriolytic activity of prolysostaphin and mutant prolysostaphin (ie lacking the C-terminal domain) were compared with that of the mature enzyme. It was observed that the target specificity of the proenzyme was somewhat reduced (5.6×10^2) compared to that of the mature bacteriocin while in the case of mutant (lacking the C-terminal domain) prolysostaphin the ability of the protein for target cell specificity was virtually lost (1.3). These values suggested that the degree of enzymatic endopeptidase activity of lysostaphin against *S. aureus* is dependent on the presence of the C-terminal domain that provides target specificity.

4.1.7 Other roles of the C-terminal domain of lysostaphin

In an attempt to identify other possible roles for the C-terminal targeting domain of lysostaphin, Baba and Schneewind (1996) expressed lysostaphin lacking the targeting domain in *S. aureus*. The results indicated that the mutant protein was successfully secreted into the *S. aureus* culture medium and that the mutant precursor was also converted into its proform but at a rate which was significantly slower ($t_{1/2}$ = 2 min) than the processing of the wild type enzyme ($t_{1/2}$ < 30 sec). These results indicated that the C-terminal targeting domain of lysostaphin is also required for rapid processing of the preproenzyme to its proform.

In order to find out more information about the lysostaphin targeting domain it was considered important to initially clone and express the domain. Furthermore, purification, use and assay of the underlying protein would allow us to determine whether the targeting domain can be cloned on its own, still retain its activity and thus be capable of protecting EMRSA-16 cells in the presence of wild type

lysostaphin. A spot test was carried out for this purpose while a broth dilution assay was used to determine the minimum concentration of lysostaphin targeting domain required to protect EMRSA-16 cells in the presence of the mature protein.

4.2 RESULTS

4.2.1 Cloning, expression and purification of the targeting domain of lysostaphin.

In order to clone the targeting domain of lysostaphin on its own, forward primer EA2 (CCC ACC ATG GGT TGG AAA ACA AAC AAA TAT GGC) and reverse primer DW41 (G CTC GAG CTT TAT AGT TCC CCA AAG) were used in a PCR reaction using pEA3 as a template. Primer EA2 was used in order to introduce an *Nco*I site, and thus an ATG codon at the translational start of the targeting domain whilst primer DW41 was used to introduce an *Xho*I site in place of the stop codon so that the translated protein would contain a C-terminal His-tag fusion. The resulting PCR produced a 270 bp fragment, which was gel purified and then cloned "blunt-ended" (see section 3.2.3) into pUC18 to produce pEA8. The 270 bp fragment of pEA8 was double-digested with *Nco*I and *Xho*I and sub-cloned into pET21d also restricted with the same enzymes. The recombinant plasmid, pEA7 was transformed into *E. coli* B834 [DE3] cells and recombinant targeting domain polypeptide was over-expressed on induction with 1 mM IPTG. The cloned gene was DNA sequenced to confirm the fidelity of the PCR. The polyhistidine tagged targeting domain (fig. 4.5) was purified to approximately 90% purity using metal chelate chromatography. Two millilitres fractions were collected from a 5-85% imidazole gradient column and fractions 12-17 were pooled together and dialysed overnight (fig.4.6 A and 4.6 B).

MGWKTNKYGTLYKSESASFTPNTDIITRTTGPFRSMPQSGVLKAGQTIHYDEVMKQDGHVWVGYTG
NSGQRIYLPVRTWNKSTNTLGVLWGTIKALHHHHHH

Fig. 4.5 The aa sequence of the lysostaphin targeting domain indicated in red. The methionine (M) and the his-tag sequence added at the N-terminal and C-terminal domains respectively are shown in blue. The exact molecular weight of the his-tagged lysostaphin T-domain is 11565 Da.

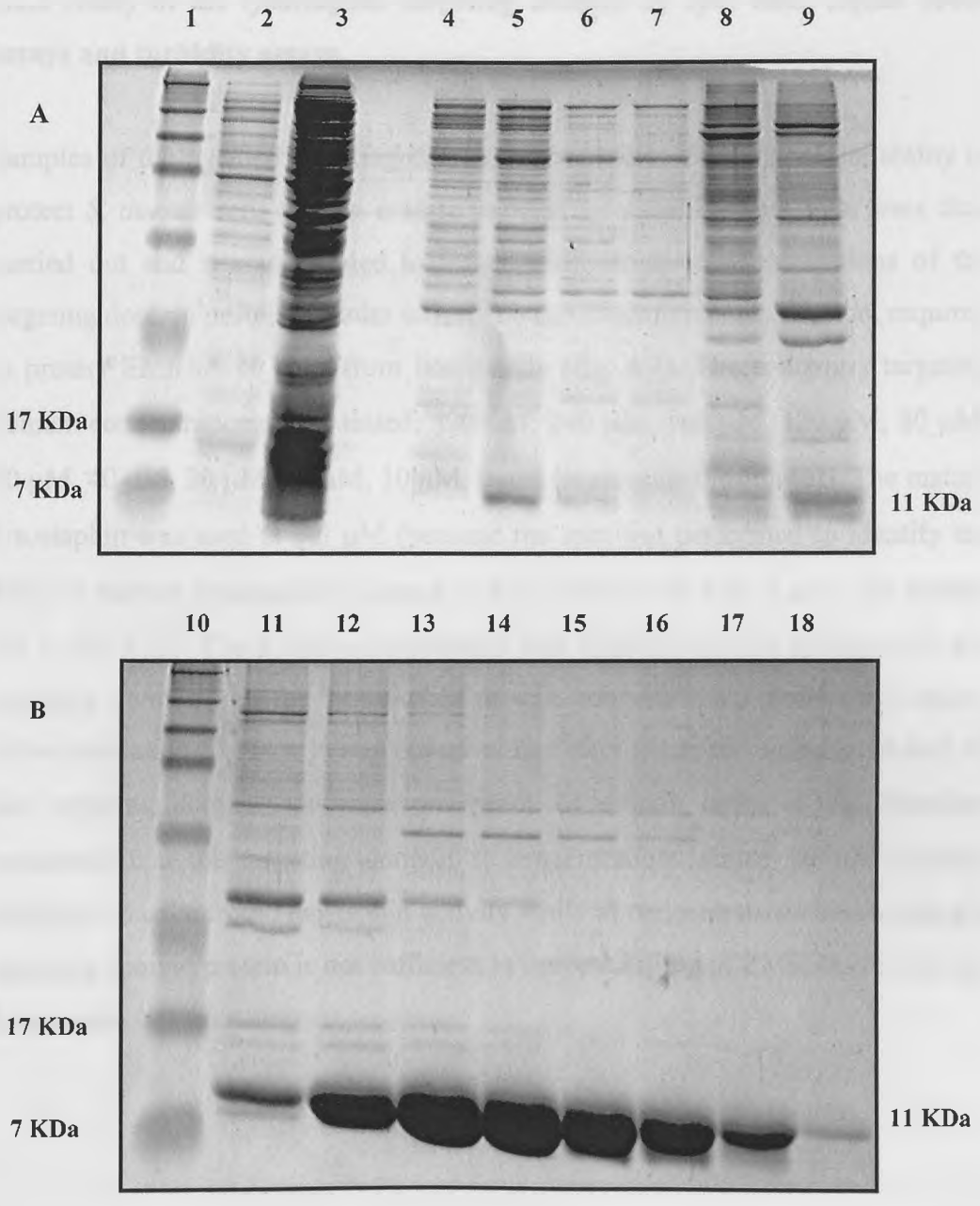

Fig. 4.6A, B 16% SDS-PAGE of total cell proteins produced by B834 (DE3) (pEA7) after 3 hr induction with 1 mM IPTG. Lanes: 1 and 10, Protein marker (83 kDa, 62 kDa, 48 kDa, 33 kDa, 25 kDa, 17 kDa and 7 kDa). Lane: 2, Flowthrough sample containing material that did not bind to the column. Lane: 3, Dilute soluble fraction of sonicated cells loaded onto the column. Lanes: 4-9 and 11-18: Indication of the targeting domain polypeptide. Fractions: 12-17 were pooled together and dialysed for further analysis.The final protein yield was ~120 mg per 500 ml of induced culture.

4.2.2 Assay of the lysostaphin targeting domain by spot tests, liquid broth assays and turbidity assays.

Samples of the purified targeting domain were used to demonstrate its ability to protect *S. aureus* cells against mature purified lysostaphin. Spot tests were thus carried out and it was decided to test a wide range of concentrations of the targeting domain protein in order to identify the minimum concentration, required to protect EMRSA-16 cells from lysostaphin (fig. 4.7). The following targeting domain concentrations were tested: 320 μM, 240 μM, 160 μM, 120 μM, 80 μM, 60 μM, 40 μM, 30 μM, 20 μM, 10 μM, 0 μM (represents the control). The mature lysostaphin was used at 3.5 μM (because the spot test performed to identify the MIC of mature lysostaphin required to kill EMRSA-16 was 3 μM, see section 3.2.1, fig. 3.13). The plates were overlaid with EMRSA-16 and spotted with the targeting domain and the lysostaphin protein concentrations mentioned above. After incubation for 24 h it was observed that only the spots with the 10 and 20 μM targeting domain concentrations produced a faint zone. It was therefore postulated that the targeting domain at concentrations above 20 μM protects EMRSA-16 cells from lysostaphin activity while at concentrations below that the targeting domain protein is not sufficient to prevent killing of EMRSA-16 cells by lysostaphin.

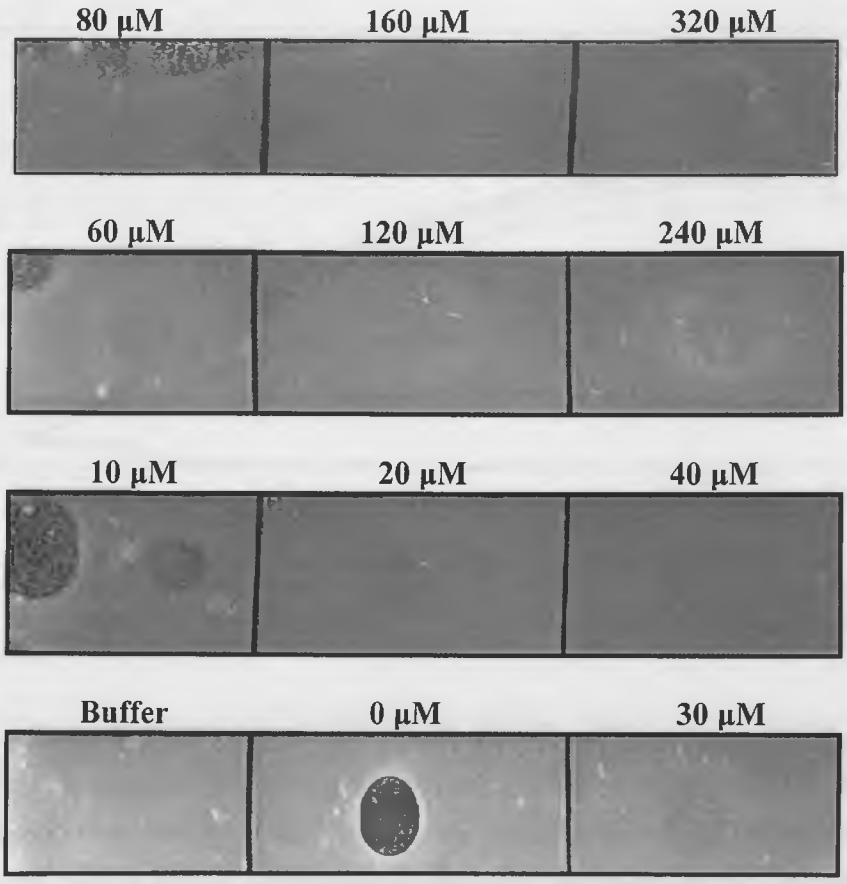

Fig. 4.7 Spot test indicating zones of inhibition at targeting domain concentrations of 10 and 20 µM, suggesting that purified targeting domain protein can protect EMRSA-16 cells from lysostaphin activity at concentrations above 20 µM.

In order to obtain a more accurate value for the minimum concentration of the targeting domain protein that is required to protect EMRSA-16 cells from killing by lysostaphin, it was decided to test the purified protein with a broth dilution assay (fig 4.8). Purified targeting domain was therefore used at concentrations of 25 µM, 10 µM, 5 µM, 2.5 µM and 0.5 µM while purified lysostaphin was used at a final concentration of 3.5 µM (because the liquid broth test performed to identify the MIC of mature lysostaphin on EMRSA-16 was 40 nM, see section 3.2.1, fig 3.14).

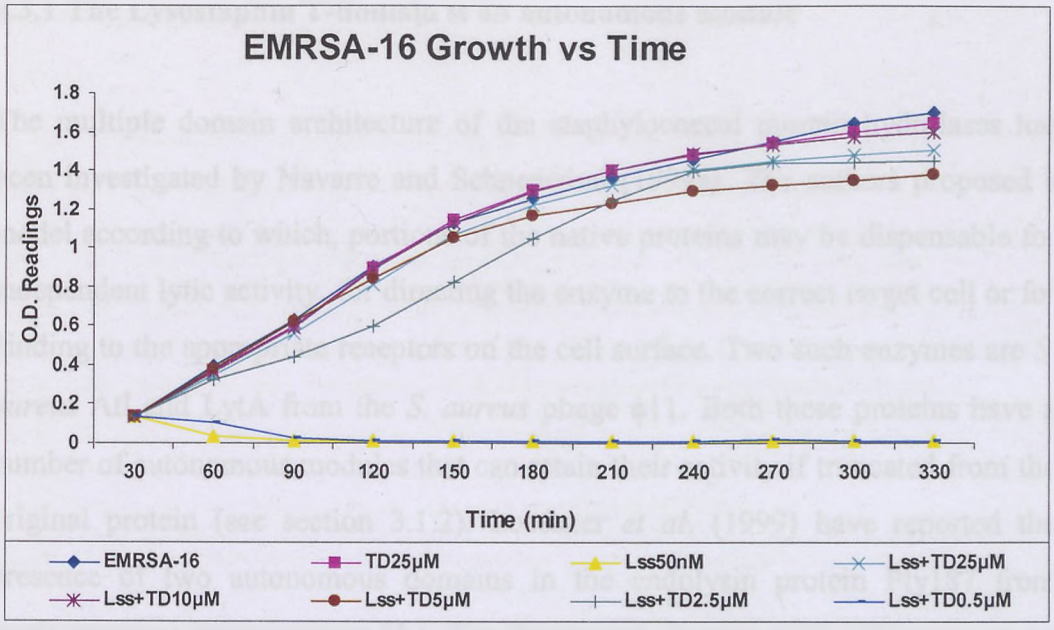

Fig 4.8 Graph indicating the results of the dilution broth assay carried out with lysostaphin, the lysostaphin targeting domain and EMRSA-16. According to the results, the minimum concentration of targeting domain protein required to protect EMRSA-16 cells from the killing activity of lysostaphin appears to be approximately 2.5 μM that is a 50 fold excess of the wild type protein used.

According to these results, when the targeting domain protein is at concentrations of ≥ 2.5 μM (or ≥50-fold excess) of lysostaphin it can sufficiently protect EMRSA-16 cells from cell killing by lysostaphin. Therefore, the targeting domain of lysostaphin can function as a separate domain on its own and offer biological protection to EMRSA-16 cells from lysostaphin.

4.3 DISCUSSION

4.3.1 The Lysostaphin T-domain is an autonomous module

The multiple domain architecture of the staphylococcal murein hydrolases has been investigated by Navarre and Schneewind (1999a). The authors proposed a model according to which, portions of the native proteins may be dispensable for independent lytic activity, for directing the enzyme to the correct target cell or for binding to the appropriate receptors on the cell surface. Two such enzymes are *S. aureus* Atl and LytA from the *S. aureus* phage ϕ11. Both these proteins have a number of autonomous modules that can retain their activity if truncated from the original protein (see section 3.1.2). Loessner *et al.* (1999) have reported the presence of two autonomous domains in the endolysin protein Ply187 from staphylococcal bacteriophage 187. The N-terminal domain of Ply187 contains an enzymatically active site that resembles an amidase and exhibits striking homology to that of LytA while the C-terminal domain of Ply187 has been assumed to code for glucosaminidase activity even though there was no evidence of staphylolytic activity when the protein was separately expressed and assayed in an overlay assay (Loessner *et al.*, 1999). The C-terminal domain was not found essential for staphylolytic activity of the N-terminal domain, confirming the suggestions of Navarre and Schneewind (1999a).

In hydrolases with autonomous domains, the presence of a specific domain architecture has been also suggested by Navarre and Schneewind (1999a). According to this, the N-terminal end of the protein harbours the catalytic activity, whereas the C-terminal domain contains the information for specific recognition of a cell-wall ligand. For example, an endolysin from staphylococcal phage Twort, called PlyTW has been found to exhibit a similar module organisation to lysostaphin (Loessner *et al.*, 1998) with an N-terminal domain coding for catalytic activity (and exhibiting significant homology to the amidase domain of staphylococcal LytA) and a C-terminal targeting domain responsible for target recognition and binding that is strongly homologous to that of lysostaphin (fig. 4.9). However, upon investigation of the functionality of the two domains of

