Jéssica Rodrigues Gomes

Licenciatura em Biologia Celular e Molecular

The role of a new *S. aureus* hydrolase in Peptidoglycan degradation

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

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Resumo

Staphylococcus aureus é um dos mais comuns microrganismos patogénicos humanos, sendo responsável por várias doenças, tais como a pneumonia e a sepsia. Infeções causadas por este microrganismo têm se tornado progressivamente mais difíceis de combater devido ao aparecimento de resistência a diversos antibióticos.

Staphylococcus aureus é uma bactéria gram-positiva, cuja principal característica é uma grossa camada de Peptidoglicano que se mostra importante para as bactérias e, portanto, defeitos na sua manutenção (degradação e síntese) são prejudiciais à integridade da parede celular. As proteínas que fazem a degradação do Peptidoglicano, o elemento mais preponderante da parede celular, designam-se Autolisinas (ou hidrólases do peptidoglicano).

Atl é a autolisina mais influente de *S. aureus*, sendo responsável pela separação das células filhas aquando da divisão celular. Sle1é outra autolisina conhecida produzida por este microrganismo que, em conjunto com o Atl, são responsáveis pela completa separação das células filhas, dado que o duplo mutante, o que não expressa ambas as proteínas, apresenta um maior número de células filhas semi-conectadas.

Este trabalho foca-se numa proteína de *S. aureus* NCTC 8325, codificada pelo gene *saouhsc_00773*, que tem uma sequência peptídica semelhante à de Sle1. A partir desta sequência foi possível expressar uma proteína com aproximadamente 29kDa que que foi purificada com sucesso. Esta proteína apresentou uma atividade hidrolítica contra o peptidoglicano em Zymografia e quando incubada diretamente com peptidoglicano purificado. No entanto, o mutante de *S. aureus* que foi construído durante este projeto de modo a ter *saouhsc_00773* deletado não apresentou diferenças em termos de taxa de crescimento relativamente à estirpe parental.

Termos-chave: Peptidoglicano, Autolisinas, *S. aureus*, *saouhsc_00773*, Hidrolases do Peptidoglicano

Abstract

Staphylococcus aureus is one of the most common human microbial pathogens, responsible for several diseases such as pneumonia and sepsis. Infections caused for this microorganism have become progressively harder to treat due to the arising of antibiotic resistance observed in clinical strains.

Staphylococcus aureus are Gram-positive bacteria. Its cell wall is characterized by a thick peptidoglycan layer that is important to bacteria as defects in its maintenance (degradation and synthesis) compromise the cell wall structure and result in bacteria death. Proteins responsible for Peptidoglycan (PGN) degradation, the major cell wall element, are called autolysins (or PGN hydrolases).

Atl is the major *S. aureus* autolysin and is responsible for daughter cell separation in cellular division. Sle1 is another known peptidoglycan hydrolase produced by *S. aureus* that, together with Atl, is involved in the cell separation of *S. aureus* bacteria, as the double mutant presents an increased number of irregular clusters of bacteria.

In this work, I have studied another *S. aureus* NCTC 8325 PGN hydrolase, which is encoded by *saouhsc_00773*, that has a peptide sequence similar to that of Sle1. From this gene, a 29kDa protein was expressed and successfully isolated. This protein presented PGN hydrolytic activity in Zymography and also in direct incubation with purified PGN. However, the *S. aureus* mutant strain that lacks this gene, which was also produced during the framework of this project, did not present observable differences in growth rate relatively to the parental *S. aureus* strain.

Keywords: Peptidoglycan, Autolysins, *S. aureus*, *saouhsc_00773*, PGN hydrolase

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Abbreviatures and Symbols

% – Percentage

:: – Insertion

773 – protein expressed from the gene *saouhsc_00773* from NCTC 8325-4 that was cloned to the expression plasmid

Amp100 – Ampicillin at 100μg/mL (Stock solution at 100mg/mL)

CFU – Colony Forming Units

CS-PCR – Colony Screening PCR

ddH2O – bi-distilled, autoclaved water

ddNTPs – Dideoxynucleotides triphosphate

DNA – Deoxyribonucleic acid

DNA LB – DNA Loading Buffer

DNAse – Deoxyribonuclease

dNTPs – Deoxynucleotides

EDTA – Ethylenediamine tetra-acetic acid

Eri10 – Erythromycin at 10μg/mL (Stock solution at 10mg/mL)

FW – Forward primer

gDNA – Genomic DNA

GFP – Green Fluorescent Protein

LA – Luria Bertani Agar

LB – Luria Bertani Broth

M – Molar

MCS – Multiple Cloning Site

O/D – Overday (approximately 8h)

O/N – Overnight (approximately 16h)

ºC – Celsius Degrees

OD_{600} – Optical Density at 600nm wavelength

ORF – Open Reading Frame

pb – Nucleotide base pairs

PCR – Polymerase Chain Reaction

PFU – Plaque Forming Units

PGN – Peptidoglycan

RNA – Ribonucleic acid

RNase – Ribonuclease

Rpm – Rotations *per* minute

RT – Room Temperature (approximately 22ºC)

RV – Reverse primer

SDS-PAGE LB – SDS-PAGE Loading Buffer

SDS-PAGE native LB – SDS-PAGE Loading Buffer without *β*-mercaptoethanol

TP – Top Speed at the bench microcentrifuge (~16000g)

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

Vs – Versus

w/v – Wight *per* volume

wt – Wild Type

X-gal – 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

X-gal100 – X-gal at 100μg/mL (Stock at 40mg/mL)

Δ – Deleted gene

1. Introduction

1.1. The bacterial model of *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) are Gram-positive bacteria which have been identified by Anton J. Rosenbach, after the isolation of a colony with an intriguing pigmented appearance ("aureus" derives from the Latin *aurum* for gold) (Licitra, 2013).

S. aureus is one of the most common human bacterial pathogens, being responsible for serious infections such as pneumonia, meningitis, and sepsis (Franklin, 1998). Despite the ability to cause different diseases in the human host, *S. aureus* bacteria can also act as commensal microorganisms, i. e. they are capable of colonizing and residing in healthy humans' nostrils and respiratory tract without triggering the appearance of any symptoms of disease. These unaware carriers can contribute to the spread of the bacteria (Chambers *et al*., 2009) among the population.

