Chapter 1

Introduction
1.1 Staphylococci

*Staphylococcus aureus* is an opportunistic pathogen belonging to the genus *Staphylococcus*. It is a gram-positive, non-spore forming bacterium, first observed in the 1880s (Ogston, 1984). Cells of this organism arrange as clusters of round golden spheres, or cocci; and have a range in diameter of between 0.7 μm to 1.2 μm.

About fourteen human specific genome sequences of *S. aureus* strains and one bovine specific strain are currently available (Herron-Olson et al., 2007). All *Staphylococcal* genomes are approximately 2.8 Mega bases (Mb) to 2.9 Mb in size, with relatively low G+C content (33 %) (Kuroda et al., 2001; Gill et al., 2005). Nearly, 2,600 open reading frames have been identified which constitute nearly 75 % of the genome. The remaining part of the genome is highly variable and consists of mobile genetic elements like pathogenicity islands, chromosomal cassettes, plasmids and transposons that are horizontally transferred across different strains (Fitzgerald et al., 2001; Lindsay and Holden, 2004). The striking features which make this organism adaptable to new conditions are the property of inter-strain genetic variability and the ability to selectively evolve certain pathogenesis-related genes.

![Scanning electron micrograph of Staphylococcus aureus](image)

*Figure 1.1 Scanning electron micrograph of Staphylococcus aureus* This image shows the bacteria at about 9,500 times their normal size.
S. *aureus* appears as spherical grape-like clusters when viewed through a microscope thus leading to its nomenclature- *Staphyl* refers to a bunch of grapes in Greek whereas the Latin term *coccus* refers to its spherical shape. They form large, round golden-yellow colonies often seen with β-hemolysis when grown on blood agar plates. The golden appearance is the etymological root for the species *aureus* which means golden in Latin. *S. aureus* is non-motile, non-sporulating and is catalase positive. They can be differentiated from most other *Staphylococci* by the coagulase test; *S. aureus* is coagulase-positive, while most other pathogenic *Staphylococcus* species are coagulase-negative (Ryan *et al*., 2004). *S. aureus* can respire in the presence of oxygen, or undergo fermentation in the absence of oxygen since they are facultative anaerobes. *S. aureus* show optimal growth at temperatures of 25 °C to 43 °C and at pH levels of 4.8 to 9.4 (Novick, 2006).

1.1.1 Manifestation of *S. aureus* infection

*S. aureus* is a versatile pathogen causing a wide variety of diseases in both community and hospital settings. Human beings are the natural reservoir of *S. aureus*. They are ubiquitous throughout nature as both a pathogen and commensal organism of humans (Lowry, 1998). Nearly 30 % of the adult population harbors this microbe asymptomatically in the anterior of their nares. This carrier population contributes to the transmission of *S. aureus* mainly through direct contact with sites of infection or areas of colonization (Wertheim *et al*., 2004).

*S. aureus* are further subdivided into two subspecies: *S. aureus aureus* and *S. aureus anaerobius*. The latter requires anaerobic conditions for growth and is an infrequent cause of infection since they are rarely encountered in nosocomial settings. In humans, skin and nasopharynx are the natural habitat of *S. aureus*. They are known to cause a wide variety of infections involving skin and soft tissues, endovascular sites and internal organs. This organism can circulate from a superficial site via the blood stream to internal organs where it can set up a metastatic focus of infection (Lowry, 1998). In case of hospital patients, surgical wounds and indwelling medical devices are the major sites of their infection.

Localized cutaneous infection of *S. aureus* causes furuncle, carbuncle, impetigo (Noble, 1997), mastitis (Sutra and Poutrel, 1994), surgical wound infection (Kernodle and Kaiser, 1997), scaled skin syndrome (Gemmell, 1995), toxic shock syndrome
(Bohach et al., 1990; Schlievert, 1993; Chesney et al., 1997), endophthalmitis (Mao et al., 1993) and corneal keratitis (Liesegang, 1988) while the more severe internal organ infections of *S. aureus* include osteomyelitis (Cunningham et al., 1996), septic arthritis (Gentry, 1997), pneumonia, septicaemia and endocarditis (Maki, 1981; Scheld and Sande, 1995; Ing et al., 1997). Severe *S. aureus* infections can also manifest as necrotizing diseases for e.g. necrotizing fasciitis and necrotizing pneumonia which can often result in the irreversible degradation of host tissues and muscle (Fowler et al., 2005).

### 1.1.2 Pathogenesis of *S. aureus*

The pathogenic success of *S. aureus* is largely based on regulatory networks that coordinate the expression of bacterial virulence factors. This is achieved by global regulators that modulate temporal gene expression according to changes in the environment (Chan and Foster, 1998; Novick, 2006; Yarwood et al., 2001). The pathogenesis of *S. aureus* is complex and involves the coordinated synthesis of cell wall-associated proteins and extracellular toxins. The diverse symptoms and manifestations of *S. aureus* infection and disease have been attributed to its ability to produce an array of *virulence factors* that are associated with specific diseases. These include:

- **Surface proteins** that promote colonization of host tissues;
- **Invasions** that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase);
- **Surface factors** that inhibit phagocytic engulfment (capsule, protein A);
- **Biochemical** properties that enhance their survival in phagocytes (carotenoids, catalase production);
- **Immunological** disguises (protein A, coagulase, clotting factor);
- **Membrane-damaging toxins** that lyse eukaryotic cell membranes (hemolysins, leukotoxin, leukocidin);
- **Exotoxins** that damage host tissues or otherwise provoke symptoms of disease (toxic shock syndrome toxin, exfoliatin toxin). (Peng et al., 1988; Janzon et al., 1989; Novick, 1993, 2006).
1.1.3 Resistance of *S. aureus* towards antibiotics

*S. aureus* is an astonishingly successful pathogen, with overwhelming resistance to antibiotic therapies. The occurrence of infections due to antibiotic resistant strains has achieved epidemic proportions, and is primarily the result of methicillin resistant *Staphylococcus aureus*, or MRSA, strains (Chambers, 2001; Kaplan *et al.*, 2005; Klevens *et al.*, 2007). In 2004, MRSA accounted for nearly 60% of all *S. aureus* infections reported for patients staying in intensive care units [National Nosocomial Infections Surveillance (NNIS) System Report, 2004]. Methicillin resistant *S. aureus* is now believed to be the leading cause of death by a single infectious agent in the United States (Kobayashi and DeLeo, 2009).