PlyTW in relation with each other, it was found that the C-terminal module was not essential for staphylolytic activity of the protein, which is not however the case with lysostaphin.

```
LytA     1  MQAKLTKNEFIERLKTSEGKQFNVDLWYGFQCFDYANAGWKVLFGLLLKGLGAKDIPFANNF
PlyTW    1  -MKTLKQAESYIKSKVNTGTDFD-GL-YGYQCMDLAVDYIYHVTDGKIRMWGNAKDAINNSF

LytA    63  DGLATVYQNTPDFLAQPGDMVVFGSNYGAGYGHVAWVI----EATLDYIIVYEQNWLGGGWT
PlyTW   60  GGTATVYKNYPAFRPKYGDVVVWTTGNFATYGHIAIVTNPDPYGDLQYVTVLEQNWNGNG--

LytA   122  DGIEQPAGVGKKLQDDNMLMISLCGLSVRILKVRQRHDQFNLLHKHPKKETAKPQPKAVELK
PlyTW  121  --IYKTELATIRTHDYTGITHFIRPNFATESSVKKKDTK-----KKPKPSNRDGINKD---K

LytA   185  IIKDVVK-GYDLPKRGSNPKGIVIHNDAGSKGATAEAYRNGLVNAPLSRLEAGIAHSYVSGN
PlyTW  174  IVYDRTNINYNMVKRGYNPVGVILHNDAGSM--TGLQYKNNLQNAGYNRWAQGIAHSYISEG

LytA   247  TVWQALDESQVGWHTANQIGNKYYYGIEVCQSMGADNATFLKNEQATFQECARLLKKWGLPA
PlyTW  235  QVWQALGESRIAWHCANQWGNKNLYGIEICQSMTASDEQFLKNEQTAFYEASRMLKKWGLKP

LytA   310  NRNTIRLHNEFTSTSCPHRSSVLHTGFDPVTRGLLPEDKRLQLKDYFIKQIRAYMDGKIPVA
PlyTW  298  DKNTVRLHMEYYQTACPHRSMKLHVGKDP-TKTSITQADIEKLKEYFIKQIKMYYEGKTPVP

LytA   373  TVSNESSASSNTVKPVASAWKRNKYGTYYMEESARFT-NGNQPITVRKVGPFLSCPVGYQFQ
PlyTW  360  TVVNQKAKTKPVKQSSTSGWNVNNYGTYYKSESATFKCTARQGIVTRYTGPFTTCPQAGVLY
Lys    291                VTPTPNTGWKTNKYGTLYKSESASF--TPNTDIITRTTGPFRSMPQSGVLK

LytA   432  PGGYCDYTEVMLQDGHVQVGY-TWEGQRYYLPIRTWNGSAPPNQILGDLWGEIS
PlyTW  423  YGQSVTYDTVCKQDGYVWISWTTNGGQDVWMPVRTWD---KNTDIMGQLWGDIY
Lys    340  AGQTIHYDEVMKQDGHVWVGYTGNSGQRIYLPVRTWN---KSTNTLGVLWGTIK
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Fig. 4.9 Sequence alignment indicating identical residues between PlyTW and LytA and PlyTW and the C-terminal targeting domain of lysostaphin (Lys). The identical residues between PlyTW, LytA and Lys are highlighted in purple whereas those identical only between PlyTW and Lys are highlighted in blue.

Baba and Schneewind (1996) have reported that the C-terminal domain of lysostaphin is necessary and sufficient in order to direct the protein to its substrates. In this study it was decided to find out whether the C-terminal

targeting domain can function as an individual module and therefore bind to the appropriate receptors on the cell surface of *S. aureus* cells in the presence of wild type lysostaphin. In order to achieve this, the C-terminal domain of the mature gene was initially cloned and the expressing protein was purified. An agar diffusion assay and a liquid broth assay were carried out with the purified protein and mature lysostaphin (both proteins used mixed together) against EMRSA-16. The purpose of these tests was to find out whether the C-terminal domain could confer protection to EMRSA-16 cells from the enzymatic activity of the mature protein and to determine the minimum concentration of protein required to achieve that. The results of this study indicated that at concentrations of 2.5 μM and higher the C-terminal targeting domain confers protection to EMRSA-16 cells in the presence of 40 nM of mature lysostaphin. These results also indicated that the domain is capable of binding to the appropriate receptors on the surface of *S. aureus* without the presence of the endopeptidase domain. However, the exact mechanism by which T-domain can offer protection to EMRSA-16 remains to be established. It is possible that on its own the lysostaphin T-domain locates and permanently binds to the appropriate receptors on the *S. aureus* cells, which once occupied cannot be identified by mature lysostaphin and thus the protein is unable to kill the cells. Furthermore, it may be that once the *S. aureus* cell receptors are bound to the T-domain of the truncated lysostaphin, the protein molecule degrades and therefore fresh supplies of T-domain are required to occupy the receptors before identified by the mature protein.

Similar liquid broth assays have been performed with colicins to establish the number of colicin receptors in *E. coli* cells and the amount of mutant colicin protein (the molecule retaining the R-binding and translocation domains but lacking the appropriate DNase domain) required to block the receptors so that wild type colicins cannot kill the *E. coli* cells (Duche *et al.*, 1995). Duche and colleagues (1995) concluded that there are 200-400 cell receptors/cell while 10-100 fold excess of mutant protein is required to bind over 90% of the receptor sites on *E. coli*. This is in contrast to our findings for the amount of lysostaphin T-domain required to block the appropriate binding sites on *S. aureus*. This is because the number of lysostaphin receptors on *S. aureus* has been estimated to be

10^6, per colony forming unit (Baba and Schneewind, 1996) and the liquid broth assay carried out in this study indicated that only 50 fold excess of T-domain mutant protein is required to protect *S. aureus* growth from the wild type lysostaphin. One reason why more amount of mutant colicin protein is required to block the binding sites of *E. coli* despite the presence of a smaller number of receptors could be because of the different mechanisms employed by the two proteins. Colicins have a much more efficient way of lysing the target cells as they destroy the DNA and hence become directly lethal. As a result all the receptor sites on *E. coli* must be blocked in order for the cells to be able to survive. Lysostaphin however, lyses *S. aureus* cells by attacking their peptidoglycan and even though it causes structural damage to the cells it cannot cause instant cell death (as is the case with colicins). Therefore, it maybe that *S. aureus* can survive from lysostaphin without having all its binding sites blocked hence why less amount of protein is required to block the *S. aureus* binding sites.

Similar studies have been performed by Lai *et al.* (2002) who attempted to characterise the two domains identified in Zoocin A (the N-terminal catalytic domain and the C-terminal targeting domain) and establish whether each of those can maintain their biological functions independent of each other. In order to achieve this purpose, each domain as well as the wild type protein were his-tagged and expressed in *E. coli*. The purified products were used in a dye release assay (similar to that described by Zhou *et al.*, (1988) for details see section: 6.3.4) while wild type protein was also purified by its native producer organism and was used as a control. Both wild type proteins were tested against indicator strains to confirm that the his-tag added to ZooA produced by *E. coli* did not alter its spectrum of activity compared to ZooA produced by *S. zooepidemicus*. The results of the dye release assay indicated similar high levels of dye release for both wild type proteins, which however were reduced when the N-terminal catalytic domain was tested. This suggested that the catalytic domain is capable of retaining its activity without the presence of the targeting domain and can thus act against target organisms on its own. In the case of the C-terminal targeting domain no dye release was detected and therefore no activity, as expected, probably due to the inability of attaching itself to the substrate. There could be

other possible explanations for the absence of activity though, such as a loss of conformation or the loss of ability of the C-terminal domain to bind an essential target.

Similar experiments have been also described with two *L. monocytogenes* bacteriophage murein hydrolases called Ply118 and Ply500 by Loessner and co-workers (2002) who identified the presence of an N-terminal catalytic domain and a C-terminal cell wall binding domain in both these proteins (Loessner *et al.*, 1995). Ply118 and Ply500 share a unique enzymatic activity and specifically hydrolyse *L. monocytogenes* cells at the completion of virus multiplication in order to release progeny phage with very few exceptions among closely related bacteria. The authors investigated the amino acid sequences of the C-terminal domains of the two proteins and found out that there was no significant sequence homology between them. This indicated the possibility that these regions might be involved in the specific recognition of the *Listeria* cell wall (Loessner *et al.*, 1995). However, in order to establish whether both domains of the two murein hydrolases are required for lytic activity, Loessner and co-workers (2002) tested truncated versions of Ply115 and Ply500 proteins for IPTG-inducible lytic activity against *L. monocytogenes*. Their results indicated that only full-length Ply118 and Ply500 produced lytic zones, no lysis was detected when the N-terminal catalytic domains of both proteins were screened alone. The corresponding C-terminal domains of Ply118 and Ply500 were also not able to hydrolyse *Listeria* cell walls.

4.3.2 Construction of chimeras with the T-domain of lysostaphin

Due to the worldwide increase of resistance to multiple antibiotics in several microorganisms, notably in *S. aureus*, new adjuncts or alternatives to antibiotics are imperative. A very promising new group of molecules for further investigation and development are the bacteriophage endolysins, which constitute one of the most ubiquitous and successful natural antimicrobials on earth (Schuch, *et al.*, 2002). Their great potential lies in their modular design which contains independent functional domains thus rendering these proteins ideal for domain-swapping studies. In this way, bacterial specificities and catalytic activities may

be improved or adopted for use with other pathogens (Lopez *et al.*, 1997). Two endolysins that exhibit such domain architecture and could therefore be used for this purpose are the staphylococcal PlyTW and Ply187 from bacteriophages Twort and 187 respectively. In addition, the *S. aureus* autolysin (Atl) and LytA produced by staphylococcal phage ϕ11 (see section 3.1.3) display a structure that reflects a combination of individual functional domains that also renders them possible candidates for the construction of chimeras. Further to this, the fact that the lysostaphin targeting domain can function as a separate domain suggests that this protein could be used in combination with enzymatic domains from other proteins as well. Therefore, isolation and fusion of the *S. aureus* amidase gene with the targeting domain of lysostaphin may result in active chimeras specifically targeted to *S. aureus* cells. Conversely, the enzymatic domains of Ply TW, Ply 187 and LytA could be utilised in order to replace the enzymatic domain of lysostaphin, in combination with its T-domain for the production of chimeras with different enzymatic activities, such as amidase or glucosaminidase, but all targeted against *S. aureus*.

The production of such proteins may be very beneficial for the current antibiotic resistance problem because resistance to endolysins is assumed to be an unlikely possibility. This has been suggested by Fischetti (2001) because the catalytic and binding targets of these proteins are essential for the viability of the cells, therefore intrinsic resistance should not develop. Experiments carried out with *Bacillus anthracis* by Schuch and co-workers (2002) confirm these assumptions. The researchers identified an endolysin from the [gamma] bacteriophage of *B. anthracis*, called PlyG that specifically kills *B. anthracis* and certain *Bacillus cereus* isolates. When isolates of a streptomycin-resistant *B. cereus*, called RSVF1 was exposed to high amounts of PlyG as well as novobiocin and streptomycin, resistant mutants were readily identified for novobiocin and streptomycin, nevertheless no significant resistant mutants were found for PlyG. In addition, when RSVF1 isolates were subjected to mutagenesis, an approximate 1,000-fold and 10,000-fold increase in novobiocin and streptomycin resistance respectively were observed. However, no PlyG-resistant derivatives were identified. These results suggested that the peptidoglycan catalytic target and carbohydrate-binding

sites may not be easily modified to prevent endolysin action. Similar experiments have been also carried out by Loeffler *et al.* (2001), with *Streptococcus pneumoniae*. The researchers identified a bacteriophage endolysin, called Pal, that can hydrolyse the cell wall of *S. pneumoniae*. They too, exposed the bacterium to low and increased concentrations of the Pal hydrolase in both agar diffusion and liquid broth assays and found that no resistant strains to Pal had been developed. Thus, the production of chimeric proteins from the two functional domains of cell wall hydrolases may prove to be efficient agents for the control of human pathogens.

4.3.3 Fusion of the T-domain with GFP (Green Fluorescent Protein) for labelling and detection of *S. aureus*.

The T-domain of lysostaphin could also be used for the construction of chimeras with GFP (Green Fluorescent Protein) for labelling and detection of *S. aureus* cells. GFP was originally isolated from the jellyfish *Aequoria victoria* in 1960s and has many applications as a marker in living cells. Its amazing ability to generate a highly visible, efficiently emitting internal fluorophore has been tremendously valuable for visualising physiological processes, monitoring subcellular protein localisation, distinguishing successful transfection or reporting in gene expression (Billinton *et al.*, 2001). Further to this, GFP is highly stable with minimal toxicity while the ability to clone and efficiently express the GFP genes in a diverse range of organisms and cells (from bacteria to mammals) render GFP the most popular fluorescent tag for the construction of chimeric proteins (Billinton *et al.*, 2001).

The production of such chimeras would involve substitution of the endopeptidase domain at the N-terminal end of lysostaphin with GFP, so that production of the fluorescent molecule can be inframe and therefore expressed as a recombinant polypeptide. Similar experiments have been performed with the cell wall binding domains of two murein hydrolases from *Listeria monocytogenes*, Ply118 and Ply 500 (Loessner *et al.*, 2002). Expression of the GFP-labelled proteins was successful and thus *Listeria monocytogenes* cells were exposed to the purified

protein. Laser scanning microscopy revealed an even, high-density green fluorescent decoration of the bacterial surface with decreased density on the septal regions for Ply500 while in the case of Ply118, fluorescent binding had taken place at the poles and the septal regions of the cells. Further to this, it was shown that the chimeric proteins did not recognise any Gram-positive or Gram-negative cells other than Listeria, confirming earlier suggestions that the targeting domains of these murein hydrolases are specific for Listeria cell walls (Loessner *et al.*, 1995). These results indicated the highly evolved, specific recognition properties of the cell wall binding domains of Ply118 and Ply 500 phage-encoded proteins of *L. monocytogenes*. Furthermore, these experiments suggested the potential usefulness of these chimeric proteins for labelling and detection of *L. monocytogenes* cells.

As opposed to the bacteriophage hydrolases of *L. monocytogenes* that are very species specific, the picture is not so clear with lysostaphin. The mature protein has been shown to lyse *S. aureus* cells (Heath *et al.*, 1987). Nevertheless, its activity is targeted at the pentaglycine in the peptidoglycan backbone that is characteristically found in many staphylococci and has therefore been reported active against those staphylococcal species (Zygmunt and Tavormina, 1972). Thus, the construction of a targeting domain::GFP chimera would be used for fluorescent assays of binding to other genera of bacterial cells. This would assess the ability of the targeting domain to bind not only to staphylococci such as *S. aureus, S. epidermidis, S. haemolyticus, S. capitis and S. sciurii* but also to other Gram-positive organisms such as *Streptococcus spp., Bacillus subtilis, Micrococcus luteus, L. monocytogenes and E. faecalis*. If the targeting domain of lysostaphin proved to be specific for *S. aureus* strains then the T-domain could be used with GFP for the rapid identification of *S. aureus* isolates. However, if the T-domain of lysostaphin was not specific for *S. aureus* then replacing the endopeptidase domain of lysostaphin with a killing activity that has a more broad spectrum activity, such as amidase activity, will produce a chimeric protein that has activity against bacterial species other than *S. aureus*.

Nevertheless, in order to carry out such experiments it is essential to gain a better understanding of the targeting mechanism behind the T-domain of lysostaphin. One method to achieve this is by identifying and subsequently mutating important residues in the T-domain that are involved in receptor-binding. Random PCR and Site-directed mutagenesis were used in this study to obtain such mutants in the C-terminal targeting domain of lysostaphin.

CHAPTER: 5

MUTAGENESIS OF THE TARGETING DOMAIN OF LYSOSTAPHIN

5.1 INTRODUCTION

During PCR amplifications undesired mutations in the amplified DNA may be often introduced if the reaction conditions and choice of polymerase enzyme favour mismatch incorporation of nucleotides (Dunning *et al.*, 1988). Nevertheless, in some cases the infidelity of PCR reactions may be desirable in order to facilitate genetic analysis of specific genes under study. For instance, defects caused by a wide range of independent mutations within a DNA fragment may indicate important residues that influence the function of the encoded protein (Kok *et al.*, 1997). In order to facilitate a genetic analysis of lysostaphin, two different mutagenesis methods were utilised in this study. These methods were Random PCR Mutagenesis and Site-directed Mutagenesis. The former technique is used in order to randomly mutate nucleotides within the template sequence and it is therefore able to produce a diverse array of nucleotide substitutions. Random PCR mutagenesis is used in cases where little information is available on the important residues of the desired gene and does not require the design of specific mutagenic primers. Site-directed mutagenesis is used to mutate specific nucleotides in a DNA fragment that have been generally identified as potentially important for the function of the gene product. Such nucleotides usually code for a specific amino acid that has been identified as a conserved residue through a multiple alignment programme. In most cases alanine scanning mutagenesis is used to mutate the desired amino acids to alanine, as alanine is deemed a neutral residue that is thought not to compromise the 3-D structure of the protein (Cunningham and Well, 1989). Nevertheless, alignment of several sequences for the location of conserved residues does not always result in identification of all the structurally and functionally important amino acids of the protein under investigation (Garinot-Schneider, *et al.*, 1996). Therefore, in order to exhaust all the possibilities of identifying important residues the method of Random PCR mutagenesis is also required. Conversely, the latter technique cannot be used alone because it is not likely that it will identify all the important residues that are crucial for the structure and function of a protein.