Infections caused by *S. aureus* are usually treated with antibiotics such as flucloxacillin and dicloxacillin (penicillinase-resistant beta-lactam antibiotics), vancomycin or erythromycin (Rayner & Munckhof, 2005). However, *S. aureus* bacteria are associated with serious health-related problems. Infections caused by a particular class of strains, the methicillin-resistant *S. aureus* (MRSA) strains, have been frequently found both in the hospital environment and in the general population (Chambers *et al*., 2009; Franklin, 1998).

It is therefore essential to learn how this microorganism thrives in the different environments where it can be found, how it is capable to infect and colonize a human host and how it evades the anti-bacterial strategies put forward by the immune system of the infected host. These objectives will be achieved with a better understanding of how the bacterial cell cycle takes place and how the bacterial cell wall, the structure that in all the above-described processes protects bacteria from the surrounding environment, is assembled and degraded.

1.2. The process of assembly of the *S. aureus* cell wall

The cell wall of *S. aureus* is organized in a manner similar to other Gram-positive bacteria. It has a thick PGN layer; which contains charged polymers, such as capsular polysaccharides and wall teichoic acids, and anchored proteins; and it is assembled at particular sub-cellular sites of the bacterial cell surface during a defined time window (Monteiro *et al.*, 2015).

The cell wall is the bacteria's primary element with stress-bearing and shape-maintaining roles and its integrity is of crucial importance to cell growth and survival. For that reason, the synthesis of cross-linked polymer peptidoglycan (PGN), which is a scaffold structure of the cell wall of both Gram-positive and Gram-negative bacteria (Kashyap *et al*., 1998; Scheffers & Pinho, 2015), is a frequent target of antibacterial products (antibiotics or animal innate immunity proteins) (Kang *et al*., 1998).

PGN basic architecture is similar among most bacteria. It is constituted by a three-dimensional flexible glycopeptide mesh of *β*-(1–4)-linked *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) subunits crosslinked by short peptides containing L- and D-amino acids (Kashyap *et al*., 1998; Scheffers & Pinho, 2015).

As illustrated in Figure 1.1, PGN is partially synthesized inside the cell and then transported from the cytoplasm, through the membrane, to the cell exterior. Here, PGN molecules are incorporated into the cell wall through a process that is aided by penicillin-binding proteins (PBP), which are enzymes involved in the last steps of peptidoglycan biosynthesis catalysing the transglycosylation and transpeptidation reactions needed for the PGN formation (Büttner *et al*., 2014; Monteiro *et al*., 2015).

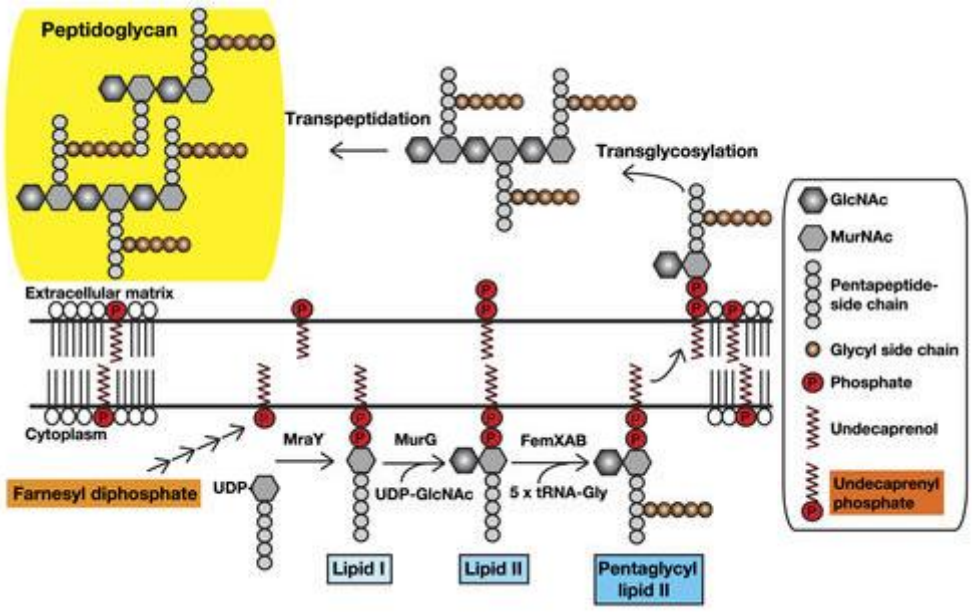

Figure 1.1 – Scheme of the synthesis of peptidoglycan. Glc*N*Ac: *β*-(1-4)-*N*-acetylglucosamine, Mur*N*Ac: *N*-acetylmuramic acid. Adapted from Yasukawa *et al*., 2016.

1.3. Peptidoglycan as the major component of the bacterial cell wall that betrays the presence of bacteria to the infected host

In an infection scenario, PGN can betray the presence of bacteria because PGN molecules can be recognized by the host innate immune system. Different receptors are able to identify PGN as a microbial-associated molecular pattern (MAMP) and initiate responses to eradicate invading bacteria: NOD-like receptors (NLRs) in mammals, LysM proteins in plants and Peptidoglycan Recognition Proteins (PGRPs) in insects (Atilano *et al*., 2014; Kang *et al*., 1998; Kashyap *et al*., 1998).

Bacteria may be identified not only through the recognition of the peptidoglycan connected to them, but also through the recognition of PGN fragments that have been released to the surrounding medium due to cell wall degradation.

Studies that have used Drosophila flies as a model for innate immunity, since mammalian proteins possesses a similar affinity to peptidoglycan than insect's PGRP, have shown that a lack of an PGN hydrolase allows the flies to recognize the presence of PGN still present at the bacterial surface (Atilano *et al*., 2014). In order to keep its concealment, bacteria may have developed strategies that prevent host receptors from recognizing PGN at the bacterial cell surface. Proteins that can degrade PGN, which are designated by autolysins, seem to have an important role in the reduction of the amount of exposed/accessible PGN (Atilano *et al*., 2011; Büttner *et al*., 2014).