In the early 1940s, antibiotic penicillin was first introduced but by 1944, the first resistant isolates of *S. aureus* emerged (Barber and Rozwadowska-Dowzenko, 1948). Such resistant strains were developed from an acquired penicillinase encoded on a plasmid (Ridley *et al.*, 1970). Penicillinase was found to inactivate the functional β-lactam ring through a targeted cleavage event, opposing the activity of the drug (Discussion on Penicillin, 1994; Wu *et al.*, 2001). Regardless of these efforts, by 1960, 80% of all *S. aureus* strains became penicillin resistant, and were spreading at pandemic proportions throughout both community and hospital settings (Ridley *et al.*, 1970). It was reported by 2008 that more than 90% of all *S. aureus* isolates possessed penicillinase-mediated penicillin resistance (Tenover, 2008).

Methicillin was developed for the treatment of penicillin resistant *S. aureus* infections in 1959 (Batchelor *et al.*, 1959). It is a semi-synthetic antimicrobial with an orthodimethoxyphenyl side group, which prevents the enzyme penicillinase access to the β-lactam ring by steric hindrance (Klein and Finland, 1963). However, the use of methicillin for treating penicillin resistant *S. aureus* infections was short lived, as within two years of its introduction the first resistant strain was isolated (Jevons, 1961).

One of the earliest targets utilized in the treatment of *Staphylococcal* infections was cell wall synthesis. In this therapeutic category, the most prominent were β-lactam and glycopeptides class of antibiotics. It was through the elucidation of the mechanism of action of these antibiotics that much of the biochemistry of peptidoglycan synthesis was established. The antibiotic vancomycin is the preferred
glycopeptide used for severe MRSA infections as it targets cell wall transpeptidation by blocking or altering the D-Ala-D-Ala motif of the glycan chains. However, in 1996, an \textit{S. aureus} strain with reduced vancomycin susceptibility (VISA) was first reported to be isolated in Japan (reduced susceptibility of \textit{Staphylococcus aureus} to vancomycin, 1997).

Hence, emergence of bacterial resistance to these antimicrobials, of last resort for example, vancomycin, underscores the need to identify and characterize new drug targets in these bacteria (Holden \textit{et al.}, 2004). Advances in genomics and bioinformatics tools have made possible the validation of a number of viable alternate drug targets. A major proportion of these targets are associated with the bacterial cell wall. These include proteins involved in the steps leading to the biosynthesis of peptidoglycan moieties UDP-N-acetylmuramyl-pentapeptide (UDP-NAM-pentapeptide) and UDP-N-acetylglucosamine (UDP-NAG) and those involved in peptidoglycan cross-linking \textit{viz.} D-alanine, D-glutamate and \textit{meso}-diaminopimelate (\textit{m}-DAP)/L-lysine biosynthesis pathways (Bugg and Walsh, 1992; Born and Blanchard, 1999; Hutton \textit{et al.}, 2003).

The focus of the work described in this thesis is on the structural and functional characterization of an enzyme involved in peptide hydrolysis from a methicillin resistant \textit{Staphylococcus aureus} (MRSA) COL strain 0085. \textit{S. aureus} is a medically important pathogen known for its ability to adapt to new conditions. The adaptability of this microbe to antibiotic stress was clearly demonstrated by a genome wide analysis (Gill \textit{et al.}, 2005; Mwangi \textit{et al.}, 2007; Baba \textit{et al.}, 2008). This analysis revealed that the transformation of a susceptible strain to one that was resistant to front-line antibiotics involved as few as 35 point mutations in 13 loci. Most of these mutations were localized to the targets of the anti-microbial agents currently employed to control \textit{S. aureus} infection. The subsequent sections in this chapter are organized to provide an introduction to peptidases involved in \textit{S. aureus} infections and to describe known catalytic mechanism of the enzymes involved in the peptidase activity.
1.2 What are peptidases?
Proteases or peptidases are proteins that catalyze the cleavage of amide bonds in peptides via exopeptidase or endopeptidase activity (Sarnovsky et al., 1929; Rawlings and Barrett, 1995). Proteases are indispensable in a wide variety of regulatory and housekeeping functions. Their importance is demonstrated by the observation that 2-3% of the total gene products in all organisms are proteolytic enzymes (Rawlings et al., 2002).

There are several types of peptidases and they all catalyze the hydrolysis of peptide bonds but the manner in which they accomplish the catalysis differs (Barrett et al., 2003). Peptidases are divided into two broad categories on the basis of type of attack on the protein:

a) **Endopeptidases**, which attack within the protein molecule,

b) **Exopeptidases**, which attack the N or C-terminal ends of the protein to produce peptides and amino acids.

Endopeptidases cleave internal peptide bonds, and release oligopeptides whereas; exopeptidases cleave peptide bonds proximal to the amino or carboxy terminus, releasing free amino acids or small peptides (McDonald, 1986).

The two groups are further sub-divided on the basis of the mechanism of action at the active site or according to the functional roles of active site residues (Taylor, 1993). These functional groups required for catalysis consist of serine, aspartic, cysteine, prolyl or metal cofactor requiring metallo amino acid residues (Rawlings et al., 2002).