5.1.1 The method of random PCR mutagenesis

Several methods for random PCR mutagenesis have been described in the literature. In some of them, mutations may be enzymatically introduced through misincorporation of nucleotides by Taq polymerases during standard PCR reactions (Keohavong and Thilly, 1989). One method is described by Zhou *et al.*, (1991) and is solely based on the inherently error-prone activity of Taq DNA polymerase that lacks the 3'→ 5' exonuclease proofreading ability (Eckert and Kunkel, 1990) that is present with other polymerases such as Pwo from Roche Diagnostics. As such, Taq DNA polymerase is an error prone enzyme and its error rate (mutations per nucleotide per cycle at 70°C) has been reported to be between 2×10^{-4} to $<1.2 \times 10^{-5}$. The average error frequency can be as low as 10^{-5} for base substitutions and 10^{-6} for frameshift errors (Eckert and Kunkel, 1990).

Zhou *et al.* (1991) used Taq DNA polymerase and PCR under standard conditions to mutagenise the *E. coli cpr* gene which consists of 633 nucleotides (encodes the catabolite gene activator protein, CAT). However, Spee *et al.* (1993) have suggested that this method is limited for use only with DNA fragments that exceed 500 bp in length, due to the fact that the resulting mutational frequency (5.5×10^{-4}) is inadequate for smaller DNA fragments. Leung *et al.* (1989) reported an improved version of the PCR mutagenesis method described by Zhou *et al.* (1991). This was still based on the error-prone activity of Taq polymerase, but incorporates Mn^{2+} (Spee *et al.*, 1993) and higher concentrations of Mg^{2+} and dNTPs to enhance the activity of the polymerase and increase the mutational frequency up to 30×10^{-4} (Eckert and Kunkel, 1990).

The PCR mutagenesis method described above, was further improved by Spee and co-workers (1993) and was used in this study because it offers several more advantages. Firstly, it allows the adjustment of the mutation frequency to the size of the targeted DNA fragment to include DNA regions <500 bp. Also, the mutational frequency obtained is not based upon addition of increased amounts of dNTP, and Mn^{2+} (that increase the infidelity of *Taq* DNA polymerase) and finally it offers the opportunity to influence the type of mutation desired by varying the

percentage of the limiting dNTP and by adding dITP simultaneously. Therefore it is cheaper, and more efficient and flexible than the other Random PCR mutagenesis methods described by Zhou *et al.*, (1991), Leung *et al* (1989) and Eckert and Kunkel, (1990). There are 3 main principles behind this mutagenesis technique (fig. 5.1). Firstly, four different PCR reactions have to take place, one for each dNTP (dATP, dCTP, dGTP and dTTP) while each reaction should contain limited amounts of one of the four dNTPs so that the probability of incorporating a dITP is increased. However, the presence of limited amounts of dNTPs will result in a dramatic reduction of the efficiency of the amplification reaction (Kuipers, 1996). Nevertheless, the efficiency of the PCR reaction can be restored due to the presence of dITP. This occurs because, misincorporation of dNTPs is enhanced in the presence of dITP due to the activity of Taq DNA polymerase, even though with a four times reduced efficiency of incorporation than that of the natural nucleotides (Innis *et al.*, 1988). As a result, in the next PCR cycle, those dITPs that will have been incorporated instead of dATPs will pair up with any of the dNTPs present. This will lead to random incorporation of dNTPs and will thus cause point mutations in the final DNA sequence that was used as a template (Spee *et al.*, 1993).

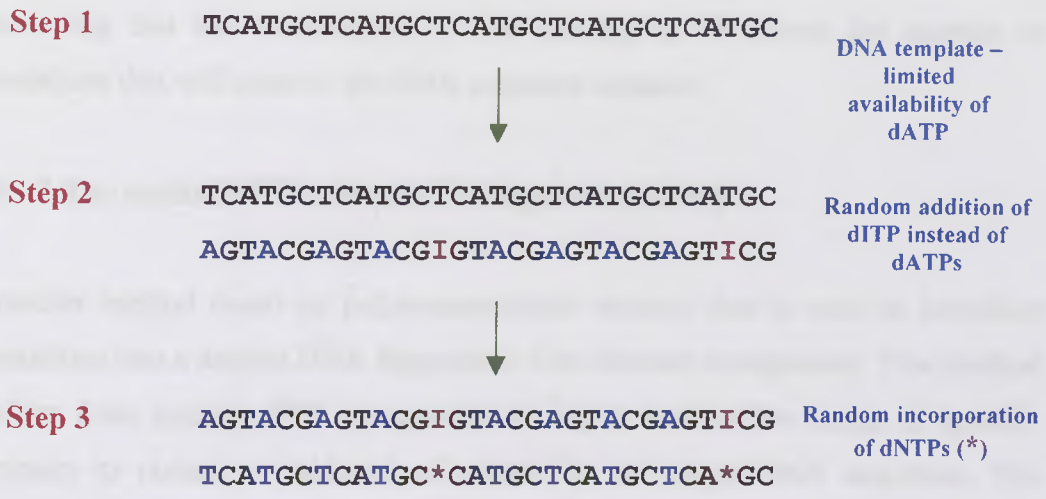

Fig. 5.1 Schematic representation of the random PCR mutagenesis used in this study. If dATP was available in limited amounts in this reaction (step 1), incorporation of dITPS would occur (step 2). In the final stage (step 3), dITP would pair up with any of the available dNTPs, leading to random mutations in the DNA template, which would result in mutations in the primary sequence of the translated protein.

A 171 bp gene from *Lactococcus lactis*, called *nisZ* (Mulders *et al.*, 1991) has been used in random PCR mutagenesis experiments to assess the mutation frequency in the DNA sequence (Spee *et al.*, 1993). Elevated levels of $[Mg^{2+}]$ and various concentrations of dNTPs were tested, with or without the addition of dITP in the final PCR reaction mix. When the concentration of the limited nucleotide in the absence of dITP was dropped to 20 μM and 14 μM, the yield of the final PCR product was reduced to 80% and 10-30% respectively compared to the yield of the control reaction, and only a minor increase in the mutation frequency was recorded. This indicated that Taq DNA polymerase cannot efficiently replace the depleted dNTP with one of the other 3 remaining dNTPs under these conditions. However, upon addition of both 20 μM of the limiting dNTP and 200 μM of dITP, the mutation frequency was increased significantly suggesting that Taq DNA polymerase could incorporate dITP to the DNA sequence. Furthermore, it was observed that upon addition of 14 μM of the limiting dNTP and 200 μM of dITP in the PCR reaction mix, the mutation frequency increased even further,

indicating that the concentration of the limiting dNTP affects the number of mutations that will occur in the DNA sequence template.

5.1.2 The method of Site-directed Mutagenesis (SDM)

Another method based on polymerase chain reaction that is used to introduce mutations into a desired DNA fragment is Site-directed mutagenesis. This method differs from Random PCR mutagenesis in that it requires the design of specific primers to mutate a residue(s) of choice in the target DNA sequence. The technique has the distinctive advantage that mutations can be targeted to anywhere in the desired gene. The technique also allows the introduction of large deletions (Bloch, 1991), DNA insertions and gene fusions (Horton *et al.*, 1989 and Yon and Fried, 1989). Higuchi *et al.*, (1988) first reported in the literature, a Site-directed mutagenesis method that required four primers and three PCR reactions in total. According to this method, two PCR reactions are initially performed to amplify two overlapping sub-fragments of the target DNA sequence (fig. 5.2). One flanking primer and one internal mutagenic primer are required for each PCR reaction. The products obtained from these two initial PCR reactions (fragments AB and CD, fig. 5.2) will be fused in a third PCR reaction as the two internal mutagenic primers are designed to overlap. Thus, in the third PCR reaction, once fragments AB and CD are mixed, denatured and reannealed, the 5' end of the upper strand of fragment AB and the 3' end of the lower strand of fragment CD hybridize. In the presence of a DNA polymerase, each of the overlapping strands acts as a primer on the other which leads to extension of the overlapping fragment and production of mutant AD. Amplification of the full-length mutant is ensured by the presence of primers a and d (Pogulis *et al.*, 1996).

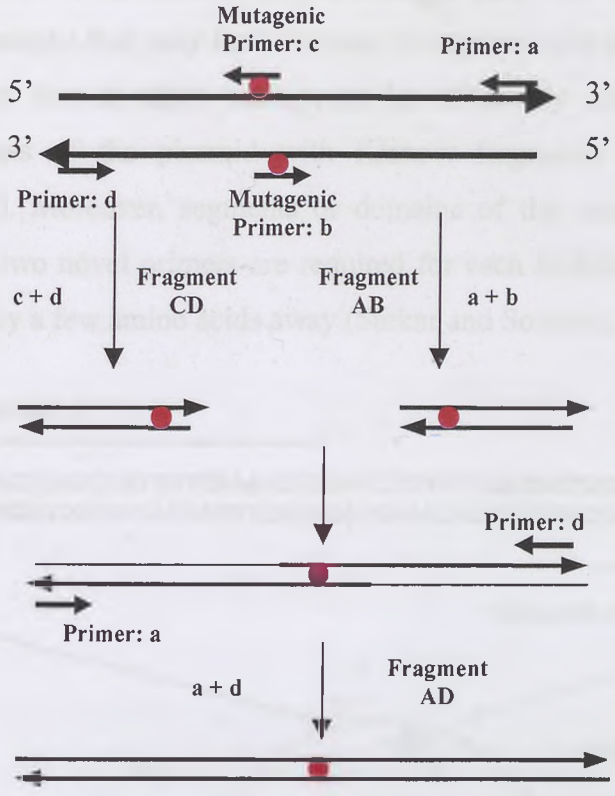

Fig. 5.2 Diagram indicating Site-directed mutagenesis with 4 primers and 3 PCR reactions. 2 PCR reactions are initially carried out requiring one universal and one mutagenic primer each. The two products, fragments AB and CD containing the desired mutation, are fused in the third PCR reaction by annealing at the overlap region. Finally, amplification of the fused product by primers a and d follows (adapted from Pogulis *et al.*, 1996).

Another method for Site-directed mutagenesis has been proposed by Hemsley *et al.* (1989). This method is based on amplification of an entire circular plasmid while a mutation may be inserted anywhere in the plasmid with no requirement for convenient restriction sites. It differs from the one described by Higuchi *et al.* (1998) in that it uses a single inverted PCR reaction and only two primers thus making it a quicker and cheaper alternative. According to this approach, amplification is initially carried out by two primers located "back to back" on the opposing DNA strands (fig 5.3). One of these primers contains the desired mismatch that will generate the Site-directed mutation in linear plasmid

molecules. Nevertheless, this technique also has a number of significant disadvantages that may limit its uses. It requires both a circular plasmid template and one that is short enough to be efficiently amplified while enzymatic treatments of the plasmid with Klenow fragments and phosphorylation are essential. Moreover, segments or domains of the protein cannot be made and finally, two novel primers are required for each additional mutation that may be even only a few amino acids away (Sarkar and Sommer, 1990).

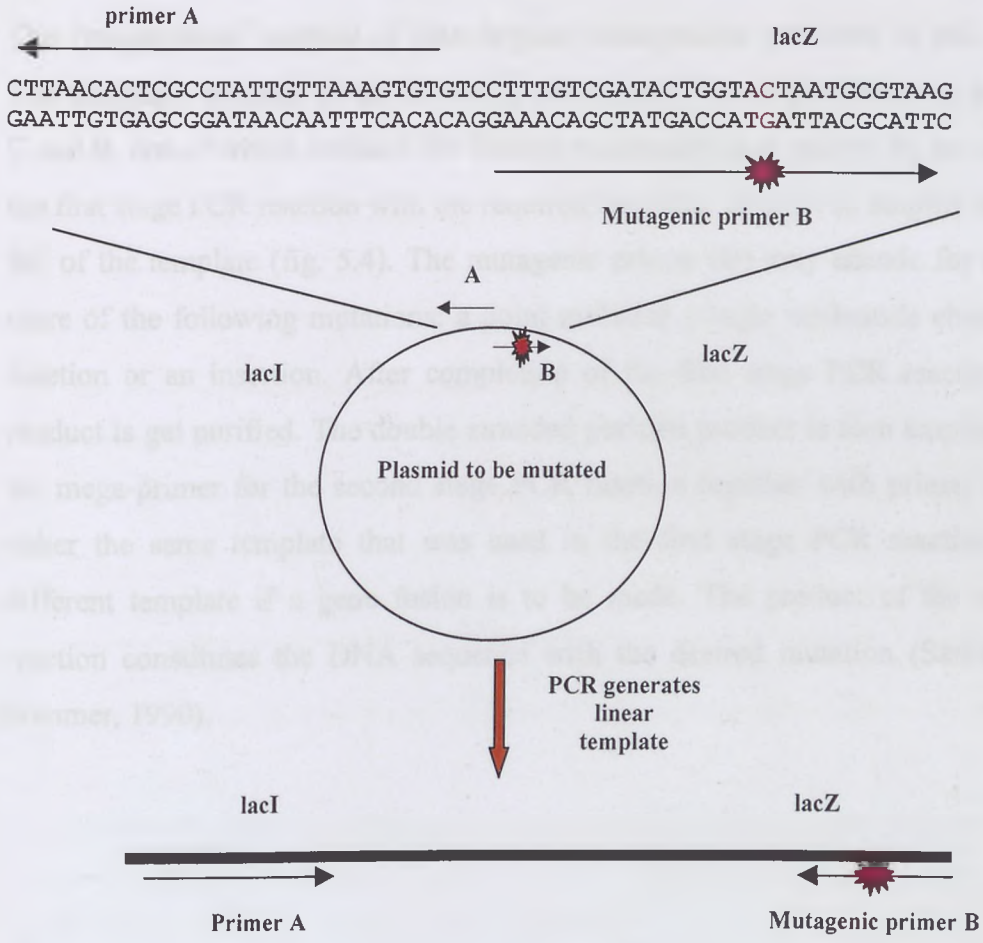

Fig. 5.3 Diagram illustrating inverse PCR Site-directed mutagenesis. Circular plasmid and two primers are required, one of which is the mutagenic primer (B). Located "back-to-back" one primer introduces the desired mutation during the PCR reaction that generates linear product. The product can then be re-circulated by treatment with Klenow fragments and phosphorylation (Hemsley *et al.*, 1989).

However, the above limitations may be overcome by another Site-directed mutagenesis method that has been proposed by Sarkar and Sommer (1990). This

method is called the "megaprimer" method and requires two PCR reactions and three primers. It is therefore, less complicated and cheaper than the one reported by Higuchi *et al.* (1988). Furthermore, the method of Site-directed mutagenesis described by Sarkar and Sommer (1990) is more rapid, because once the primers are ready, incorporation of the desired mutation may be performed in one day and confirmation by sequencing can be achieved on the next day, which is important when results need to be generated quickly.

The "megaprimer" method of Site-directed mutagenesis was used in this study. The technique is based on the following procedure: Two of the necessary primers C and B, one of which contains the desired mutation(s) (e.g. primer B) are used in the first stage PCR reaction with the required template, in order to amplify section BC of the template (fig. 5.4). The mutagenic primer (B) may encode for one or more of the following mutations: a point mutation (single nucleotide change), a deletion or an insertion. After completion of the first stage PCR reaction, the product is gel purified. The double stranded purified product is then employed as the mega-primer for the second stage PCR reaction together with primer A and either the same template that was used in the first stage PCR reaction or a different template if a gene fusion is to be made. The product of the second reaction constitutes the DNA sequence with the desired mutation (Sarkar and Sommer, 1990).

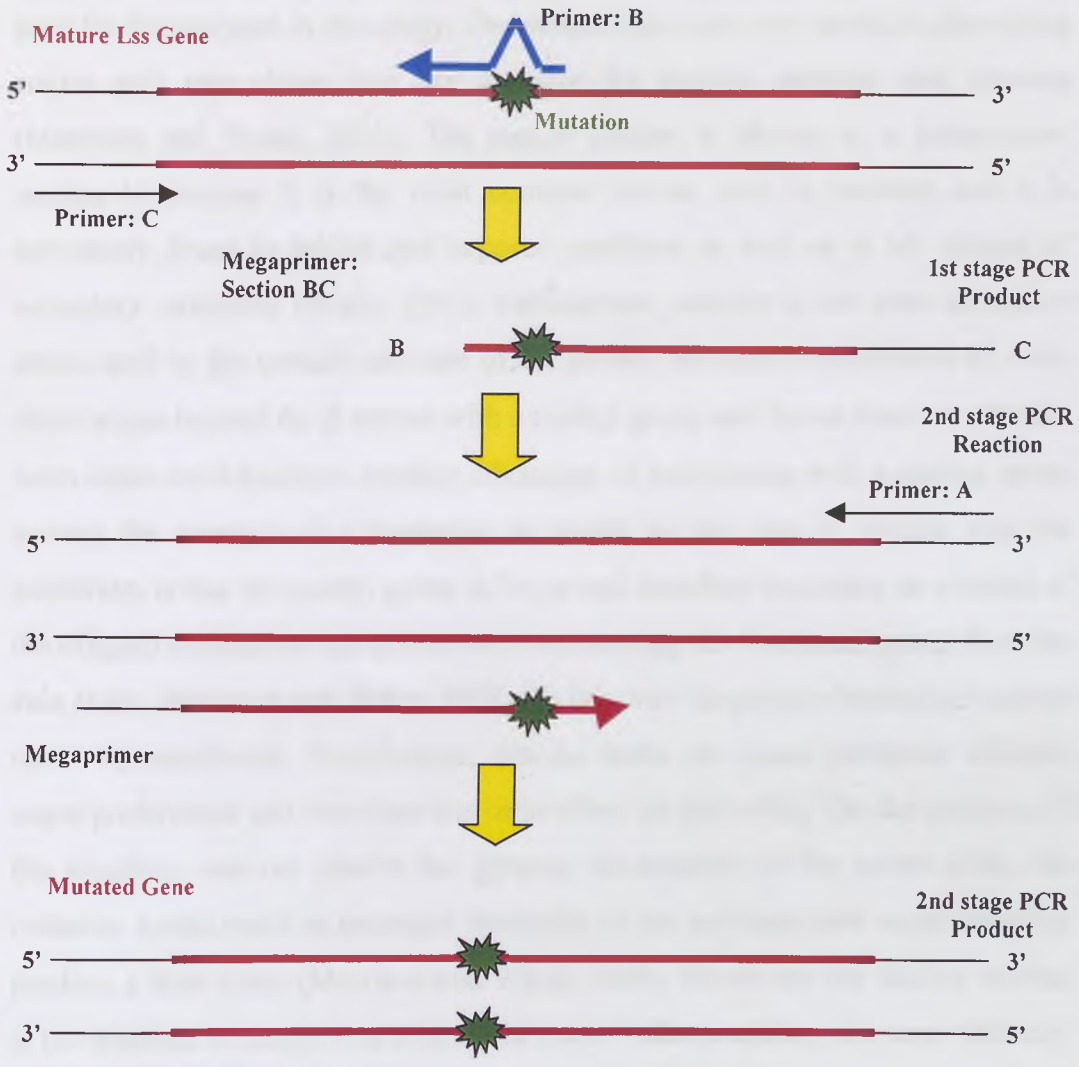

Fig. 5.4 Diagram indicating the Site Directed Mutagenesis Method, using a mega-primer. In the 1st stage PCR, the mutagenic primer B, is used with a suitable flanking primer C to amplify fragment BC that contains the desired mutation. In the 2nd stage reaction, the 1st stage PCR product is used as one primer (the mega-primer) with a suitable primer A, to generate the DNA fragment containing the desired segment.

5.1.3 Alanine scanning mutagenesis

In order to identify candidate residues for the functionality of lysostaphin through Site-directed mutagenesis, it is essential to identify first which residues need to be

mutated. Once the target amino acids have been identified the nature of substitution must be decided. A method called alanine-scanning mutagenesis was used for this purpose in this study. The method has been very useful in identifying amino acid side chains that are essential for protein structure and function (Morrison and Weiss, 2001). The reason alanine is chosen as a replacement residue is because it is the most common amino acid in proteins and it is commonly found in buried and exposed positions as well as in all manner of secondary structures (Wells, 1991). Furthermore, alanine is the least disruptive amino acid to the overall structure of the protein because it substitutes all side-chain atoms beyond the β carbon with a methyl group and hence does not alter the main chain conformation. Another advantage of substituting with a methyl group instead for example of a hydrogen as would be the case if glycine was the substitute, is that the methyl group is larger and therefore maintains an element of the original structure of the protein whilst removing the functional group from the side chain (Morrison and Weiss, 2001). In this way the peptide backbone remains relatively unaffected. Furthermore, alanine lacks the usual backbone dihedral angle preferences and therefore has little effect on flexibility. On the contrary, if the substitute was not alanine but glycine, the simplest of the amino acids, the mutation would result in increased flexibility of the backbone and could therefore produce a false result (Morrison and Weiss, 2001). Moreover, the alanine residue is the simplest to interpret as it removes atoms without adding new ones that may be the cause for favourable or unfavourable novel interactions (Cunningham and Wells, 1989). In this way, alanine substitutions can facilitate the identification of structurally critical or functionally important residues within the target protein. The method of alanine substitutions has to be treated with caution when the substituted amino acid has similar chemical properties to that of alanine. In that case it is possible that the difference between the wild type amino acid and alanine is not of sufficient magnitude to result in loss of activity or function.

5.1.4 Aims

In order to identify important residues in the targeting domain of lysostaphin two different mutagenesis protocols were used, that is Random PCR and Site-Directed mutagenesis. Cloning and expression followed and a stab test was subsequently carried out against EMRSA-16 to establish whether the cloned mutants produce an active protein. Once the proteins expressed by the cloned mutants were identified as inactive, purification of the mutant proteins followed so that further experiments could be performed to study their activity in much more detail.

5.2 RESULTS

5.2.1 Cloning, expression and purification of mutants obtained by random PCR mutagenesis

Random PCR mutagenesis was used to induce mutations in the mature lysostaphin gene of pEA3 as described in section 2.10. The PCR products were restricted with enzymes *Nde*I and *Xho*I and ligated into vector pET21a, also restricted with the same enzymes. Transformation of the ligated vector followed into *E.coli* B834 [DE3] cells. Out of a total of 70 colonies obtained after transformation of the mutated pEA3 plasmid, two were found to produce inactive lysostaphin in a stab test (frequency of ~3%). However, when the mutant proteins were expressed only one of the two produced a full size mature lysostaphin on SDS-PAGE. Sequencing of the mutant plasmid revealed two mutations, one at P152 where the CCA codon had been mutated to CCC, and is thus silent, and the other at residue F172 where the TTC codon had been mutated to TCC, which results in a phenylalanine to serine mutation at this position (pEA9). The mutant protein was purified using metal chelate chromatography (fig. 5.5A and B) and mutant protein fractions 8 and 10-18 were pooled together and dialysed overnight. The purification process produced very concentrated samples (~10 mg/ml) suggesting that the protein was stable and the possibility that the F172S mutation did not have a dramatic effect on the folding of the protein and did not cause critical disruptions on the overall structure of the protein.

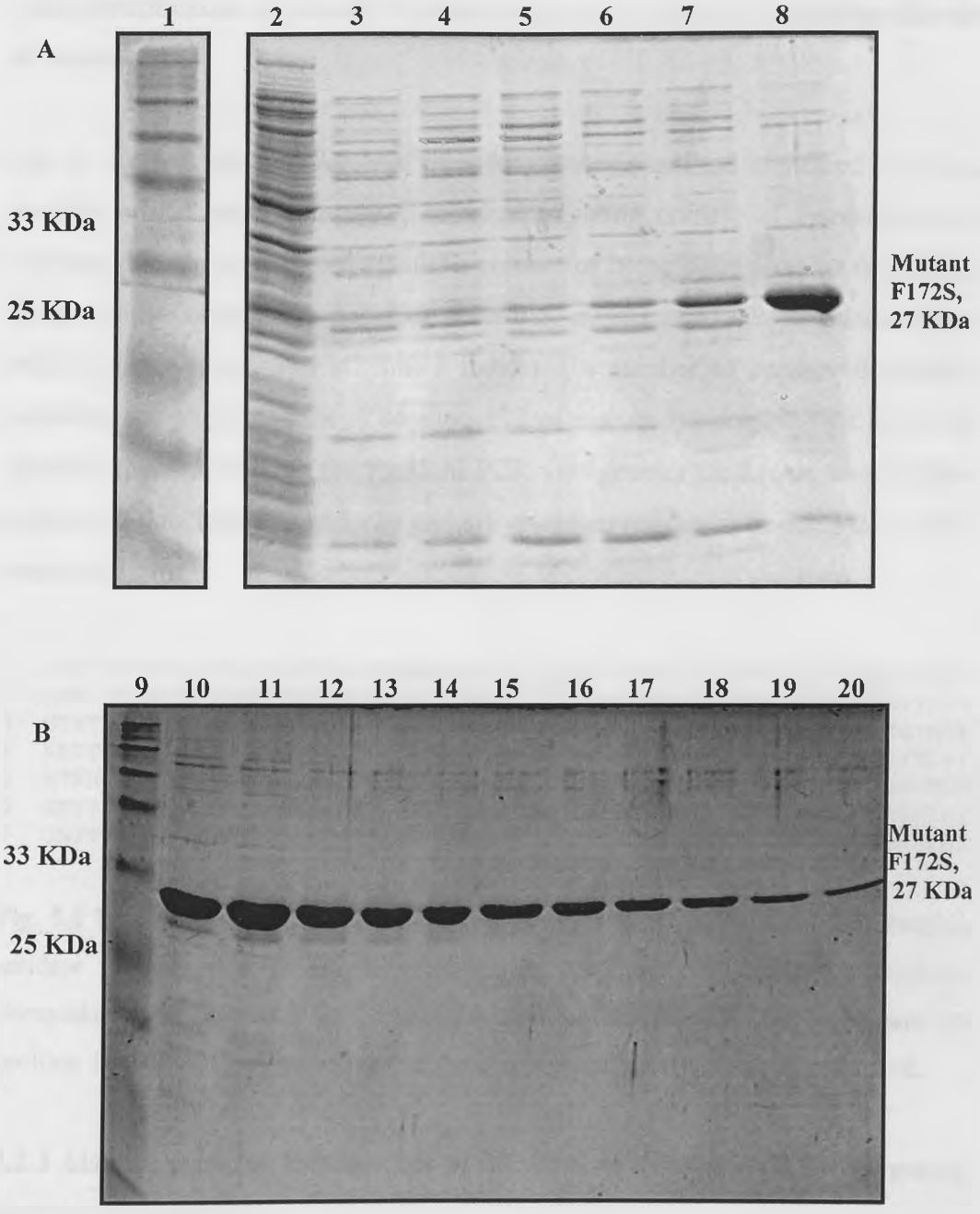

Fig 5.5A, B 16% SDS-PAGE of total cell proteins produced by B834 (DE3) (pEA9) after 3 h induction with 1 mM IPTG. Lanes: 1 and 9, Protein marker (83 kDa, 62 kDa, 48 kDa, 33 kDa, 25 kDa, 17 kDa and 7 kDa). Lane: 2, Flowthrough sample containing material without his-tag and therefore unbound to the column. Lanes: 3-8 and 10-20, Mutant protein F172S fractions ranging from very concentrated samples (~10 mg/ml) to less concentrated ones. Samples 8 and 10-18 were pooled together and dialysed for further analysis.

5.2.2 Identification of conserved residues in the C-terminal targeting domain of lysostaphin.

Due to the fact that the Random PCR Mutagenesis method identified only one possibly critical residue in the C-terminal targeting domain of lysostaphin the targeting domain was aligned against a number of homologue proteins from other Gram-positive organisms, including several *S. aureus* amidases, *S. aureus* LytM and *S. capitis* Ale-1. The alignment indicated a number of conserved residues including the phenylalanine at position 172 of mature lysostaphin that had been mutated to serine through the Random PCR mutagenesis technique, two tyrosine residues at positions 203 and 226 and a tryptophan residue at position 214 of the mature protein.

