A NEW BACTERIAL PEPTIDOGLYCAN PEPTIDASE LYTU AND INSIGHTS INTO SUBSTRATE RECOGNITION BY LYSOSTAPHIN FAMILY

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ABSTRACT

Staphylococcus aureus is a pervasive pathogen, whose infections frequently result in serious medical complications and death. Its encounters are yet more perilous in clinical settings where professional care and financial resources alone do not suffice to ensure successful treatment results. The virulence of the bacteria is enforced by numerous cellular mechanisms that have allowed it to develop resistance to every drug used to this date. The bacterial cell wall (CW) is the primary line of defense, the most common target in treatment strategies, and is likely to remain the prioritized candidate for future therapeutic solutions.

The main structural component of bacterial CW is peptidoglycan (PG) that forms protective layers. PG is administered by a large number of enzymes that are involved in its synthesis, maintenance, and cleavage. One family of enzymes, M23 peptidases, cleaves pentaglycine bridges that link chains of PG and are specific to *S. aureus*. These enzymes can be used by the bacteria to manage its own PG in a controlled manner or, alternatively, by hostile microorganisms and cause cell death. Therefore, M23 peptidases of *S. aureus* are important as potential targets for drugs as well as pharmacological tools themselves that are already employed by nature. Substrate recognizing SH3b domains enhance the effectiveness of M23 endopeptidases.

Previous research had identified a putative M23 peptidase gene, transcription of which is upregulated under *S. aureus* exposure to compounds harmful to cell wall. We examined and characterized the product of the gene. The protein, which we named LytU, is an M23 family zinc-dependent enzyme that cleaves pentaglycine. It is anchored in plasma membrane and is extracytoplasmic, residing in a periplasm-like space. The physiological role of LytU is not confirmed, but evidence suggest it can recycle PG fragments and participate in daughter cell separation. A distinct feature of the enzyme is its ability to strongly bind a second zinc ion, which incapacitates catalytic residues. We propose that together with pH, the binding of second ion serves a regulatory function *in situ*. Solution structure of the LytU catalytic domain has been determined.

Binding of substrate pentaglycine to catalytic M23 domain is very transient at least *in vitro*. The binding, nevertheless, is accomplished by SH3b domain of enzymes bearing it. Contrarily to previous beliefs, we found that SH3b domain binding to substrate is primarily driven by interactions with PG branching peptides, rather than by weaker interaction with pentaglycine. The binding of SH3b to substrate is independent of catalytic domain and it targets and binds the PG peptide moieties that are proximal to but different from the pentaglycine cleaved by catalytic domain.

In summary, we have introduced and characterized a new M23 family endopeptidase, proposed a regulation mechanism, and changed the paradigm of substrate binding by M23 peptidases. Our results are expected to contribute to a better understanding of *S. aureus* physiology and provide means for the development of cures.

ORIGINAL PUBLICATIONS

I. **Raulinaitis, V.,** Tossavainen, H., Aitio, O., Seppala, R., & Permi, P. (2017). 1H, 13C and 15N resonance assignments of the new lysostaphin family endopeptidase catalytic domain from staphylococcus aureus. *Biomolecular NMR Assignments, 11*(1), 69-73.

II. **Raulinaitis, V[#].,** Tossavainen[#], H., Aitio, O., Juuti, J. T., Hiramatsu, K., Kontinen, V., & Permi, P. (2017). Identification and structural characterization of LytU, a unique peptidoglycan endopeptidase from the lysostaphin family. *Scientific Reports, 7*(1), 6020.

III. Tossavainen, H., **Raulinaitis, V**., Kauppinen, L., Pentikäinen, U., Maaheimo, H., & Permi, P. (2018). Structural and functional insights into lysostaphin-substrate interaction. *Frontiers in Molecular Biosciences, 5*, 60.

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Kyburz, A., **Raulinaitis, V**., Koskela, O., Kontinen, V., Permi, P., Kilpelainen, I., & Seppala, R. (2010). 1H, 13C and 15N resonance assignments of the major extracytoplasmic domain of the cell shape-determining protein MreC from bacillus subtilis. *Biomolecular NMR Assignments, 4*(2), 235-238.

Muona, M., Aranko, A. S., **Raulinaitis, V.**, & Iwaï, H. (2010). Segmental isotopic labeling of multi-domain and fusion proteins by protein trans-splicing in vivo and in vitro. *Nature Protocols, 5*(3), 574-587.

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ABBREVIATIONS

CBD	Cell wall binding domain
CHAP	Cysteine, histidine-dependent amidohydrolases/peptidases
CW	Cell wall
ITC	Isothermal titration calorimetry
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NMR	Nuclear magnetic resonance
PG	Peptidoglycan
SAXS	Small-angle X-ray scattering
SH3b	Bacterial SH3 (src Homology-3) domains
PBP	Penicillin-binding protein
TCS	Two-component system
VISA	Vancomycin-intermediate *S. aureus*
VRSA	Vancomycin-resistant *S. aureus*
VSSA	Vancomycin-susceptible *S. aureus*

1. INTRODUCTION

Staphylococcus aureus [from Greek and Latin *staphylo* – "resembling cluster, bunch of grapes", *coccus* – "berry", i.e. round*, aureus* – "golden", i.e. yellow color], a Gram-positive bacterium, is one of over 2000 human bacteria species and one of just under 100 species that cause infectious diseases to their host (McFall-Ngai, 2007). Its medical impact is aggravated by the pervasive presence, morbidity of bacteremia cases, and readiness to develop drug resistance.

Already upon the discovery of *Staphylococcus* in 1878, Alexander Ogston noted "micrococci so deleterious when injected, and so harmless on the surface of wounds and ulcers" (Ogston, 1881). Although it must be noted that *S. aureus* itself can cause skin infections, it is indeed its entrance into the bloodstream (i.e. bacteremia) that triggers the most severe and lethal clinical events. The array of *S. aureus* caused diseases, to name a few, includes food poisoning, cellulitis, abscesses, pneumonia, osteomyelitis, endocarditis, and toxic shock syndrome (Lowy, 1998; Staphylococcus aureus infections – infections, Merck Manuals).

Many healthy individuals carry the bacteria as well, predominantly in their nostrils, without any symptoms. It is estimated that persistent and intermittent carriers account for 20 and 60% of population, respectively, and 20% never carry the pathogen (Kluytmans *et al.*, 1997). Bacteremia afflicts 20 – 50 individuals per 100,000 of global populations, with Scandinavian rates being among the best and the US among the worst, and 10 – 30 percent of these cases are fatal (van Hal *et al.*, 2012). *S. aureus* can be contracted in hospitals where drug resistant strains become particularly frequent and concerning. Formation of durable biofilms enables transmission of the bacteria via medical devices and contamination of intravenous catheters carries high risks (Brooks & Jefferson, 2012).

The toughness of *S. aureus* against treatment has been noted by Ogston himself, who wrote: 'Once established the micrococci are hard to kill – the only thing I found effective was cauterisation with a strong solution of chloride of zinc' (Ogston, 1881). The adversity continues to this date and will in the foreseeable future. Every introduction of a major anti-staphylococcal agent was followed by the discovery of resistant strains: penicillin in 1940s, methicillin (MRSA) in 1960s, and vancomycin (VRSA) upon the increase of its use in 1990s. The origins of infections gradually moved beyond hospitals and into communities and livestock (Chambers & Deleo, 2009; Knox *et al.*, 2015). The medical challenges translate into an enormous economic drawback: in European Union alone, hospital acquired MRSA infections affect 150,000 patients and cost additional 380 million euro (Köck *et al.*, 2010), and the total inflicted costs in the US are in billions (Lee *et al.*, 2013). Calls for new therapeutic approaches have been made (World Health Organization, 2014; Centers for Disease Control and Prevention, (U.S.), 2013). New drugs are continuously proposed (Bal *et al.*, 2017; Choo & Chambers, 2016; Purrello *et al.*, 2016). Nevertheless, the time-proven tendency of *S. aureus* to develop resistance to small molecules is troubling and urges to develop new treatment paradigms (Taubes, 2008).

1.1. *Staphylococcus aureus* drugs and resistance

Antibacterial agents can be grouped into three main classes, based on their intracellular targets and interference objects: cell envelope (including cell wall and plasma membrane), protein synthesis, and DNA synthesis. **Table 1** lists currently used anti-staphylococcal agents. Cell wall synthesis targeting β-lactam class antibiotics like penicillin/methicillin and glycopeptides related to vancomycin account for nearly a half and are most commonly used (Bassetti *et al.*, 2013). New generation β-lactams ceftaroline and ceftobiprole as well as glycopeptides dalbavancin, oritavancin, and telavancin were approved by drug regulatory agencies in the last decade, daptomycin and linezolid have gained prominence and the development of tetracyclines, fluoroquinolones, and oxazolidinones is ongoing (Bal *et al.*, 2017).

Spearheaded by the availability of new anti-staphylococcal agents follows a horde of studies on combinations in their utilization. Composite application of antibiotics allows for a range of benefits. On one end, for example, usage of β-lactam amoxicillin is enforced by clavulanate, which inhibits β-lactamase that inhibits the degradation amoxicillin (Sader *et al.*, 2007). On another end, the binding of dalfopristin to ribosomal 50S subunit induces conformational changes that facilitate the 100-fold binding increase of quinupristin, making the synergetic effect bactericidal, i.e. lethal, as opposed to only bacteriostatic, i.e. preventing cellular reproduction, for each antibiotic alone (Allington & Rivey, 2001). Literature is galore for such microbial and pharmacokinetic studies and, while the combination of vancomycin or daptomycin with β-lactam antibiotics has been gaining traction (Bal *et al.*, 2017), arguably mainly due to their own potency, the consensus on preferred therapeutical methods remains to be a subject for debates (Liu *et al.*, 2011) with ever-growing resources and seemingly unlimited boundaries. Meanwhile, prudent administration of *S. aureus* antibiotics must be exercised due their toxicity to humans and adverse effects that all medications display to a certain degree. Moreover, careful selection of concentrations is necessary for optimal effect and heterogeneous resistance of the bacteria.

Multi-drug resistance is the central challenge of *S. aureus* problem. The resistance mechanisms are yet more diverse, complex, and multilayered than the action mechanisms of anti-staphylococcal agents.

The two main classes of anti-staphylococcal agents are β-lactam class antibiotics and glycopeptides. β-lactams bind and inhibit enzyme transpeptidase, also known as penicillin-binding protein, PBP, because they are structural analogues of D-alanyl-D-alanine residues that are the substrate for the transpeptidase in cross-linking of *S. aureus* peptidoglycan chains during the synthesis of cell wall (**Figure 1**). Alternatively, glycopeptides thwart bridge formation of these chains by binding and covering D-Ala-D-Ala residues. In addition, glycopeptide oritavancin possesses a 4'-chlorobiphenylmethyl group, which is capable to permeabilize and depolarize plasma membrane (Belley *et al.*, 2010). Consequently, the resistance mechanisms against these drugs are most studied and rather well understood. At the core of β-lactam resistance is a SSC*mec* genetic element, which carries gene *mecA* that encodes a PBP2a protein, which has a reduced susceptibility to β-lactam binding (Hartman & Tomasz, 1981; Peacock & Paterson, 2015). Resistance to vancomycin and related glycopeptides primarily relies on mechanisms producing and maintaining a thicker cell wall, which leads to the trapping of glycopeptides at the naturally available free D-alanyl-D-alanine residues (~20 % of the total) in the outer layers of cell wall, thus, preventing their access to deep layers where the linkages are formed (Hiramatsu, 2001) as well as

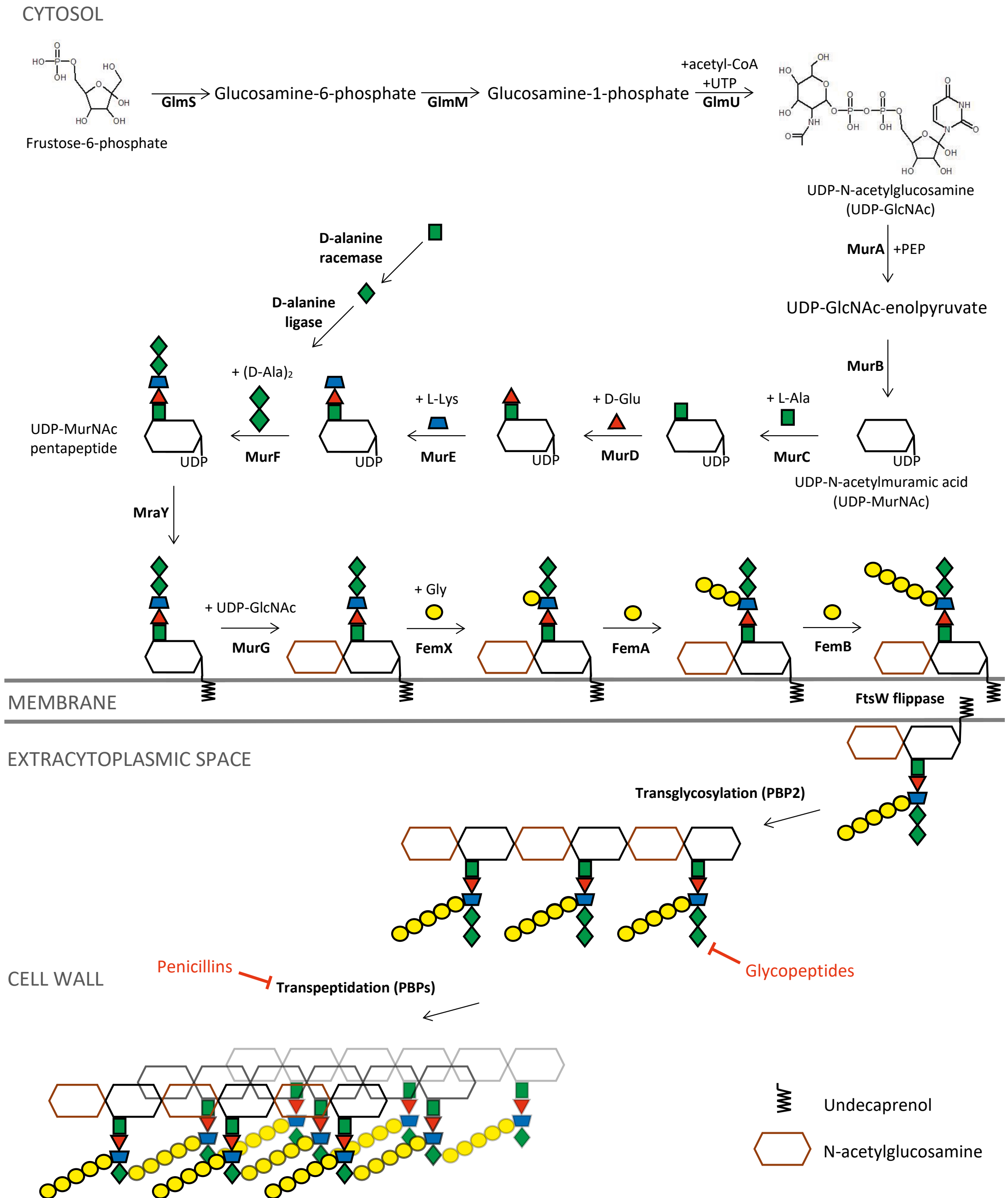

Figure 1. Synthesis of *S. aureus* peptidoglycan. Inhibition targets by most common classes of antistaphylococcal agents is shown. See text for more details. Adapted from (McCallum *et al.*, 2011; McCallum *et al.*, 2010; van Heijenoort, 2001).

substitution of the terminal D-Ala to D-lactate or D-serine, which modifies the drug target (Courvalin, 2006).