**Serine proteases:** They contain a serine residue at their active site that covalently binds and processes substrates of broad specificity (Rawlings and Barrett, 2004).

**Aspartic proteases:** These proteases catalyze the hydrolysis of peptide bonds from each end of aromatic or bulky amino acid residue containing 7 substrates. These proteases catalyzed acid-base reactions by virtue of two aspartic residues located at their active site (Rawlings and Barrett, 1995).

**Cysteine proteases:** An active site cysteine residue is present in these proteases, which requires a reducing agent for enzymatic activity (Rawlings and Barrett, 2004).

**Prolyl proteases:** These are proline specific peptidases that catalyze the cleavage of amide bonds following a proline residue or an imide bond that precedes it. These
proteases demonstrate full activity in the presence of manganese ions, and maintain substantial sequence homology to various peptidases that require divalent metal ions for catalysis (Yaron and Naider, 1993; Bazan et al., 1994).

**Metallo proteases:** These types of proteases require divalent metal ions at their active site to drive peptide hydrolysis, and are highly diverse. These proteases are organized according to the conserved sequences that bind metal ions at their active sites, often HEXXH (Taylor, 1993; Bazan et al., 1994; Rawlings et al., 2002).

### 1.2.1 Functional significance of proteases

Proteases are further differentiated according to their cellular location, either intracellularly, membrane/wall associated or secreted into the external environment. This largely depends on the target substrates, enzymatic specificity and fate of the peptides released during hydrolysis.

Intracellular proteases function in processes such as cell metabolism or sporulation (Sussman and Gilvarg, 1971; Dancer and Mandelstam, 1975). For example, the Lon and ATP-dependent proteases of *Escherichia coli* regulate the destruction of damaged proteins in response to environmental stress (Chung and Goldberg, 1981). This type of response is necessary to prevent the aggregation of hydrophobic residues that are exposed when proteins denature due to heat or acid shock.

Membrane bound proteases are known to display a range of functions in the microbial cell, in addition to regulatory and nutritional roles. For example, the membrane bound protease of *E. coli*, RseP. RseP functions in a two-step proteolytic cleavage process, known as regulated intramembrane proteolysis, and assists in the induction process of σE under conditions of stress, which allows the rapid modification of gene transcription profiles (Akiyama et al., 2004).

Secreted proteases are associated with nutritional roles. Moreover, they regulate protein maturation and activation events. This can be achieved at the post translational level, when proteins are secreted in inactive zymogen configurations (Drapeau et al., 1972; Schneewind et al., 1992; Rice et al., 2001; Shaw et al., 2004). An example to quote is seen in the programmed cell death and/or apoptosis of eukaryotic cells. Apoptosis is mediated by caspase proteins which are synthesized and secreted in an inactive form. Post translational proteolytic processing is needed
to achieve a functional state. Such regulations ensure that cell death signals are not prematurely released (Kerr et al., 1972).

Proteases were initially characterized as non-specific degradative enzymes associated with protein catabolism. However, it is becoming increasingly evident that proteolysis could be another mechanism for achieving precise cellular control of biological processes in all living organisms, through the highly specific hydrolysis of peptide bonds. This highly specific and limited substrate cleavage is termed proteolytic processing (Barrett et al., 1998).

Proteases, through their ability to catalyze irreversible hydrolytic reactions, regulate the fate and activity of many proteins by controlling appropriate intra- or extracellular localization; shedding from cell surfaces; activation or inactivation of proteases and other enzymes, cytokines, hormones or growth factors; conversion of receptor agonists to antagonists; and exposure of cryptic neoproteins (which is when the proteolytic cleavage products are functional proteins with roles that are distinct from the parent molecule). Hence, proteases initiate, modulate and terminate a wide range of important cellular functions by processing bioactive molecules, and thereby directly controlling essential biological processes, such as DNA replication, cell-cycle progression, cell proliferation, differentiation, morphogenesis, tissue re-modelling, neuronal outgrowth, homeostasis, wound healing, immunity, angiogenesis and apoptosis (Sternlicht and Werb, 2001).

1.2.2 Peptidases in bacterial pathogenesis

Proteolytic enzymes have many physiological roles and are essential factors for homeostatic control in both eukaryotes and prokaryotes. Similarly, microbial proteases play several roles during their life cycle, however, the enzymes produced by pathogenic microorganisms, especially by opportunistic pathogens, occasionally act as toxic factors to the host. Many of the toxic proteases are metalloproteases having divalent metal ion (commonly zinc) in the catalytic site (Miyoshi and Shinado, 2000; Botelho et al., 2011).

These toxic proteases act as virulence factors in case of many infectious microorganisms, viruses and parasites. Animal venom also contains proteases that effect tissue destruction or evade host responses. Hence, many proteases or their
substrates are an important focus of attention for the pharmaceutical industry as potential drug targets (Lopez-Otin and Overall, 2002).

Understanding the reaction mechanism of metallopeptidases will aid in the design and synthesis of new molecules that can be screened as potential pharmaceuticals since most of these enzymes play important roles in cellular processes such as tissue repair, protein maturation, hormone level regulation, cell-cycle control and protein degradation. Therefore, these enzymes are significant in several disease states including cancer, HIV, stroke, diabetes, bacterial infections, neurological processes, schizophrenia, seizure disorders, and amyotrophic lateral sclerosis (Holz et al., 2003).

There has been an increasing interest in the identification and functional characterization of the many proteases that are present in various organisms, from bacteria to man. Advancement in several large-scale genome-sequencing projects has provided new opportunities to realize the complexity of protease systems. Regardless of these advances, the substrates and in vivo roles for newly identified proteases are unknown. Even for proteases that have been well characterized, their biological functions are often not fully understood. Hence, it becomes inevitable to develop new techniques to identify the protease repertoire that is expressed and active in a cell, tissue or organism, as well as to identify all the natural substrates of each protease (Lopez-Otin and Overall, 2002).