1.4. *S. aureus* enzymes that are involved in the degradation and trimming of the PGN macromolecule

Autolysins, enzymes capable of degrading the PGN macromolecule, are involved in several cellular essential processes: (1) cell division, as bacteria need, first, to break the existing PGN covalent bonds in order to insert the newly synthesized PGN in the growing cell wall; (2) cell separation, as degradation of PGN is required to separate two daughter cells from each other; (3) maintenance of the plasticity of the cell wall, as PGN degradation is involved to changes in bacteria morphology required in their adaptation to different environment; (4) in the antibiotic-induced bacterial lysis, as PGN hydrolases, which are normally present inactive in bacteria seem to be activated in the presence of certain antibiotics; (5) in the pathogenesis of bacteria, as they are capable of modulating the levels of inflammatory PGN components released to the surrounding medium or of trimming the old PGN at the bacterial cell surface (Büttner *et al*., 2014; Kashyap *et al*., 1998).

PGN hydrolases (autolysins) are specific for a certain covalent ligation of the PGN, and these enzymes can be classified accordingly with the bond they degrade as muramidases, glucosaminidases, amidases, endopeptidases, and carboxypeptidases (Büttner *et al*., 2014).

Peptidoglycan hydrolases must be highly regulated to prevent accidental cell lysis as these enzymes are potentially lethal and able to destroy the cell wall of producing organisms. This regulation occurs at several levels from the transcriptional to the post-translational (Vollmer *et al*., 2008).

Defects in autolysins production, in some species, generate aberrant cell separation. Hydrolysis of the nascent septa is required for the cells' proper division. It therefore seems likely that PGN synthesis needs to occur across the cell surface concomitantly with autolysis. The interaction among the enzymes and the cell wall is crucial for their activity and several preserved mechanisms have been found for a number of enzymes (Monteiro *et al*, 2015; Vollmer *et al*., 2008).

Targeting these critical enzymes represents an attractive strategy for the development of new antibacterial compounds such as antibiotics (Büttner *et al*., 2014).

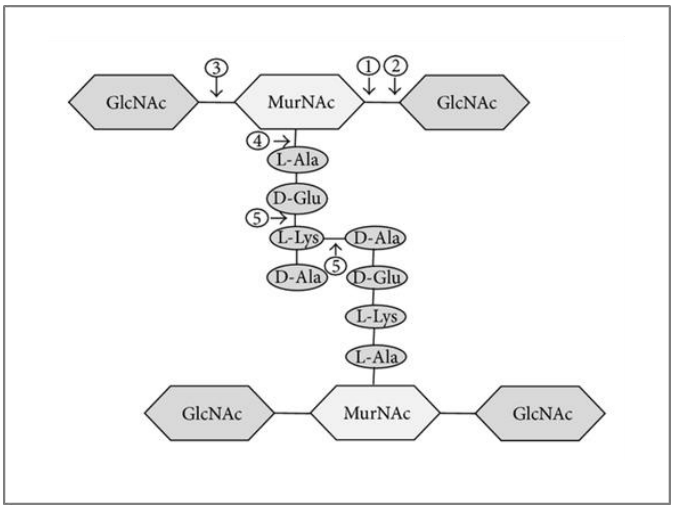

Figure 1.2 – Scheme that represents the cleavage sites of PGN hydrolases in the peptidoglycan macromolecule. The cleavage sites are indicated: (1) *N*-acetyl-*β*-D-muramidase (lysozymes), (2) lytic transglycosylase, (3) *N*-acetyl-*β*-D-glucosaminidase, (4) *N*-acetylmuramoyl-L-alanine amidases, and (5) endopeptidase. Legend: Glc*N*Ac (*N*-acetyl glucosamine); Mur*N*Ac (*N*-acetyl muramic acid) (Elbreki *et al*, 2014).

1.4.1. Atl, the major *S. aureus* autolysin

Atl is considered to be the major *S. aureus* autolysin. Loss of this enzyme results in cell cluster formation and impairment in its ability to avoid recognition by PGRP.

Atl is produced as a 138 kDa proenzyme that includes a signal peptide, a pro-peptide, a catalytic domain with *N*-acetylmuramyl-L-alanine amidase activity, three repeats (R1-R3), and a C-terminal catalytic domain with *N*-acetylglucosaminidase activity (Zoll *et al*., 2010).

The Atl proenzyme experiences proteolytic processing to generate the two extracellular mature lytic enzymes identified as the 51 kDa glucosaminidase (containing the repeat R3 and the catalytic domain) and a 62 kDa amidase (containing the catalytic domain and repeats R1R2, which are responsible for attaching the enzyme to the cell wall). Both subunits show activity in Zymogram analysis (Vollmer *et al*., 2008; Zoll *et al*., 2010) by cleaving the PGN at distinct positions. The amidase cleaves amide bond between peptides and glycans (number 4 of Figure 1.2) while the glucosamidase cleaves glycan strands (number 3 of Figure 1.2).

By immunoelectron microscopy, Atl was found to localize on the cell surface at the next cell division septal region, which agrees with the proposed function of Atl in the hydrolysis of the PGN for the separation of daughter cells (Büttner *et al*., 2014). Also, Monteiro *et al.* reported Atl as an enzyme exclusively localized at the external edge of the septum.

The role of Atl in the concealment of *S. aureus* from the Drosophila's innate immune receptors has been recently described (Atilano *et al*., 2014). Although it seemed that the major lytic activity resided in the amidase domain, mutants having only glucosaminidase activity (AM^- mutant) or only amidase activity (GL^- mutant) were still able to avoid strong binding with Drosophila's PGRP-SA, indicating that either amidase or glucosaminidase enzymatic activity was sufficient to impair recognition of *S. aureus* by PGRP-SA. Only when both activities were lost (Atl AM^-GL^- double mutant) the receptor

was capable of easily recognizing the bacterial cell surface by direct bind to PGN. These results acknowledge Atl as an essential protein in the concealment of bacterial PGN at the cell surface from host recognition (Atilano *et al*., 2014).