In addition to these systematic resistance mechanisms, *S. aureus* has an arsenal of approaches to evade specific drugs. The disruption of cell plasma membrane is the distinct and primary action mechanism of daptomycin (Pogliano *et al.*, 2012). Mutations in several unrelated genes have been linked to increased resistance to daptomycin and expression of multiple peptide resistance factor, MprF, which couples lysine to phosphatidylglycerol thereby changing the acidity of membrane and prevents Ca^{2+} ion-bound daptomycin from interacting with the membranes (Baltz, 2009). Linezolid acts by arresting peptidyl-transferase center in ribosome and the resistance to it is promoted by a single amino residue deletion 24 Å away from its binding center, subsequently triggering a cascade of structural rearrangements that prevent its binding (Belousoff *et al.*, 2017). Tedizolid, which is up to four times more potent than linezolid does not yet have a clarified resistance mechanism (Bensaci & Sahm, 2017). Tetracyclines act by preventing the attachment of aminoacyl-tRNA to ribosomal acceptor and the resistance occurs by mediating their efflux or ribosomal protection, although additional mechanisms may exist (Chopra & Roberts, 2001). Clindamycin disrupts peptide chain initiation and its effect is halted by ribosomal methylation processes (Spížek & Řezanka, 2017). Dalfopristin/quinupristin obstruct the release of newly synthesized peptide chains and the resistance to them also relies on facilitation of their efflux, enzymatic modification, or alterations of their binding targets (Nailor & Sobel, 2009). Fusidic acid loses its competence to bind ribosomal elongation factor G upon point mutations in its gene *fusA* (Besier *et al.*, 2003). Likewise, the resistance to rifampicin, which inhibits the synthesis of RNA by binding to the RNA polymerase is caused by point mutations in the polymerase gene *rpoB* (Aubry-Damon *et al.*, 1998). Gentamycin is rendered inactive by staphylococcal aminoglycoside 6′-*N*-acetyltransferase or gentamicin phosphotransferase (Dowding, 1977). Mupirocin is a unique antibiotic interfering with the activity of isoleucyl-tRNA synthetase and *S. aureus* has three known pathways to its resistance: one encoding a point mutation in the native synthetase, one encoding for another variant of synthetase almost always linked to a gene acquired through a plasmid, and one other linked to another likely plasmid-acquired gene (Hetem & Bonten, 2013). Quinolones exert their effect on DNA by converting their targets gyrase and topoisomerase IV to destructive enzymes that fragment DNA. *S. aureus* has three general types of resistance mechanisms to them: (i) target-mediated, when enzymes are modified for lower binding affinity, (ii) plasmid-mediated, which can lead to specific proteins reducing drug binding, or inactivating the drug, or promoting its efflux, and (iii) chromosome-mediated, by down-regulation of porin proteins during drug uptake or efflux protein expression for drug clearance (Aldred *et al.*, 2014). For example, *S. aureus* has been found to have mutations in both DNA gyrase and topoisomerase IV as well as a chromosomal efflux pump NorA (Tanaka *et al.*, 2000). Cotrimoxazole is a combination of sulfonamide sulfamethoxazole and trimethoprim that bind to dihydropteroate synthase and dihydrofolate reductase, respectively, inhibiting *de novo* synthesis of folate and its biologically active form tetrahydrofolate, thus acting synergistically (Wormser *et al.*, 1982). Yet, as with other anti-staphylococcal agents, just three years after the introduction of the drugs, cotrimoxazole-resistant bacteria were reported (Nakhla, 1972) and the mechanism of resistance has been explained by amino acid mutations in the enzymes (Dale *et al*., 1997; Sköld, 2000).

Table 1. Currently used anti-staphylococcal agents.

Agent	Family/Class	Target	Reference
Amoxicillin	β-lactam antibiotics	Cell wall synthesis (transpeptidase)	Sader *et al*., 2007
Cefazolin			Li *et al*.,2017
Cephalexin			Sader *et al*., 2007
Ceftobiprole			Scheeren, 2015
Ceftaroline			Scheeren, 2015
Dicloxacillin			Miranda-Novales *et al*., 2006
Methicillin			Newsom, 2004
Nafcillin			Sakoulas *et al*., 2014
Oxacillin			Best *et al*.,1974
Dalbavancin	Glycopeptides	Cell wall synthesis (D-Ala-D-Ala residues)	Chen *et al*.,2007
Oritavancin			Belley *et al*., 2010
Teicoplanin			Peetermans *et al*.,1990
Telavancin			Das *et al*., 2017
Vancomycin			Choo & Chambers, 2016
Daptomycin		Plasma membrane disruption	Rybak, 2006
Doxycycline	Tetracyclines	Protein synthesis (30S rRNA-mRNA complex)	Cunha, 2013
Minocycline			Cunha, 2014
Tigecycline			Rose & Rybak, 2006
Linezolid	Oxazolidonones	Protein synthesis (50S subunit)	Brickner *et al*., 2008
Tedizolid			Rybak & Roberts, 2015
Clindamycin	Lincosamides	Protein synthesis (50S rRNA)	Daum, 2007
Dalfopristin+ Quinupristin		Protein synthesis (50S subunit)	Allington & Rivey, 2001
Fusidic acid		Protein synthesis (ribosomal elongation factor G)	Spelman, 1999
Gentamicin	Aminoglycosides	Protein synthesis (30S subunit)	Chen *et al*.,2014
Mupirocin		Protein synthesis (Isoleucine tRNA synthetase)	Gilbart *et al*.,1993
Rifampicin		RNA synthesis (RNA polymerase)	Hackbarth *et al*., 1986
Ciprofloxacin	Quinolones	DNA separation (inhibition of DNA gyrase and topoisomerases II and IV)	Oliphant & Green, 2002
Quinolone			Takei *et al*., 2001
Cotrimoxazole		DNA and RNA synthesis (folic acid synthesis inhibition)	Lyon & Skurray, 1987

Evidently, the introduction of new drugs to treat *S. aureus* infections and continuously emerging resistance to them merely fuels “a rat race” with no foreseeable end. The use of anti-staphylococcal agents has not lowered the global prevalence of the bacteria, which continues to co-evolve with human host. To this end, a strategy to curtail infections by maintaining the homeostasis of the ecosystem, rather than futile efforts to exterminate natural flora has been proposed (Hiramatsu *et al.*, 2014). The concept is based on the findings that strains previously resistant to certain drugs can revert and lose their acquired resistance. Therefore, the engineering of so called “reverse antibiotics” that, when used in combination empower the previously used ones, may be a more rational approach for future treatments rather than a vain pursuit of new “silver bullets”. The validity of this unconventional tactics remains to be seen, yet it still calls for a continuation of discoveries of new anti-microbials.

To break this vicious cycle less orthodox approaches can be effective, and the vast diversity and ingenuity of the nature may provide tips for solutions and offer targets and instruments. The resilience of *S. aureus* cell wall has established and proven it as the cellular system that is not only the first frontier in drug resistance but is also elaborate in its maintenance and robust in defense mechanisms. Drawing from a proverb that enemy fortifies or threatens with what it is afraid of, it can be deduced that the enforcements of the cell wall of *S. aureus* hold the most plausible key to unlock weakness of the bacteria.

1.2. *S. aureus* cell wall and its main components

S. aureus is a Gram-positive bacterium and its cell envelope is constituted from plasma membrane and cell wall, which has only one thick layer of peptidoglycan polymer, also known as murein. The thickness of peptidoglycan layer is about 20 nm, however, it can be thicker, for example, in vancomycin resistant strains, where it exceeds 30 nm (Hiramatsu, 2001). Although not typical to Gram-positive bacteria, *S. aureus* cell wall has been shown to also include a periplasmic space, which separates its peptidoglycan layer from membrane (Matias & Beveridge, 2006). This compartment, about 16 nm wide, is substantially thinner than a typical periplasmic space in Gram-negative cells and, therefore, is not a conventional periplasm and is better distinguished by referring to it as an extracytoplasmic space, periplasm-like space, or simply the interface between plasma membrane and peptidoglycan. Herein, the terms “cell wall” and its dense section of “peptidoglycan layers” will be used interchangeably and, though technically a part of the cell wall, the periplasmic compartment will be referred to separately.

The cell wall of bacteria takes part in countless metabolic and anabolic functions that are facilitated by its components, including transport and immunogenicity, and plays roles in numerous cellular processes, e.g. division. The scope of the studies in this thesis is one class of hydrolytic enzymes involved in structural integrity of cell wall.

The primary roles of cell wall are to maintain shape of bacteria as well as to provide the protection and participate in cell division. The main two constituents of *S. aureus* cell wall are peptidoglycan and teichoic acids, each accounting for nearly a half of the dry cell wall weight (Sarvas, *et al.*, 2004). Additional components are capsular polysaccharides, surface proteins and phospholipids (comprehensively reviewed in Rajagopal & Walker, 2017).

1.2.1. Peptidoglycan

Peptidoglycan (PG) (**Figure 2A**) network provides a sturdy structure and support for cell wall that is able to sustain intracellular turgor pressure reaching nearly 2 MPa (Francius *et al.*, 2008), for comparison, exceeding the regular car tyre pressure almost 10-fold. A remarkably distinct feature of *S. aureus* and just few other staphylococci PG is bridges formed by pentaglycine, whereas other bacteria have other types of connectors (Vollmer *et al.*, 2008). The typical length of PG chain in *S. aureus* cell wall is 3 – 10 monomer residues, only less than 15% of chains are estimated to be longer than 25 monomer residues (Boneca *et al.*, 2000), and factors determining the length of chain remain unknown. Consensus exists on glycan mainchain forming a helical twist (Labischinski *et al.*, 1979). However, contradicting models have been presented to depict peptide stems, which flank pentaglycine bridges, within a plane perpendicular to the glycan mainchain, as anti-parallel or parallel (Dmitriev *et al.*, 2004; Sharif *et al.*, 2009), and both models could justify high cross-linking degree, >80%, found in the bacteria (Cegelski *et al.*, 2006; Sharif *et al.*, 2009; Snowden *et al.*, 1989; Vollmer & Seligman, 2010). The most recent work by solid state NMR suggests glycan helix with disaccharide unit periodicity of 40 Å and each successive peptide stem rotating 90º in respect to previous stem and cross-linked stems having parallel orientation (Kim *et al.*, 2015). Cringeworthy is the fact that even the latter study was still compelled to provide evidences to support seemingly intuitive planar model of PG glycan strand arrangement parallel to cytoplasmic membrane. For the lack of unequivocal experimental evidences, a challenging alternative scaffold model, which posits that the glycan strands extend perpendicularly to the membrane, has been proposed and earnestly considered (Dmitriev *et al.*, 2003; Scheffers & Pinho, 2005; Vollmer & Höltje, 2004).

In spite of PG importance, significant gaps of understanding remain in its network construction and turnover. Peculiarly, the mechanisms of the network synthesis during cell division, growth, and in stationary phases are better investigated and understood in more complex oval and rod-shaped bacteria, like *Bacillus subtilis* and *Escherichia coli*, than the simplest geometrical form of all – spherical cocci (Pinho *et al.*, 2013). It is helpful to remind that phylogenetic studies found cocci dispersed in various branches of prokaryotic tree and its deepest branches contained only rod-shaped bacteria, thus implying seemingly counterintuitive: cocci are degenerate forms of elongated bacteria rather than the latter being advanced species of the former (Siefert & Fox, 1998; Stackebrandt & Woese, 1979; Woese *et al.*, 1982). A study using super-resolution microscopy discovered that *S. aureus* cells briefly elongate before division and called for reassessment of previous cell division model (Monteiro *et al.*, 2015). Meanwhile, the investigations of PG network cleavage and turnover are hindered by multiple hydrolase enzymes that may compensate for each other's function (*see* "1.2.1.2. Cleavage of peptidoglycan network").

1.2.1.1. Synthesis of peptidoglycan monomer and its network

The synthesis of PG is carried out in a long series of steps, which can be grouped into cytosolic, membrane-associated, and extracytoplasmic stages (**Figure 1**). Biosynthesis of PG monomers begins in cytosol (van Heijenoort, 1998). The precursor of monomers is fructose-6-phosphate. The

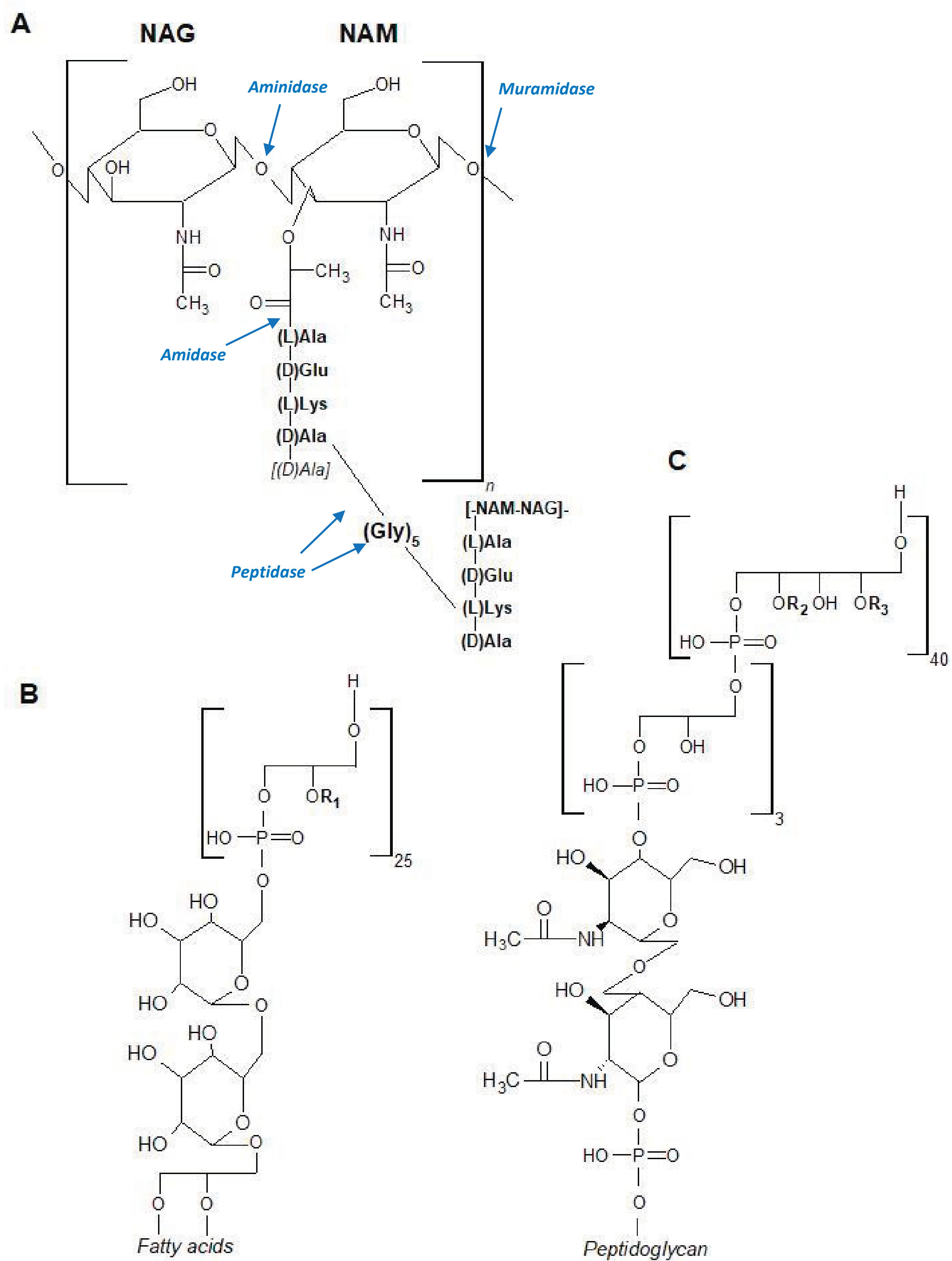

Figure 2. Structures of peptidoglycan monomer (**A**), wall teichoic acid (**B**) and lipoteichoic acid (C). R = H, D-glucosyl, or D-alanyl.

enzymes carrying out the synthesis are glucosamine-6-phosphate synthase, GlmM mutase, bifunctional GlmU enzyme catalyzing acetylation and uridylation, MurA transferase, MurB reductase, MurC, MurD, MurE, and MurF synthetases. Availability UDP-GlcNac is a limiting step for the subsequent pathways (Mengin-Lecreulx *et al.*, 1989), and it should be noted that it is also required downstream in the formation of lipid II from lipid I.