Identifying the substrate degradomes of one such peptidase from S. aureus COL0085 forms the important part of the study carried out in this thesis which will facilitate our understanding of their physiological and pathological roles and thereby point to new drug targets. Such information, along with knowledge of the protease degradome of a cell, will increase our understanding of the biological roles of this peptidase in the cellular context with respect to cell function and pathology.

1.2.3 Proteases of S. aureus
The S. aureus genome encodes 132 putative proteases and 42 non-peptidase homologs (Rawling, Barrette and O’Brien, 2002). Some of the putative or characterized proteases are secreted into the external environment in a temporally-regulated manner. For example; aureolysin and the serine-protease like enzymes (Chan and Foster, 1998; Rice et al., 2001; Karlsson and Arvidson, 2002; Shaw et al.,
Many of these secreted proteases have been characterized extensively and have been shown to contribute to the progression of disease in *S. aureus* (McGavin *et al.*, 1997; Lindsay and Foster, 1999; McAleese *et al.*, 2001; Karlsson and Arvidson, 2002). The switch of infectious states from adhesion to invasion has been suggested to be primarily driven by the interplay of these secreted proteases, and cell adhesion and colonization factors (McGavin *et al.*, 1997) (pathogenic mechanism of *S. aureus* described earlier in section 1.1.2).

### 1.3 Metallopeptidases

Metalloproteases (metallopeptidases or metalloproteinases) form the largest and the most homogenous family of proteases. They represent an extensive class of hydrolases which bring about the cleavage of peptide bonds by the action of a water molecule which is in turn activated by complexing to bivalent metal ions (Johanna Mansfeld, 2007). The majority of metalloproteases are characterized by a catalytic zinc ion (Becker and Roth 1992; Hopper 1994). However, in some enzymes, manganese, cobalt, nickel or even copper ions execute the catalytic reaction (Johanna Mansfeld, 2007). Additionally, metalloproteases with two metal ions acting co-catalytically are also observed. The metal ion is complexed by three conserved amino acid residues that can be His, Asp, Glu or Lys (Johanna Mansfeld, 2007). Metalloproteases cleave proteins and peptides, and deregulation of their function leads to pathology. An understanding of their structure and catalytic mechanisms is necessary for the development of strategies for their regulation (Xavier Gomes-Ruth, 2009).

Hydrolysis of peptide bonds is vital for life and the factors responsible for it are ubiquitous. Metallopeptidases are among those which participate in metabolism through both extensive and unspecific protein degradation and controlled hydrolysis of specific peptide bonds (Neurath and Wash, 1976). Deregulation of such vast degrading potential leads to pathologies as these metallopeptidases act as virulence factors during poisoning and microbial infections. These array of biological functions makes structural studies of these proteins crucial to any understanding of their function and to the design of novel, highly-specific therapeutic agents to modulate their activity (Lopez-Otin and Overall, 2002; Xavier Gomes-Ruth, 2009).
1.3.1 Family of metallopeptidases

Metallopeptidases are regarded as the most diverse of the catalytic types of peptidases. About 30 families are recognized so far with regard to three conserved active site residues, histidine, glutamine, lysine and/or aspartate. According to the classification of proteases based on protein structure and homology implemented in the MEROPS database (merops.sanger.ac.uk), metalloproteases are found in 14 different clans (Rawling and Barrette, 2004). In addition, clan M - contains metalloprotease families not yet assigned to a clan. Proteases from clans MA, MC, MD, ME, MJ, MK, MM, MO and MP require only one catalytic metal ion, in most cases zinc ion, whereas clans MF, MG, MH, MN and MQ contain two metal ions acting co-catalytically on the substrate (M stands for metalloprotease).

Family and Clan The term family is used to describe a group of enzymes in which each member shows evolutionary relationship to at least one other, either throughout the whole sequence or at least in the part of the sequence responsible for catalytic activity. A clan comprises a group of families for which there are indications of evolutionary relationship, despite the lack of statistically significant similarities in sequence. Such indications of distant relationship come primarily from the linear order of catalytic-site residues and the tertiary structure. Distinctive aspects of the catalytic activity such as specificity or inhibitor sensitivity may also contribute occasionally (Rawlings and Barrett, 1995).

Among the 30 families recognized so far, half of the families comprise enzymes containing the His-Glu-Xaa-Xaa-His (or HEXXH) motif as shown by X ray crystallography to form the binding site of divalent metal ion. The “HEXXH” pentapeptide is very common in protein sequences and SwissProt database reports about one fifth of the occurrences of this motif in proteins that are known to be metallopeptidases. The motif is small enough to have arisen independently on several occasions. Hence, additional evidence would be needed before one could suggest that all HEXXH containing enzymes are evolutionarily related (Bode et al., 1992).

A consensus sequence containing the HEXXH pentapeptide can be defined more precisely as abXHEbbHbc, in which b is an uncharged residue, c is hydrophobic, and
X can be almost any amino acid. The families of metallopeptidases can be divided into five groups, the first three of which contain the HEXXH motif.

First set of family comprises a glutamic acid residue completing the metal-binding site, and hence the families in this “HEXXH + E” group show signs of a relationship with each other and they form clan MA. In the second set, a third histidine residue is a ligand and hence “HEXXH + H” families also become interrelated forming clan MB. The third set of HEXXH families is that in which additional metal ligands are present which are yet to be identified. The fourth group is a heterogeneous one wherein the essential metal atoms are bound at motifs other than HEXXH. The fifth and final group comprises those in which the metal ligands are quite unknown (Jongeneel et al., 1989; Rawlings and Barrett, 1995).