```
1  ASF--TPNTDIITRTTGPFRSMPQSGVLKAGQTIHYDE-VMKQDGHVWVGYTGNSGQRIYLPV
2  ASF--TANTDIITRLTGPFRSMPQSGVLRKGLTIHYDE-VMKQDGHVWVGYNTNSGKRVYLPV
3  GTFTLN--TTINLRT-APFSNAPLIATLSKGQQVSYDGYGIELDGHVWIRQPRANGTYGYMAT
4  ARFT-NGNQPITVRKVGPFLSCPVGYQFQPGGYCDYTE-VMLQDGHVWVGYTWE-GQRYYLPI
5  ATFKCTARQGIVTRYTGPFTTCPQAGVLYYGQSVTYDT-VCKQDGYVWISWTTNGGQDVWMPV
6  GNYTVANVKGNNVRD-GYSTNSRITGVLPNNATIKYDG-AYCINGYRWITYIANSGQRRYIAT
7  GNYTVANVKGNNVRD-GYSTNSRITGVLPNNTTITYDG-AYCINGYRWITYIANSGQRRYIAT
```

Fig. 5.6 Multiple alignment of *S. aureus* lysostaphin (1), *S. capitis* Ale-1 (2), *Bacillus* amidase (3) and four *S. aureus* amidases (4, 5, 6 and 7). Conserved residues: phenylalanine (at position 172), tyrosine (at positions 203 and 226) and tryptophan (at position 214) in the C-terminal targeting domain of lysostaphin are highlighted in red.

5.2.3 Alanine scanning mutagenesis of the conserved residues in the targeting domain of lysostaphin.

Alanine scanning mutagenesis was carried out in order to establish the importance of the conserved amino acid residues to the biological activity of lysostaphin. Site-directed mutagenesis was thus performed to introduce alanine residues at each of the four, among others, conserved amino acids (F172, Y203, Y226, and W214) in the targeting domain of the protein. It was further decided to introduce a tyrosine residue at position 172 as well. Both phenylalanine and tyrosine are two very similar amino acids harbouring an aromatic ring (as opposed to alanine

which does not have a side chain group). Their only structural difference is the presence of an –OH group that occurs in tyrosine. Thus, mutation of phenylalanine to tyrosine would confirm the importance of the presence of an aromatic ring in the structural and functional properties of lysostaphin.

5.2.3.1 Cloning, expression and purification of mutant F172A

Plasmid pEA3 was used in a PCR reaction in order to obtain the desired lysostaphin mutant. The process was carried out in 2 stages (as described in Materials and Methods) using forward primer CNP110 (G TCA GCT AGC GCC ACA CCT AAT ACA) and reverse primer T7 terminator (CTA GTT ATT GCT CAG CGG TGG). Primer CNP110 was specifically designed in order to mutate the phenylalanine residue at position F172S to an alanine residue.

In the first stage of the PCR reaction primers CNP110 and T7 terminator were used to produce the megaprimer fragment. In the second stage PCR the product of the first reaction was used as the reverse primer and T7 promoter was used as the forward primer to produce the desired mutated fragment. The product was gel purified and restricted with *Nde*I and *Xho*I. The restricted mutated lysostaphin gene was then ligated into expression vector pET21a also restricted with *Nde*I and *Xho*I. Transformation of the ligated vector followed into *E. coli* B834 [DE3] cells to demonstrate that expression of the mature protein was IPTG inducible. Plasmid isolation showed that the desired fragment had been successfully cloned and sequencing confirmed the presence of the mutation F172A in the targeting domain of lysostaphin (pEA10). The mutant protein was purified using metal chelate chromatography and the most concentrated fractions were pooled together and dialysed overnight. However, the purification process produced significantly less concentrated samples (fig. 5.7A and B) compared to those obtained by the wild type protein and by the F172A mutant (fig. 5.5A and B), suggesting a possibly critical change in the structure and therefore the activity of lysostaphin.

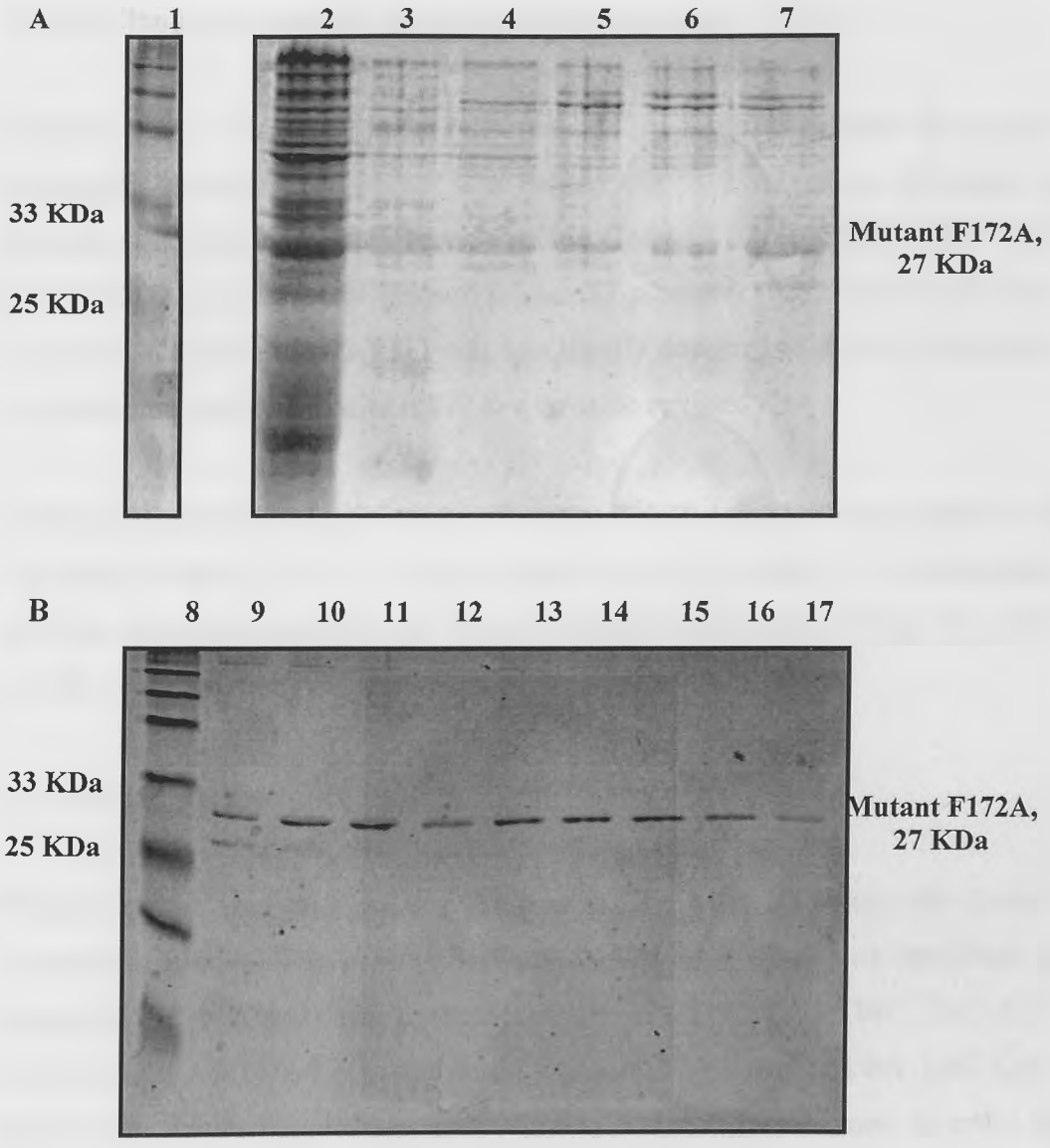

Fig. 5.7A, B 16% SDS-PAGE of total cell proteins produced by B834 (DE3) (pEA10) after 3 hr induction with 1 mM IPTG. Lanes: 1 and 8, Protein marker (83 kDa, 62 kDa, 48 kDa, 33 kDa, 25 kDa, 17 kDa and 7 kDa). Lane: 2, Flowthrough sample containing material without his-tag hence unbound to the column. Lanes: 3-7 and 9-17, Mutant protein F172A. Samples 9-17 were pooled together and dialysed for further analysis.

5.2.3.2 Cloning, expression and purification of mutant F172Y.

Plasmid pEA3 was used in a PCR reaction in order to obtain the desired lysostaphin mutant. The process was carried out in 2 stages (as described in Materials and Methods) using reverse primer CNP111 (G TCA GCT AGC TAC ACA CCT AAT ACA) and forward primer T7 promoter (TAA TAC GAC TCA CTA TAG GG). Primer CNP111 was specifically designed in order to mutate the phenyalanine residue at position 172 to a tyrosine residue.

Cloning, expression and purification of mutant F172Y (pEA11) was carried out as described in section 5.2.3.1. The purification process produced very concentrated samples (data not shown) similar to those obtained from mutant F172S (fig. 5.5A and B).

5.2.2.3 Cloning, expression and purification of mutant W214A

Plasmid pEA3 was used in a PCR reaction in order to obtain the desired lysostaphin mutant. The process was carried out in 2 stages (as described in Materials and Methods) using reverse primer CNP113 (CAT CAC TTC ATC AGC ATG AAT TGT TTG) and forward primer T7 promoter (TAA TAC GAC TCA CTA TAG GG). Primer CNP113 was specifically designed in order to mutate the tryptophan residue at position 214 to an alanine residue.

Cloning, expression and purification of mutant W214A (pEA13) was carried out as described in section 5.2.3.1. In this case, the purification process did not produce very concentrated samples (data not shown) suggesting that the substitution of tryptophan with alanine at position 214 of the mature protein has had a dramatic effect on the structure of lysostaphin.

5.2.3.4 Cloning, expression and purification of mutants Y203A and Y226A

Plasmid pEA3 was used in PCR reactions in order to obtain the desired lysostaphin mutant. The process was carried out in 2 stages (as described in

Materials and Methods) using reverse primers CNP114 (CC TGT ATA ACC TAC CGC AAC ATG ACC GTC) for mutation Y203A and CNP115 (TCT TAC AGG CAA GGC AAT ACG TTG GCC) for mutation Y226A and forward primer T7 promoter (TAA TAC GAC TCA CTA TAG GG). Primer CNP114 was specifically designed in order to mutate the tyrosine residue at position 203 to an alanine residue while primer CNP115 was designed in order to mutate tyrosine at position 226 to an alanine.

Cloning, expression and purification of mutants Y203A (pEA14) and Y226A (pEA15) were carried out as described in section 5.2.3.1. The mutant proteins were purified using metal chelate chromatography and the most concentrated fractions of each mutant were pooled and dialysed overnight. The purified fractions of both these proteins were very concentrated (data not shown) equal to those obtained by purification of mutant F172S (fig. 5.5A and B).

5.2.4 Stab test with lysostaphin mutants against EMRSA-16

A stab test with all lysostaphin mutants was carried out immediately after the cloned mutants were expressed and shown to produce full size lysostaphin. Mutants that do not inhibit the growth of EMRSA-16 are those of interest because the full size lysostaphin they produce has become inactive as a result of the mutational changes that occurred by the two mutagenesis methods used in this study.

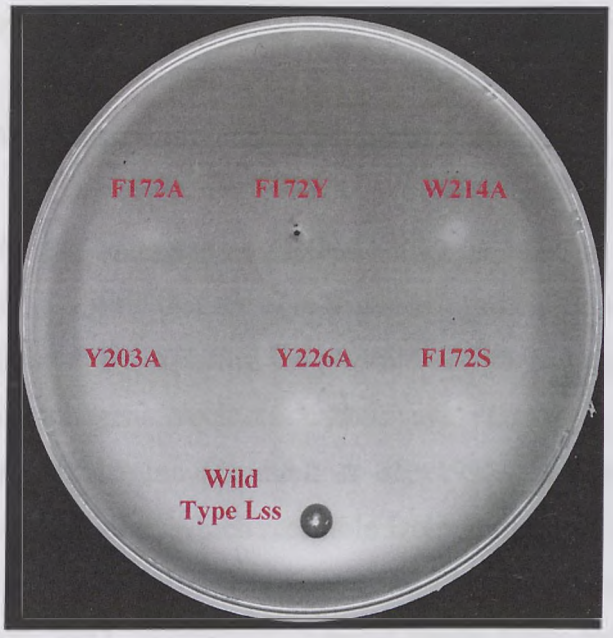

Fig. 5.8 Stab test carried out with all lysostaphin mutants and wild type protein with EMRSA-16 as the overlay, indicating that the mutants did not produce zones of inhibition, similar to those produced by wild type lysostaphin, which is indicated at the bottom of the plate.

E. coli B834 [DE3] expressing mutants F172A, F172Y, W214A, Y203A, Y226A F172S and wild type lysostaphin were stabbed onto an LB plate and left to grow at 37°C overnight. The plate was exposed to chloroform and then overlayed with EMRSA-16. The results (fig. 5.8) suggested that the cloned mutants were all inactive against EMRSA-16 because they did not produce any zones of inhibition against the pathogen. If the mutants were active then they should have produced zones of inhibition similar to those produced by the wild type protein. Therefore this is a first indication that the cloned mutants produce an inactive lysostaphin as a result of the mutational changes in the lysostaphin T-domain.

5.3 DISCUSSION

5.3.1 Random PCR mutagenesis using chemical mutagens

A number of different mutagenesis methods may be used in order to identify important residues in a gene that are able to abolish protein function. One way to cause such mutations is by exposing the plasmid carrying the gene of interest to various chemical mutagens such as nitrous acid (HNO_2), sodium bisulfite ($NaHSO_3$) and hydroxylamine (Fromant *et al.,* 1995). A variety of chemical mutagens are base analogues, which resemble DNA purine and pyrimidine bases in structure yet showing faulty pairing properties. When one of these base analogs is incorporated into DNA, occasional copying errors occur and as a result the wrong base is incorporated into the copied strand. For example, nitrous acid deaminates adenine to hypoxanthine, a base analogue that resembles guanine. Hypoxanthine may thus pair up with cytosine, which will finally pair with guanine, eventually resulting in an A to G mutation (Voet and Voet., 1995). On the other hand, hydroxylamine, converts cytidine to hydroxyaminocytidine, a base analogue of thymine. Thus, hydroxyaminocytidine will eventually pair with adenine, resulting in a C to T mutation. Similarly, hydroxylamine may cause G→A mutations in double stranded DNA (Busby *et al.*, 1982). However, in all cases, PCR chemical mutagenesis produces a certain range of mutations depending on the chemical properties.

5.3.2 Assessment of the mutation frequency obtained by random PCR mutagenesis by using dITP in the PCR reactions

In order to be able to identify a wide range of putative active site residues in the C-terminal targeting domain of lysostaphin it was decided to use the method of random PCR mutagenesis. Sequencing of the resulting plasmids obtained from random PCR mutagenesis revealed two mutations located within two different plasmids. Thus, two out of a total of seventy screened colonies produced inactive mutant proteins, which corresponded to a mutation frequency of approximately 3%. Zhou and co-workers (1991) have reported that for their 633 nucleotide gene,

11% of their mutagenised transformant clones were phenotypically mutant. Therefore, the 3% mutant frequency that was achieved in this study with the 245 amino acid long lysostaphin is in good agreement with the mutant frequency described by Zhou *et al.* (1991).

The 3% mutant frequency that was achieved in this study with lysostaphin corresponded to two clones each harbouring a different mutation in the T-domain. One of the two mutants had a silent mutation (at position P152 of the full size gene, from CCA to CCC) but did not produce full size lysostaphin while in the other mutant clone, codon TTC had been changed to TCC at position 172 of mature lysostaphin and the gene expressed the full size protein. As a result phenylalanine had been replaced by serine at that position due to the T→C mutation. It has been suggested that substitution of A and T residues with G and C nucleotides, appears to be favoured during the Random PCR mutagenesis method (Bloch, 1991). Similar observations have been made by Tindall and Kunkel (1988). In their attempt to determine the fidelity of DNA synthesis with Taq DNA polymerase, they found that the lack of exonuclease activity of the enzyme resulted in A +T to G + C mutations. Furthermore, experiments carried out by Eckert and Kunkel (1990) indicated that the fidelity of Taq DNA polymerase responds strongly to changes in $MgCl_2$ concentration and pH. More specifically, the authors reported an increase in base substitution frequency from 40 x 10^{-6} when 1 mM $MgCl_2$ was used in a PCR reaction to 340 x 10^{-6} when 20 mM $MgCl_2$ were used. Furthermore, in the case of pH, the same authors reported that the more acidic during a PCR reaction the higher the fidelity of DNA synthesis by Taq polymerase. For example, at pH values ranging from 5-6 low mutant frequencies were observed, varying from 14-66 x 10^{-6}. Further experiments have been carried out by Fromant and co-workers (1995) who varied the concentration of manganese ions and found out that the fidelity of Taq DNA polymerase was decreased when the concentration of manganese ions was increased. Also, Tindall and Kunkel (1988) have reported that in the presence of increased amounts of manganese ions the range of mutations caused by Taq DNA polymerase was much more diverse than the AT → CG substitutions that are usually observed under standard PCR conditions.

Spee *et al.*, (1993) also produced similar results when performing Random PCR mutagenesis with the 171 bp *Lactococcus lactis nisZ* gene, with 60% of the mutations obtained being either A to G or T to C mutations (with only 26% of G to A and C to T mutations). Also, it has been claimed (Kuipers, 1996) that the yield of T and C depleted reactions are frequently lower than that of A and G depleted reactions, due to the lower pairing ability of dITP with A and G residues of the template DNA. Further to this, it has been reported that the lower the yield of the PCR product in a depleted (of dNTPs) reaction the higher the mutation frequency will be (Kuipers, 1996). These three suggestions taken together indicate that the mutation range obtained with the Random PCR mutagenesis method is also somewhat restricted even though to a smaller extent than the hydroxylamine mutagenesis method that almost exclusively result in A or T residues (Busby, *et al.*, 1982).

5.3.3 Site-directed mutagenesis of conserved amino acids to alanine residues

In order to be able to achieve a more diverse range of mutations it was decided to carry out Site-directed mutagenesis to replace the desired residues with residues of choice, namely alanine. Thus, the conserved residues that were identified after multiple sequence alignment as well as the residue that was substituted with serine (at position 172) after Random PCR mutagenesis were all mutated to alanine in order to find residues that are critical for the activity of the lysostaphin targeting domain. The purification process indicated that mutants F172S, F172Y, Y203A and Y226A gave soluble proteins while mutants W214 and F172A did not produce very concentrated yields. Similar experiments were recently carried out by Odintsov and co-workers (2004) with LytM. The LytM protein together with lysostaphin belong to a group of metalloproteases (Park, *et al.*, 1995) with a characteristic HxH motif (Hooper, 1994) that occurs in the endopeptidase domain of lysostaphin. Similarly, in this study, the group attempted to identify functional roles for several conserved amino acid residues by substituting them with alanine. One of these residues included a histidine at position 291. This amino acid occured within this characteristic HxH motif and was found in many lysostaphin-

type peptidases, which suggests that the underlying histidine may play a very important role in the function of the protein. Further to this, another three residues, H210, D214 and H293 of the full length LytM were mutated to alanine because they were found to be conserved in lysostaphin-type sequences and were therefore suspected to have critical roles in the activity of the LytM protein. The four mutant proteins were expressed and purified. All purified proteins were soluble apart from H293 that was insoluble. This insolubility indicated that the mutation of histidine to alanine at position 293 of mature LytM affected significantly the folding of the protein, abolishing its function. Further tests were carried out in order to confirm the role of each mutant protein. The results indicated that all of the mutants were inactive, even though H293A had produced soluble and very concentrated protein, suggesting the importance of the original residues in the structure and function of the LytM protein.