Membrane-associated stage begins with membrane-anchored MraY transferase docking the cytosolic product to the membrane acceptor undecaprenylphosphate and producing MurNac(pentapeptide)-pyrophosphoryl undecaprenol. Next, transferase MurG yields GlcNac-MurNac(pentapeptide)-pyrophosphoryl undecaprenol. In literature, the afore two products are commonly referred to as lipid 1 and lipid II, respectively. Subsequently, peptidyl transferases FemX, A, and B, add first, second and third, and fourth and fifth glycine residues, respectively (Ehlert *et al.*, 1997; Rohrer & Berger-Bächi, 2003). This concludes the synthesis of PG monomer. The monomer is then transferred across plasma membrane into extracytoplasmic space by mechanism not yet understood and pyrophosphorylase recycles the lipid carrier.

The PG network synthesis takes place in extracytoplasm in two steps: at first the monomers are linked into chains and then the chains are cross-linked (**Figure 1**). The first step is transglycosylation and produces glycan strands while bonding N-acetylglucosamine and N-acetylmuramic acid of separate monomer units. In the following step, the chains are cross-linked via pentaglycine bridges by transpeptidation. These reactions are carried out by a bifunctional enzyme, a penicillin-binding protein, PBP2, named for its predisposition to bind penicillins. *S. aureus* has four PBPs (for comparison, *B. subtilis* and *E. coli* have 16 and 12, respectively) and others possess only transglycosylase function. PBP1 is involved in cell division and separation and possibly belongs to a divisome complex that may also include autolysis enzymes (Pereira *et al.*, 2009). The function of PBP3 is not well understood and in an oval pneumococcal it is found dispersed along cell wall (Morlot *et al.*, 2004; Pinho *et al.*, 2000). PBP4 is responsible for producing highly cross-linked PG network (Curtis *et al.*, 1980). Notably, strains resistant to penicillins, like methicillin resistant MRSA, possess the fifth enzyme, PBP2A, which has a diminished antibiotic sensitivity and can substitute for PBP2 (Pinho *et al.*, 2001).

Presently, based on localization of PBPs at a division site in intact cell, it is commonly believed this to be likely the only location for new peptidoglycan chain incorporation, although peripheral synthesis is not ruled out and small-scale repair-related synthesis may accompany scission during the peptidoglycan turnover (Giesbrecht *et al.*, 1998; Pinho *et al.*, 2013). Peptidoglycan synthesis probably begins with PBP1. PBP2 is directed to the septum by recognizing its lipid II substrate and PBP4 is gathered by intermediate of locally synthesized wall teichoic acids (Atilano *et al*., 2010; Pinho & Errington, 2005).

After formation of *S. aureus* division septum and before the splitting of daughter cells, new layers of cell wall PG are formed and they are separated by low density PG layer in between (Matias & Beveridge, 2007). Since this fragile PG layer does not extend to the surface of parental cell, it is possible that autolysins for cell splitting are only needed along the edges of septal ring.

S. aureus bacterial tubulin homolog FtsZ assembles a ring of cell division machinery. In the cells devoid of FtsZ, PG synthesis is delocalized and leads to cell enlargement and lysis (Pinho & Errington, 2003). The localization of FtsZ in the midsection of the cell is secured by essential protein GpsB, which also stimulates its GTPase activity and promotes lateral bundling of the polymers (Eswara *et al*., 2018; Santiago *et al*., 2015). GpsB deletion mutants are unable to divide and formed enlarged cells that eventually rupture. Another essential *S. aureus* protein, EzrA, was

shown to interact with cytoplasmic FtsZ as well as nearly all other putative constituents of divisome in periplasm (Steele *et al.*, 2011). This study found EzrA to be required for GpsB localization, divisome formation and even PG synthesis. However, another study, while confirming its importance in properly localized FtsZ and PBP2, at the time did not find the protein to be essential, but rather responsible for cell size homeostasis (Jorge *et al.*, 2011).

At least two models have been proposed how nascent PG strands get incorporated in rod-shaped bacteria and they both rely on "make-before-break" strategy, which postulates that new chains are incorporated before the cleavage of the old linkages. The first model, designed for Gram-positive bacteria and called "inside-to-outside", emphasizes the unextended conformation of PG layer laid immediately outside the plasma membrane and increasing stretching, which leads to its cleavage, as the new layers are produced and older ones are pushed further away (Koch & Doyle, 1985). The second model, suggested for Gram-negative *E. coli* and called "three-for-one", states that three new cross-linked strands replace one old one, upon their covalent bonding to the free amino groups in the cross bridges on both sides of the old one and the scission of the latter (Höltje, 1998). It is not known if either of the models is applicable to *S. aureus*.

1.2.1.2. Cleavage of peptidoglycan network

Cleavage, or lysis, of microbial cell-wall peptidoglycan is a biological process as critical as its synthesis. Firstly, it is vital for cell division and growth when new strands need to be incorporated. Secondly, it takes place during cell life cycle in PG recycling and after its death. Thirdly, it is used by competing strains, own parasites (e.g., *S. aureus* bacteriophage phi MR11) as well as in medical applications. PG hydrolysis participates in a variety of other more specific functions. For instance, PG cleavage fragments, commonly known as muropeptides, may trigger immune system response in host organisms. In *S. aureus* this mechanism remains under scrutiny, since newer reports argue that intact PG is more effective than muropeptides (Fournier & Philpott, 2005; Humann & Lenz, 2009; Wolf & Underhill, 2018).

Virtually every bond between PG constituents can be cleaved and specific enzymes are known. There is a large diversity of PG compositions and structures among bacteria and this differentiation is pronounced in the amino acid sequences of peptide stems and bridges that link disaccharide chains (Schleifer & Kandler, 1972; Turner *et al.*, 2014; Vollmer & Seligman, 2010). The assortment of PG hydrolysis enzymes across the species, strains, and even isogenous cells is yet more vast and difficult to comprehend. Arguably, the most recent extensive review article undertook the challenge over a decade ago and additional enzymes are continuously added (Vollmer *et al.*, 2008; Vermassen *et al.*, 2019). The authors conceded to the enormity of information about the PG hydrolases, which "would fill many bookshelves" and the impossible task to summarize it in a single article. We narrow our focus to *S. aureus*-specific peptidoglycan and pertinent hydrolases (PGHs).

Commonly cleaved PG bonds are shown in **Figure 2A** and the enzymes can be divided into groups cleaving glycan strand and branches supporting their network. The scission of glycan chain is carried out by glycosidases that include *N*-acetylglucosaminidases and *N*-acetyl-β-D-muramidases. The branches with bridges in between them are cleaved by *N*-acetylmuramyl-L-Ala amidases and peptidases. Remarkably, some PGHs may have more than one and distinct function domain. Furthermore, the enzymes display a variety of additional domains, conserved sequences, and targeting signal fragments that facilitate localization and substrate recognition for the enzymes

to carry out their physiological roles and catalytic functions (Sharma *et al.*, 2016; Vermassen *et al.*, 2019).

PGHs that target *S. aureus* PG have two types of origins: endogenous and exogenous. Endogenous enzymes perform essential autolytic functions during cell growth, division and death or are introduced by bacteriophages and allow their progeny to leave bacteria after breaching cell wall from inside. The enzymes are called autolysins and endolysins, respectively. Exogenous enzymes have only a detrimental effect on bacteria and can be produced to suppresses the bacteria by its hosts or released by competing strains. Studies from bioinformatics to microbiology reveal a vast array of putative and new PGHs found in nature, many of which do not have an apparent link to *S. aureus*, although some can be expected to eventually appear relevant. To illustrate the diversity, **Table 2** presents a group of enzymes that have been directly shown to lyse peptidoglycan of *S. aureus*.

Lysozyme is by far the best known and studied among PGHs. Lysozyme from hen egg white was the first enzyme to have had its 3D-structure determined (1965) and at the time of this writing, search for "lysozyme" in the Protein Data Bank (www.rcsb.org) returned 2033 related 3D-structures. The characterization of other PGHs is not only not nearly as copious but also incomplete. Lysozyme was discovered in 1922 by Alexander Fleming, who later shared Nobel prize for the discovery of the world's first antibiotic (Penicillin G) and was proven to be an antimicrobial enzyme of innate immune system, present in mucus, tears, milk, and egg's white. Remaining true to its elasticity, *S. aureus* produces PG with the *O*-acylated, by O-acetyltransferase, *N*-acetylmuramic acid at C_6-OH position (Bera *et al.*, 2005; Brott & Clarke, 2019; Pushkaran *et al.*, 2015). This allows it to evade the lysozyme activity and is a likely contributor for resistance while colonizing mucosal membranes.

Lysozyme structure consists of a muramidase catalytic domain connected to a transmembrane signal peptide. The majority of *S. aureus* PGHs have more complex structure than that of lysozyme and substrate targeting domains are frequent. Other antimicrobial enzymes (lysostaphin and ALE-1) use M23 family peptidases as their catalytic domains. Autolysins may possess M23 peptidase (LytM), amidase and glucosaminidase (AtlA), as well as cysteine, histidine-dependent amidohydrolase/peptidase, CHAP (Sle1 and LytN). Endolysins of bacteriophages typically have one or more of CHAP, amidase, and glucosaminidase domains (**Table 2**).

Four classes of lysozyme-type muramidases have been shown to be active against peptidoglycan but muramidases are not present in *S. aureus* genome (Baba *et al.*, 2008; Vollmer *et al.*, 2008). Although lacking significant sequence identity, they have similar three-dimensional conformations. M23 family peptidases are zinc-ion dependent enzymes that distinctly cleave between the second and third as well as third and fourth glycine in the pentaglycine bridge (Warfield *et al.*, 2006). Amidases are found in autolysin (AtlA) as well as in endolysins. Atl is a prepro-protein which, following membrane transfer and removal of signal peptide, has its amidase and glucosaminidase domains separated and each directed to their targets in PG by repeat sequence domains (Baba & Schneewind, 1998). When an endolysin, amidase does not have a signal sequence, but is rather working in tandem with phage holin protein that creates a pore and allows it to transfer through plasma membrane (Loessner, 2005; Young, 1992). Glucosaminidase domain has been also identified in putative LytX, LytY, and LytZ enzymes, all of which possess membrane signal sequences (Frankel *et al.*, 2011). CHAP domains are present in many proteins that typically

Table 2. Enzymes confirmed to cleave *S. aureus* peptidoglycan. Note: Listed are only enzymes that have been isolated and do not include predicted enzymes, enzymes shown to be active against PG of other bacteria and artificial chimeric proteins. sp, signal peptide, SH3b, src Homology-3 bacterial domain, LysM, lysin motif, CHAP, cysteine, histidine-dependent amidohydrolase/peptidase.

Enzyme	Host organism	Localization	Targeting domain	Function	Catalytic domain	References
Lysostaphin	*S. simulans*	secretion	SH3b	antimicrobial	M23/M37	(Schindler & Schuhardt, 1964)
ALE-1	*S. capitis*	secretion	SH3b	antimicrobial	M23/M37	(Sugai *et al*., 1997a)
AtlA	*S. aureus*		repeat sequences	autolysin	amidase, glucosaminidase	(Oshida *et al*., 1995)
Sle-1	*S. aureus*		LysM	autolysin	CHAP	(Kajimura *et al*., 2005)
LytM	*S. aureus*		sp	autolysin	M23/M37	(Ramadurai & Jayaswal, 1997)
LytN	*S. aureus*		LysM	autolysin	CHAP	(Frankel, Hendrickx, Missiakas, & Schneewind, 2011)
HydH5	phage IPLA88	virion-associated	unknown	virion release	CHAP, glucosaminidase	(Rodríguez *et al*., 2011)
LysGH15	phage GH15	virion-associated	SH3b	virion release	CHAP, amidase	(Gu *et al*., 2011)
LysH5	phage H5	virion-associated	SH3b	virion release	CHAP, amidase	(Obeso, Martínez, Rodríguez, & García, 2008)
LysK	phage K	virion-associated	SH3b	virion release	CHAP, amidase	(O'Flaherty, Coffey, Meaney, Fitzgerald, & Ross, 2005)
LysWMY	*S. warneri* M prophage		SH3b		CHAP, amidase	(Yokoi *et al*., 2005)
phi 11 endolysin	phage phi 11	virion-associated	SH3b	virion release	CHAP, amidase	(Navarre, Ton-That, Faull, & Schneewind, 1999)
MV-L	phage phi MR11	virion-associated	SH3b	virion release	CHAP, amidase	(Rashel *et al*., 2007)
PlyGRCS	phage GRCS	virion-associated	SH3b	virion release	CHAP	(Linden *et al*., 2015)
PlySs2	*S. suis* prophage		SH3b		CHAP	(Gilmer, Schmitz, Euler, & Fischetti, 2013)
Trx-SA1	phage IME-SA1	virion-associated	SH3b	virion release	amidase	(Fan *et al*., 2016)

have a role in PG hydrolysis (Bateman & Rawlings, 2003; Rigden *et al.,* 2003). The domains have conserved cysteine and histidine residues and display a dual activity: L-muramoyl-L-alanine amidase and D-alanyl-glycyl endopeptidase. Analysis of 12 *S. aureus* and 44 staphylococcal bacteriophage genomes revealed that *S. aureus* genome encodes at least 10 and each phage 1 or 2 proteins bearing CHAP domains (Zou & Hou, 2010). In autolysins and endolysins CHAP domains are associated with SH3b and LysM targeting domains, respectively.