From the evolutionary standpoint it may be noted that families of metallopeptidases are represented in both prokaryotes and eukaryotes. Moreover, the prokaryotic and eukaryotic enzymes often show similarity in terms of both structure and properties. This observation was suggested by Barrett and Rawlings (Barrett and Rawlings, 1993) since metallopeptidases of higher organisms were introduced into the eukaryotic cell relatively recently, at the time of the endosymbioses that led to the formation of mitochondria and other organelles, rather than being derived from the ultimate common ancestor of prokaryotic and eukaryotic cells.

The study carried out in this thesis is for a putative peptidase from MRSA strain *S. aureus* COL0085 which is found to be highly conserved within the peptidase M20 family in the MH clan of metallopeptidases. This was observed when a homology search for this polypeptide sequence was done with National Center for Biotechnology Information TBLASTN, against the EMBL, DDBJ, GenBank, and PDB databases.

1.3.2 Why study enzymes with respect to M20 family of peptidases

Clan MH enzymes form the most heterogeneous group. Enzymes reported in this clan include aminopeptidases, carboxypeptidases, dipeptidases and aminocyclases (Franzetti *et al.*, 2002; Russo and Baumann, 2004; Perrier *et al.*, 2005; Odintsov *et al.*, 2005). The M20 family of metallopeptidase clan includes (for example) ACY1 from human and porcine, carboxypeptidase (CP) S precursor from *Saccharomyces*
cerevisiae, succinyl-diaminopimelate desuccinylases from Escherichia coli,
Corynebacterium glutamicum and Haemophilus influenzae, acetylor-ornithine
deacetylases from Escherichia coli and Dictyostelium discoideum, and CPG2
precursor from Pseudomonas sp. strain RS-16 (Boyen et al., 1992). Most of these
are found to be soluble dimeric zinc- or cobalt-coordinated enzymes showcasing
similar biochemical properties with three conserved amino acid motifs in the active
site. The available crystal structures for microbial M20 family of enzymes show a
dizinc-binding domain, which is characteristic of all the MH clan of zinc peptidases,
and a typical smaller domain, which is inserted in the middle of the metal-binding
domain which mediates homodimerization (Biagini and Puigserver, 2001).

1.3.3 Summary of M20 family of metallopeptidases

Statistics for M20 family of metallopeptidases gives the following data:

| Sequences | : 7503 |
| Identifiers | : 38 |
| Identifiers with PDB entries | : 12 |

The notable peptidases in family M20 are carboxypeptidase such as the glutamate
carboxypeptidase from Pseudomonas, the thermostable carboxypeptidase Ss1 of
broad specificity from the archaea such as Sulfolobus sp. and the yeast Gly-X
carboxypeptidase. The dipeptidases include bacterial dipeptidase, peptidase V, a
eukaryotic, non-specific dipeptidase, and two Xaa-His dipeptidases (carnosinases).
There is also the bacterial aminopeptidase, peptidase T that acts only on tripeptide
substrates and has therefore been termed a tripeptidase. Dug1p from
Saccharomyces cerevisiae which is a Cys-Gly peptidase is the only
metallopeptidase belonging to M20 family since members of the M19, M1, and M17
family was known to carry such Cys-Gly peptidase activity (Kaur et al., 2009). A
detailed table showing the members of M20 family of metallopeptidases reported so
far has been described below (Table 1.1).
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</tr>
<tr>
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Table 1.1 Subfamilies of M20 family of metallopeptidases (adapted and modified from MEROPS: the protease database (merops.sanger.ac.uk) (Rawlings, O’Brien and Barrett, 2002; Rawlings, Morton and Barrett, 2006).
1.4 Catalytic mechanism of the metallopeptidases

Proteases belong to the International Union of Biochemistry class I group of hydrolase enzymes. Divalent zinc metal is found in most metalloproteases. Besides this, metal ions like Mn, Co, Ni and Mg are also found. Therefore, the function of these enzymes is related to the chemistry of these divalent metal ions with which they are associated (Irving and Williams, 1952; Rulísek and Vondrasek, 1998). For example, zinc metallopeptidases are quite versatile as they have a remarkable coordination sphere which is adaptable to accommodate a broad range of coordination numbers and geometries while forming complexes (Vallee and Auld, 1990a). Zinc can act as a Lewis acid in catalysis if it retains a positive charge after ligating to protein side chains. It can also act as a base or nucleophile in catalysis if water bound to zinc is converted to hydroxide. Hence, catalytic zinc sites can be regarded as amphoteric. The oxidation/reduction properties of its neighboring transition metals are a major cause of their ligand/exchange rates, amphoteric properties and coordination geometries. Given that, zinc has a filled d-shell it does not have oxidation/reduction properties. Hence, a stable metal ion species is provided in a biological medium whose redox potential is in constant flux (Rawlings and Barrett, 2004).

The most common amino acids that supply ligands to the catalytic sites of metallopeptidases are His, Glu, Asp and Cys. In zinc metalloproteases, the catalytic site contains zinc which forms complexes with any of the three nitrogens (His) and oxygen (Glu or Asp) donors, with His being the predominant amino acid chosen by a ratio of about 2:1 over Glu plus Asp (Rawlings and Barrett, 2004). Histidine (usually the N\text{e}^{2} nitrogen) may be chosen because of its capacity to disperse charge through hydrogen bonding of its non-liganding nitrogen. The carboxylate anion of Glu and Asp ligands will reduce the charge on the metal, making it more difficult for the metal-bound water to ionize and for the metal to act as a Lewis acid catalyst (Rawlings and Barrett, 2004).
1.4.1 Metal sites in metalloproteases

![Figure 1.2 Zinc-binding sites in metalloproteases. Schematic shown for A) catalytic site for carboxypeptidase A. B) structural site for the matrix metalloproteases. C) cocatalytic site for Aeromonas aminopeptidase.](image)

The metal-binding sites in the proteases can be classified as catalytic, co-catalytic and structural (Figure 1.2 A, B and C) (Vallee and Auld, 1990b and 1993b). These can be explained taking zinc metalloenzymes as an example.