In experiments done in COL, other *S. aureus* strain, the lack of Atl amidase and glucosaminidase activities led to larger cells that are less elongated when compared with the parental strain, indicating that autolytic activity of Atl is involved not only in cell separation but also in cell size homoeostasis and shape maintenance (Monteiro *et al*., 2015).

1.4.2. Peptidoglycan hydrolase: Sle1

Complete separation of the daughter cells of *S. aureus* seems to be mediated by other autolysins besides Atl. It has been shown that SleI has an important role in daughter-cells separation as in its absence, or targeted inhibition, the daughter cells remain attached to one another by a marginal peptidoglycan bridge (Kajimura *et al*., 2005; Scheffers *et al*., 2015).

Sle1 (also known as Aaa) is a 32kDa protein that contains: (1) one N-terminal signal peptide, (2) three repeated sequences, all showing high similarity with the lysin motif (LysM), which is known for conferring cell wall attachment to various surface-associated proteins and (3) one C-terminal located cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain, which has bacteriolytic activity in numerous proteins and has been confirmed to specifically cleave various amide bonds of PGN (Heilmann *et al*., 2005).

Sle1 mutant did not differ from the wild type in colony morphology, growth rate and cell cluster formation, proposing that Sle1 does not play a role in cell separation or, most likely, that this function of Sle1 have been taken over by other autolysins such as Atl, which seemed to be more strongly expressed in the Sle1 mutant (Heilmann *et al*., 2005). This fact has been confirmed through the Atl/Sle1 double mutant (Kajimura *et al*., 2005).

The cell surface localization by Immunofluorescence microscopy of Sle1 and the impaired adherence to surface-adsorbed plasma proteins suggest that Sle1 might be involved in the colonization of host tissue by *S. aureus*, with both LysM domain and the CHAP domain mediating the binding (Heilmann *et al*., 2005; Hirschhausen *et al*., 2012; Vollmer *et al*., 2008). *S. aureus* Sle1 mutant also has significantly decreased virulent in comparison to the wild type in a murine acute infection (Hirschhausen *et al*., 2012; Kajimura *et al*., 2005; Pilgrim *et al*., 2003; Rupp *et al*., 2001).

1.5. Sle1-like proteins

At least 13 genes of the *S. aureus* genome encode known or putative peptidoglycan hydrolases, although the products of only three of these genes (*atl*, *sle1* and *lytM*) have been characterized.

Analysis of the *S. aureus* NCTC genome has identified two genes that may be responsible for the production of proteins with peptide sequence similar to that observed in Sle1:

- SAOUSH_00671 that possesses two LysM domains and one CHAP domain,
- SAOUSH_00773 that possesses one LysM domain and one CHAP domain.

This project tried to study the role of SAOUSH_00773 in propagation of *S. aureus* bacteria, in the determination of its ability to degrade PGN and its role in the concealment of bacteria from the host PGN receptors. It involved an analysis of the sequence that encodes SAOUSH_00773, as well as its surrounding regions, to determine the presence of predicted protein domains; and, at a laboratory level, it involved procedures of DNA manipulation to clone and express the target gene, in order to purify the SAOUSH_00773 protein and test its activity, and to delete the gene from the *S. aureus* NCTC genome, in order to study the impact of its absence in the propagation of bacteria.

2.Materials and Methods

2.1. Biological Material: Strains & Plasmids

E.coli strains (DH5α, DC10B, BL21(DE3)) were provided from the research Laboratory that host the Master student.

S. aureus strains (RN4220 and NCTC 8325-4) were provided from the research Laboratory that host the Master student. Mutant *S. aureus* strains (NCTC 8325-4 ΔSle1, NCTC 8325-4 ΔAtl, NCTC 8325-4 AM-, NCTC 8325-4 GL- and NCTC 8325-4 AM-GL-) are resultant of the work of former or current PhD students at the research Laboratory that host the Master student.

2.1.1. *Escherichia coli* strains

E. coli are gram-negative bacillus bacteria that reproduce easily in laboratory conditions with a generation time of approximately 30 minutes and an optimal growth at 37ºC.

DH5α strain was used for plasmid transformation due to its efficiency in DNA uptake;

BL21(DE3) strain was used to induce protein expression since it carries the gene for the IPTG-inducible T7 RNA polymerase, needed to express recombinant genes that are under the influence of a T7 or T7-lac promoter, such as the one existent in the pET21a plasmid (New England Biolabs, at 24/09/17).

2.1.2. *Staphylococcus aureus* strains

S. aureus NCTC 8325-4 is a descendant of S. aureus NCTC 8325 from which three phages have been removed by UV radiation (Novick, 1967).

S. aureus RN4220 is a descendant of the NCTC 8325-4 strain, whose DNA restriction enzymes have been inactivated, that is capable of being transformed with DNA from *E. coli*; this strain is mostly employed only as a subcloning host (Berscheid *et al*., 2012).

2.1.3. Plasmids

Plasmids are small circular DNA fragments that have one replication origin (ori) and some type of selective mark, such as antibiotic resistance.

pET21a is a cloning plasmid usually used to clone and express proteins (together with BL21(DE3)). It has a T7 promoter sequence, as well as a Multi Cloning Site (MCS) and Ampicillin resistance as a selective mark.

pMAD was created by Arnaud & Débarbouillé, 2004 to simplify gene deletion. It has two replication origins, one of them thermo-sensitive in *S. aureus*. This plasmid also has two selective resistance marks: spectinomycin and erythromycin and presents, as well, a thermostable β-galactosidase

that allows an easy screening of transformants on X-Gal plates by white/blue screening (Arnaud & Débarbouillé, 2004).

2.2. Protocols

S. aureus genomic DNA extraction

A 10µL loop of cells from confluent growth or pellet from overnight culture was resuspended in 100µL 50mM EDTA and 1µL Lysostaphin (10mg/mL) and 2µL RNAse (10mg/mL) were added and incubated at 37ºC for 30 minutes. 400µL of 50mM EDTA and 250µL of Nuclei Lysis Solution were added and incubated at 80ºC for 5 minutes. After the samples cooled to room temperature, 100µL of Protein Precipitation Solution was added and vortexed vigorously for 20 seconds, followed by a 10 minutes ice incubation and a 20 minutes centrifugation (TS, RT). The supernatant was transferred to a microcentrifuge tube containing 840µL of room temperature isopropanol, mixed and centrifuged for 30 minutes (TS, RT). The supernatant was drained, 500µL of 70% ethanol added to wash the pellet and centrifuged for 3 minutes (TS, RT). The tube was drained and the pellet air-dry for 5 minutes and resuspend in 100µL of ddH_2O.