Delivery of the PGHs to their substrate, can be achieved in several ways. LytM is directed to plasma membrane by its 1-25 amino acid residue signal peptide (Sabala *et al*., 2012). To provide passage of out cytoplasm through cell wall for freshly constructed virion particles, endolysins follow the plasma membrane permeabilizing holin proteins. Once in the vicinity of a target, PG, effectiveness of the enzymes can be enhanced by substrate binding structures. There is a variety of these, some interactions are not yet understood. Covalent anchoring of proteins to cell wall, via LPxTG domain and by sortase A is a common mechanism in Gram-positive bacteria, yet unlikely applicable to PGHs (Schneewind & Missiakas, 2012). Most prominent *S. aureus* cell wall binding domains (CBDs) include ~ 90 aa residue SH3b, a bacterial analogue of src Homology-3 domain, and 45-65 - residue long LysM, lysin motif. Already before the start of the studies described hereafter, 3D-structure of ALE-1 SH3b domain has been determined, its importance was not questionable, yet the mechanism of binding remained to be elucidated (Lu *et al.*, 2006). LysM domains, discovered in 1986, were found in over 4000 thousand proteins in merely a decade (Buist *et al.*, 2008). The domains were shown to bind to *N*-acetylglucosamine moieties of PG and have been tested for numerous industrial and medical applications (Ohnuma *et al.*, 2008; Visweswaran *et al.*, 2014).

Two PGHs, LysWMY and PlySs2, are encoded by *S. aureus* prophages (**Table 2**). Prophages are mobile genetic elements incorporated into genome and are considered to be responsible for genetic diversity of the bacteria, which is a clonal species with conserved core genome (Feil *et al*., 2003). *S. aureus* genomes have one to four prophages that supply additional virulence and fitness factors (Azam & Tanji, 2019; Lindsay, 2010). Lysostaphin immunity factor, *lif*, acquired by *S. simulans* via horizontal gene transfer provides serine substitutions in pentaglycine bridge during PG synthesis, making the host bacteria resistant to lysostaphin, which is excreted to eliminate competing *S. aureus* (Thumm & Götz, 1997; Tschierske *et al.*, 1997). The pentaglycine bridge is the most distinct feature of *S. aureus* PG and, therefore, is a target for species-specific lysis.

1.2.1.3. Regulation of peptidoglycan synthesis and autolysis

Synthesis and autolytic hydrolysis of PG must be tightly regulated in order for bacteria to remain viable during its cell cycle and under external stress factors. A significant portion of *S. aureus* enzymes involved in these processes has been identified and their functions investigated. The mechanisms governing them, and their topology remain to be elucidated and their understanding presently is fragmentary. Rod-shaped *B. subtilis*, posing a lower health hazard than *S. aureus*, became a primary model for Gram-positive bacteria. Yet, the information obtained from its studies cannot be directly translated to *S. aureus*, for their differences in shape and underlying structural systems (Pinho *et al.*, 2013).

Activities of *S. aureus* PG synthetic and hydrolytic enzymes are coregulated. Although widely recognized in other bacteria, direct evidence for this in *S. aureus* arrived not long ago

(Antignac *et al*., 2007). Cells grown in the presence of subinhibitory concentrations of β-lactam antibiotics specific to PBPs 1 – 4 displayed decreased expression of autolytic enzyme genes, namely *atl*, *sle1*, and *lytM* and were substantially less susceptible to autolysis. In addition, reduced transcription of *pbpB* gene, which encodes PBP2, reduced amount or activity of autolysins and lowered transcriptions levels of *atl* and *sle1*, but the latter were restored upon stimulation of the former. The same year another study showed that modulation of PG synthesis gene *murF* had genome-wide impact and altered 668 out of 2740 open reading frames of *S. aureus* strain COL (Sobral *et al*., 2007). Heretofore, several global mechanisms were shown to participate in PG modulation.

The complexity of regulation of *S. aureus* physiological processes, including synthesis and lysis of PG, manifests in two main classes of actors that can act alone or in combination: small size broad range (so called global) transcription regulators and two-component signal transduction systems (TCSs) along with factors influencing them (Haag & Bagnoli, 2017). Due to the involvement of a large amounts of genes, some overlap and synergistic effects exist and information to expose cross-interaction is continuously pouring in. Noteworthy for a literature examining reader is a caution that some of the nomenclature of the same regulation factors and TCSs varies between researchers or is updated by the same authors during the course of studies, for instance, factor MgrA/RAT/NorR (Ingavale *et al*., 2003; Luong *et al*., 2003; Truong-Bolduc *et al*., 2003) and bacitracin and nisin-sensitive TCS BraRS/BceRS/NsaRS (Blake *et al*., 2011; Hiron *et al*., 2011; Kawada-Matsuo *et al*., 2013; Ohki *et al*., 2003; Yoshida *et al*., 2011).

Multiple-gene regulator protein MgrA has the most evident function in autolysis regulation among global cytoplasmic transcriptional regulators. Overall, it was shown to positively regulate 175 and negatively 180 genes in *S. aureus* strain Newman, including virulence factors like toxins and protein A (Jenul & Horswill, 2018; Luong *et al*., 2006). It was discovered to suppress cell autolysis by decreasing transcription of *lytM* and *lytN*, but did not have effect on *atl*, *pbp2* and *pbp4* (Ingavale *et al*., 2003; Luong *et al*., 2003). TCS ArlRS, which shares 70% of affected genes with MgrA, has been shown to directly recruit the regulator for virulence factor expression (Crosby *et al*., 2020). MgrA is a dimeric winged helix protein with 147 residue domains. It likely acts by directly binding promoters of target genes and is considered to be an oxidation sensor for its cysteine residue (Chen *et al*., 2006; Somerville & Proctor, 2009). The transcription factor is a homolog of SarA protein family, which is responsible for regulation a wide spectrum of *S. aureus* genes, primarily virulence factors (Cheung *et al*., 2008; Jenul & Horswill, 2018). Interestingly, *sarA* was found to be involved in a positive regulation of *mecA*, which encodes PBP2A that is responsible for β-lactam antibiotic oxacillin resistance (Li *et al*., 2016). In addition, SarA binds to a *fmtA* promoter and directly regulates expression of FmtA esterase, which modulates charge of cell surface by removing D-alanine from lipoteichoic teichoic acids and is increased under oxacillin stress (Rahman *et al*., 2016; Zhao *et al*., 2012).

1.2.2. Two-component systems

TCSs consist of transmembrane histidine kinase, cytosolic response regulator and phosphorylation driven signaling cascade affecting expression of a set of genes. *S. aureus* core genome has 16 such receptor-sensor systems that respond to stimuli and govern virulence genes, cell wall metabolism as well as react to environmental factors such as nutrients and antimicrobial agents. TCSs provide bacteria with features that allow to adapt to certain conditions and were generally considered to be

required for a free-living cell. However, a recent study has challenged this presumption and has shown that all TCSs are dispensable under constant environmental conditions and when growth is arrested only one, namely WalKR, previously called YycG/YycF, is necessary and sufficient to retain viability and bacterial growth (Villanueva *et al*., 2018). They have also been found to be self-sufficient entities.

Although broad reach of TCSs does not allow to yet completely exclude any of them affecting regulation of *S. aureus* PG synthesis and lysis, involvement of several has been determined. The most important among the TCS, WalKR (aka WalK/WalR, WalRK, VicRK) plays a major role in cell wall metabolism and was named accordingly. Genes positively regulated by the TCS in *S. aureus* include *atlA*, *lytM* , *sle1* as well as two genes encoding muramidase (*isaA* and *sceD*) and five genes with CHAP domains (*ssaA*, *sa0620*, *sa0710*, *sa2097*, and *sa2353*) (Delauné *et al*., 2012; Dubrac & Msadek, 2004; Dubrac & Msadek, 2008; Dubrac *et al*, 2007). WalKR-depeleted cells clustered and died but did not lyse, showed increased resistance to lysostaphin-induced lysis and their peptidoglycan had modestly increased cross-linking (Dubrac *et al*., 2007). *S. aureus* cells with inactive WalKR had thicker cell wall, impaired defective division septa, but LytM protein and *ssaA* gene each were able to restore cell growth and reduce wall thickness, yet abnormalities in septa remained (Delaune *et al*., 2011). WalKR activity was shown to positively correlate with biofilm formation and expression of genes of TCS SaeSR (Delauné *et al*., 2012; Beltrame *et al*., 2015; Dubrac *et al*., 2007). Incubation of vancomycin-susceptible *S. aureus* (VSSA) strains with vancomycin or daptomycin was shown to generate mutations in WalKR that could reduce its activity, but also increased antibiotic resistance and led to appearance of vancomycin-intermediate (VISA) strains (Hafer *et al.*, 2012; Howden *et al*., 2011; Shoji *et al*., 2011). Insertion IS256 known to contribute antibiotic resistance in *S. aureus* has been found both to increase and decrease of *walKR* expression (Kuroda *et al*., 2019; Lyon *et al.*, 1987; McEvoy *et al*., 2013). Proteins YycH and YycI have been shown to activate WalKR and increase vancomycin susceptibility in methicillin resistant strains (Cameron *et al*., 2016).

Autolysis regulated locus ArlRS is another TCS substantially involved in PG metabolism. Originally, its mutant *arlS* was discovered to exhibit increased autolysis and thought to downregulate *atlA* (Fournier & Hooper, 2000). Later ArlRS was shown to downregulate *lytN*, but have no effect on *atlA* , *sle1*, *lytM* and have a positive regulation on another autolysis repressing TCS LytSR and global regulator MgrA (Crosby *et al*., 2016; Crosby *et al*., 2020; Memmi *et al.*, 2012; Liang *et al*., 2005). ArlRS knockout mutant of methicillin resistant *S. aureus* strains had significantly increased sensitivity to oxacillin, which had a synergistic effect with oritavancin (Bai *et al*., 2019).

Other TCSs have been shown to be involved in PG metabolism as well and some of their roles may not be obvious. For example, AirSR identified as oxygen sensing regulator was shown to bind and upregulate promoters of *lytM* and *pbp1* (Sun *et al.*, 2013). Activity of *S. aureus* exoprotein expression SaeRS TCS correlated with *atlA* expression levels and a model for their involvement in biofilm formation was proposed (Mashruwala, Gries, Scherr, Kielian, & Boyd, 2017). Strains lacking staphylococcal respiratory regulator SrrAB displayed reduced expression of AtlA impeded biofilm formation, while wild-type bacteria were increasing *atlA* transcription and cell lysis under impaired respiration (Mashruwala, Guchte, & Boyd, 2017). LytSR originally was identified as a TCS affecting autolysis of *S. aureus* cells (Brunskill & Bayles, 1996a). Later studies have found that LytSR controls expression of a large number of genes, many of them metabolic, and its suppressive effect on cell lysis is exerted by positive control of *lrgAB* operon products that inhibit proteins of *cidAB* operon, which encodes holin-type proteins permeabilizing

plasma membrane and facilitating autolysin access to PG (Brunskill & Bayles, 1996b; Groicher *et al.*, 2000; Rice *et al*., 2003; Sharma-Kuinkel *et al*., 2009; Yang *et al*., 2013).

Certain antimicrobial compounds, expectedly, have impact on all TCSs. Three TCSs are considered to be tasked with combating their deleterious effects: BraSR, GraSR, and VraSR, named after bacitracin, glycopeptide, and vancomycin associated resistance, respectively. Bacitracin binds tightly to undecaprenyl pyrophosphate and inhibits PG synthesis at the step of monomer translocation from cytosol to extracellular surface. BraSR initiates response that leads to an active elimination of the antibiotic by an ABC transporter and so far there is no evidence of cell wall or PG modifications that would facilitate the resistance (Blake *et al*., 2011; Hiron *et al.*, 2011; Kawada-Matsuo *et al.*, 2011; Kawada-Matsuo *et al*., 2013).

GraSR (aka antimicrobial peptide sensing ApsSR or GraXRS for the need of third scaffold component X) defense mechanism is more elaborate. It upregulates *dlt* operon responsible for teichoic acid D-alanylation which reduces negative charge of cell wall and repels cationic antimicrobial peptides (Li *et al*., 2007; Yang *et al*., 2012). GraSR also helps *S. aureus* to survive under oxidative stress, at pH lower and temperature higher than physiological (Falord *et al.*, 2011; Muzamal *et al.*, 2014; Villanueva *et al*., 2018). Notably, cells grown in the presence of last-resort antibiotic colistin and lacking GraSR had reduced expression rates of PGHs *atlA*, *sle1*, *isaA*, *ssaA*, *sceD* as well as five additional putative CHAP amidase genes, which meant this TCS was partially overlapping with the WalKR gene regulation (Falord *et al*., 2011).

VraSR is quite distinct among TCSs in PG modulation by primarily providing PG synthesis, not lysis enzymes. It was shown to not affect transcription of *atl*, *ssaA* and *isaA*, instead upregulating genes *pbpB* (PBP2), *murZ* (analog of MurA) as well as PBP-complementary glycosyltransferase gene *sgtB* and *prsA*, which ensures proper folding of PBP proteins (Gardete *et al.*, 2006; Jousselin *et al*., 2012; Kuroda *et al*., 2003; Liang *et al*., 2018; Sengupta *et al.*, 2012; Tajbakhsh & Golemi-Kotra, 2019). In addition to vancomycin, VraSR increases resistance to many other glycopeptides, β-lactams and other antibiotics (Cui *et al.*, 2009; Doddangoudar *et al.*, 2011; Kato *et al.*, 2010; Mensa *et al.*, 2014; Pietiäinen *et al*., 2009). Intriguingly, among the genes in its regulon, *sa0205* was found to be upregulated up to 50-fold by a set of antimicrobial agents, including cationic antimicrobial peptides, vancomycin, teicoplanin, and bacitracin (Pietiäinen *et al*., 2009). Sequence analysis of the gene indicated it to encode a protein with an M23 endopeptidase domain, which is responsible for cleaving pentaglycine bridges specific to *S. aureus* PG.

1.2.3. Teichoic acids

Teichoic acids (TAs) are cell wall neighbors of peptidoglycan and some of their functions are connected. They are a major constituent of *S. aureus* CW and may account for as much as 50 – 60% of its mass (Hancock, 1997; Sarvas *et al.*, 2004). These anionic polymers belong to one of the two main classes: wall teichoic acids (WTAs) and lipoteichoic acids (LTAs). Their structures are shown in **Figure 2**. WTAs are covalently bound to PG via phosphodiester between their disaccharide ManNac(β1-4)GlcNAc and MurNAc (NAM) of PG chain (Araki & Ito, 1989). The bulk of WTAs is composed of glycerol and ribitol phosphates each having 2 – 3 and 30 – 50 repeats, respectively (Coley *et al.*, 1976; Endl *et al.*, 1983; Navarre & Schneewind, 1999; Sanderson *et al.*, 1962). Ligation is carried out by LytR-CpsA-Psr family enzymes (Chan *et al.*, 2013). LTAs are anchored in the plasma membrane lipid layer to Glc(1-4)Glc-(1-3)diacylglycerol

and their moiety is composed of disaccharide and 16 – 40 repeats of glycerol phosphate (Koch *et al.*, 1984; Navarre & Schneewind, 1999). The attachment of glucose units to diacyl glycerol is performed by YpfP (Kiriukhin *et al.*, 2001).