**Catalytic sites** are generally four or five coordinated in metallopeptidases. In the free state the geometry is often distorted-tetrahedral or trigonal-bipyramidal. Water always acts as a ligand for these sites (Figure 1.2 A). The metal-bound water (in this case zinc) is activated for ionization, polarization or displacement by the identity and arrangement of ligands co-ordinated to zinc (Vallee and Auld, 1990a). Generally, in metalloproteases Glu or His residue within hydrogen-bonding distance of the metal-bound water play the role of a general acid/base catalyst or may stabilize the
transition state of a tetrahedral intermediate. Otherwise, the direct ionization of the metal-bound water will lead to nucleophilic catalysis by zinc hydroxide, whereas displacement of water or expansion of the co-ordination sphere can result in Lewis acid catalysis by the catalytic zinc atom. Thus structure of the active site shows that the identity of the three protein ligands, their spacing and secondary interactions with neighboring amino acids in conjunction with the vicinal properties of the active center created by protein folding are all critical for the various mechanisms through which zinc can be involved in catalysis (Rawlings and Barrett, 2004).

**Structural metal site** consists of four protein ligands with no bound water molecule. Cys, followed by His are found to be the favored ligands in such sites in metalloenzymes (*Figure1.2 B*) (Auld, 2001a). It has been observed that metalloproteases normally use either disulfides or calcium ions to help in stabilizing the structure of the enzyme. For example almost all matrix metalloprotease sequences (Morgunova *et al*., 1999) are known to have such sites.

**Cocatalytic sites** in metalloenzymes contain two or three metals in close proximity with two of the metals bridged by a side-chain moiety of a single amino acid residue, such as Asp, Glu, His and sometimes a water molecule (Auld, 2001a). Asp and Glu and sometimes an additional water molecule are the preferred bridging ligands for these sites (*Figure 1.2 C*). No Cys ligands have been reported for these sites. The only unusual amino acid ligand found is the Lys in the bovine lens family of aminopeptidases (Kim and Lipscomb, 1994). The ratio of Asp and Glu to His is about 2:1.

The bridging amino acids and water play important roles in catalysis as their dissociation from either or both metal atoms during catalysis could change the charge on the metal thus promoting its action as a Lewis acid or allowing interaction with an electronegative atom of the substrate. On the other hand, the bridging ligand might contribute quickly in the reaction as a nucleophile or general acid/base catalyst. In this fashion the metal atoms and their coupled ligands play specific roles in each step of the reaction to bring about catalysis (Rawlings and Barrett, 2004).

The total number of protease side chain ligands to the co-catalytic metal sites is five instead of three and four for the catalytic and structural zinc sites respectively. The metals in these sites may therefore be important to the overall fold and stability of the
protein as well as catalytic function. Only a few of the co-catalytic sites contain only zinc ions. Several of the aminopeptidases have been characterized as the di-zinc, di-cobalt or di-manganese complexes. However the native metal is sometimes still in question. For example, the *E. coli* methionine aminopeptidase 1 (Roderick and Matthews, 1993), the hyperthermophile *Pyrococcus furiosus* methionine aminopeptidase 2 (Tahirov et al., 1998b) and human methionine aminopeptidase 2 (Liu *et al*., 1998) have been isolated as di-cobalt enzymes, and the *E. coli* proline aminopeptidase as the di-manganese enzyme (Wilce *et al*., 1998). The physiological metal for these enzymes is still not certain. Zinc works as well as cobalt in the yeast aminopeptidase 1 (Walker and Bradshaw, 1998) and recent studies of the *E. coli* MetAP-1 indicate that it functions as a Fe (II) enzyme (D'Souza and Holz, 1999).

### 1.4.2 Catalytic mechanisms of metalloproteases

Addition of a water molecule across a peptide bond forms the basis of catalytic activity by all metalloexo- and metalloendoproteases. Hence, the products of the reaction are a protonated amine and an ionized carboxylate group at neutral pH values. Mechanism of catalysis can be explained by taking zinc metallopeptidases as an example with 2His1Glu/Asp zinc sites at the catalytic sites, also with cocatalytic zinc sites.

### 1.4.2.1 Catalytic zinc site with 2His and 1Glu/Asp residues

Metallopeptidase like carboxypeptidase A is the best studied enzyme to describe the catalytic mechanism involving 2His and 1Glu/Asp catalytic zinc sites (Auld and Vallee, 1987; Auld, 1997). $k_{cat}$ and $K_m$ profiles of this enzyme when analyzed showed a three protonation state model $\text{EH}_2 \leftrightarrow \text{EH} \leftrightarrow \text{E}$ (Auld and Vallee, 1970). Hence, it was predicted that the enzyme can bind substrates but not hydrolyze them when it is in its $\text{EH}_2$ form below the acidic $pK_1$. The ionization of the group $\text{EH}_2$ with a $pK_a$ of 6.0 transforms the enzyme to its active form $\text{EH}$. Further ionization of the enzyme to the $\text{E}$ form, occurring with a $pK_2$ of 9.0, markedly reduces substrate binding and therefore catalysis. To discuss the mechanism of such enzymes $pK_1$ is assigned to the ionization of the carboxyl group of Glu270 ([Figure 1.3](#)) and its subsequent interaction with the water ligand of zinc, stabilizing the active-site structure and $pK_2$ to ionization of the zinc-bound water.
Initially in step (1) (Figure 1.3) the Glu270 acts as general base catalyst by removing a proton from the metal-bound water allowing the metal bound hydroxide to attack the peptide carbonyl. In case of carboxypeptidase A, Arg127 plays a role in stabilizing the negative charge in the transition state (Phillips et al., 1992). Other active-site residues such as Arg145, Asn144 and Tyr248 play roles in binding the substrate carboxyl group and thus aiding in positioning the substrate for catalysis (Christianson and Lipscomb, 1989). In the second step (2) Glu270 acts as a general acid catalyst as it donates a proton to the leaving amino group causing the metal bound tetrahedral intermediate to collapse forming products, which is shown in step (3). Finally, in step 4, the N-terminal product leaves and water returns to the metal.