S. aureus genomic DNA extraction for colony screening

A 10µL loop of cells from confluent growth or pellet from overnight culture was resuspended in 500µL Alkaline wash solution (0.05M Sodium citrate, 0.5M NaOH), incubated for 20 minutes (RT) and centrifuged (TS) in a microcentrifuge for 1 minute. The pellet was washed in 500µL 0.5M Tris-HCl (pH 8.0), centrifuged again, resuspend in 100µL sterile Milli-Q water, boiled for 10 minutes and centrifuge (TS) for 5 minutes. The supernatant was transferred to a clean microtube and store at -20ºC.

Colony Screening PCR

In each PCR, a reaction lacking DNA template was done acting has a blank control.

In *E.coli* screening, a sample of the colony was directly added to the reaction mix, while in *S. aureus* screening genomic DNA needed to be extracted beforehand thus *S. aureus* cells do not lysis through boiling. The primers used in CS-PCR are presented in Table 2.1 and the PCR conditions in Tables 2.2 and 2.3.

Table 2.1 – Primers' sequences used in Colony Screening PCR.

Primer	*Sequence (5´- 3')*
T7 promoter primer	TAATACGACTCACTATAGGG
T7 terminator primer	TAGTTATTGCTCAGCGGTGG
pMAD I	CTCCTCCGTAACAAATTGAGG
pMAD II	GTCCAGGCAGGTAGATGACG

Table 2.2 – Reagents' concentration used in Colony Screening PCR to search for DH5α pET21a 773 positive colonies, DH5α pMAD del 773 positive colonies, NCTC 8325-4::pMAD del 773 and NCTC 8325-4 Δ773 positive colonies (to search for *S. aureus* positive colonies, gDNA has to be previously extracted).

	Mix
Dream Taq Buffer	1x
dNTPs	200µM
FW primer	0.2µM
RV primer	0.2µM
Dream Taq	0.5U
DNA	*E.coli* colony sample / *S. aureus* gDNA

Final volume: 20µL

Table 2.3 – PCR cycles' conditions used in Colony Screening PCR. *Extension time for each template: pET21a 773 – 1min; pMAD del 773 & NCTC:: pMAD del 773 – 2:30 min; NCTC Δ773 – 3:00.

		Temperature	*Time (min)*
	Initial Denaturation	95ºC	05:00
x30	*Denaturation*	95ºC	00:30
	Annealing	53ºC	00:30
	Extension	72ºC	~1min/Kb *
	Final Extension	72ºC	05:00

Restriction Enzyme Digestion (*Nae*I and *Hind*III)

Table 2.4 – Digestion conditions when using *Nhe*I and *Hind*III Restriction Enzymes.

	Mix
NaeI	1µL
CutSmart Buffer	1x
DNA	~1200ng pET21a 773

Volume: 20µL; Digestion Temperature: 37ºC; Digestion Time: O/N

	Mix
HindIII	1µL
2.1 NEBuffer	1x
DNA	~1000ng pMAD del 773

Volume: 20µL; Digestion Temperature: 37ºC; Digestion Time: O/N

Preparation of *E. coli* Competent Cells

A single colony of the pretended *E. coli* strain was inoculated overnight at 37ºC, 180rpm in 3mL LB medium. The overnight culture was diluted 1:1000 in 100mL of LB medium, gowned until OD_{600} reaches 0.35 – 0.45, placed on ice for 10 – 15 minutes and then transferred to pre-cooled 50mL falcon tubes and centrifuge at 4ºC for 15 minutes at 3500 rpm. The pellet was resuspended in 15mL of RF1, placed 15 minutes on ice and centrifuged at 4ºC for 15 minutes at 3500 rpm. The same step was repeated with 4mL of RF2. To the final washed pellet was added 800µL 50% glycerol and it was divided into 200µL aliquots and stored at -80ºC.

Solutions in ddH_20; Filter sterile (0.2µm):

RF1 pH5.8: 100mM RbCl, 60mM $MnCl_2 \cdot 4H_2O$, 10mM $CaCl_2 \cdot 2H_2O$, 1.6M glycerol, 30mM KAc pH7.5
RF2 pH6.8: 10mM RbCl, 75mM $CaCl_2 \cdot 2H_2O$, 1.6M glycerol, 10mM MOPS pH6.8

Transformation of *E. coli* Competent Cells

To defrosted competent cells, ~500 ng of DNA was added and incubated on ice 10 minutes, at 42ºC 1 minute and on ice 5 minutes. Is was added 1 mL of LB and incubated at 37ºC with shaking for 1 hour or at 30ºC with shaking for 2 hours (when transforming thermosensitive plasmids, i.e. pMAD).

Cells were plated in LB medium suplemented with 100µg/mL ampicillin for pET21a or 10µg/mL erythromycin for pMAD in several dilutions (1µL transformed cells + 99µL LB; 10µL transformed cells + 90µL LB; 100µL transformed cells; pelleted cells + 100µL LB), The plaques were incubated at 37ºC for 16h.

Protein purification from inclusion bodies from *E. coli* cells

BL21a(DE3) *E. coli* strain was transformed with a pET21a derivative (pET21a 773) carrying the gene for SAOUHSC_00773. An overnight culture of a single colony of the transformed strain was diluted to 0.05 OD_{600} and grown on 1L of LB supplemented with 100µg/mL ampicillin at 37ºC, 180 rpm until reaches an OD_{600} ~0.5 and the culture was induced by the addition of 1mM IPTG and incubated O/N at 37ºC, 180 rpm.