Despite structural similarities between WTAs and LTAs, their synthesis pathways are separate and performed by different enzymes (Gründling & Schneewind, 2007a; Gründling & Schneewind, 2007b; Brown *et al.*, 2008;). The key difference is that WTAs are synthesized in cytoplasm, thereupon flipped to the surface of membrane and ligated to PG in the last step, whereas LTAs only have two glucose units added to diacylglycerol in cytosol, and addition of glycerol phosphate is carried out after this anchor is flipped across the membrane. *S. aureus* WTAs are not essential for survival *in vitro* and their synthesis genes can be deleted altogether, however, inhibition of only later steps is lethal, since it leads to ”poisoning” of plasma membrane with WTA intermediate, depletion of carrier lipid and obstruction of PG synthesis (Chaudhuri *et al.*, 2009; D'Elia *et al.*, 2009; Qiao *et al.*, 2014; Weidenmaier *et al.*, 2004). *In vivo* WTAs are important for colonization and infection, LTAs appear to be dispensable only at temperatures below 30 ℃ and both types of TAs cannot be deleted at the same time, probably because they compensate for each other’s functions (Oku *et al.*, 2009; Schirner *et al.*, 2009; Weidenmaier *et al.*, 2005).

The most common modifications of TAs are D-alanylation and glycosylation (**Figure 2 B** & **C**). It is estimated that in *S. aureus* LTAs 70% of glycerol phosphates carry D-alanine, 15% have GlcNAc and 15% are not modified (Schneewind & Missiakas, 2014). D-alanine is added by proteins of *dlt* operon in cell envelope, but the steps are not yet understood. Enzymes glycosylating WTAs but not LTAs are known, and the process is thought to take place in cytoplasm (Brown *et al.*, 2012; Sobhanifar *et al.*, 2015). The main purpose of alanylation is to regulate the net charge of cell wall as the addition of positive charges reduces overall negative charge. Glycosylation has been shown to be instrumental in adhesion, colonization and interaction with phages (Winstel *et al.*, 2015; Xia *et al.*, 2011).

The many roles of TAs include immune response, biofilm formation, adhesion, virulence, antibiotic resistance (Rajagopal & Walker, 2017; Xia *et al.*, 2010). TAs also have functions in *S. aureus* cell division, morphology, and PG autolysin regulation. WTAs and LTAs are necessary for proper division septa formation (Campbell *et al.*, 2011; Gründling & Schneewind, 2007; Oku *et al.*, 2009). FtsZ division ring is not assembled in the absence of both LTAs and WTAs and for the cells grown in the presence of tunicamycin, with WTA synthesis inhibited, D-alanylated LTAs were able to come to cell rescue (Santa Maria *et al.*, 2014). WTAs were found necessary for localization of lytic AtlA and synthetic PBP4 at the cell division septum (Atilano *et al.*, 2010; Schlag *et al.*, 2010). Blocking WTA synthesis decreased methicillin resistant *S. aureus* resistance to β-lactams, although PBP2A synthesis was not impaired (Campbell *et al.*, 2011). Impeded synthesis of LTAs and inhibited D-alanylation of TAs has yielded reduced autolysis acitivity (Fedtke *et al.*, 2007; Peschel *et al.*, 2000).

1.2.4. M23 peptidases

S. aureus pentaglycine bridges that link PG chains are cleaved by M23 endopeptidases. The MEROPS database of peptidases distinguishes M23 family as a group of enzymes, their homologues, and putative proteins most of which share a ~130 amino acid residue domain with active sites residues in motifs HXXXD and HxH, incorporate a zinc ion in their catalytic site, and typically have preference for Gly-Gly bonds (Rawlings *et al.*, 2018). The database identifies over 11 000 M23 gene sequences and the family members can be found in bacteria, archaea, protozoa,

plants, animals, and viruses. The family is divided into A and B subfamilies, the former has a wider range of substrates and they also differ in metal coordination and sequence alignment. However, M23B members constitute the vast majority of the family (>95%) and herein will be the focus. Concomitantly, the enzymes are frequently referred to as the LytM family enzymes, although their earliest described and most-studied representative is lysostaphin (Schindler & Schuhardt, 1964). Doubtless, this is because LytM was the first enzyme identified with a single well-defined domain, which was M23 endopeptidase, whereas lysostaphin has an additional domain with a different function.

1.2.4.1. Composition and roles of M23 peptidases

The apparent function of known M23 peptidases is the cleavage of *S. aureus* PG and their origins can be internal (autolysins, endolysins) or external (bactericidal). Lysostaphin and ALE-1, secreted by *Staphylococcus simulans* and *Staphylococcus capitis*, respectively, are two examples of the latter. Both proteins have a single M23 domain, which is flanked by repeat sequences upstream and a C-terminal SH3b domain downstream that is a bacterial analog of SRC Homology 3 domain facilitating substrate recognition. Lysostaphin is produced as a preproprotein, including a 36 amino acid residue signal peptide directing it to plasma membrane, 15 tandem repeats each consisting of 13 residues, and a mature protein part of 246 residues that carries M23 and SH3b domains. Although active in proprotein form, lysostaphin is 4.5-fold more active after the repeat sequences are removed, a process catalyzed in cell cultures by extracellular cysteine protease (Neumann *et al.*, 1993; Thumm & Götz, 1997). The role of the repeats remains unclear, their deletion does not obstruct secretion and folding of active lysostaphin and, in general, activity of the enzyme increases by reducing the number of repeats attached to mature protein (Thumm & Götz, 1997). The 12-residue fragment between the signal peptide and the first repeat sequence, however, appears to be important for secretion, but does not affect the synthesis of enzyme or cell growth. ALE-1 shares 91% and 84% sequence identity with lysostaphin M23 and SH3b domains, respectively (BLAST, National Center for Biotechnology Information). It is 362-residue long and shorter than 493-residue lysostaphin. It has only six 13-residue repeats and these do not need to be cleaved off for the full enzymatic activity (Sugai *et al.*, 1997a). Both lysostaphin and ALE-1 are encoded by plasmids acquired by staphylococci, not necessary for normal cell growth, and are accompanied by genes encoding resistance factors, *lif* and *epr* respectively, that replace glycine with serine residues in PG bridges at positions 3 and 5 to make hosts immune to these lytic enzymes (Ehlert *et al.*, 2000; Heath *et al.*, 1989; Sugai *et al*, 1997b; Thumm & Götz, 1997). Likely orthologs of these lysis enzymes and their immunity factors, zoocin A and *zif*, have been found in *Streptococcus equi* subsp. *zooepidermicus* (Beatson *et al.*, 1998; Lai *et al.*, 2002; Simmonds *et al.*, 1996). *Staphylococcus sciuri* was the first staphylococcus found to possess the immunity factor alone, without carrying the gene for enzyme against lysis of which it would protect (Heath *et al.*, 2005).

LytM is a well-studied and until research described hereafter was the only known M23 family autolysin of *S. aureus*. It is a 316-residue protein that has a single endopeptidase domain in its C-terminus, which shares 50% identity with lysostaphin and ALE-1 catalytic domains. The first studies reported the gene to be located in chromosome and conserved only in *S. aureus* species. The protein caused cell lysis, contained one zinc atom per molecule, and its even distribution across cell suggested role in cell growth (Ramadurai & Jayaswal, 1997; Ramadurai *et al.*, 1999). Subsequently, a full-length LytM was demonstrated to be inactive and required cutting of the

catalytic domain away from the N-terminal section that was blocking active site and adding additional coordination to the zinc cofactor (Firczuk *et al.*, 2005; Odintsov *et al.*, 2004). Later study provided rather controversial results finding *lytM* deletion mutant to have no effect on cell autolysis, rather promoting it under oxacillin stress, and authors questioned LytM function as an autolysin (Singh *et al.*, 2010). Nevertheless, it has been shown that overexpression of LytM can restore growth of WalKR-depleted *S. aureus* cells, it is regulated by this TCS as well as global regulator RNAIII, it is suppressed under stationary growth, and generally accepted as autolysin (Chunhua *et al.*, 2012; Delaune *et al.*, 2011; Lioliou *et al.*, 2016). Release of the characteristic *S. aureus* protein A has also been attributed to lytic capability of LytM (Becker *et al.*, 2014).

There is a wide range of M23 family proteins that are neither involved with *S. aureus* PG pentaglycine cleavage nor even peptidases. For instance, *B. subtilis* phage φ29 gene 13 product gp13 is a structural component of phage tail, shares 14% identity with LytM catalytic domain and has several possible functions, including D,D-endopeptidase (Cohen *et al.*, 2008; Cohen *et al.*, 2009). Short 99-residue M23 domain of EnpA$_C$ from *Enterococcus faecalis* shares 40% sequence identity and cleaves D-Ala-L-Ala bonds in a variety of bacteria (de Roca *et al.*, 2010). EnvC and related factors from *E. coli* share ~30% identity with LytM but lack peptidase function and instead act as cell wall separation regulators (Uehara *et al.*, 2009; Uehara *et al.*, 2010). Related recruitment proteins recently were found in *Haemophilus influenzae*, *Caulobacter crescentus*, *Xanthomonas campestris*, and multicellular cyanobacterium *Anabaena* (Bornikoel *et al.*, 2018; Ercoli *et al.*, 2015; Yang *et al.*, 2018; Zielińska *et al.*, 2017).

1.2.4.2. Characteristics of M23 peptidase domains

M23 domains share similar structural fold not only among members of the subfamilies and within the family, but also have similar core elements with M15 peptidases that include D-Ala-D-Ala peptidases. Based on their common coordination of Zn^{2+} ion by Nε, Oδ and Nδ atoms of histidine, aspartate and histidine, respectively, a joint classification "LAS enzymes" has been proposed (Bochtler *et al.*, 2004). However enticing search for similarities, patterns, and new classifications may be, any inclusion of peripheral proteins complicates specifications within the group and should be avoided if not necessary. The unifying features of active M23 peptidases is their conserved active site residues and overall fold. Known to the author 3D-structures of active M23 domain include LytM (PDB ID 1QWY, 2B0P, 2B13, 2B44, 4ZYB), lysostaphin (4LXC, 4QP5, 4QPB), zoocin A (5KVP), gp 13 (3CSQ), LasA from *Pseudomonas aeruginosa* (3IT5, 3IT7), NMB0315 from *Neisseria meningitidis* (3SLU), and D,D-endopeptidase from *Vibrio cholerae* (2GU1). The proteins have a characteristic groove formed by β-sheet and bordered by fours loops (**Figure 3A,** blue and red arrows respectively). The catalytic Zn^{2+} ion is coordinated by histidine and aspartate from HXXXD motif as well as one histidine from HXH motif. The second histidine from the latter fragment together with a remote conserved histidine are understood to be catalytical. Mutation of these residues to alanine abolishes catalytic activity of ALE-1 (Fujiwara *et al.*, 2005). It has been noticed that a tyrosine residue is always present in the first loop of catalytic domain of LytM and closely related enzymes (**Figure 3B**), and its mutation to other residues significantly lowers activity, hence implying a role in catalytic mechanism (Grabowska *et al.* 2015; Spencer *et al.*, 2010). Presence of one zinc ion per molecule has been confirmed in these enzymes (Odintsov *et al.*, 2004; Sugai *et al.*, 1997a; Trayer & Buckley, 1970; Wang *et al.*, 2011). Other metal ions, Co^{2+}, Mn^{2+}, Cu^{2+}, have been shown to activate zinc-depleted LytM, although the full activity

observed with zinc was not restored (Firczuk *et al.*, 2005). Metal ion chelators, e.g. EDTA, are potent and reversible inhibitors. The pH optima for lysostaphin, ALE-1, LytM, and LasA are 7.5, 7-9, 7.5, and 8-9, respectively (Browder *et al.*, 1965; Firczuk *et al*., 2005; Spencer *et al.*, 2010; Sugai *et al*., 1997a).

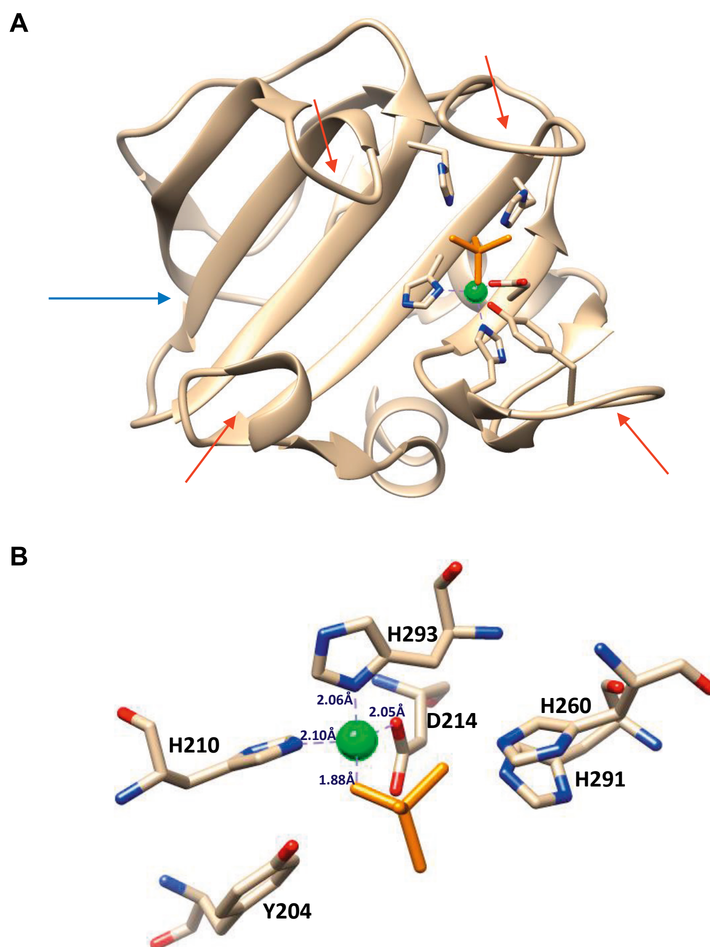

Figure 3. Overall structure of LytM catalytic domain (188-316) (**A**) and close-up view of the active site residues with zinc ion (green) coordinated in the center (**B**). Catalytic groove (blue arrow) is formed by four flanking loops (red arrows). Residues H210, D214, H293 coordinate zinc ion together with cacodylate ($C_2H_6AsO_2^-$) ion (orange). Residues H260 and 291 are catalytic and Y204 is implied in catalytic mechanism as well. Figures are based on PDB ID 2B0P (Firczuk *et al*., 2005).