Figure 1.3 Mechanism of hydrolysis in carboxypeptidase A.
with the C-terminal product still bound in a salt-bridged manner to Glu270 completing the catalytic reaction (Rawlings and Barrett, 2004).

1.4.2.2 Proteases containing co-catalytic zinc sites

Proteases with cocatalytic metal sites are also reported to be widely distributed in bacteria, yeast, plant and animal sources, especially aminopeptidases which catalyze the hydrolysis of a wide variety of N-terminal peptides and amino acid derivatives. The structures of metallopeptidases with co-catalytic Zn, Co and Mn sites have been reported. For example, bacterial carboxypeptidase G2 (Rowsell et al., 1997), a tripeptidase (Hakansson and Miller, 2002) and a dipeptide transport protein (Remaut et al., 2001) contain cocatalytic zinc sites. Bovine lens leucine aminopeptidase (BLAP), *Aeromonas proteolytica* aminopeptidase (AAP) and *Streptomyces griseus* aminopeptidase (SGAP) are the ones in which most extensive steady-state and pre-steady state kinetic, spectral and structural studies have been carried out. AAP and BLAP exhibit similar kinetics (Taylor, 1993b) and their C-terminal catalytic domains have similar folds.

![Figure 1.4 Possible mechanisms for aminopeptidases. Here X represents the general acid/base catalyst.](image-url)
Exemplifying AAP and SGAP enzymes, potential mechanism for the co-catalytic aminopeptidases enzymes that are similar in nature to the one for the 2His1Glu catalytic zinc sites can be formulated (Holz, 2002). In this mechanism (Figure 1.4) the scissile peptide carbonyl disrupts the bridging water and interacts with Zn1 in the first step (1). Thus Glu151/Glu131 acts as a generalized base first by removing a proton from the Zn2-bound water. The Zn2-bound hydroxide then attacks the carbonyl of the scissile peptide bond to form the tetrahedral intermediate step (3). Finally the protonated form of Glu131/Glu151 acts as a general acid catalyst by donating a proton to the scissile amide nitrogen allowing the tetrahedral intermediate to collapse forming the product complex step (4) (Figure 1.4).

Similar type of catalytic mechanisms could be expected in case of the putative peptidase under study as it has been found to be classified as a metallopeptidase belonging to M20 family of metallopeptidases wherein the crystal structures of several peptidases and their catalytic mechanism have been reported.

1.4.3 Structural aspect of proteins belonging to M20 family of metallopeptidases

The available crystal structures of M20 enzymes (Table 1.1) shows that, in general, these proteins adopt a two-domain structure and are either monomers or homodimers in solution. The structures are found to be well conserved despite poor sequence similarity. However, aminopeptidase characterized from Aeromonas proteolytica and Streptomyces griseus become the only known exceptions where the protein is folded into a single α/β globular domain (Chevrier et al., 1996; Greenblatt et al., 1997).

Of the two domains in M20 proteins, the first domain is referred to as the catalytic domain and the second domain is referred to as the lid domain in monomeric M20 proteins (Jozic et al., 2002) or as the dimerization domain in homodimeric enzymes (Rowsell et al., 1997; Hakansson and Miller, 2002; Badger et al., 2005; Lundgren et al., 2007). The catalytic domain harbors residues essential for catalysis and metal binding. However, the active site is located between the two domains and contains a di-metallic center coordinated by residues from the catalytic domain. Few residues from the lid domain also play a role in substrate binding and catalytic activity (Lindner et al., 2003; Lundgren et al., 2007). Even if, most enzymes of the M20 family bind
zinc or cobalt; manganese, copper and magnesium are also preferred metal cofactors (LeClere et al., 2002; Munih et al., 2007; Bitto et al., 2009).

Crystal structures are available for peptidases from subfamilies M20A, M20B and M20D, showing protein folds typical of clan MH, which represents the largest group of metallopeptidases based on sequence similarity. For example, glutamate carboxypeptidase (CPG2) belonging to subfamily M20A exists as a homodimer, and the structure shows that each monomer consists of two domains, of which the N-terminal is catalytic and the C-terminal links the monomers in the dimer (Figure 1.5) (Rowsell et al., 1997). The three-dimensional structure determination of CPG2 was undertaken to identify the active site of the enzyme and to allow modification of the protein and/or substrate with a view to improving the therapeutic properties of the system (Pauptit et al., 1997).

![Figure 1.5 Ribbon diagram of the CPG2 dimer. One subunit is coloured blue and the other is coloured yellow. The active-site zinc ions are represented by magenta spheres (PBD id: 1CG2).](image)

The crystal structure of PepV (subfamily M20A) in complex with the phosphinic inhibitor AspΨ[PO2CH2]AlaOH, a dipeptide substrate mimetic, elucidated the substrate binding geometry and identified the critical residues involved in substrate binding and catalysis (Jozic et al., 2002). It also explained the different functions of the two zinc ions coordinated with the active site residues involved in catalysis. This structure revealed a catalytic domain and a lid domain, which together form an internal active site cavity that traps the inhibitor. The catalytic domain is topologically similar to catalytic domains from amino- and carboxypeptidases. However, the lid
domain was unique among the related enzymes compared to the other related exopeptidases (Jozic et al., 2002).