In this study, all six mutants proteins were also subjected to further tests in order to confirm their importance in the structure and function of lysostaphin. A stab test was carried out to assay the mutants for lysostaphin production and activity against EMRSA-16. The plate (fig. 5.8) was examined 24 h later, however no zones of inhibition were observed for any of the mutants. This absence of bacteriolytic activity suggested the importance of performing further tests to assess these mutants and investigate the reasons behind their lack of activity against EMRSA-16. It was suspected that the mutations have affected the receptor binding of lysostaphin to the cell walls as the mutations were all located in the T-domain. Thus, a spot test, a turbidity assay and the FRET assay would be carried out in order to investigate the effect of these mutations on the activity of the proteins. These tests would also reveal, to what extent the activity of each mutant had been affected compared to the wild type lysostaphin.

CHAPTER: 6

BIOLOGICAL ACTIVITY OF THE LYSOSTAPHIN C-TERMINAL TARGETING DOMAIN MUTANTS.

6. 1 INTRODUCTION

The two mutagenesis methods, described in the previous chapter, produced six lysostaphin mutants, which were tested for staphylolytic activity with a stab test and showed no zones of inhibition as opposed to the wild type protein. In this chapter, the mutants were tested with three more methods, in order to obtain more specific information about their activity, using wild type lysostaphin as the control. The turbidimetric assay, the spot test and the fluorescence resonance energy transfer (FRET) method were used to evaluate the cleavage activity of each mutant and enable us to comment on the consistency of the results produced by the three assays and draw conclusions about the importance of the effect of the various mutations on the activity of the mutant lysostaphin. Finally, a reversion experiment was carried out with mutant F172A in order to confirm the importance of the phenylalanine residue at position 172 of mature lysostaphin.

6.1.1 Turbidimetric method for detection of lysostaphin activity

Evaluation of the activity of a protein against a microorganism may be determined by means of turbidimetry. This method is based on recording the changes in turbidity of a suspension of test bacteria after addition of the enzyme as compared with the turbidity of the bacterial suspension in the absence of the protein (Marova and Kovar, 1993). Turbidity may be measured by means of spectrophotometry and fluorimetry. Turbidimetric determination with the aid of a spectrophotometer is the most popular method for detection of turbidity changes in microbial suspensions between 600 and 650 nm, due to its accessibility and accuracy.

The method of turbidimetry has been employed by Iversen and Grov (1973) in order to determine the activity of lysostaphin. Robinson and colleagues (1979) used the turbidity test to investigate the endopeptidase activity of lysostaphin against the cell wall of its producing organism. Finally, Marova and Kovar (1993) have reported spectrophotometric detection of the bacteriolytic activity of diluted lysostaphin solutions against *S. aureus* cells, by recording changes in turbidity

Here, the turbidity test that was utilised in order to measure the bacteriolytic activity of lysostaphin and its mutants against EMRSA-16 was carried out according to Kessler *et al.* (1997) who used the technique to measure the staphylolytic activity of the protein LasA, produced by *Pseudomonas aeruginosa*. The results of the effects of the mutations in the targeting domain of lysostaphin on the cleavage specificity of the mutant and wild type enzymes were compared for evaluation of the impact of the mutation.

6.1.2 The agar diffusion assay

The agar diffusion assay is a quick and easy way to detect antibacterial activity or lack of it in any mutants by comparing their activity with that of the wild-type protein. Pre-defined aliquots of serially diluted protein antibiotic are spotted onto a freshly overlaid lawn of a sensitive indicator strain which are left to grow for a period of time at the required incubation temperature. Zones of clearance in the vicinity of the spot and the surrounding area represent cell death. Resistence to the protein antibiotics is shown by the absence of any zones of inhibition. The protein titre is the lowest concentration of protein that produces a clear zone of inhibition. The spot test has been successfully used elsewhere to assess the structure to function relationships of other antibacterial proteins and their mutants. In an attempt to investigate the mechanism of action of the receptor binding domain of the protein antibiotic colicin E9 against sensitive *E. coli*, Penfold *et al.* (2004) engineered disulphide bonds across both arms of the helical hairpin of the receptor binding domain of colicin E9. The spot test was used to show that the disulphide bonds rendered the mutated colicin inactive as a result of its 'locked' conformation. In another study, Wallis *et al.* (1995) used the spot test assay to test the specificity of the cognate Im9 and non-cognate Im2, Im6, and Im8 immunity proteins for the colicin E9 DNase domain. Im9 offered full protection against the DNase domain of colicin E9 to the sensitive indicator strain as shown by no zones of inhibition whereas zones of inhibition produced by the colicin E9 DNase domain in the presence of the non-cognate immunity proteins showed that they were unable to completely neutralise the toxic effects of the DNase domain. The spot test data was also consistent with subsequent data gained on the affinities of

binding of the cognate and non-cognate immunity proteins for the ColE9 DNase domain.

6.1.3 The Fluorescence Resonance Energy Transfer (FRET) assay

One of the most important challenges for modern molecular and cellular biology is to understand the complex interactions that take place within a living cell during normal and abnormal growth. For instance, in order for extracellular messages to be transferred from the cell membranes to the cell nucleus, various signal transduction pathways must intervene, the ultimate result being the activation of cellular functions. These pathways involve a number of coordinated and sequential interactions of many macromolecules, which may include protein-protein and protein-DNA interactions. In the past, protein-protein interactions were studied with standard biochemical techniques. However, more sophisticated and analytical assays are required if one wishes to examine these interactions within the living cell. One such approach is Fluorescence Resonance Energy Transfer (FRET), which has become increasingly popular due to its vast number of applications in many areas including structure and conformation of proteins receptor/ligand interactions and enzyme kinetics (Selvin, 2000).

6.1.3.1 Fluorescence

Fluorescence occurs when a molecule is excited by light of a specific wavelength and emits light at a longer wavelength. Molecules have specific spectra that describe the range of light that excites them and the range over which they emit light. For example, due to the presence of tryptophan and tyrosine residues, when lysostaphin is excited at a wavelength of 310 nm or lower it yields increasing fluorescence at 420 nm, while at wavelengths of 320 nm or higher no light is emitted by these amino acids. The observed intensity of fluorescence is determined by the number of molecules emitting light within a given time period. Intensity is dependent upon concentration of fluorescent molecules in a sample, as well as upon factors that affect the rate of emission of individual molecules, such as the solvent.

6.1.3.2 Mode of action of the FRET assay

Fluorescence Resonance Energy Transfer (FRET) occurs when a "donor" (fluorophore) molecule has an emission spectrum that overlaps the excitation spectrum of an "acceptor" (quencher) molecule, and these two molecules are within a distance of 10-100 Å (or up to 10 nm) of one another (fig. 6.1). Under these conditions and when excited by incident light the donor molecule will transfer the excited state energy to the acceptor. This leads to a reduction in the donor's fluorescence intensity and excited state lifetime, and an increase in the acceptor's emission intensity (Selvin, 2000). The FRET assay has been used in many studies that require the development and characterization of specific substrates for enzymes such as cysteine and serine proteases (Melo *et al.*, 2001) and the enzyme cruzipain from *Trypanosoma cruzi* (Del Nery *et al.*, 1997).

In the present study, the use of a Perkin Elmer LS50b fluorimeter (running software; Luminescence Spectroscopy: FL Winlab) allowed examination of the ability of lysostaphin to cleave a FRET substrate. This method has several advantages. It is very fast and convenient requiring only small amounts of samples. Furthermore, it is a direct assay that can measure fluorescence very accurately and objectively, providing real-time information and continuous measurements that allow one to follow kinetics of the desired protein. However, it is important to make sure that the fluorescence readings are not impaired by the presence of molecules other than the substrate that absorb or fluoresce light.

Quenched fluorescence peptides have been previously synthesized and used as substrates in order to perform FRET assays. In these peptides, the energy transfer from the donor to the acceptor, which are separated by a peptide chain, occurs either intermolecularly if cleavage takes place at either ends of the peptide chain or intramolecularly if digestion takes place within the peptide chain, leaving each end of the substrate attached to a number of amino acids (Gershkovich and Kholodovych, 1996). Examples of enzymes that cleave the peptide chain intermolecularly are proteases such as Angiotensin I-Converting enzyme (Araujo *et al.*, 2000). Experiments involving quenched fluorescence peptides have been

carried out with proteases that cause cleavage intramolecularly as well. Melo and colleagues (2001) have reported the synthesis and hydrolysis of such peptide substrates by cysteine and serine proteases. In this study, the substrate was chemically synthesized (by Rachel Warfield, School of Chemistry) and consisted of 5 glycines, a donor fluorophore attached to the N-terminal glycine, called ortho-aminobenzoic acid (Abz), and an acceptor or quenching fluorophore located at the C-terminal glycine residue, called N-(2,4-dinitrophenyl)ehtylenediamine (EDDnp). Fluorescence appeared when Abz and EDDnp were separated by hydrolysis of one of the peptide bonds between the amino acids (fig. 6.1).

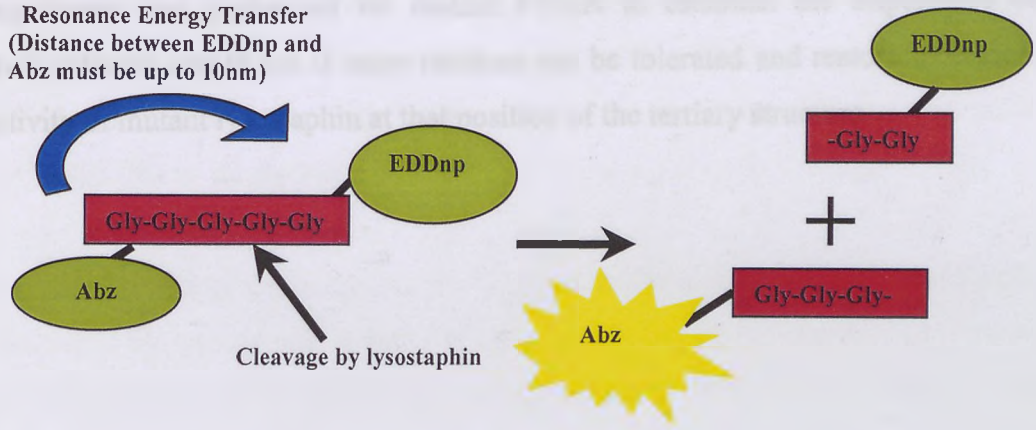

Fig. 6.1 Diagram indicating the resonance energy transfer of the Abz component of the Abz-Gly5-EDDnp substrate to the EDDnp component, and subsequent loss of quenching of the fluorescence after cleavage by lysostaphin and the active mutants. An important condition for FRET to occur is for the two fluorophores to be within a distance of 10 nm/100 Å.

These particular donor and acceptor fluorophores were chosen because of their FRET efficiency and their suitability for the pH 7.5 optimum for lysostaphin endopeptidase activity. The Abz group is a very popular donor because of its high hydrophilicity, its small size and high quantum yield. Gershkovich and Kholodovych (1996) have synthesized peptide substrates using the Abz group as the donor and other groups including EDDnp as the acceptors. The results indicated that the increase in fluorescence intensity by EDDnp after complete

hydrolysis of the substrate was 8.5 times higher than with the other acceptors. The reason for this is the significant overlap between the emission spectrum of Abz and the absorption spectrum of EDDnp.

6.1.4 Aims

The aims of this chapter were to assess the impact of the mutations introduced in the targeting domain of lysostaphin on its ability to locate the target substrate so that cleavage can then occur by the endopeptidase domain. The mutants were assayed by a spot test, a turbidity and a FRET assay. Finally, a reversion experiment was performed for mutant F172A to establish the importance of phenylalanine and to see if other residues can be tolerated and restore biological activity of mutant lysostaphin at that position of the tertiary structure.

6.2 RESULTS

6.2.1 Turbidity assays with the mutant lysostaphin proteins.

Turbidity assays were carried out as described in section 2.17 with lysostaphin and its mutants. Three micromoles of the purified mutant lysostaphin proteins and lysostaphin were used to challenge EMRSA-16 cells. The turbidity of the cells alone was monitored for 5 minutes at an OD_{600}. After the first 5 minutes, mutant/wild type lysostaphin was added and the changes in the turbidity of the cells were monitored over a period of 15 minutes. The activity of each of the mutant proteins was indicated by the decrease in the OD_{600} readings and compared to those obtained by wild type lysostaphin. All proteins were run on an SDS-PAGE immediately prior to the experiment and their full-sized presence confirmed that no degradation had taken place during dialysis the night before.

According to the graph in figure 6.2, the drop in absorbance of EMRSA-16 cells challenged by lysostaphin mutant F172A is almost negligible compared to that of EMRSA-16 cells challenged by the wild type protein. For the first 5 minutes, the turbidity of EMRSA-16 cells remained stable (OD_{600} ~ 0.8-0.9) in both cases, however, upon addition of wild type lysostaphin the absorbance readings decreased dramatically and dropped to OD_{600} ~0.14 as opposed to those obtained after addition of the mutant protein, which did not fall below an OD_{600} ~0.6. Therefore, it may be concluded that lysostaphin mutant F172A does not affect the turbidity of EMRSA-16 and is inactive against EMRSA-16 cells.

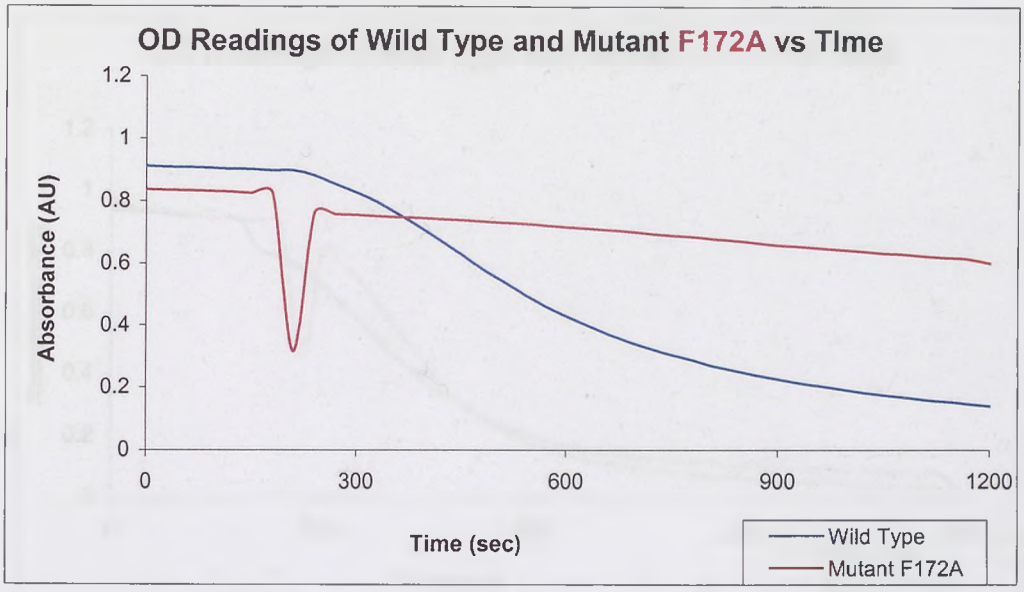

Fig. 6.2 Turbidity assay indicating the changes of OD_{600} readings of EMRSA-16 cells over a time period of 1200 sec in the presence of mutant protein F172A (red) and lysostaphin (blue).

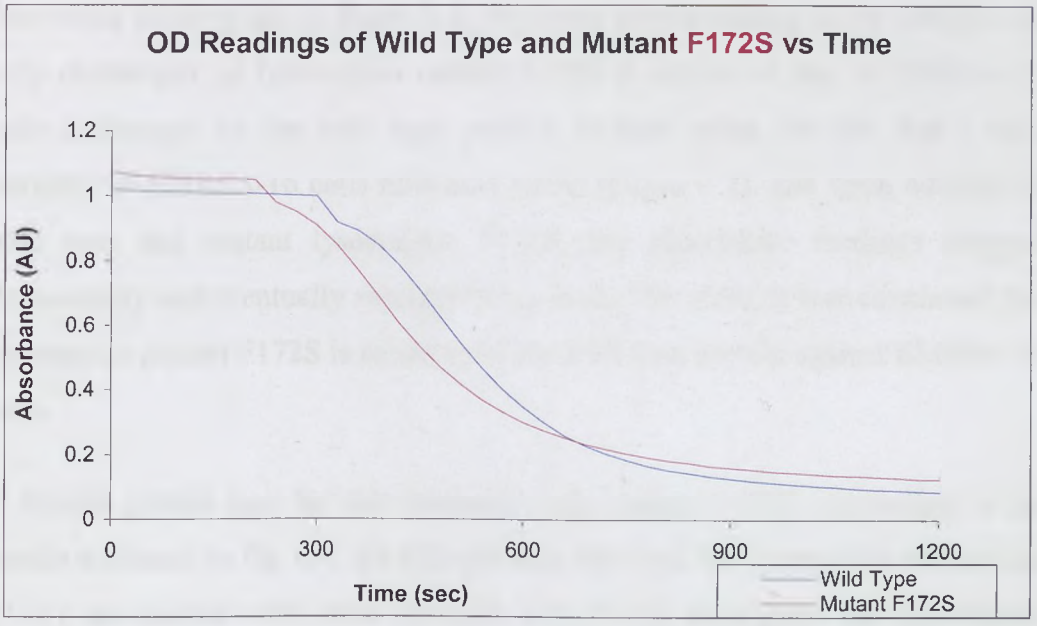

Fig. 6.3 Graph indicating the changes of OD_{600} readings of EMRSA-16 cells over a time period of 1200 sec in the presence of mutant protein F172S (red) and lysostaphin (blue).

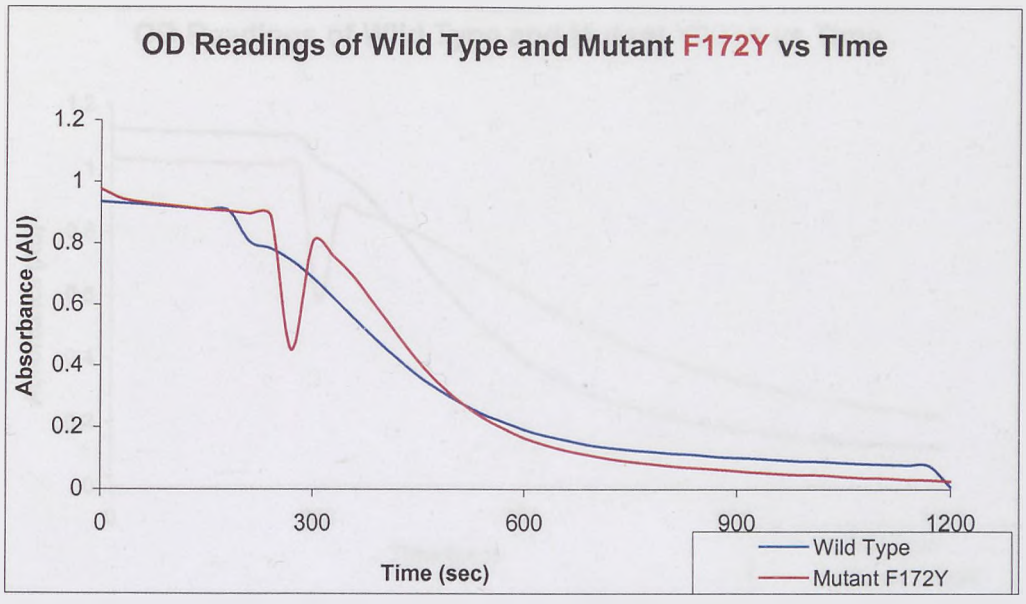

Fig. 6.4 Graph indicating the changes of OD_{600} readings of EMRA-16 cells over a time period of 1200 sec in the presence of mutant protein F172Y (red) and lysostaphin (blue).

According to the graph in figure 6.3, the curve corresponding to the EMRSA-16 cells challenged by lysostaphin mutant F172S is similar to that of EMRSA-16 cells challenged by the wild type protein. In both cases, for the first 5 min, turbidity of EMRSA-16 cells remained stable ($OD_{600} \sim 1$), and upon addition of wild type and mutant lysostaphin F172S, the absorbance readings dropped dramatically and eventually reached $OD_{600} \sim 0.1$. Therefore, it was concluded that lysostaphin mutant F172S is as active as the wild type protein against EMRSA-16 cells.

A similar picture may be also observed with mutant F172Y. According to the results indicated in fig. 6.4, the OD readings obtained for lysostaphin and mutant F172Y are similar with those obtained with F172S throughout the experiment. Therefore, it was concluded that lysostaphin mutant F172Y is as active as the wild type protein against EMRSA-16 cells.

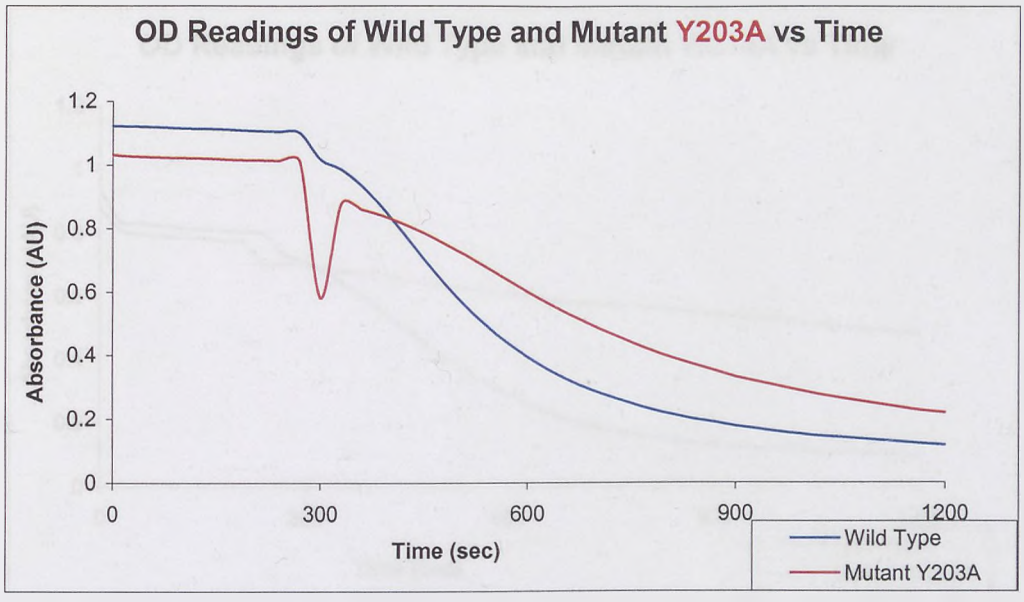

Fig. 6.5 Graph indicating the changes of OD_{600} readings of EMRA-16 cells over a time period of 1200 sec in the presence of mutant protein Y203A (red) and lysostaphin (blue).

According to the graph in figure 6.5, there is slight difference between the bacteriolytic activity of mutant protein Y203A and that of the wild type protein. Even though the OD readings indicate that there is activity in the case of the mutant, this is limited and not as efficient as that of wild type lysostaphin, because the two curves do not overlap at all, which was the case with F172S and F172Y. The final OD_{600} readings for mutant Y203A and wild type were approximately 0.1 and 0.2 respectively, indicating a dramatic reduction in the absorbance values, which suggests that there is definitely some killing activity against the EMRSA-16 cells.