1.2.4.3. SH3b domains of M23 peptidases

SH3b domains are ~ 90-residue long bacterial analogs of smaller ubiquitous eukaryotic SH3 domains that are involved in signaling pathways and interact with proline-rich fragments (Ponting *et al.*, 1999; Saksela & Permi, 2012; Whisstock & Lesk, 1999). Yet the binding of typical PXXP site in SH3bs is partially obstructed and instead these domains have 20-residue extension, which creates an interface for interaction with pentaglycine (Gu *et al*., 2014; Hirakawa *et al.*, 2009; Lu *et al.*, 2006).

This property of SH3b domains has been recruited by some M23 peptidases that can take advantage of substrate recognition. The enzymes include lysostaphin and ALE-1, and the structures of their SH3b domains are known (Baba & Schneewind, 1996; Hirakawa *et al*., 2009; Lu *et al*., 2006). Before this study, however, little has been understood about the interaction of SH3b domains and peptidoglycan. It has been accepted that pentaglycine bridges are essential for the interaction of lysostaphin SH3b with cell wall PG, but other cell wall components, like proteins and teichoic acids, were not involved (Gründling & Schneewind, 2006). Additional support for the central role of pentaglycine was provided in case of ALE-1, and in a separate study interaction between ALE-1 catalytic and SH3b domains was observed *in vitro* (Lu, H. *et al*., 2013; Lu, J. Z. *et al*., 2006). Towards the end of the study described herein, a 3D-structure of SH3b-pentaglycine complex was released (PDB ID: 5LEO, 2017). Pentaglycine was shown to occupy the predicted N-groove of the domain (**Figure 4**) and it furthered the focus on pentaglycine as the key factor in substrate binding.

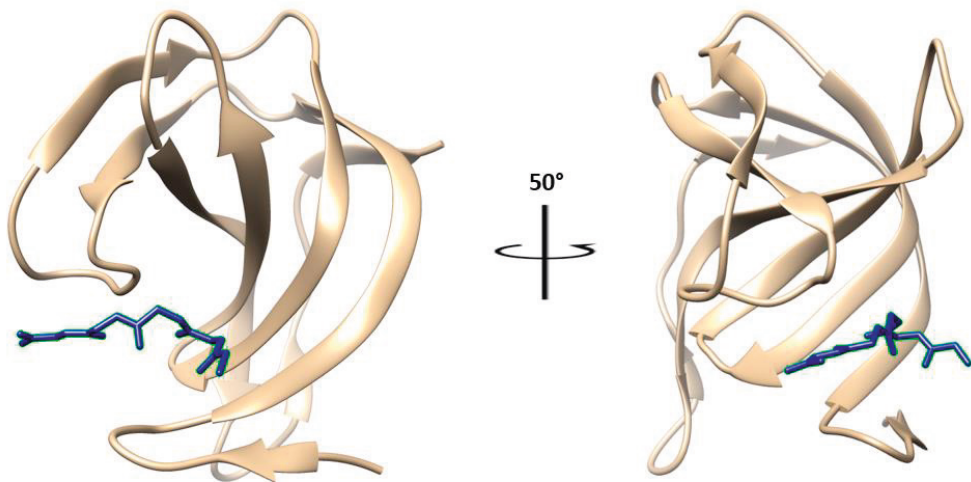

Figure 4. SH3b domain from lysostaphin with co-crystalized pentaglycine (PDB ID: 5LEO, Jagielska *at al*., 2017).

1.2.4.4. Application of M23 peptidases

All *S. aureus* peptidoglycan hydrolases are of interest and importance for two main reasons: (i) they provide insight into the pathogen's physiology and (ii) can potentially be used in antibacterial therapies. As such, they are not only prospective targets, but are also conspicuous instruments themselves. Already the first study identifying lysostaphin asserted its anti-staphylococcal potential (Schindler & Schuhardt, 1964).

Vast research showing lysostaphin activity against *S. aureus in vitro*, *in vivo*, in biofilms, and different infection models alone and in combination with other antimicrobials has been reviewed (Bastos *et al.*, 2010; Kumar, 2008; Septimus & Schweizer, 2016; Suresh *et al.*, 2019; Wittekind & Schuch, 2016). The main advantage of lysostaphin is that it is active against the antibiotic-resistant strains, because it targets the characteristic pentaglycine bridge in cell wall PG and not the enzymes or steps leading to cell wall synthesis that have evolved to be drug-resistant. Native LytM has been reported to have a very low anti-staphylococcal activity in physiological conditions (Sabala *et al.*, 2012). This activity, however, can be enhanced up to 540-fold when the M23 domain is fused with the cell wall recognition SH3b domain and the enzyme becomes therapeutically relevant (Osipovitch & Griswold, 2015). Moreover, in low ionic environment, catalytic domain of LytM is more active than catalytic domain of lysostaphin (Jagielska *et al.*, 2016). Phage endolysins with catalytic domains from different families have received a lot of attention, but the potential of some enzymes remains untapped and M23 domains with their *S. aureus* specificity demand for special consideration (Kashani *et al.*, 2018; Nelson *et al.*, 2012; Pastagia *et al.*, 2013). To this end, molecular engineering of known and hypothetical autolysins can provide a wealth of resources (Blazanovic *et al.*, 2015; Jagielska *et al.*, 2016; Osipovitch *et al.*, 2015).

2. AIMS OF THE STUDY

The research was triggered by the finding of *S. aureus* gene *sa0205* upregulation under stress of antimicrobials which interfere with cell wall integrity. We postulated that the gene encodes a protein important to maintain cell wall. Analysis of the gene sequence indicated it to contain an M23 peptidase domain. This led us to hypothesize that the protein is an active enzyme. Consequently, we have set out to investigate the properties and functions of the protein.

During progression of the studies, new questions continuously arose and to address them approaches were designed and tasks were undertaken. The main aims of the study were the following:

1. To obtain the protein in a form suitable for studies, including the structural ones by NMR (Article I)
2. To investigate its physiological role (Article II)
3. To reveal its enzymatic properties (Article II)
4. To determine its structural features as the basis for 2 and 3 (Article II)
5. To clarify substrate binding (Article III)

The overall goal was two-fold: (i) characterization of a new member of the family and (ii) increased knowledge about peptidoglycan cleavage in *S. aureus* cell wall.

3. METHODS AND MATERIALS

The following is the summary of methods used during the studies. For detailed information a reader is invited to visit the Materials and Methods' sections of the original publications as referenced below.

The applied methods are conventional and did not require specific adaptations. Nonetheless, in our work we have introduced a new approach to monitor reaction and measure activity of pentaglycine-cleaving enzymes.

Table 3. Methods used to obtain the results covered in the thesis.

Method	Article
Cloning, expression, and purification of LytU	I, II
Cloning, expression, and purification of LytU mutants and LytM	II
Cloning, expression, and purification of lysostaphin	III
Protein NMR spectroscopy	I, II, III
Generation of LytU deletion mutant	II
Generation of LytU overexpression construct	II
Construction of GFP-LytU fusion protein	II
Cell fractionation	II
Immunolocalization of proteins	II
Microscopy	II
Cell lysis measurement	II
Enzyme activity measurement by NMR spectroscopy	II, III
ITC	II
Molecular dynamics simulations	III
SAXS	III

Chromosomal DNA from *Staphylococcus aureus* strain Newman was used as the gene template for proteins LytU and LytM. Gene of mature lysostaphin (residues 251-493) was synthesized *de novo* at GenScript (NJ, USA). For purification, all proteins were cloned into pGEX-2T vector (GE Healthcare Life Sciences) and expressed with GST tag in *E. coli* BL21 (DE3) cells (Merck Biosciences, Germany).

4. RESULTS

This section provides condensed main findings and the results that are discussed in greater detail in the original publications.

4.1. Identification of LytU protein

In the beginning, the sequence of putative protein encoded by gene *sa0205* was analyzed by HMMTOP, MODELLER, and SignalIP tools (Nielsen *et al.*, 1997; Sali & Blundell, 1993; Tusnády & Simon, 2001). The protein, consisting of 192 amino acid residues, was predicted to have a membrane-spanning fragment (residues 7-25), a disordered region (26-48), and an M23 catalytic domain in its C-terminus (**Figure 5A**). Its catalytic domain region (residues 49-182) has 41.9, 43.2, and 42,4% sequence identity with its counterparts in LytM, lysostaphin, and ALE-1, respectively (**Figure 5B**). The protein possesses conserved residues Y 67, H76, D80, H125, H157, and H159 characteristic to M23 peptidases. Sequence alignment revealed a distinct insertion at position 151, which, depending on the *S. aureus* strain, can be isoleucine or lysine.

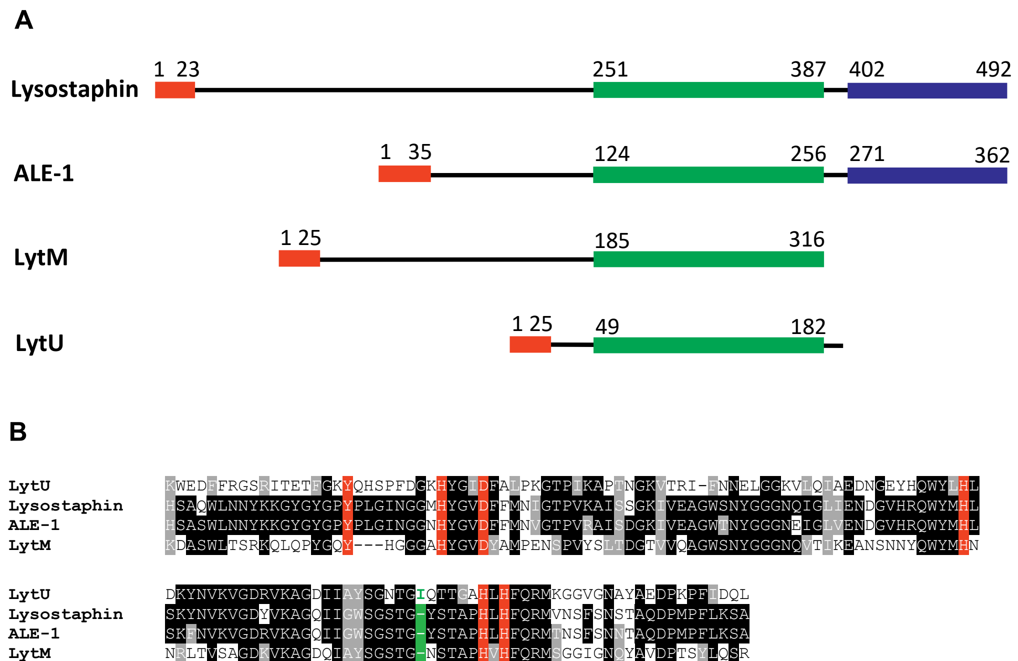

Figure 5. **A**. Comparison of the full-length sequences of lysostaphin, ALE-1, LytM, and LytU. Red, signal sequence; green, catalytic domain; blue, SH3b domain. The exact limits of domains may vary in literature and among research groups. **B**. Sequence alignment of the catalytic domains corresponding to 49-182 residues in LytU. Conserved active site residues Y67, H76, D80, H125, H157, and H159 are highlighted in red. The unique LytU insertion at position 151 is highlighted in green. (Adapted from Article II.)

We cloned the gene lacking hydrophobic transmembrane peptide into pGEX-2T plasmid and expressed it in *E. coli* with a GST-tag, which assisted in purification and subsequently was removed. The purified protein was not homogeneous and had a varied length with several evident proteolytic cleavage sites in N-terminal fragment, the furthest downstream occurring between residues 69 and 70. In addition, the full-length (26-192) soluble portion of the protein delivered poor quality NMR $^{1}H,^{15}N$-HSQC spectrum, expectedly caused by flexible N-terminal fragment. We produced several proteins of different length based on these cleavage sites. Truncation at the start of catalytic domain (49-192) allowed to obtain a mostly uniform length protein, yielded satisfactory NMR spectra with well-dispersed peaks, and was suitable for structural studies (**Figure 6**). By following existing nomenclature, the next available identifier was LytU and we named the protein accordingly.

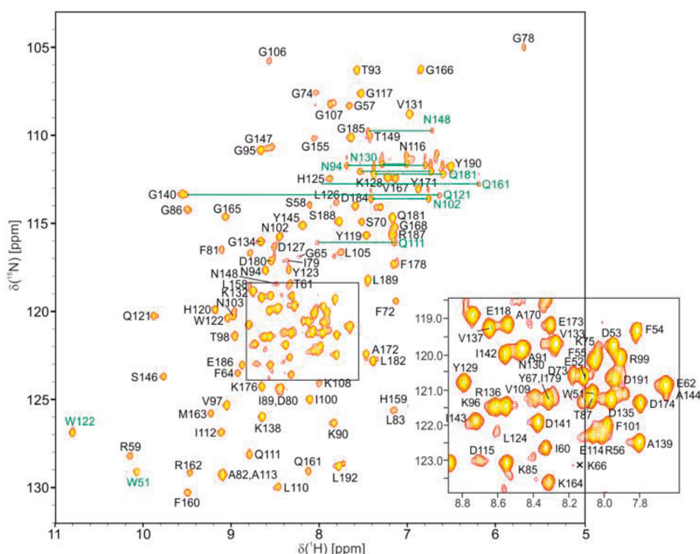

Figure 6. ^{1}H, ^{15}N-HSQC spectrum of one zinc-bound LytU 49-192. (Adapted from Article I.)

4.2. Physiological role of LytU

To grasp the physiological role of LytU, we have investigated its cellular location and impact of expression on cell growth. For the first purpose, LytU was expressed in *S. aureus* cells with Strep-tag and separately fused with GFP protein. Immunoblotting against Strep-tag showed that LytU was present in cell membrane fractions. Removing of cell wall peptidoglycan layer with lysostaphin left cell protoplasts with LytU bound, but it was removed by trypsin, indicating that soluble part of the protein is extracytoplasmic. Expression of LytU-GFP fusion showed that LytU is found across cell membrane, including septa between dividing daughter cells (**Figure 7A**).

LytU deletion and overexpression constructs were produced to explore its *in situ* function. LytU deletion did not have a clear effect on cellular viability and growth, thus, the protein was determined to be non-essential. During careful examination of cells, small flaws resembling

tearing edges could be seen on the surface. These edges arguably corresponded to previous division planes and thus indicated that LytU plays a role in cell separation, which is not executed properly in its absence.

Overexpression of LytU affected cells profoundly. Overexpression of *lytU* gene led to cell death in cultures (**Figure 7B**). Inspection of individual cells by electron microscopy showed that individual cells were dividing, yet, their daughter separation was hindered and eventually occurred by the rupture at septum.