Peptidase T characterized from *Escherichia coli* is a tripeptidase that hydrolyzes tripeptides at their N-termini (Strauch and Miller, 1983; Miller et al., 1991; Hakansson and Miller, 2002). Peptidase T is classified into the M20B family of metallopeptidases. Peptidase T has the same basic fold as CPG2 and comprises two domains; a catalytic domain with an active site containing two metal ions, and a smaller domain formed through a long insertion into the catalytic domain.

The X-ray crystal structure of ILL2 from *Arabidopsis thaliana* shows it to be a dinuclear metallopeptidase from the M20 peptidase family (Bitto et al., 2009). This structure also consists of two domains, a larger catalytic domain and a smaller satellite domain. The metal-coordinating residues in the active site of ILL2 include a conserved cysteine that clearly distinguishes this protein from previously structurally characterized members of the M20 peptidase family and classifying them as M20D subfamily. Other members belonging to this family whose crystal structures are available are HmrA, an antibiotic resistance factor of methicillin-resistant *Staphylococcus aureus* and YxeP protein from *Bacillus subtilis*.

Structural comparison showed close overall similarity between several members of the M20 family of peptidases. Hence, we could expect a similar structural fold for the putative peptidase reported in this thesis. These structures could be used as a template for interpreting its structure and active site residues involved in catalysis.

### 1.4.4 Inhibitor studies involved in M20 classes of metallopeptidases

An enzyme inhibitor is defined as a molecule which can bind enzymes and decrease their activity. Since blocking an enzyme’s activity can kill a pathogen or correct a metabolic imbalance, many drugs are enzyme inhibitors. The binding of an inhibitor can stop a substrate from entering the enzyme’s active site and/or hinder the enzyme from catalyzing its reaction. Several such studies had been reported in M20 family of metallopeptidases. Already it has been discussed in section 1.2.2 of this chapter about the importance of understanding the catalytic mechanism of metallopeptidases. Such studies will aid in designing and synthesis of new
molecules to act as potential pharmaceuticals as they have become the target of intense efforts in inhibitor design.

Information regarding the active site residues of an enzyme will help in understanding the specificity of such enzymes. Hence, substrate analogues can be used as inhibitors or mutagenesis could be carried out to bring down the activity. For example, reports are available from M20 family, where mutations to His397 of ILL2, lead to a complete loss of the enzymatic activity (Bitto et al., 2009). This shows the crucial role of such residue in catalysis. Serine- and cysteine peptidase inhibitors exerted no relevant inhibitory activity in case of HmrA protein. In contrast, it was efficiently inhibited by general zinc-chelating agents such as EDTA, o-phenanthroline, tetraethylenepentamine, as well as by excess zinc (Botelho et al., 2011). Therefore, the regulatory mechanism of this enzyme could potentially be exploited for the development of selective inhibitors as in case of the protein tyrosine kinases (Schindler et al., 2000) and other metal dependent peptidases.

1.5 Objectives

It is evident from literature that microbial metallopeptidases are the main cause of pathogenesis in human beings since they have proteolytic activity towards many of the host proteins. Such enzymes could be targeted to counteract the infectious state by developing specific inhibitors. The enzyme under study is from a nosocomial pathogen Staphylococcus aureus strain COL0085 which is found to be classified as a metallopeptidase belonging to M20 family of metallopeptidases. However, there are no reports on this enzyme which suggests that the structure-catalytic activity correlations of this putative peptidase are not known. The clone of this peptidase was available and its crystal structure not known.

The main objective of the present work was:

1. Cloning, expression and purification of putative peptidase from Staphylococcus aureus strain COL0085 in E.coli strain.
2. Biochemical characterization of the protein expressed, such as,
   - Establishing a biochemical assay system to assess enzyme activity.
   - Enzyme kinetics.
   - Substrate specificity.
• Metal ion co-factor preferences of the enzyme.
• Effect of regulators and inhibitors on enzyme activity.

3. Studying the oligomeric status of the protein.

4. Homology modeling and crystallization trials.

To achieve the above objectives, the following methodology was adopted.

1.6 Methodology

• The putative peptidase gene was amplified from the genomic DNA of *Staphylococcus aureus* strain COL0085 by PCR and cloned into pET15b vector using standard cloning techniques to generate pET15b-SACOL0085 putative peptidase plasmid DNA. In this construct, the plasmid open reading frame is fused to a vector-encoded N-terminal histidine tag, and placed under the control of the T7 promoter.

• Cloning and expression of the plasmid DNA will be carried out in *E.coli* strain and the protein will be purified using Ni-NTA affinity chromatography.

• The putative peptidase activities will be studied using colorimetric assay techniques and enzyme kinetics will be established.

• Metal ion occupancy issue and substrate specificity will be studied by spectroscopic and chromatographic techniques.

• Effect of regulators and inhibitors on enzyme activity will be screened with different chelating chemical agents.

• The oligomeric status of the protein will be studied using gel filtration techniques and Dynamic Light Scattering experiments.

• Homology modeling

• Crystallization trials will be carried out with the purified protein using Hampton crystallization kits (Hampton Research, Inc.). The conditions of crystallization will be examined using both hanging-drop and the sitting drop method under oil.
1.7 Organization of the thesis

The work carried out in the present doctoral work has been organized into six chapters.

Chapter 1  Introduction

Chapter 2  Isolation and purification of putative peptidase from *Staphylococcus aureus* COL0085

Chapter 3  Metal ion dependency and enzyme characterization

Chapter 4  Influence of cysteine residues and determination of kinetic constants

Chapter 5  Analysis of cysteine residues and inhibitor studies

Chapter 6  Determination of oligomeric status, homology modeling and crystallization trials of SACOL0085 peptidase.

Conclusions

References