A similar picture to that of mutant F172A may be also observed with mutant W214A (fig. 6.6). For the first 5 min. turbidity of EMRSA-16 cells remained stable ($OD_{600} \sim 0.9$) in the case of both wild type and mutant proteins, however, upon addition of the former the absorbance readings dropped dramatically and reached $OD_{600} \sim 0.1$ as opposed to those obtained after addition of the mutant protein, which reached $OD_{600} \sim 0.5$. Therefore, it may be concluded that lysostaphin mutant W214A is significantly less active than the wild type against EMRSA-16 cells.

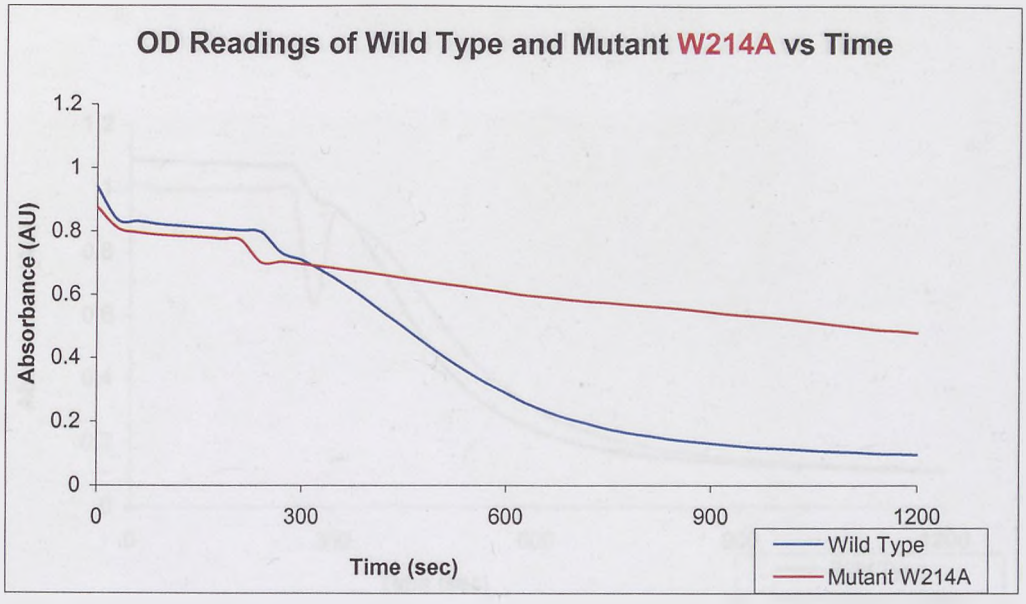

Fig. 6.6 Graph indicating the changes of OD_{600} readings of EMRA-16 cells over a time period of 1200 sec in the presence of mutant protein W214A (red) and lysostaphin (blue).

Finally, the activity of mutant Y226A is similar to that of F172Y and F172S, according to the graph in fig. 6.7. The OD readings of mutant Y226A and wild type lysostaphin are similar throughout the experiment and in the end the two curves overlap, indicating that the mutant achieved the same turbidity OD readings as lysostaphin. Therefore, the bacteriolytic activity of the Y226A mutant protein against EMRSA-16 cells is as efficient as that of wild type lysostaphin.

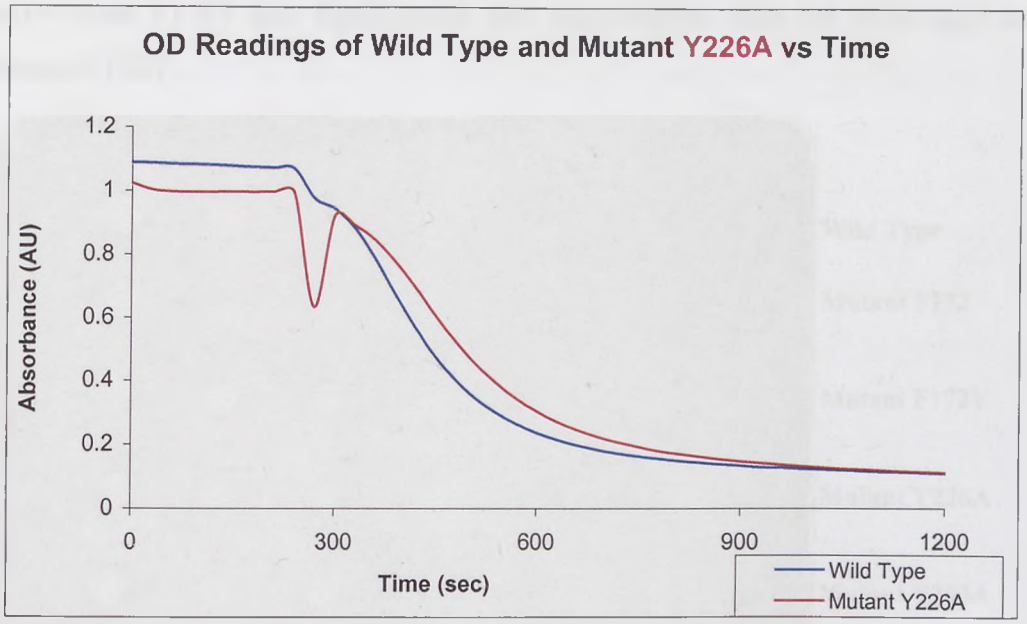

Fig. 6.7 Graph indicating the changes of OD_{600} readings of EMRA-16 cells over a time period of 1200 sec in the presence of mutant protein Y226A (red) and lysostaphin (blue).

6.2.2 Spot test

A spot test was carried out with purified wild type lysostaphin and its targeting domain mutant proteins to examine their activity against EMRSA-16 cells. The results and the various concentrations that each protein was tested at are shown in fig. 6.8. It was observed that wild type lysostaphin produced clear zones of inhibition at concentrations higher than 2.5 μM. At lower concentrations hazy zones were produced while at 0.5 μM it appeared that the protein is completely inactive. Mutant protein F172S was slightly less active than the wild type. Clear zones of inhibition were observed at concentrations 20 μM, 10 μM, 5 μM and 4 μM while at 2.5 μM a hazy zone was produced and at 1 μM and 0.5 μM the mutant protein showed no activity against EMRSA-16. Mutant F172Y appeared more active than F172S; the protein exhibited similar activity to that of the wild type and thus at concentrations of 2.5 μM and higher F172Y produced clear zones of inhibition. In the case of mutant Y226A, clear zones of inhibition were observed at concentrations higher than 5 μM. Thus, mutant Y226A is slightly less

active than F172S and significantly less bacteriolytic than the wild type and mutant F172Y.

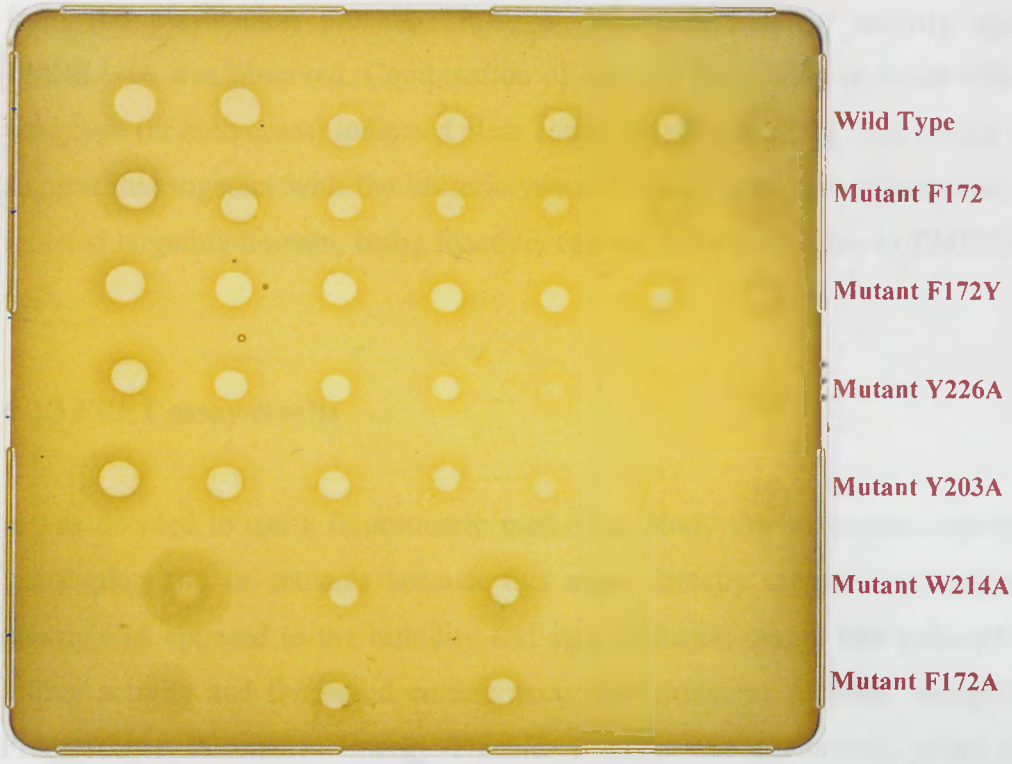

Fig. 6.8 Spot test indicating the activity of wild type lysostaphin and its mutants. The concentrations used for wild type and mutants: F172S, F172Y and Y226 are as follows (from left to right): 20 μM, 10 μM, 5 μM, 4 μM, 2.5 μM, 1 μM 0.5 μM. For Y203A (from left to right): 30 μM, 20 μM, 15 μM, 10 μM, 5 μM, 3 μM, 1 μM. For W214A (from left to right): 43 μM mutant protein, 2.5 μM wild type, wild type and mutant at concentrations 2.5 μM and 43 μM respectively. For F172A (from left to right): 108 μM mutant protein, 2.5 μM wild type, wild type and mutant at concentrations 2.5 μM and 108 μM respectively.

A similar picture may be observed with mutant Y203A, which was tested at slightly different concentrations (30 μM, 20 μM, 15 μM, 10 μM, 5 μM, 3 μM and 1 μM). The results showed that mutant protein Y203A produced clear zones at concentrations of 10 μM or higher and a hazy zone at 5 μM while at concentrations 3 μM and 1 μM the mutant is completely inactive against EMRSA-16. Therefore, this mutant is even less active than mutant Y226A.

Mutants W214A and F172A were tested undiluted at concentrations 43 μM and 108 μM respectively, which represent the most concentrated samples obtained from the purification process. However, complete lack of activity against EMRSA-16 was observed. Combination of each of the mutant proteins with the wild type (in both cases) indicated clear zones of inhibition (fig. 6.8), which may suggest that together with the bacteriolytic activity of wild type lysostaphin, the mutated targeting domain, being inactive, can not offer protection to EMRSA-16 cells.

6.2.3 FRET assay results

It was decided to use a fluorometric method to study the enzymatic activity of lysostaphin and its mutants because this assay directly measures pentaglycine cleavage as opposed to the turbidity and agar diffusion assays that measure cell killing activity and the liquid culture assay that measures receptor recognition. Fluorescence Resonance Energy Transfer (FRET) was performed, using Abz-Gly5-EDDnp as the substrate, which is however, highly insoluble. Thus, in order to use it, Abz-Gly5-EDDnp had to be diluted in pure DMSO. However, DMSO can affect the structure and hence the activity of lysostaphin. In order to measure cleavage of the substrate where DMSO does not effect lysostaphin activity and the substrate is high enough for the assay, 10 μM Abz-Gly5-EDDnp substrate were used in ~2% DMSO and buffer A (50 mM Tris pH 7.0, 150 mM NaCl) or buffer B (50 mM sodium-phosphate pH 7.0, 150 mM NaCl). These two buffers were used in the FRET assay because they had been also used during the turbidity assay where we demonstrated that lysostaphin is able to lyse *S. aureus* cells. Buffers A and B were chosen for the turbidity assay in accordance with Schindler (1965) and Trayer *et al.* (1970) because of their similar composition to LB agar; both are NaCl-based, a requirement for lysostaphin to be able to lyse *S. aureus* cells.

An experiment was initially performed in order to decide which of the two buffers (A or B) should be used with the substrate and DMSO. The fluorescence of the two buffers was thus compared and it was observed that buffer B did not fluoresce at all unlike buffer A which would have masked the fluorescence of Abz once

released from the quenching effect of EDDnp. Therefore buffer B was used for dilutions with the Abz-Gly5-EDDnp substrate.

It was then decided to examine the fluorescence of 1 μM lysostaphin at different excitation wavelengths in order to determine which excitation wavelength was more suitable for the most reliable fluorescence measurements. Excitation was performed at wavelengths of 300, 310, 320, 330 and 340 nm. The results in fig 6.9 indicated that excitation of lysostaphin at 310 nm or lower (300 nm) yielded increasing fluorescence as expected, due to the presence of tryptophan and tyrosine residues while excitation at 320 nm or higher wavelength (330, 340 nm) yielded essentially no fluorescence. DMSO that was required for the solubility of the substrate, yielded fluorescence when excitation wavelength approached 350 nm. It was therefore decided that excitation at 325 nm was most suitable for fluorescence measurements.

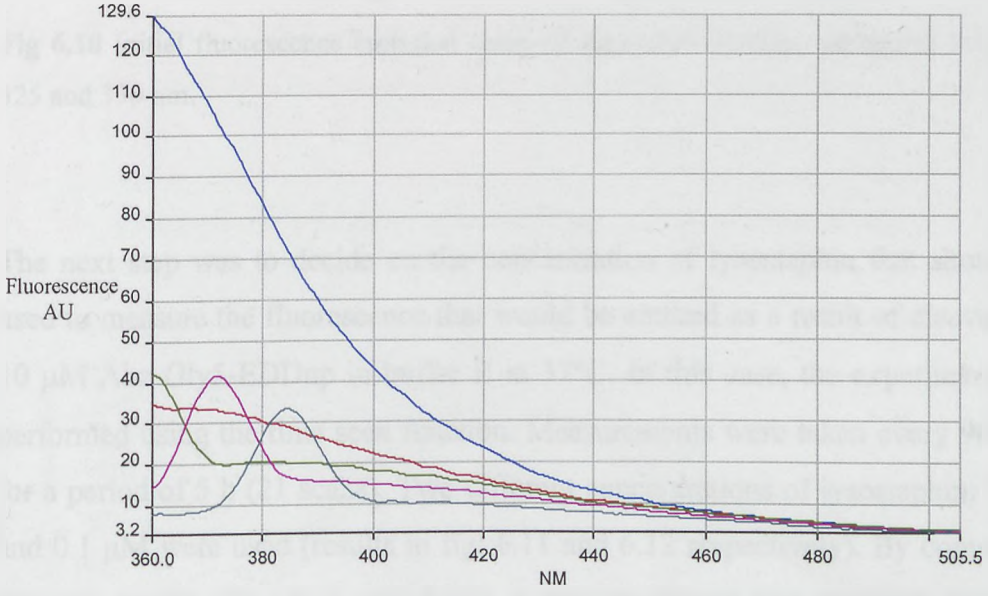

Fig 6.9 Initial fluorescence scans of lysostaphin at 300 (blue), 310 (orange), 320 (green), 330 (pink) and 340 (navy) nm.

In order to examine the fluorescence of Abz-Gly5-EDDnp diluted in DMSO, when excited at different wavelengths, it was decided to excite the substrate at

310, 320, 325 and 330 nm. There were only slight differences between the different excitation wavelengths and therefore an excitation wavelength at 325 nm could also be used with the substrate (fig. 6.10).

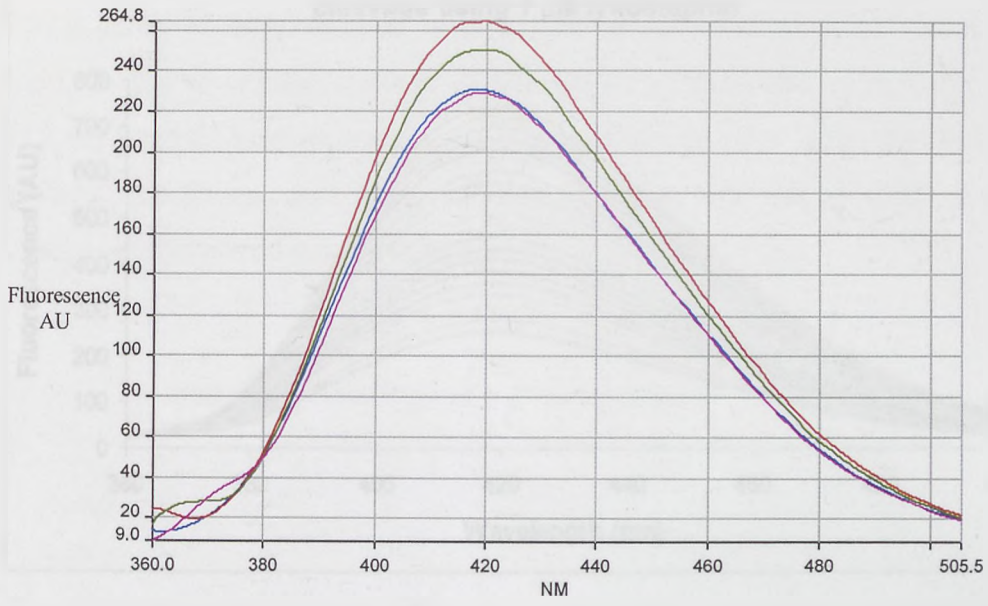

Fig 6.10 Initial fluorescence emission scans of Abz-Gly5-EDDnp, excited at 310, 320, 325 and 330 nm.

The next step was to decide on the concentration of lysostaphin that should be used to measure the fluorescence that would be emitted as a result of cleavage of 10 μM Abz-Gly5-EDDnp in buffer B at 37°C. In this case, the experiment was performed using the time scan function. Measurements were taken every 900 sec for a period of 5 h (21 scans). Two different concentrations of lysostaphin: 1 μM and 0.1 μM were used (results in fig: 6.11 and 6.12 respectively). By comparing the two graphs (fig.: 6.11 and 6.12), it was confirmed that substrate cleavage activity was dependent upon the concentration of the enzyme added and it was observed that the use of the higher concentration of lysostaphin (1 μM) resulted in considerably more substrate cleavage. Also, the mutant proteins were expected to either show the same or less activity than wild type lysostaphin. Therefore, by assaying the mutants at the higher concentration of 1 μM it was made possible to observe cleavage equal to and down to one tenth the activity of wild type

lysostaphin. Hence it was decided to perform the FRET assay with 1 μM of protein.

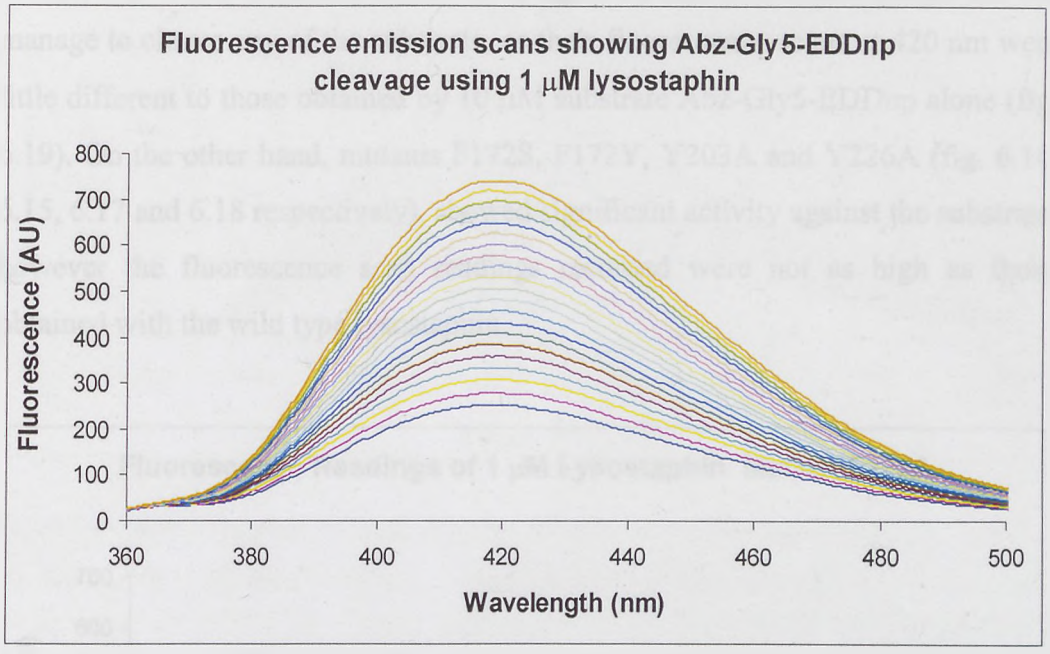

Fig. 6.11 Graph indicating cleavage activity of 1 μM lysostaphin against 10 μM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

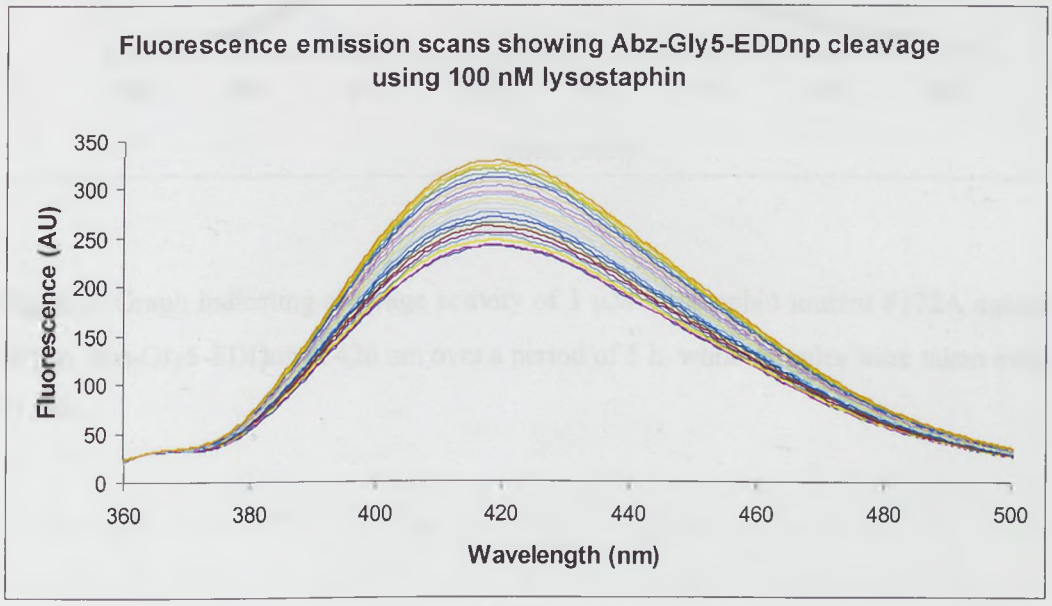

Fig. 6.12 Graph indicating cleavage activity of 0.1 μM lysostaphin against 10 μM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

The same experiment was repeated for the lysostaphin targeting domain mutants again using 1 μM of each protein. The results indicated that mutant proteins F172A and W214A (results indicated in fig. 6.13 and 6.16 respectively) did not manage to cleave any of the substrate, as their fluorescence scans at 420 nm were little different to those obtained by 10 μM substrate Abz-Gly5-EDDnp alone (fig. 6.19). On the other hand, mutants F172S, F172Y, Y203A and Y226A (fig. 6.14, 6.15, 6.17 and 6.18 respectively), showed significant activity against the substrate, however the fluorescence scan readings recorded were not as high as those obtained with the wild type lysostaphin.

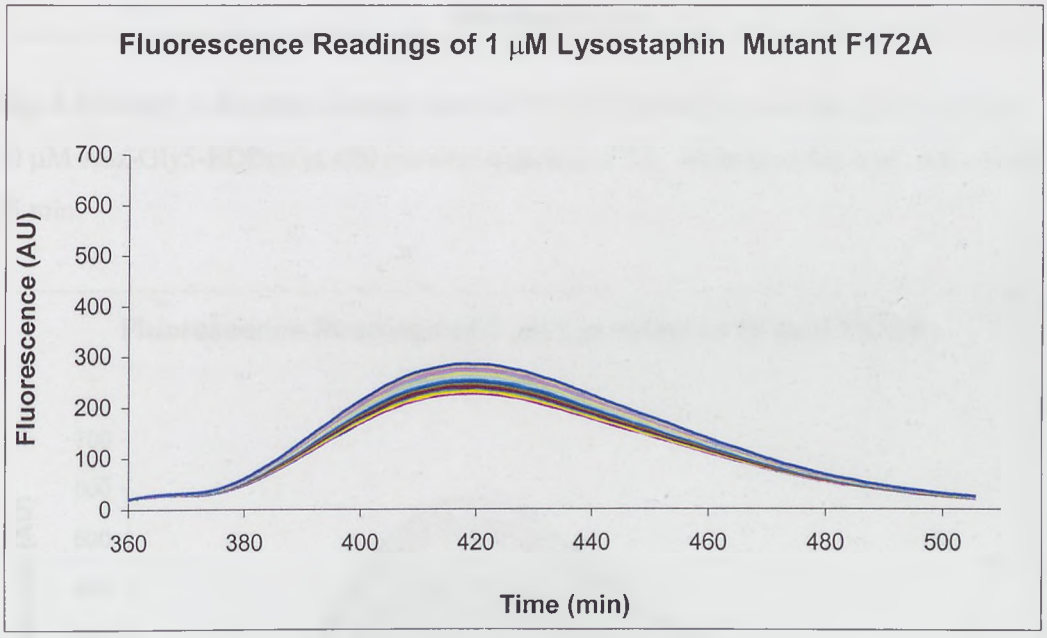

Fig. 6.13 Graph indicating cleavage activity of 1 μM lysostaphin mutant F172A against 10 μM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

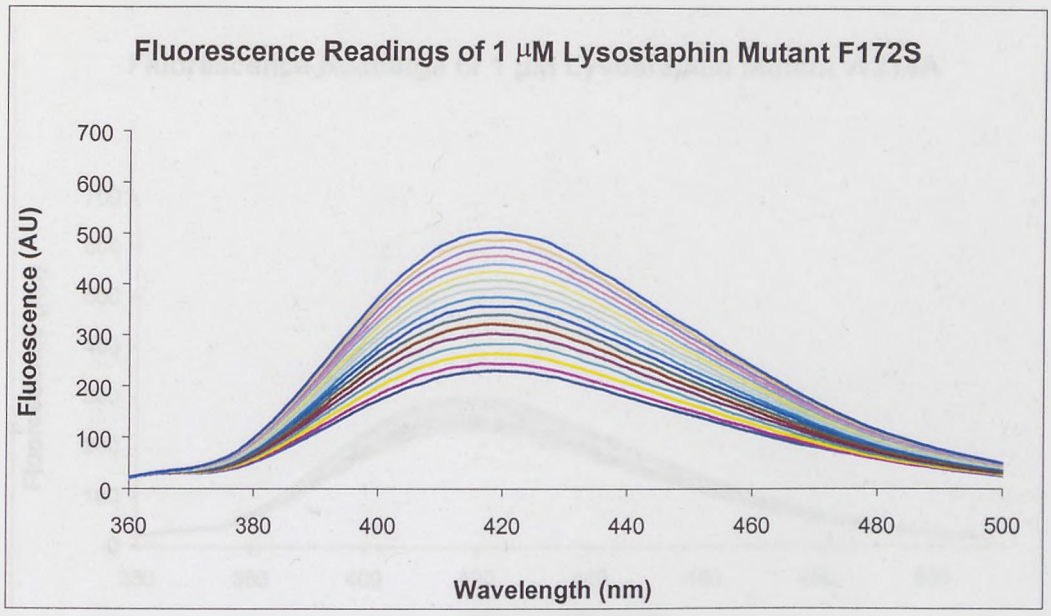

Fig. 6.14 Graph indicating cleavage activity of 1 μM lysostaphin mutant F172S against 10 μM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

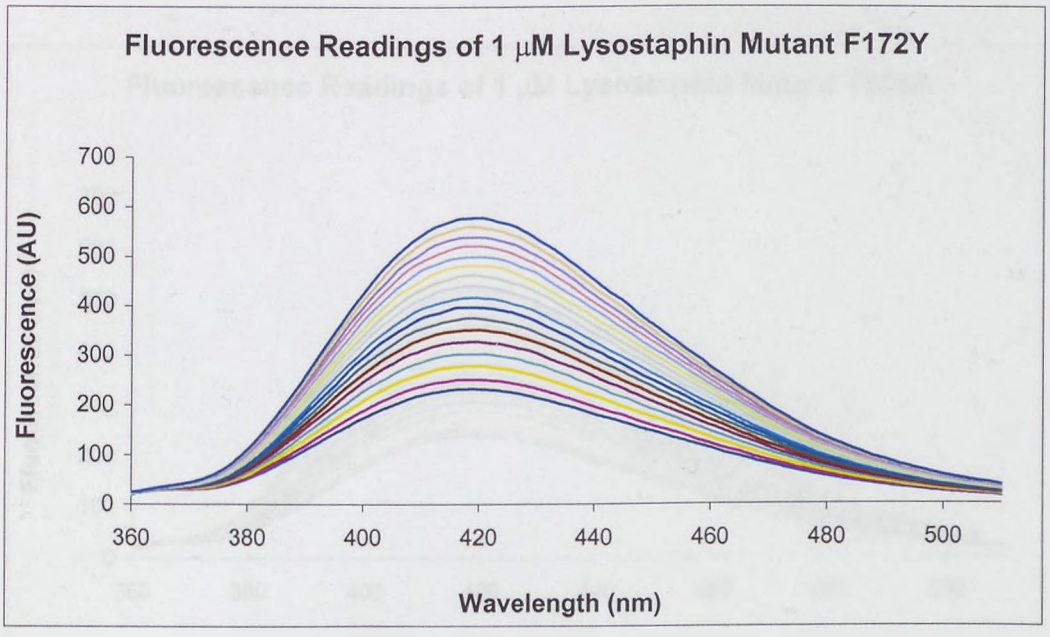

Fig. 6.15 Graph indicating cleavage activity of 1 μM lysostaphin mutant F172Y against 10 μM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

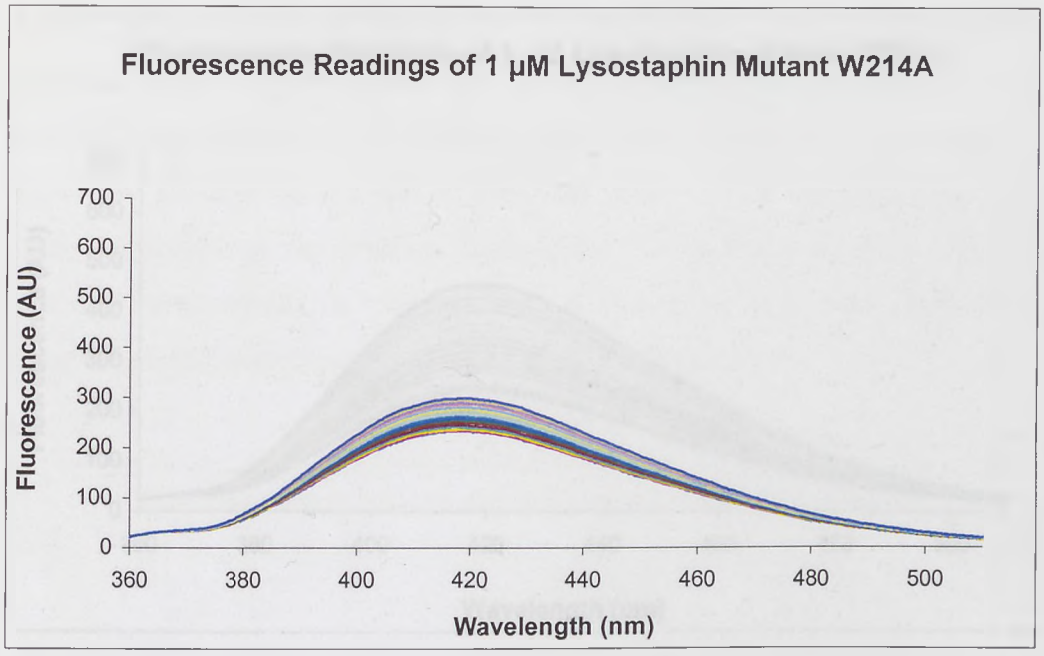

Fig. 6.16 Graph indicating cleavage activity of 1 µM lysostaphin mutant W214A against 10 µM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

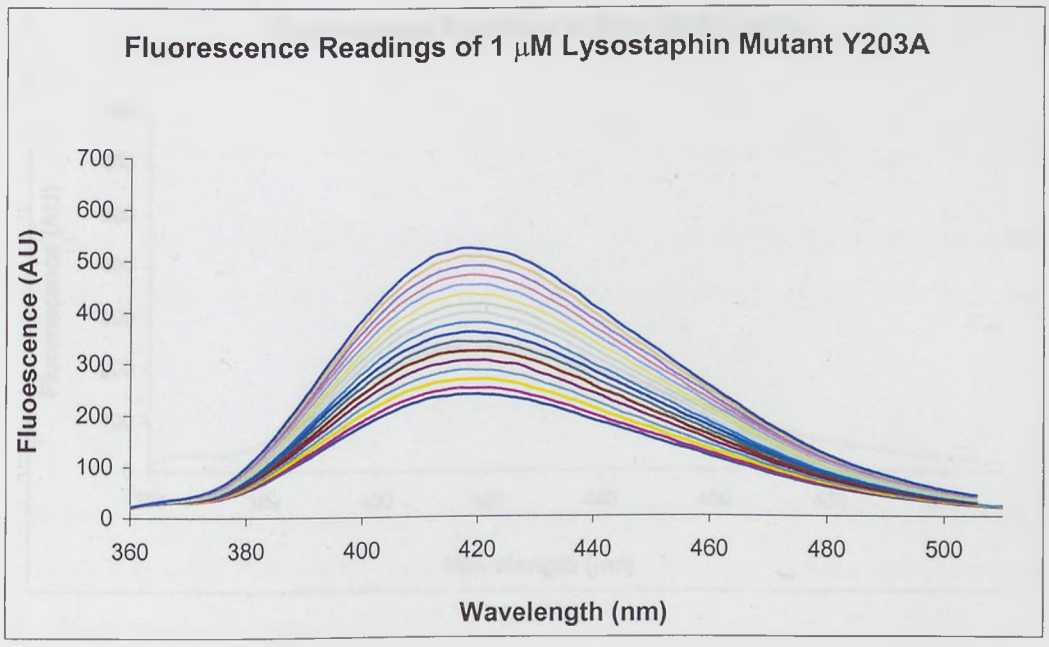

Fig. 6.17 Graph indicating cleavage activity of 1 µM lysostaphin mutant Y203A against 10 µM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

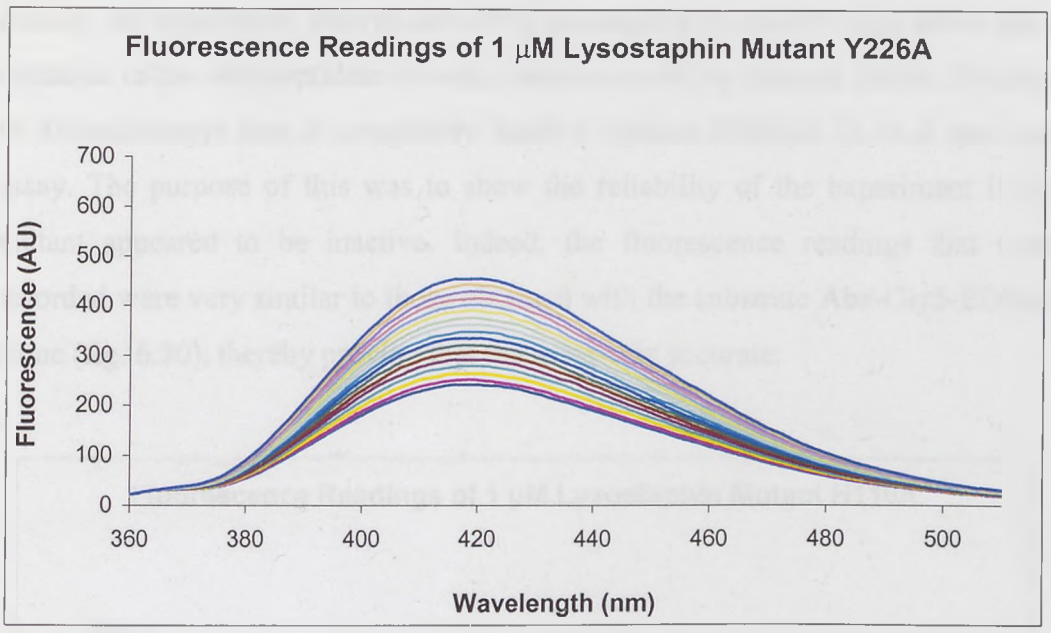

Fig. 6.18 Graph indicating cleavage activity of 1 μM lysostaphin mutant Y226A against 10 μM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

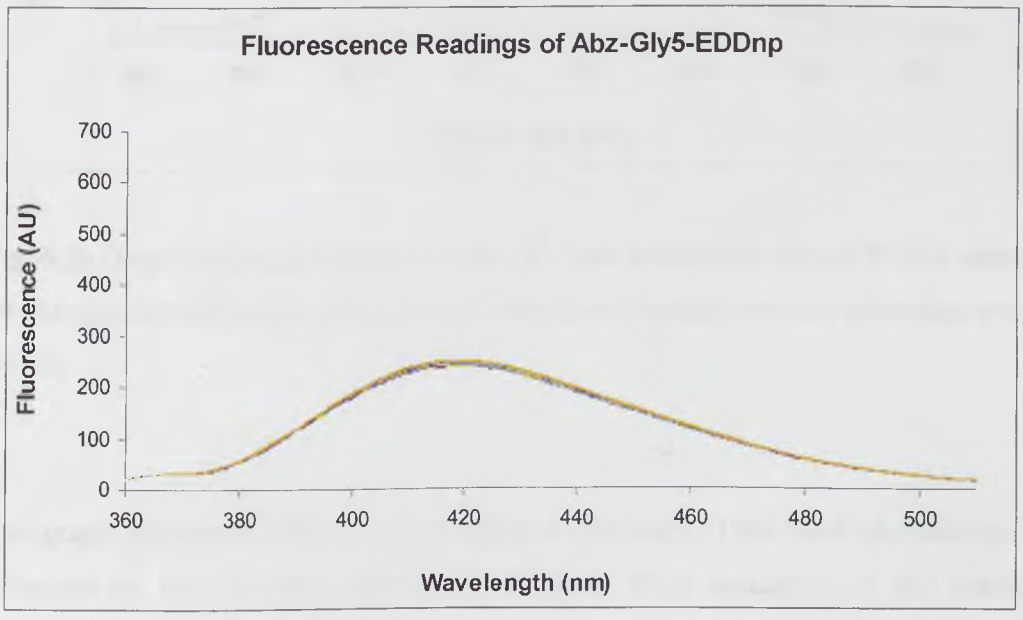

Fig. 6.19 Graph indicating fluorescence readings of 10 μM substrate Abz-Gly5-EDDnp in the absence of lysostaphin over a period of 5 h, while samples were taken every 15 min.

Finally, the experiment was repeated with lysostaphin mutant H116A, which has a mutation in the endopeptidase domain (mutant cloned by Hannah Jarvis, Division of Microbiology) that is completely inactive against EMRSA-16 in a spot test assay. The purpose of this was to show the reliability of the experiment if the mutant appeared to be inactive. Indeed, the fluorescence readings that were recorded were very similar to those obtained with the substrate Abz-Gly5-EDDnp alone (fig. 6.20), thereby proving that the assay was accurate.

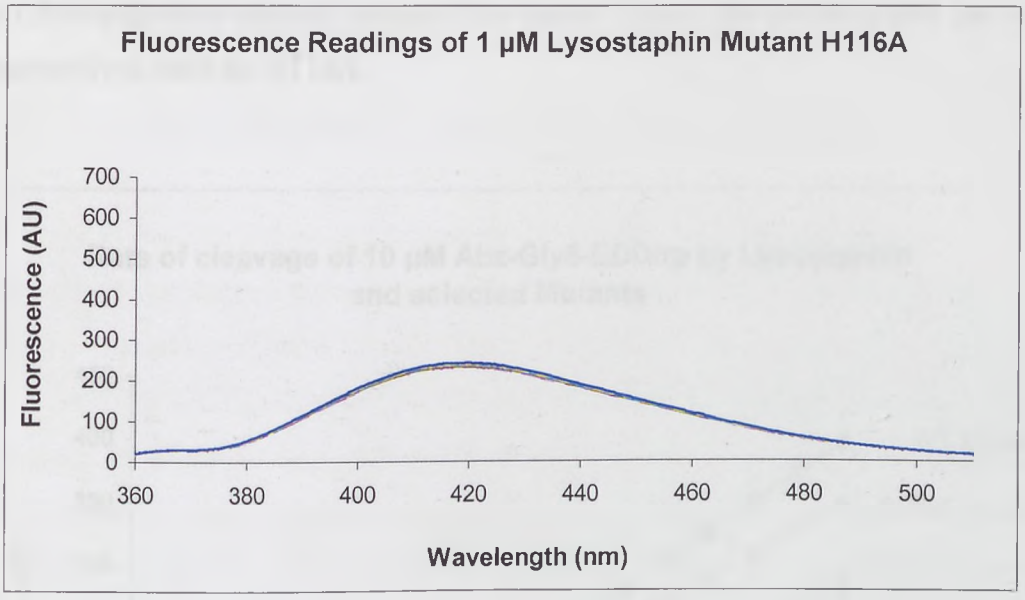

Fig. 6.20 Graph indicating cleavage activity of 1 µM lysostaphin mutant H116A against 10 µM Abz-Gly5-EDDnp at 420 nm over a period of 5 h, while samples were taken every 15 min.

The graph indicated in fig. 6.21 constitutes a summary of the rates of cleavage of substrate by the different lysostaphin mutants. Thus according to the results, obtained from the agar diffusion assay, the turbidity test and the FRET assay, lysostaphin mutants may be divided into three groups, based on their enzymatic activity:

(a) Targeting domain mutants that cleave the substrate less efficiently than wild type, but are apparently still able to be bacteriolytic in the agar diffusion assays and turbidity test. These are: F172S, F172Y, Y203A and Y226A

(b) Targeting domain mutants that cleave the substrate less efficiently than wild type and are not bacteriolytic in the agar diffusion assays and turbidity test. These are: W214A and F172A

(c) Endopeptidase domain mutants that cannot cleave the substrate and are not bacteriolytic such as: H116A.

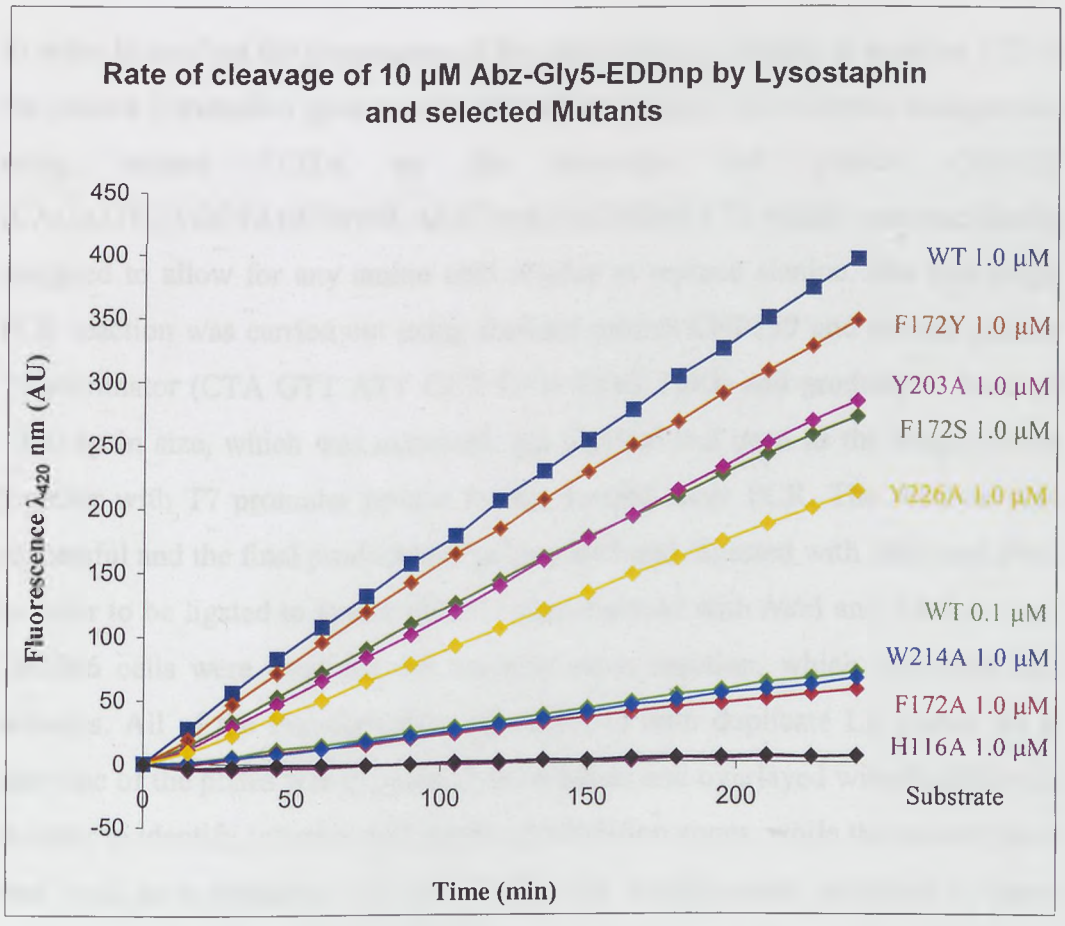

Fig. 6.21 Graph indicating the rate of cleavage of 10 µM substrate Abz-Gly5-EDDnp achieved by lysostaphin and its mutants.

It is speculated that each domain of lysostaphin has its own recognition binding site. Thus, when the targeting domain recognises and binds to its target (possibly on the cell wall) the endopeptidase domain may be brought closer to its own recognition binding site that is, the pentaglycine substrate where it can bind to and subsequently cleave it. The results of the FRET assay showed that a mutation in the T-domain resulted in lysostaphin mutants with reduced endopeptidase activity against the pentaglycine substrate. Therefore, it may be speculated that the two domains may interact with each other.

6.2.4 Reversion of lysostaphin mutant F172A back to any residue.

In order to confirm the importance of the phenylalanine residue at position 172 of the mature lysostaphin gene, it was decided to perform Site-directed mutagenesis using mutant F172A as the template and primer CNP139 (CAGAGTCAGCTAGC**NNN**CACCTAATACAGATAT), which was specifically designed to allow for any amino acid residue to replace alanine. The first stage PCR reaction was carried out using forward primer CNP139 and reverse primer T7 terminator (CTA GTT ATT GCT CAG CGG TGG) and produced a band of ~300 bp in size, which was extracted, gel purified and used as the mega-primer together with T7 promoter primer for the second stage PCR. The reaction was successful and the final product was gel purified and digested with *Nde*I and *Xho*I in order to be ligated to vector pET21a also digested with *Nde*I and *Xho*I. *E. coli* ER2566 cells were used for the transformation reaction, which produced 120 colonies. All of the transformants were stabbed onto duplicate LB plates. 24 h later one of the plates was exposed to chloroform and overlayed with EMRSA-16 in order to identify colonies that produced inhibition zones, while the second plate was used as a reference. 17 out of the 120 transformants appeared to have bacteriolytic activity against EMRSA-16 and sequencing of these revealed that in one of the sequenced plasmids, a methionine residue at position 172 had been incorporated, which corresponds to the start codon ATG while in another case a tyrosine residue at the same position (TAC) had replaced alanine. The vast majority of the plasmids (15 out of 17) that were sequenced revealed the

substitution of A172 with a phenylalanine codon TTC and one with a phenylalanine TTT codon. The fact that in nearly all of the mutants the alanine residue at position 172 was reversed back to the original phenylalanine residue supports the importance of phenylalanine at that position, although there is still some biological activity with a methionine or tyrosine residue.

6.3 DISCUSSION

6.3.1 Absence of zones of inhibition by the mutants in the stab test