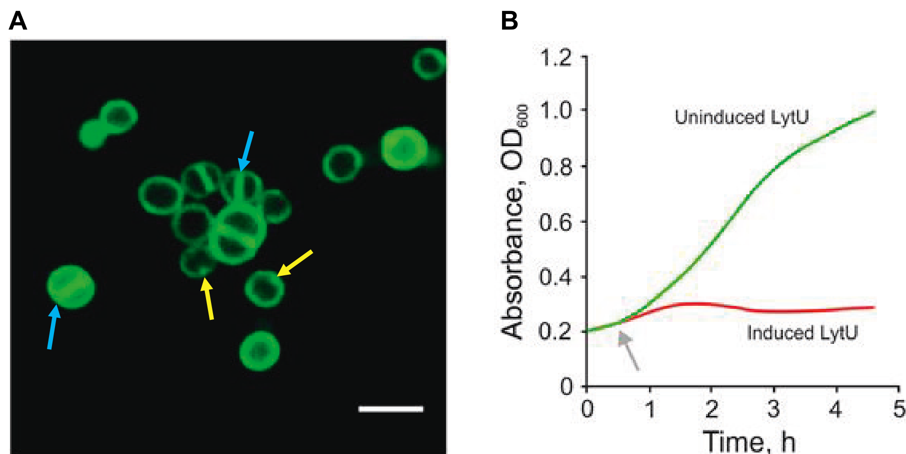

Figure 7. **A**. Localization of LytU-GFP fusion protein. Yellow arrows point to LytU accumulation at the start of division septum formation. Blue arrows show high LytU density in septa. Scale bar is 2 μm. **B**. Induction of LytU overexpression. The arrow indicates induction point. (Adapted from Article II.)

4.3. Enzymatic properties of LytU

Expectedly, in our initial experiments, lysis of stationary and logarithmic growth phase cells was observed upon addition of the recombinant LytU to cell cultures. The results, however, did not provide unequivocal conclusions, since the activity was observed also with metal ions that were determined to not be cofactors of active enzyme. Free metal ion concentrations, their binding by cell wall components, exchange, any additional lytic processes, and vague microenvironments rendered cell culture to be too complicated and with too many variables for a system intended for accurate enzyme study. Adoption of other techniques using insoluble and non-physiological substrates was not optimal as well. Therefore, we have devised the minimalistic system, using pentaglycine as a substrate that allowed for direct reaction monitoring and convenient product quantification by NMR (**Figure 8**).

LytU cleaves pentaglycine. We have tested the activity in the pH range from 6.2 to 7.9 and found it highest at the pH 7.9. Higher pH values were not explored due to the limitations of the essay at the time. We also found that LytU activity at 25 °C drops to just over 20% of that at 37 °C. The observation implies that LytU does not provide any advantages for cells at the temperature that would be encountered by biofilms on medical equipment.

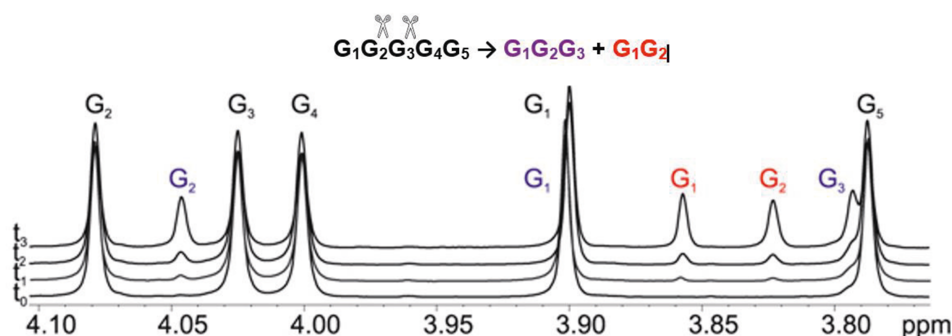

Figure 8. Cleavage of pentaglycine by LytU as observed by NMR. (Adapted from Article II.)

LytU can use several different metal ions as cofactors. Besides Zn^{2+}, these include Mn^{2+}, Cu^{2+}, and Co^{2+}. Mg^{2+}, Fe^{2+}, and Ca^{2+} did not confer activity to the enzyme. Interestingly, activities with copper and cobalt ions were several-fold higher than that with zinc ion. Although Mn^{2+} and Co^{2+} were also suitable cofactors for lysostaphin, they were less optimal than Zn^{2+}. We have found that lysostaphin replenishment with molar excess of Cu^{2+} allowed to exceed activity of zinc-bound enzyme, which could be explained by copper being a more suitable cofactor with a lower protein affinity.

Full-length soluble LytU has the same pentaglycine cleavage activity as the truncated catalytic M23 domain, suggesting that N-terminus does not need to be cleaved off *in vivo*. LytU isoform Lys151 is about 40% more active against pentaglycine than Ile151, which is as active as catalytic domain of LytM (**Figure 9**). Most strikingly, these residues, insertions when compared to M23 domains in other related proteins, appear essential for catalytic activity, since their deletion inactivates the enzyme, while keeping the overall structural fold intact. On the other hand, the introduction of these insertions into LytM made the protein insoluble and obstructed its purification.

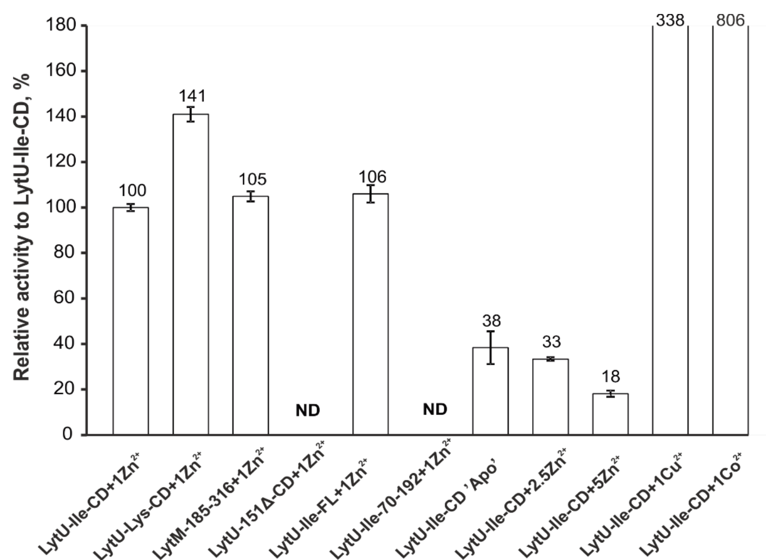

Figure 9. Comparative catalytic activity of selected proteins. (Adapted from Article II.)

Our robust catalytic activity monitoring setup system allowed us to detect the effect of excess zinc ions on LytU catalysis. The binding of second zinc ion is inhibitory. These inhibitory effects were significantly smaller in LytM and lysostaphin. The binding of second zinc is pH-dependent and NMR titration experiments show that at pH 6.5 and higher, in the presence of second zinc, catalytic histidines are arrested. K_d values, determined by isothermal titration calorimetry, for LytU-Ile and LytU-Lys binding of the first and second zinc ions were 0.26 and 0.22 nM and 0.32 and 0.49 μM, respectively.

4.4. Structural features of LytU catalytic domain

The catalytic domain fold of LytU is akin to those of its M23 family and superposition of its backbone atoms in secondary structure elements with LytM catalytic domain results in RMSD of 0.9 Å (**Figure 10A**). The apparent substrate accommodating groove is shaped by five β-strands and four loops, namely, N-terminal, β3-β4, β6-β7, and C-terminal. We have determined one- and two-zinc bound structures of LytU and the average displacement of loop β6-β7 is smaller in the two-zinc form, most likely due to rigidity induced by the coordination of second zinc atom (**Figure 10B**). The catalytic site is formed by residues H76, D80, and H159 that coordinate catalytic zinc ion and residues H125 and H157 believed to be directly involved in catalysis or, alternatively, inhibited by the second zinc. The loop β6-β7 is the residence of residue 151, however due to the flexibility, its precise side chain conformation cannot be determined.

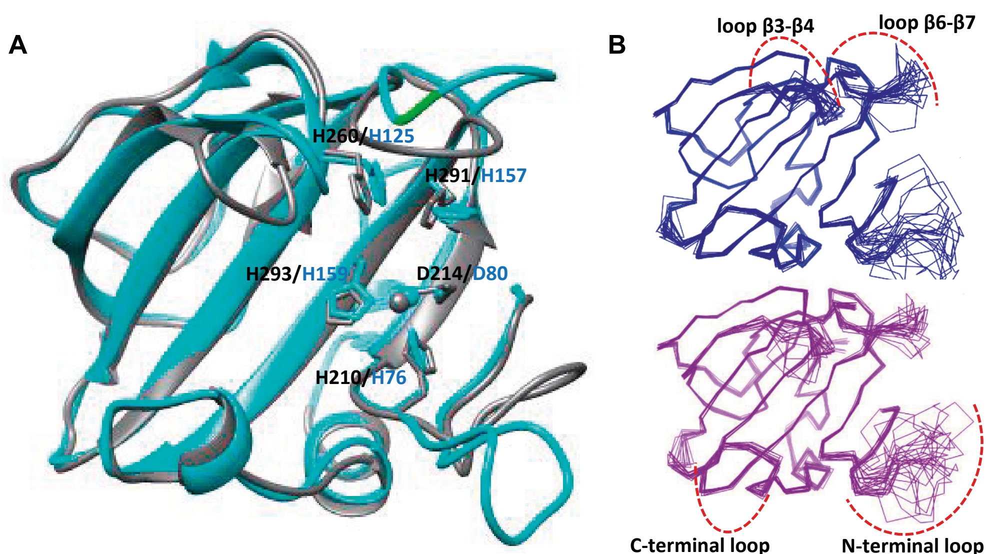

Figure 10. **A**. Superposition of LytU (cyan) and LytM (gray) catalytic domains, corresponding to LytU residues 60-192 and LytM residues 197-316 (PDB IDs: 5KQB and 2B0P, respectively). The green fragment indicates the backbone of residue 151. **B**. Ensembles of fifteen structures of the least restraint violation of one- and two-zinc bound LytU, blue and purple, respectively (PDB IDs: 5KQB and 5KQC). Residue 151 is located in the loop β6-β7, which remains flexible after binding the second zinc ion. The second inhibitory zinc ion is not illustrates bound to LytU, due to the inability to determine its third ligand and not fully clear location. (Adapted from Article II.)

Regrettably, we were not able to determine the third ligand of second zinc. Proximal candidates N148 and Q152 were shown to not be involved, since mutations N148A and N148S/Q152A did not cancel the binding of second zinc. Nevertheless, the second zinc seemed to stabilize hydroxyl protons of S146 and T149 as well as region 64-75, which contains tyrosine 67, the analogs of which in LytM and LasA were implicated in catalytic mechanism. The Y67 role in the LytU catalysis is supported by our finding that the shortest proteolytically stable LytU (70-192) retained the overall structure but was completely inactive.

4.5. Substrate binding by SH3b domain

We were not able to map substrate, pentaglycine, binding to LytU catalytic domain or its analogs of lysostaphin and LytM. Hundred-fold excess of substrate did not induce detectable shift perturbations in NMR spectra. This led us to hypothesize that the interaction between the catalytic domain and the substrate is very transient and only occurs during the reaction between the catalytic site residues and the scissile bond. Consequently, in search for the more pronounced enzyme-substrate interaction, our attention shifted to the SH3b domain of lysostaphin.

In our experiments, SH3b did bind pentaglycine in the N-terminal groove similarly as determined previously. The binding is not dependent on the presence of catalytic domain and the 14-residue linker in between remains flexible despite presence or absence of the substrate. The binding, however, is exceptionally weak with K_d estimate larger than 10 mM. We hypothesized that additional interactions must be taking place. Indeed, we have found that lysostaphin SH3b domain binding had a significantly higher affinity when using a synthetic A-d-EK-GGGGG-A-d-EK-d-A PG fragment, which would resemble the stem-cross-bridge-stem sequence in native peptide. The peptide interaction sites on the SH3b surface were inferred from an NMR chemical shift perturbation assay (**Figure 11**). Structural models generated from existing structures and NMR binding data suggested that the SH3b domain recognizes pentaglycine and branch peptide moieties that are not directly linked and compose a larger structural unit in the complex PG architecture.

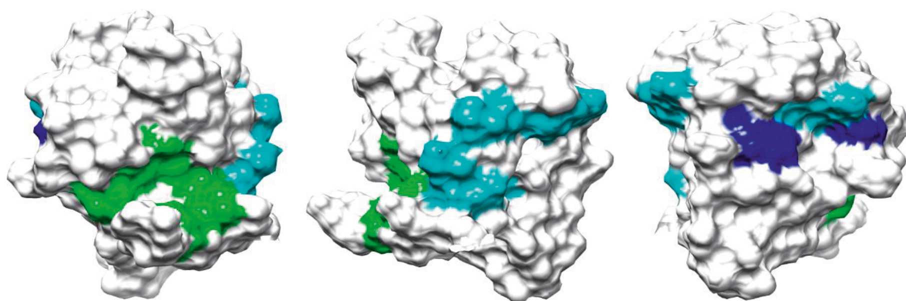

Figure 11. Surface of the lysostaphin SH3b domain from different angles. The surface of G_5K peptide binding groove is shown in green. Lysine was included to solubilize pentaglycine. Additional cyan and blue binding surfaces were mapped when A-d-EK-GGGGG-A-d-EK-d-A peptide was used. Blue surfaces represent residues R427 and W489 that are strictly conserved in *S. aureus*-targeting SH3b domains and their mutations R296A and W358A in ALE-1 reduced binding 3- and 2-fold, respectively, when compared to wild type binding (Lu *et al*., 2006). PDB ID: 5NMY. (Adapted from Article III.)

We found no evidence of interaction between lysostaphin catalytic and SH3b domains *in vitro*. Replacement of zinc ion with paramagnetic Mn^{2+}, which induces transverse paramagnetic relaxation enhancement and as a result "bleaches" or broadens peaks in NMR spectra within up to 35 Å distance, showed no effect on peaks in the catalytic domain beyond 23.6 Å including SH3b domain. Overall rotational correlation times, τ_c, derived from relaxation data were different for catalytic and SH3b domains: 7.8 and 6.0 ns at 35°C, respectively. Although isolated SH3b had τ_c 4.1 ns, indicating some motion restriction from the attached catalytic domain, the τ_c estimate for a unified complex was 13.4 ns and suggested flexibility between partly independent domains. Small-angle X-ray scattering showed a full-length lysostaphin to form two main conformation populations – a more extended and a more compact one – the latter one being preferred.

5. DISCUSSION

The large number of peptidoglycan hydrolases tempts to downgrade the importance of any single enzyme. In fact, so far only one gene, *pcsB* in *Streptococcus pneumoniae*, has been shown to be essential for the viability of its host (Sham *et al.*, 2011; Wheeler *et al.*, 2015). The author of this thesis would like to invite quite an opposite perspective and bolster a different viewpoint: the large repertoire of the enzymes is not to be considered as a mere redundancy, but rather exemplifies the critical value of their shared functions that cells cannot afford to lose. The costly energetic and genomic resources are justified and there is no "just another peptidase in a wall" (Scheffers & Tol, 2015). Moreover, employment of separate enzymes allows for dedicated roles and accurate control spatially and temporally. Full comprehension of the network is inevitably compulsory to appreciate role of each constituent and recognition of each and every member and its individual function are important to understand the global mechanism.