Pelleted cells were washed twice in 20mL of Equilibration Buffer and lysis was carried out twice by French Press at 1000 psi. The centrifuged resultant pellet was retained and was resuspended in 20mL of Resuspension buffer. After approximately 60 hours at 4ºC homogenization, the solution was diluted to 4 M urea and left on a rocker at 4ºC for further 24 hours. The supernatant was mixed with 3mL Talon resin and after 1 hour on a rocker at 4ºC, the resin was washed with crescent level solutions of Imidazole Wash solution. Finally, the resin was eluted with 5mL Elution Buffer for 1 hour at 4ºC on the rocker and dialysed overnight against PBS.

Solutions in MiliQ H_2O and 0.2µm Filtered; Store at 4ºC;
Equilibration Buffer: 50 mM Na2PO4 pH 7.4; 300 mM NaCl
Resuspension buffer: 20 mM Na2PO4 pH 7.4; 500mM NaCl; 8 M urea
Dilution buffer: 20 mM Na2PO4 pH 7.4; 500mM NaCl
Wash solution: 50 mM Na2PO4 pH 7.4; 300 mM NaCl; 4 M urea
Elution Buffer: 50 mM Na2PO4 pH 7.4; 300 mM NaCl; 150 mM Imidazole
PBS 10x pH 6.0: 1.4M NaCl, 25mM KCl, 100mM $Na_2HPO_4.7H_2O$, 20mM KH_2PO_4

Preparation of the crude autolytic extracts from *S. aureus* cells

An overnight culture of a single colony of the *S. aureus* strain of interest was diluted to 0.05 OD_{600} and grown on 250mL of TSB at 30ºC until the cultures reached an OD_{600} ~0.3. Pelleted cells were washed in 20mL of cold Washing buffer, resuspended in 250µL 4% SDS (w/v) and incubated at 25°C

for 30 min. After centrifugation, the supernatant containing the crude enzyme autolytic extracts, was stored at 4ºC.

Solution in MilliQ water; store at 4ºC:

Washing buffer pH 7.5: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl.

Preparation of substrate cells for gel zymograms

An overnight culture of a single colony of *S. aureus* NCTC 8325-4 strain was diluted to 0.05 OD_{600} and grown on 250mL of TSB at 37ºC until the cultures reached an OD_{600} ~1.0. The resultant pellet was washed with 250mL of Milli-Q water and resuspended in 30 mL of Milli-Q water before being autoclaved for 15 min, 121°C and the suspension lyophilized in the speed vac. Finally, bacterial substrate cells were resuspended in Milli-Q water at 100mg/mL and stored at -20 °C.

Analysis of the crude autolytic extracts by gel zymography

Acrylamide SDS-PAGE gels were prepared accordingly with Table 2.15 and 12µL of *S. aureus* crude autolytic extracts mixed with 8µL 5x SDS-PAGE native LB (samples were not heated/boiled) and loaded into the gel. The electrophoresis was carried using Tris-Glycine-SDS buffer as Running buffer, RT and 70 V. The gel was rinsed once and washed 3 times with Milli-Q for 15 min at RT with gentle agitation and incubated O/N at 37 °C with gentle agitation in Renaturation buffer (several concentrations were tested). Finally, zymograms were stained in Methylene Blue Solution for half an hour and destained in MilliQ until clear bands are visible.

Solutions in MilliQ water:

Renaturation buffer pH 7.5: 50mM Tris-HCl pH 7.5, 0.1% (v/v) Triton X-100, 10mM $CaCl_2$, 10mM $MgCl_2$;

Methylene blue solution: 0.1% (w/v) methylene blue, 0.01% (w/v) potassium hydroxide.

Preparation of *S. aureus* RN4220 Competent Cells

An overnight culture of a single colony of *S. aureus* RN4220 strain was diluted to 0.05 OD_{600} and grown on 100mL of TSB at 37ºC until the cultures reached an OD_{600} 0.4 - 0.6. Cells' pellet was washed twice in 0.5M Sucrose, incubated on ice for 15 minutes before resuspension in 600µl of 0.5 M Sucrose and division into 50µl aliquots (-80ºC).

Electroporation of *S. aureus* RN4220 Competent Cells

To thaw *S. aureus* competent cells 500ng DNA was added. The mixture was transferred to a 0.2cm BioRad Gene Pulser cuvette, incubated on ice for 5 mins, electroporated (2500V, 25µF and 100Ω) and immediately rescued with 1mL TSB. After 2 hours incubation at 30ºC, the cells were plated in TSA appropriate medium and incubated at 30ºC.

Transduction Protocol

Phage Assay

A cell suspension of the indicator strain (RN4220) was mixed with several phage 80α dilutions in Phage top Agar and poured into pre-prepared Bottom Phage Agar plates. After allowing the top agar to set, the plates were inverted and incubated overnight at 30ºC.

Count the phage plaque and calculate the titre of the lysate (PFU/mL= N / (I x D) = 1.07x10^11

PFU – plaque forming units; N= number of plaques; I=volume of phage used (mL); D=dilution of the phage.

Making the Phage Lysate

A cell suspension of the donor strain (RN4220 pMAD del 773) was mixed with several phage 80α dilutions in Phage Top Agar and poured into pre-prepared Bottom Phage Agar plates. After allowing the top agar to set, the plates were inverted and incubated overnight at 30ºC. To the plate showing almost confluent lysis was added 3mL of Phage Buffer. After 1 hour at 4ºC, the top agar layer was scrapped to a falcon tube and incubated upsidedown at 4ºC for 1 hour. The resultant supernatant was filtered with a 0.45µm filter to a sterile tube and stored at 4ºC. The phage lysate sterility was tested by plating 100µL on a TSA plate and incubate overnight at 37ºC. 0.5 mM of $CaCl_2$ was added to the cell suspension as well as the Top and Bottom agar media.

Transduction

The cell suspension of the receiving strain (NCTC 8325-4) was mixed with Phage Buffer and several 80α phage lysate amounts and incubated for 20 minutes at 37ºC before combining with 0.3GL Phage Agar and pour into pre-prepared 0.3GL Bottom Agar plates (no antibiotic layer on top of a 3x antibiotic concentration layer). After allowing the Top Agar to solidify, plates were incubated for 48 hours at 30ºC. 0.5 mM of $CaCl_2$ was added to the cell suspension as well as the phage buffer solution.