The results of the protection assay described in chapter 4, indicated that excess of purified targeting domain protein in the presence of lysostaphin can protect EMRSA-16 cells from lysostaphin's bacteriolytic activity. It was thus assumed that once the targeting domain finds the appropriate receptors on the staphylococcal cell wall it binds and therefore prevents binding and subsequent killing from mature wild type lysostaphin. The various mutations introduced in the C-terminal targeting domain of lysostaphin were expected to have an effect on the targeting domain binding specificity and therefore the biological activity of lysostaphin. The stab test, to test the activity of the lysostaphin mutants (described in Chapter: 5) indicated the absence of zones of inhibition and suggested that the mutants were inactive against EMRSA-16. This result showed that the mutations brought about critical changes in the structure of the molecule that rendered the targeting domain unable to direct the enzyme to its target cells and it was thus considered important to conduct further experiments in order to investigate the possibility of some of these mutants to be essential residues for lysostaphin activity. Another possibility is that the mutant protein is unstable in *E.coli* and therefore gives no zones of inhibition in a stab test. In order to distinguish between these possibilities it was required to test further the activity of purified proteins.

6.3.2 The turbidity assay indicated that mutations W214A and F172A abolished bacteriolytic activity

The first assay that was performed with the purified mutant proteins was a turbidity test. This assay is a very easy, quick and accurate way to measure enzyme activity because it generates real-time results. Spectrophotometric detection of the bacteriolytic activity of lysostaphin against *S. aureus* cells has been performed in the past (Marova and Kovar, 1993). The experiment was based on recording the changes of the absorbance values of a suspension containing *S.*

aureus cells and purified lysostaphin over various periods of time in order to determine the optimum conditions for similar assays. Even though the growth curves of bacteria are usually measured between 600 and 620 nm, the results of the experiments indicated that the ideal wavelength to measure turbidity changes was 540 nm as opposed to 620 nm reported by Robinson *et al* (1979) and 600 nm reported by Iversen and Grov (1973). The range of absorbances tested by Marova and Kovar was between 460 and 620 nm, however at A_{540} the activity rate obtained was 2.5 times higher than that observed at A_{620}.

Here, the turbidity test was carried out in order to compare the activity of the mutant proteins to that of the wild type and obtain information about the importance of the mutations introduced in the targeting domain of lysostaphin. The assay was performed spectrophotrometrically with a Shimatzu UV-160 while changes in turbidity were measured at 600 nm over a period of 20 min as described by Kessler *et al.*, (1997) who also performed the same experiment in order to measure staphylolytic activity of LasA (a staphylolytic protease produced by *P. aeruginosa*). All mutant proteins as well as the wild type were tested at 3 μM against EMRSA-16 cells. The results of the experiment showed that mutants F172S, F172Y and Y226A did not exhibit reduced activity compared to the wild type as indicated by graphs in fig. 6.3, 6.4 and 6.7 respectively. Hence serine and tyrosine at position 172 and alanine at position 226 may be tolerated. On the other hand, mutants W214A and F172A did not show any activity against EMRSA-16, indicating that tryptophan and phenylalanine could be important for lysostaphin activity at positions 214 and 172 respectively. Finally, mutant Y203A showed some bacteriolytic activity, however the protein was not as active as mutant proteins F172S, F172Y and Y226A.

6.3.3 Comparison of the results obtained by the three assays-Spot test, Turbidity assay and Stab test

A spot test was also carried out with lysostaphin and its mutants. A wide range of concentrations were used to test the purified proteins against EMRSA-16, in order to obtain a more specific picture of their range of activity. The results of the spot

test (fig. 6.8) indicated that clear zones of inhibition for the wild type existed at concentrations of 2.5 μM or higher, which was consistent with the first spot test described in chapter: 3, where lysostaphin was active against EMRSA-16 at concentrations of 3 μM or higher. Mutant F172Y was found to be almost as active as lysostaphin while mutants F172S and Y226A were found to be less active than the wild type and produced clear zones of inhibition at concentrations of 4 μM and 5 μM respectively. Finally, mutant Y203A was only active against EMRSA-16 at concentrations of 10 μM or higher, while mutants W214A and F172A were completely inactive against the test organism.

The results obtained from the spot test come in contrast with those obtained from the stab test (which showed that the mutant proteins did not produce any zones of inhibition) that was initially carried out with the lysostaphin mutants. The reason for this is because the conditions under which the activity of the proteins was tested were very different between the two tests. In the stab test, the activity of the mutants was based only on exposure of the cells carrying a mutant plasmid to chloroform in order to break them open and release the protein. On the other hand, the bacteriolytic activity of the mutant proteins in the spot test was tested after the mutants had been induced and their proteins purified. Another explanation for the different results between the two tests could be that the local concentration of lysostaphin in the spot test is a lot higher than in the stab test. Further to this, the conditions under which the mutant proteins were used in the stab test were not ideal because they had to be expressed by *E. coli* along with several other proteins which might have affected their bacteriolytic activity. These differences in the conditions between the two tests explain why the mutant proteins were active against EMRSA-16 in the spot test and not in the stab test.

The results obtained from the turbidity assays and the spot test appear to be quite consistent. In both tests, mutant proteins F172S and F172Y are very active against EMRSA-16 and in some cases as bacteriolytic as the wild type. Again, consistent results were obtained with mutant Y203A. Moderate activity of the mutant protein against EMRSA-16 and significantly less than wild type lysostaphin was observed

in both tests. Only, in the case of mutant Y226A, the results between the two tests varied slightly. According to both the turbidity assay and the spot assay mutant Y226A retains some bacteriolytic activity, however, the readings obtained from the former assay indicated high degree of activity, compared to that of the wild type, while in the spot test the zones of inhibition produced suggested activity similar to that of Y203A, which was significantly lower than that of the wild type. This variation between the two assays may be explained by the fact that the spot test does not produce real-time results as is the case with the turbidity assay. Finally, mutants W214A and F172A appear completely inactive against EMRSA-16 cells in both tests.

6.3.4 Assay of the mutants with the FRET assay

Lysostaphin activity has been also investigated turbidimetrically by using a dye release assay (Zhou *et al.*, 1988). Ramazol Brilliant Blue R (RBB)[3]-dyed staphylococcal cells or peptidoglycan were used as the substrate and the activity of the enzyme was measured by the colour product originating from the breakdown of the substrate. The soluble hydrolytic products released by lysostaphin were measured spectrophotometrically at 595 nm after the insoluble substrate was removed by filtration or centrifugation. This method constitutes a more sensitive and accurate test because it generates more reproducible results than the turbidimetric method described above. Zhou *et al.*, (1988) have reported that the dye release assay is 3x more sensitive than the turbidity test described by Iversen and Gron (1973).

A colorimetric microtiter plate assay has been also developed in order to assay the activity of lysostaphin (Kline *et al.*, 1994). The group reported the synthesis of a chemically defined substrate, *N*-acetylhexaglycine, to measure enzymatic activity by recording the changes in optical density at 405 nm. This assay has several advantages over the tests described above. It is rapid, easier, more straightforward than the dye release assay and more accurate. Further to this, the assays that have been used to measure lysostaphin activity and are described above measure lytic activity on whole cell substrates. Consequently, the results cannot be very

reproducible while a number of complex variables may intervene between the measured event and the activity of the enzyme. With the assay described by Kline and colleagues (1994) construction of a suitable substrate allows direct measurement of the activity of the protein.

Here, a similar oligopeptide was constructed and used in a FRET assay to measure the cleavage of a pentaglycine substrate by lysostaphin and compare it to that of mutants. The FRET assay constitutes the most sophisticated and efficient method for measuring enzymatic properties to date. The test uses a pentapeptide substrate to which a donor fluorophore and a quenching fluorophore are attached at opposite ends of the peptide and produce measurable fluorescence after cleavage of the peptide by the enzyme. In this study, the substrate consisted of a pentaglycine, a donor fluorophore (*ortho*-aminobenzoic acid (Abz)) at the N–terminal glycine and an acceptor or quenching fluorophor N-(2, 4-dinitrophenyl)ethylene diamine (EDDnp) at the C-terminal glycine residue. The efficiency of this pair has been demonstrated by several groups in peptide analogues of Abz-Phe-Arg-Arg-Val-EDDnp for kallikreines (Chagas *et al.*, 1991), in substrates for papain (Guathier *et al.*, 1993), endooligopeptidase A (Juliano *et al.*, 1990) and for human rennin (Oliveira *et al.*, 1992).

The easiest and most straightforward way to analyse whether a FRET interaction is taking place and therefore if the substrate is being cleaved by the enzyme, is to excite the donor fluorophore and measure the acceptor fluorescence. Kramer and colleagues (2000) have reported excitation of Abz of a similar substrate (Abz-Ala-Arg-Arg-Ala-Tyr(NO_2)-NH_2) at 325 nm that resulted in a fluorescence signal with an emission maximum at 430 nm. Johanning and colleagues (1998) have attached Abz and EDDnp to various polypeptides in order to investigate the specificity of prohormone convertase 2. The excitation wavelength used was 320 nm and Abz fluoresced at 420 nm. Similarly, in order to investigate substrate specificity of the major cysteine protease cruzipain, Del Nery and coworkers (1997) excited their substrates (bound to Abz and EDDnp) at 320 nm and followed fluorescence emissions at 420 nm. In accordance with these studies, we tried a range of excitation wavelengths at 310, 320, 325 and 330 nm. The results showed that the

differences in fluorescence were not significant. However, because the tyrosine and tryptophan residues of lysostaphin yield fluorescence at wavelengths of 310 nm or lower and due to the fact that DMSO (that is required because the peptide substrate is highly insoluble) yields fluorescence when excitation approaches 350 nm, it was decided to excite the substrate at 325 nm and record fluorescence emissions at 420 nm.

The pentaglycine substrate was also tested in order to measure the fluorescence it yields. Thus the activity of the mutants and that of the wild type was calculated as the producing fluorescence emission minus the fluorescence emission of the substrate alone. According to the results in fig. 6.11 and 6.13 – 6.18, mutants F172Y, F172S and Y203A produced high fluorescence readings, indicating that they had retained most of their endopeptidase activity, while mutants W214A and F172A yielded low fluorescence, suggesting that they cleaved the substrate less efficiently than the wild type. Mutant Y226A appeared to have retained some activity as indicated by the turbidity test as well, however the fluorescence levels were lower than those obtained by F172S, F172Y and Y203A. None of these three mutants had lost all of their enzymatic activity therefore none of the mutated residues are directly involved in the catalytic mechanism of lysostaphin. There are a number of possibilities as to why some of the targeting domain mutants have a somewhat reduced catalytic activity when compared to lysostaphin. The targeting domain mutations could indirectly reduce catalysis by altering the 3D structure of the protein and hence the conformation of key catalytic active site residues and/or important substrate binding site residues in the endopeptidase domain. They may be important for targeting of the endopeptidase domain to the pentaglycine substrate binding or both the targeting domain and the endopeptidase domain may have exclusive binding pockets. One way to investigate these possibilities is by CD (circular dichroism) spectra. This method provides information on the secondary structure of proteins, allowing the detection of possible secondary structure perturbations in a protein caused upon site-directed mutagenesis. By comparing the Far UV spectra of wild-type lysostaphin with that of mutants F172S, F172Y, Y203A and Y226A of the targeting domain it is possible to assess the effect of the mutations on the global secondary structure of lysostaphin. If the

spectra of the mutants superimpose with that of the wild-type then it is reasonable to say that the mutations had no effect whereas if the spectra are shifted then the secondary structure might be significantly affected so as to affect the activity of the endopeptidase domain.

The results of the FRET assay were surprisingly similar to the results gained from the whole cell turbidity and spot test assays. In the turbidity and the spot tests, the results indicated that mutants W214A and F172A were inactive against EMRSA-16 while F172S, F172Y, Y203A and Y226A retained bacteriolytic activity similar to that of the wild type. It was assumed that the mutations W214A and F172A had rendered the mutants inactive because the T-domain was no longer able to bind to its substrate. Thus, in the FRET assay in which cell wall binding of the T-domain is by-passed, it was thought that as both domains are independent of each other then the W214A and F172A mutations would not affect the endopeptidase activity of the mutant proteins. However, the results of the FRET assay showed that the catalytic activity of the W214A and F172A mutant proteins was indeed affected by the mutations in the T-domain. Thus, the endopeptidase and T-domains may not be completely autonomous and, in the absence of a 3D crystal structure of lysostaphin, we speculate that both targeting and endopeptidase domains may interact in some way with each other. If this is correct then the mutations in the T-domain may affect the correct folding of the mutant lysostaphin, which disrupts the interaction between the targeting and endopeptidase domains therefore preventing the endopeptidase domain from recognising, binding and cleaving the pentaglycine substrate. Another possibility might be that the T-domain mutations compromise the activity of the mutants by making them more insoluble and therefore reducing their concentration in solution during the FRET assay. This would also result in mutants with reduced catalytic activity against the substrate.

Finally, mutant H116A (developed by Hannah Jarvis, Division of Microbiology) was also tested with the FRET assay. This protein has a mutation in the endopeptidase domain that rendered lysostaphin inactive in a turbidity and a spot test. Fig. 6.20 shows that the fluorescence produced during the FRET assay was

the same as that of the substrate alone, suggesting that the pentaglycine was not cleaved and thus the reliability of the method was confirmed.

6.3.5 Indication of the importance of phenylalanine at position 172 of mature lysostaphin by a reversion experiment with mutant F172A