A prototypical *S. aureus* strain was found to have up to 13 known or putative PGHs (Antignac *et al.*, 2007). In this study we introduce LytU, a previously unreported peptidase and the second representative of the M23 family. The enzyme is encoded in chromosome but is not essential. It is anchored in the membrane and its soluble part is extracytoplasmic. Evidence shows LytU is involved in daughter cell separation and its gene transcription previously was found upregulated upon exposure to certain antibacterial compounds (Pietiäinen *et al.*, 2009). The enzyme is metal ion-dependent and can successfully use Zn^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+} ions, the latter two making it hyperactive when compared to ostensibly physiological zinc. LytU can bind a second zinc ion in a process that is pH-dependent and results in locked catalytic residues. Enzymatic activity is expressed by cleaving pentaglycine.

Our findings achieved initial aims, but they also prompt new questions and their contribution to the larger goal – to enhance knowledge on peptidoglycan cleavage in *S. aureus* cell wall – is anything but complete. The imminent discussion topics could be grouped under two general questions: What does LytU do *in situ*? What properties of LytU define its role?

In situ role. The presence of LytU in plasma membrane facing cell wall is expected from an autolysis enzyme. Its even dispersion in adult and dividing cells precludes from extracting specific clues about its function. It is counted on LytU-GFP fusion to not interfere with native LytU localization. It could be argued that the amount of the LytU is slightly higher in septa between dividing cells (**Figure 7A,** blue arrows), but this observation can be attributed to the double membrane layer at the septum as well as the point of view not precisely perpendicular to the septum and hence occupying a broader angular view and visible area. Increase in density of LytU can be seen at the newly emerging division sites (**Figure 7A**, yellow arrows), but this also cannot answer whether the larger quantities of the enzyme are required or are only a lateral result of formation of additional membrane layer. The detrimental effect of LytU overexpression on cell separation seems counterintuitive, yet the explanation can be as simple as LytU overloading and incapacitating separation mechanism, an event even more likely if LytU was indeed involved in cell separation and formed complexes with other relevant proteins.

The functions of autolysins in living cells commonly are considered during cell division and growth. The third, less glamorous function, is the maintenance of existing PG layers and recycling of its fragments. It is a well-established process in Gram-negative bacteria and *E. coli* has been estimated to recycle over half of its PG in each generation (Park & Uehara, 2008). PG recycling, however, has received substantially less attention and, after a prolonged debate, has been confirmed to occur in Gram-positive bacteria only very recently (Mayer *et al.*, 2019). It would

be reasonable to consider the involvement of LytU, the gene of which is upregulated upon the damage of CW.

The overall structure of LytU may provide more information for interpretation of its function. LytU is anchored in membrane and the 20-residue linker between the anchor and the catalytic domain has neither a strict structure nor a known function. Although it may be imagined that it forms interaction with other CW components, any evidence is lacking and it may simply be a swivel for the broader reach of the catalytic domain. LytU is localized in periplasm-like space between cell membrane and PG layers. This space was measured to be ~ 16 nm wide (Matias & Beveridge, 2006). Our calculations show that with LytU linker even in the fully stretched conformation, LytU would extend only to about 2/3 of the compartment width and not be able to reach the layers of PG. This would be consistent with LytU cleaving fragments of CW that are floating in periplasm-like space. It also cannot be excluded that brief conditions exists when this space narrows and LytU has access to the layered PG. The C-terminus of LytU ends with a 10-residue tail that does not appear to belong to the catalytic domain. Curiously, the first residues of the tail are LPDG, somewhat reminiscent of characteristic LPXTG fragment which bears scissile bond T-G used by sortase A to anchor proteins to PG (Navarre & Schneewind, 1994; Schneewind & Missiakas, 2012). Evidently, this anchoring does not occur, and it is not clear if the sequence is an evolutionary rudiment or just a coincidence.

Metal ions. The ability of some enzymes to use different metal ions as cofactors is well known and this feature of LytU is not surprising. The hyperactivation by Cu^{2+} and Co^{2+} is unlikely to play role *in vivo* yet should be taken into consideration when designing antimicrobial enzyme applications that can be more effective with a substitute ion. Ion affinity towards native enzymes or their mutants and derivatives must be addressed before these substitutions are implemented.

The most remarkable finding was the binding of second zinc, which was inhibitory and had a high affinity. Notably, high concentrations of zinc have been shown to be inhibitory to M23s in the past, but the mechanism was not explained. In our work we saw that molar excess of zinc ion led to the binding to catalytic residues H125 and H157 in LytU as well as H329 and H360 in lysostaphin. In the previous studies, for the lack of the better knowledge, the enzymes routinely were exposed to ion concentrations that we have found already to be halting catalytic reaction. The ability to detect this effect should be partly attributed to our reaction monitoring system that we would highly recommend to research groups that have access to NMR equipment. Presently, there is no consensus system for pentaglycine cleavage monitoring. The closest to the standard procedure is the lysis of *S. aureus* cells, however, this system is too complicated to resolve delicate details.

A lot remains to be desired in understanding of ionic microenvironments of the *S. aureus* cell envelope compartments. Teichoic acids conduct processes governing charge and metal ion distribution across CW PG, and proton gradient furnishes pH at the surface of plasma membrane. Zinc concentration in blood is in 9.2 – 20 µM range and anionic TAs traffic the ions across CW. Further details are scarce, studies of zinc contents in *S. aureus* CW are lacking and conditions need to be inferred from other systems. Discrepancies among studies exist due to their methodology and different subjects. An earlier study of intact *S. aureus* walls determined K_d of 350 µM for Mg^{2+} (Lambert *et al.*, 1975). Investigation of complete CW using solid-state NMR produced K_d of 600 ± 300 µM for Mg^{2+} binding to the phosphate of the repeating unit in *B. subtilis* (Kern *et al.*, 2010). Significantly lower K_d values were obtained for isolated streptococcal LTAs: 15 and 8.9 mM for Mg^{2+} and Ca^{2+}, respectively (Rose & Hogg, 1995). The comprehensive and consolidating model of metal ion binding was proposed in isolated *B. subtilis* CW (Thomas & Rice, 2014). Two zones of binding were demonstrated: strong initial binding followed by a negative cooperativity-induced

weaker binding. The calculated K_d values for Ca^{2+} and Mg^{2+} at pH 5.65 were 1.43 and 1.49 µM, respectively (Thomas & Rice, 2014). Taking into account previously determined similar values between Zn^{2+}, Ca^{2+}, and Mg^{2+} in isolated *B. subtilis* walls (Doyle *et al.*, 1980) and our data for the first and second zinc binding sites (K_d values 0.2 - 0.3 nM and 0.32 - 0.49 µM, respectively), both LyU sites can successfully compete against CW in metal ion binding.

Regulation. Autolysins are sensitive to pH, which can be used for their regulation (Calamita & Doyle, 2002). For example, AtlA has been shown to be subject to pH regulation orchestrated by cooperation of membrane proton gradient and wall teichoic acids that contribute positive and negative charges, respectively (Biswas *et al.*, 2012). Additional dimensions to regulation mechanism are created by alanylation of teichoic acids that reduces overall negative charge and attraction of metal ions to the negative charges of phosphate network.

The binding of second zinc has been observed in several other proteases, for instance the well-known carboxypeptidase A and thermolysin, where it is inhibitory as well (Gomez-Ortiz *et al.*, 1997; Holland *et al.*, 1995). The binding of second zinc ion by LytU is apparently stronger than by lysostaphin and LytM. Such vulnerability of the enzyme to lose its activity seems evolutionary impractical. Therefore, a rationale should exist, and regulation mechanism seems to be a plausible explanation, especially, since other regulation mechanisms, e.g. inhibition by N-terminal sequence, are not known. Based on our data and results, we propose a framework model for LytU regulation (**Figure 12**). The enzyme exists in an active state only under well-balanced conditions. Lowering pH protonates and inactivates catalytic histidines. At higher pH and lower proton concentration, the inhibition is carried out by the binding of second zinc ion.

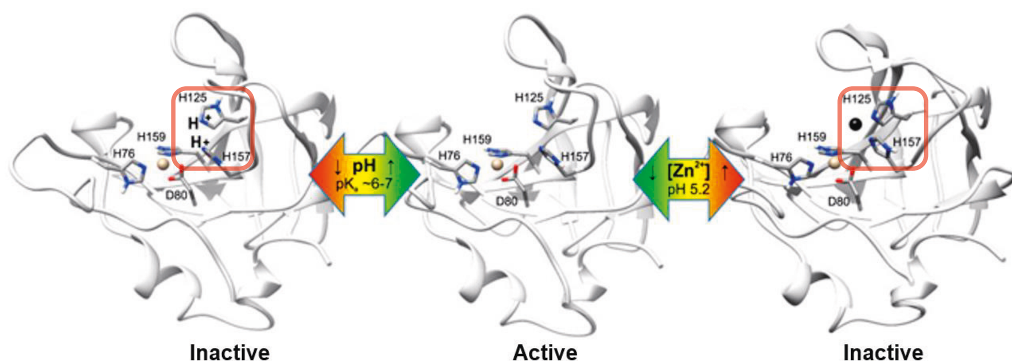

Figure 12. Regulation of LytU activity by pH and zinc ions. Catalytic histidines (H125 and H157) are emphasized in red frames when inactivated by protons at low pH (left) and the excess second inhibitory zinc ion (right). The catalytic zinc is shown in light gray, the inhibitory zinc is shown in black. (Adapted from Article II.)

*Binding of PG by SH3b.*Our research has brought a paradigm shift in PG binding and cleavage by lysostaphin. Previously, pentaglycine bridges have been the focal point in the SH3b-promoted binding to PG. We, however, showed that the SH3b binding is driven by PG branch peptides, rather than weaker binding to pentaglycine. Our data strongly indicate that SH3b binds to branch peptides and pentaglycine that are not directly linked. The flexible linker acts as an entropic spring. Furthermore, the cleavage of the bound pentaglycine seems sterically obstructed and the function of SH3b domain is to anchor lysostaphin to PG, whereas the contact with the

substrate pentaglycine is established by the catalytic domain alone, by browsing the vicinity of its anchoring site.

In accordance with our NMR peptide binding data, a very recent study proposes a two-site recognition mechanism between the lysostaphin SH3b and *S. aureus* PG (Gonzalez-Delgado *et al*., 2020). The authors solved by X-ray crystallography the structure of the complex between SH3b and a branched PG fragment, AγQK[GGGGG]A. In the complex, pentaglycine and branch peptide are recognized by different binding sites on opposite sides of the SH3b domain. The authors postulate an unusual binding mechanism, in which binding leads to clustering of SH3b domains, permitting a dynamic and synergistic target recognition.

Outlook. Several directions can be taken to advance the research on LytU and M23 peptidases in general. The most basic one is rather tedious, unlikely to result in exciting findings but could provide valuable foundation for long-term goals. That would be characterizing all currently known M23s, and possibly other PG hydrolases, in parallel, in a range of directly comparable and uniform conditions. Presently available information includes different protein expression and activity assessment systems, thus making direct comparison impossible and any extrapolated conclusions of little virtue. Recently, a separate study has also found lysostaphin to be reversibly inhibited by excess of zinc ions (Ojha *et al.*, 2018). The authors referred to the phenomenon observed in LytU, but because of the different experimental setup and context of the enzyme environment, refrained from further elaborations. Another seemingly natural inclination would be to generate *S. aureus* cells with double or multiple autolysin deletions and determine which enzymes can compensate for the function of another. Although such studies seem inevitable, one should be prepared for anticlimactic results, since many of hydrolase functions overlap and even the double deletion of major autolysins Atl and Sle1 proved non-lethal and only severely impaired daughter cell separation, which to a smaller degree can be observed upon some individual gene knockouts (Kajimura *et al.*, 2005).

Construction of LytU chimeras can prove the enzyme to be a prospective antimicrobial candidate. As a matter of fact, a very recent study has already put LytU to the task and showed that the fusion protein with the lysostaphin SH3b, had MIC against MRSA 421-fold lower than that of LytU alone (Taheri-Anganeh *et al*., 2019). LytU, as a homolog of lysostaphin, may share some medical advantages. Lysostaphin is valued for being active against dividing and non-dividing cells, not having negative effect on gut microbiota, not being toxic, and being active in human serum (Bastos *et al.*, 2010). Clearly, the biggest concern is the potential resistance acquired via *lif* or *erp* factor, which would incorporate serine residues into pentaglycine bridge making *S. aureus* defiant to lysostaphin. On the bright side, strains that have acquired such resistance have shown reduced viability and hence, could be more susceptible to treatments that combine M23 peptidase with other antimicrobial agents (Kusuma *et al*., 2007).

Upon the discovery of lysostaphin in 1964, a lot of hope has been put into its anti-staphylococcal effect. This excitement has gradually subsided because of the discovery of new antibiotics and commercial manipulations. However, with the emergence of strains highly resistant to antibiotics, the lysostaphin family is regaining attention and experiences the advent of applications.

6. CONCLUSIONS

The research described in this thesis began with the inquiry about the function of *S. aureus* gene *sa0205* and led to the investigation of its product protein LytU. Granting that it was initiated by scientific curiosity, it has been shaped by the context of peptidoglycan hydrolases and directed by pertinent hypotheses along the way. Some findings were unexpected and invited unscripted paths of study. Based on the harvested results and knowledge accumulated during the project, the following conclusions can be drawn.

1. *S. aureus* possesses previously not characterized and unreported autolysin that belongs to the M23 peptidase family. Its participation in PG cleavage must be taken into account when exploring the roles of other hydrolases and when comprehension of cell-wide processes is in question.
2. At least this particular enzyme is subject to tight regulation by pH and concentration of the physiological metal ion. Awareness and recognition of these factors are necessary when investigating PG hydrolysis.
3. The ability of certain metal ions to bestow catalytic hyperactivity when compared to natural cofactor should be considered when designing *ad hoc* therapeutic tools.
4. More research is required to gain substantially better understanding of ionic microenvironments *in situ* and *in vivo*.
5. Catalytic domain of M23 peptidases is not efficient at binding substrate *in vitro* and substrate targeting domains should be employed to enhance their function. Moreover, the interaction of these domains and substrate can be studied separately and the focus should shift from pentaglycine bridges to the 3D-architecture of PG.
6. In order to characterize and compare different enzymes *in vitro*, a reliable reaction measurement system must be utilized and the number of known variables has to be maximized.
7. The determined structures of LytU and its distinct properties, namely the insertion-bearing loop neighboring the active site, can potentially provide further insights into the catalytic mechanism.
8. In addition to contributing to innate cell processes, LytU can supplement resources in search for anti-staphylococcal remedies.

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My road to get here was long. Longer than many had. I began working on my first doctoral project in a different laboratory abroad more than half of my life ago. The third time was the charm. Time will tell if this ambition and stubbornness was worth it. Well, at least my restless and exhausted Dad and Mom can now finally feel relieved.

If only for the long duration of my journey, I have encountered numerous fascinating people. Many of them were not even friends or close colleagues, but quite often outstanding yet invisible people in administrative personnel or technical support, who went extra miles in their efforts to help and contributed in immeasurable and very valuable ways. I met many great people. To list them all is a crazy task. This Acknowledgements part would be longer than the thesis scientific part.

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Cordially, Your fan,

Vytas

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