Solutions in ddH_20; Autoclave and store at 4ºC:

0.3GL Agar pH 7.8 1L: 3g Casamino acids, 3g Yeast extract, 5.9g NaCl, 3.3mL DL-Lactate acid, 2mL 50% Glycerol, 0.5g Tri-sodium citrate, 15g Agar (Bottom Agar) or 7.5g Agar (Top Agar)

Phage Agar pH 7.8 1L: 3g Casamino acids, 3g Yeast extract, 5.9g NaCl, 15g Agar (Bottom Agar) or 5g Agar (Top Agar)

Phage Buffer 1L: 1mM $MgSO_4$, 4mM $CaCl_2$, 50mM Tris pH 7.8, 100mM NaCl, 1g Gelatin

Integration and Excision into *S. aureus* chromosome using pMAD based vectors

Integration

The overnight culture of the NCTC 8325-4 pMAD del 773 constructed strain was diluted 1:1000 into TSB supplemented with Erythromycin at 10µg/mL (Ery10) and incubated at 30°C for 8h. A second 1:1000 dilution was made, the culture incubated at 43°C overnight, plated on TSA + Ery10 + X-gal100

(supplemented with X-gal at 100 µL/mL) and growth at 43°C overnight. At this temperature the plasmid is unable to replicate on its own and only the colonies that integrated the plasmid will maintain the antibiotic resistance and survive. The blue colonies were streaked on TSA + Ery10 + X-gal100 plates and incubated at 43°C overnight in order to have single isolated colonies. *S. aureus* genomic DNA was extracted and the plasmid integration confirmed by PCR.

Excision

The overnight culture of the confirmed NCTC 8325-4::pMAD del 773 strain was diluted 1:500 into TSB and grown at 30°C for 8h before plating in TSA + X-gal100 and incubated at 43°C overnight. The obtained white colonies were stricked to TSA + Ery10 + X-gal100 and TSA + X-gal100 plates and incubated at 37°C O/N. *S. aureus* genomic DNA was extracted from the erythromycin-susceptible colonies and the excision was confirmed by PCR.

Culture Growth assay

An overnight culture of a single colony of the strain of interest was diluted to 0.05 OD_{600} and grown on 20mL of liquid media (LB, for *E. coli* or TSB, for *S. aureus*). Media was supplemented with antibiotic accordingly. Several samples were taken according to the desired time intervals and the ODs_{600} were registered.

PGN pellet degradation assay

In a microtube, 500µg of purified PGN, 50µL of 25mM $NaHPO_4$ pH 5.5 and 20µg of the purified protein (digest PGN with mutanolysin as a comparison) were mixed. MilliQ-water was added to the final volume of 100µL. For each protein/enzyme, a blank was made by replacing the volume of PGN for MilliQ water. The mixtures were incubated O/N at 37ºC 1200rpm in the Thermomixer. The pellet was obtained thru a 5-minute RT TS centrifugation.

2.3. Plasmid Construction

2.3.1. Construction of the plasmid to gene deletion using pMAD as the backbone.

2.3.1.1. Primer design

The primers were designed resorting to a Molecular Biology' software from DNASTAR called Lasergene. To the UP forward primer was added a restriction site for *EcoR*I and to the DOWN reverse primer was added a restriction site for *BamH*I. This endonucleases' restriction sites were added to enable the insertion of the PCR fragment into the pMAD plasmid in a known orientation. A start and stop codons were added to the UP overlapping primer and DOWN overlapping primer so there is a one amino acid-long "protein" and the reading grid of the surrounding genes/regulatory regions doesn't become compromised. These two primers (UP over and DOWN over) are also partially complementary in order

to do an overlapping PCR and combine the UP and DOWN fragments (without the *saouhsc_00773*). The primers are presented in Table 2.5.

Table 2.5 - Primers' sequences to amplification of *saouhsc_00773*' UP and DOWN regions.

Name	*Primers (5' - 3')*
UP FW EcoRI	CGCCGGAATTCGGATTAAAAGTGTAACATTGC
UP over RV	GATATATTACTTACATCTTGCTGTCATTCCTTTGC
DOWN over FW	CAGCAAGATGTAAGTAATATATCAAGACAAG
DOWN RV BamHI	GCGGATCCCTAATAATGACACTACATC

Added; Overlap; EcoRI; START&STOP; BamHI

2.3.1.2 PCR conditions for fragments amplification

For the amplification of the *saouhsc_00773*'s UP and DOWN regions, DNA from NCTC 8325-4 was extracted and separated PCRs were performed. The *saouhsc_00773*'s 1Kb UP region was amplified resorting to the forward primer "*UP FW EcoRI*" and the reverse primer "*UP over RV*". The *saouhsc_00773*'s 1Kb DOWN region was amplified resorting to the forward primer "*DOWN over FW*" " and the reverse primer "*DOWN RV BamHI*". PCR's Master mixes, reagent concentration and cycles conditions are at Tables 2.6 and 2.7.

Following amplification, the resulting fragments UP and DOWN were cleaned (Clean-up KIT) and another PCR (overlapping PCR) was conducted to combine the two DNA fragments using the forward primer "UP FW EcoRI" and the reverse primer "DOWN RV BamHI". The resulting amplification will consist of a unique fragment biding the UP+DOWN regions (U+D) (reagent concentration and cycles conditions are at Tables 2.6 and 2.7).

Table 2.6 – Reagents used in PCR to amplify UP and DOWN fragments and the UP+DOWN fragment.

	Mix		
Phusion Buffer	1x		
dNTPs	200µM		
FW primer	0.2µM		
RV primer	0.2µM		
Phusion	0.01U		
Template	~500ng NCTC 8325-4 gDNA	or	0.5µL UP fragment + 0.5µL DOWN fragment

Final volume: 50µL

Table 2.7 - PCR cycles' conditions used to amplify UP and DOWN fragments and UP+DOWN fragment. *Extension time for each template: UP & DOWN – 2min; UP+DOWN – 4min.