A reversion experiment was also performed to establish the importance of phanylalanine at position 172 and to see if other residues can substitute for phenylalanine and restore biological activity of the mutant lysostaphin. Site-directed mutagenesis was thus performed in order to mutate alanine at position 172 back to any residue. A stab test was carried out and revealed that 17 out of 120 colonies had produced zones of inhibition. Sequencing of these plasmids indicated that 15 of them had incorporated phenylalanine in place of alanine at position 172 of mature lysostaphin, suggesting the significance of phenylalanine at that position. However, in the absence of the crystal structure of lysostaphin it is difficult to predict the real importance of phenylalanine residue and what interactions it makes that render it important to the activity of lysostaphin. According to the results of the assays described above, it appears that serine, which constitutes a simple and polar amino acid with only a hydroxyl side chain did not affect the activity of the lysostaphin targeting domain significantly when substituted with phenylalanine that has a more complex structure due to the presence of an aromatic side chain. Furthermore, substitution of phenylalanine with tyrosine, which is very similar to phenylalanine (the only difference between the two being the presence of the hydroxy (-OH) group in tyrosine) did not abolish lysostaphin activity either. On the other hand substitution of phenylalanine with alanine that has a very simple structure did result in inactivation of lysostaphin. Therefore, it could be that the presence of an aromatic R-group (that is present in phenylalanine and tyrosine) or its substitution with a hydroxyl R-group (that is present in tyrosine and serine) are necessary for lysostaphin to retain its activity while the presence of an aliphatic R-group (that is present in alanine) brings about major conformational changes that result in loss of activity. Futhermore, it would be reasonable to assume that protein inactivity at position 172 may be a matter of size. Phanylalanine is a large aromatic acid and even

though serine and tyrosine are neither as big nor entirely aromatic, they are certainly bulkier than alanine. In the case of mutant Y226A and Y203A, the substitution of a complex amino acid bearing an aromatic ring, with alanine, did not significantly affect the activity of these lysostaphin mutants. On the other hand, in the case of mutant W214A, the results of the assays suggest that alanine is the reason for lack of activity because of the major differences in the structure of these two amino acids.

6.3.6 The crystal structure of lysostaphin could indicate more critical resudues

Recently Odintsov and co-workers (2004), reported the crystal structure of LytM and investigated the importance of certain residues in the functionality of the underlying protein. The researchers identified four amino acids in the crystal structure of LytM that were assumed to play a critical role in the activity of the protein because they constitute Zn^{2+} ligands (a characteristic feature of metallopeptidases such as lysostaphin and lytM), one of which is also involved in what has been termed as the "asparagine switch" by the authors of the study. This is analogous to the "cysteine switch" (Van Wart and Birkedal-Hansen, 1990) that describes an intramolecular complex between a single cysteine residue in the propeptide domain of a protein belonging to the MMP family (Matrix MetalloProteinase) and the essential zinc atom in the catalytic domain, a complex that blocks the active site and consequently renders the enzyme inactive. Conversely, in the case of LytM, asparagine was suspected to form a complex with Zn^{2+} and therefore to prevent the enzyme from being active in its proform. Mutation of this residue to alanine and assay of the mutant revealed an active proenzyme, confirming the role of asparagine in the activity of the proform of the LytM protein. Further mutation and assay of three more residues (two histidines and one aspartic acid) in the mature form of the enzyme, revealed inactive mature molecules as the proteins were unable to cleave the pentaglycine substrate, which confirmed the importance of these residues in Zn^{2+} chelation. Conversely, in the case of lysostaphin, identification of its crystal structure could reveal potential

Zn^{2+} ligand residues and a similar "asparagine switch", both of which would provide valuable information for the mode of action of lysostaphin.

CHAPTER: 7

GENERAL DISCUSSION

7.1 Introduction

The fact that lysostaphin is considered an antimicrobial agent with very promising potential against staphylococcal infections and especially those caused by EMRSA as well as the lack of published work on the mechanism behind its target specificity (only two group studies are available by Baba and Schneewind (1996) and Thumm and Gotz (1997)) constituted the motivation for this study. The work that is thus described here was carried out in order to investigate further and provide more information on the activity and biochemistry of the C-terminal targeting domain of lysostaphin in an attempt to understand the mechanism that the underlying protein uses to kill its target organisms.

7.2 Potential uses of the T-domain of lysostaphin

The results of this study indicated that the lysostaphin T-domain is an individually functional module that is capable of retaining its activity without the presence of the protein's enzymatic domain and can therefore protect EMRSA-16 cells in the presence of mature lysostaphin. These results suggest that the T-domain may be utilized in future studies for the construction of chimeras with other individually functional domains that exhibit enzymatic activity. This would be very useful because the production of such chimeras constitutes an important step towards the formation of broad-spectrum antibiotics. Two homologues of lysostaphin, namely *S. aureus* LytM and *E. coli* LytM were cloned, expressed and purified for this purpose. Assay of *S. aureus* LytM against two *S. simulans* and one *S. aureus* strains (see section 3.3.2) did not show any killing activity and one reason for this could be because the *S. aureus lytM* gene does not code for any known targeting domain signals (Ramadurai and Jayaswal, 1997). Hence, fusion of the lysostaphin T-domain with the enzymatically active C-terminal domain of *S. aureus* LytM could potentially result in a novel antimicrobial agent against *S. aureus*.

The fact that the lysostaphin T-domain is an individually functional module could be also used to construct an identification tool for *S. aureus* in clinical samples. As opposed to the *Listeria monocytogenes* bacteriophage hydrolases that are very

species specific, the picture is not so clear with lysostaphin yet. The protein has been reported to lyse *S. aureus* (Heath *et al.*, 1987) as it targets the pentaglycine present in many staphylococci (Zygmunt and Tavormina, 1972). Therefore, additional experiments could be carried out to establish lysostaphin specificity within the staphylococcous genus. If lysostaphin proved to be targeted only against *S. aureus* the protein could be further fused with GFP for the construction of a tool to identify *S. aureus* in clinical samples.

7.3 Identification of important residues in the lysostaphin T-domain

The comparison of amino acid and nucleotide sequences between different proteins or genes has become a standard procedure when information on specific functions of proteins or genes are required. Several computer programs are nowdays available for the identification of similarities between proteins. These programs are very popular within the scientific community as they can be used not only for the identification of functional domains that can give specific roles to uncharacterised proteins but also for the identification of specific residues that are critical for protein function. Evidence of this is seen in the case of the T-domain of lysostaphin, where according to sequence alignments with other homologues four of its residues were highly conserved. The results of the spot test, the turbidity and the FRET assay indicated that W214A and F172A resulted in inactive lysostaphin while a reversion experiment carried out with the latter mutant confirmed the importance of phenylananine at position 172 of mature lysostaphin. However, it is important to note that three of the remaining residues that were mutated that is, F172S, F172Y and Y203A had no major effect on the activity of the protein. This highlights the fact that even though sequence alignments of homologous proteins can provide important information it can also be misleading. Dorit and Riley. (2002) reported a similar situation when attempting to mutate 10 amino acids in the receptor binding domain of colicin E9. According to the study, only 6 out of the 58 different mutations resulted in inactivity, even though that particular region was highly conserved. Therefore, it may be postulated that in many cases, even strikingly conserved residues may not be critical for the function of a protein.

7.4 The crystal structure of lysostaphin could reveal more critical residues

Even though, no more lysostaphin residues that can abolish protein function have been reported in the literature, it is speculated that there are several more, notably in the endopeptidase domain of lysostaphin due to the presence of the HxH motif (a short conserved signature sequence containing histidine and glutamate residues common among metallopeptidases such as lysostaphin and *S. aureus* LytM). Identification of such conserved residues in the catalytic domain of lysostaphin based on sequence alignments is being carried out by Hannah Jarvis (University of Nottingham). However, more critical residues could be identified if the crystal structure of lysostaphin was resolved. Recently, Odintsov and co-workers (2004) revealed the crystal structure of *S. aureus* LytM, which helped them identify critical amino acids other than those indicated by sequence alignments. In particular, an asparagine residue at position 117 of the mature protein (located in the profragment of LytM) was assumed to be important (even though not conserved among homologues) because according to the crystal structure it could be easily moved without disrupting the structural integrity of the protein. Indeed, that residue proved to form a complex with the zinc atom that blocked the active site of the mature protein and in that way inactivated LytM, prior to cleavage of its proform. In the same way, the crystal structure of lysostaphin may reveal more residues that whilst not conserved may be critical sites for folding and/or activity of the protein.

7.5 Resistance to lysostaphin – possible solution

Even though it is hard to predict whether resistance to lysostaphin will develop, it is reasonably safe to assume that such a possibility is not unlikely once the protein becomes clinically available. A recent study carried out by Climo and colleagues (2001) indicated that resistance to lysostaphin is possible after prolonged exposure of their ORSA (oxacillin resistant *S. aureus*) isolates to low concentrations of the protein. In such a case, Climo and colleageus (2001) reported that the phenomenon may be suppressed by the co-administration of β-lactams such as

nafcillin or oxacillin. Another alternative to the problem would be to use directed evolution by means of gene shuffling in order to make alterations to the endopeptidase domain of lysostaphin. Since lysostaphin targets the pentaglycine of the *S. aureus* peptidoglycan backbone and immunity of the producer *S. simulans* is based on the *lif* gene that is responsible for the substitution of glycines with serines in its own pentaglycine, a lysostaphin molecule with an altered endopeptidase domain that can cleave a serine-containing cross bridge would be desirable.

The technique of gene shuffling mimics the evolutionary processes and has proved extremely successful in systems for evolving enzymes with novel specificities/enhanced activities and has been widely used in plant biotechnology. The process involves fragmentation of variants of a population of mutant genes (that can be obtained through mutagenesis) selected on the basis of their containing enhanced mutations that render them suitable for templates for the next progeny. These DNA fragments can then be recombined with each other during a PCR reaction where homologous fragments act as primers for each other to produce a protein with beneficial properties (Minshull and Stemmer, 1999). Using this technique, it may prove possible to produce an enhanced lysostaphin molecule that can cleave cross-bridges containing serine molecules that the wild type lysostaphin would be unable to attack.

7.6 Clinical trials with lysostaphin in the form of a cream to eradicate staphylococcal colonisation

Animal studies have been carried out with lysostaphin in the form of a cream for the eradication of MRSA nasal colonisation in a cotton rat model (Kokai-Kun *et al.*, 2003). *S. aureus* nasal colonisation by both MRSA and MSSA is usually treated with mupirocin for a period of 3 days and multiple applications are required in order for the infection to clear. On the contrary, when the lysostaphin cream was applied, staphylococcal colonisation was eradicated by a single dose within 4 hours of application and its antistaphylococcal activity was present even 24 h after instillation. Further to this, the lantibiotic nisin was also used to treat the

same infection in a cotton rat model, which however was found to have lost its antistaphylococcal activity 2 h after application. Taken together these results indicate that the lysostaphin cream formulation is by far the most effective comparatively to the current treatment of choice, mupirocin and the lantibiotic nisin. Kokai-Kun and colleagues (2003) also observed during the same study, that lysostaphin resistance did not emerge despite the large number of different *S. aureus* strains used. Clinical trials have therefore been initiated with the lysostaphin cream formulation with the anticipation that the product will become a valuable tool for the control of community EMRSA and the prevention of nosocomial *S. aureus* infections.

7.7 PEGylation of lysostaphin shown to increase the efficacy of the protein administered intravenously

Research is being carried out in order to improve the efficacy of intraveneously administered lysostaphin. Walsh and co-workers (2003) carried out work in order to increase lysostaphin's *in vivo* half-life to allow for more effective tissue distribution and reduce its immunogenicity. Conjugation of lysostaphin to polyethylene glycol (PEG) was performed in order to achieve these goals. Their results indicated that PEGylation of lysostaphin not only prolonged serum drug persistence for up to 24 hours (compared to 1 hour for unconjugated lysostaphin) but reduced antibody binding as well. These findings further increase the value of lysostaphin as a potential intraveneous antibiotic against EMRSA.

7.8 The potential of lysostaphin in genetic engineering

Apart from its great potential as an antibiotic, lysostaphin has been used in recent studies involving the construction of transgenic mice and cows to combat mastitis. This disease is an inflammatory reaction of the mammary gland and in addition to animal distress, is estimated to cost the US industry alone $1.7 billion annually (approximately $200 per cow). *S. aureus* is the major contagious mastitis pathogen accounting for approximately 15-30% of infections and has proved extremely difficult to be able to control under standard management practices

(Kerr *et al.*, 2001). Kerr and colleagues (2001) have reported the construction of transgenic mice that are capable of expressing lysostaphin in their mammary glands and can thus be completely resistant to mastitis while their milk protein contents were similar to those obtained by non-transgenic mice. Further to this, Fan and colleagues (2002) have successfully carried out the process of transfer of a lysostaphin gene into the mammary gland of a goat. These studies clearly demonstrate the potential of lysostaphin in genetic engineering to combat the most prevalent disease of dairy cattle.

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