		Temperature	*Time (min)*
	Initial Denaturation	98ºC	01:00
x30	*Denaturation*	98ºC	00:10
	Annealing	58ºC	00:30
	Extension	72ºC	~1min/Kb*
	Final Extension	72ºC	05:00

2.3.1.3. DNA Restriction Enzymes Digestion and Ligase Reaction

Following the amplification, the PCR product (U+D) was clean using the Clean-up KIT and pMAD plasmid DNA was extracted using the Miniprep KIT from an *E.coli* strain (Dh5α pMAD) that carried the plasmid.

Since *Eco*RI and *BamH*I are active in the same buffer, it was possible to double digest the U+D fragment and the pMAD plasmid (Table 2.8). After digestion, the resulting fragments were cleaned (Clean-up KIT).

Table 2.8 - U+D fragment and pMAD digestion conditions.

	Mix
EcoRI	1.5µL
BamHI	0.75µL
3.1 NEBuffer	1x

EcoRI has 50% activity in 3.1 NEBuffer and BamHI has 100% activity in 3.1 NEBuffer.
Volume: 20µL; Digestion Temperature: 37ºC; Digestion Time: O/N

The PCR product and plasmid DNA were then combined and bind using DNA's T4 Ligase (Table 2.9). The resulting plasmid was called pMAD del 773.

Table 2.9 - Digested PCR product and pMAD Ligase reaction conditions.

Enzyme	*Enzyme amount*	*Buffer*	*U+D digested PCR product*	*Digested pMAD*
T4 Ligase	1µL	1x T4 Ligase Buffer	3µL	10µL

Volume: 20µL; Ligation Temperature: 22ºC; Incubation Time: O/N

2.3.2. Construction of the plasmid to gene expression using pET21a as the backbone.

2.3.2.1 Primer design

The primers were designed resorting to a Molecular Biology' software from DNASTAR called Lasergene. To the forward primer was added a restriction site for *Nhe*I as well as a histidine tail (HIS-tag) to facilitate the process of protein purification. Similarly, a restriction site for *Eag*I enzyme was added to the reverse primer. Both endonucleases' restriction sites have been confirmed to be unique in the cloning vector as well as the gene fragment, which will guarantee the insertion of the PCR fragment into the expression plasmid pET21a in the correct place and direction, what is important in gene expression. The addition of a start and stop codons in the primers were not required since those are present in the plasmid and in the gene respectively. The reading frame was also taken into consideration when designing the primers. The primers' sequences are presented in Table 2.10. The cloned sequence of *saouhsc_00773* and overall structure are presented in Appendix 1.

Table 2.10 – Primers' sequences to amplification of *saouhsc_00773*.

Name	*Primers (5' - 3')*
773 FW NheI	GCCG.CTAG.CCATCACCATCACCATCACCAACAACATGGC
773 RV EagI	CGCGCGCC.GGCC.GTTAGTGGATGTAATTATATTTTCC

Added; HIS tag; NheI; EagI; **STOP**

2.3.2.2. PCR conditions for gene amplification

For the amplification of the *saouhsc_00773*, DNA from NCTC 8325-4 was extracted and PCR was performed using "*773 FW NheI*" as the forward primer and "*773 RV EagI*" as the reverse primer. The resulting PCR product has 808bp of length. PCR's reagent concentration and cycle conditions are stated in Table 2.11 and 2.12.

Table 2.11 – Reagents' concentration used in PCR to amplify *saouhsc_00773*.

	Mix
Phusion Buffer	1x
dNTPs	200µM
FW primer	0.2µM
RV primer	0.2µM
Phusion	0.01U
DNA	~500ng

Final volume: 50µL

Table 2.12 - PCR cycles' conditions used to amplify *saouhsc_00773*. Extension: 1 minute

		Temperature	*Time (min)*
	Initial Denaturation	98ºC	01:00
x30	*Denaturation*	98ºC	00:10
	Annealing	58ºC	00:30
	Extension	72ºC	~1min/Kb
	Final Extension	72ºC	05:00

2.3.2.3. DNA Restriction Enzymes Digestion and Ligase Reaction

After amplification, the PCR product was clean using the Clean-up KIT and plasmid DNA (pET21a) was extracted using the Miniprep KIT from a carrying *E.coli* strain (Dh5α pET21a).

Due to the different buffers required by *Nhe*I and *Eag*I, concomitant digestion was not possible. Therefore, the first digestion of the clean PCR product and the plasmid DNA was performed using *Nhe*I (Table 2.13), followed by a Clean-up, the second digestion with *Eag*I and finalized with a Clean-up.

Table 2.13 – S*aouhsc_00773* PCR product and pET21a digestion conditions.

	Mix
NheI	1µL
2.1 NEBuffer	1x
DNA	*773* or pET21a

Volume: 20µL; Digestion Temperature: 37ºC; Digestion Time: O/N

	Mix
EagI	1µL
3.1 NEBuffer	1x
DNA	*773* or pET21a

Volume: 20µL; Digestion Temperature: 37ºC; Digestion Time: O/N

After digestion, the PCR product and plasmid DNA were combined resorting to a DNA T4 Ligase (Table 2.14). The resulting plasmid was named pET21a 773.

Table 2.14 - Digested PCR product and pET21a Ligase reaction conditions.

Enzyme	*Enzyme amount*	*Buffer*	*773 digested PCR product*	*Digested pET21a*
T4 Ligase	1µL	1x T4 Ligase Buffer	240ng	175ng

Volume: 20µL; Ligation Temperature: 22ºC; Incubation Time: O/N

2.4. Recipes

Tris-Acetic-EDTA (TAE) 50x pH 8.3 1L: 242g Tris base, 57.1mL glacial acid, 18.61g EDTA

0.8% (w/v) Agarose gel: Dissolve 0.8g of Agarose in 100mL 1x TAE

Tris-Glycine-SDS Buffer pH 8.8 1L: 30g Tris base, 144g Glycine, 10g SDS

5x SDS-PAGE Loading Buffer (SDS-PAGE LB): 2.1mL MilliQ water, 1mL 10% (w/v) SDS, 0.8mL Tris-HCl 1M pH 6.8, 0.5mL glycerol, 0.5mL β-mercaptoetanol, 0.1mL Bromophenol blue 0.2% (w/v); (In SDS-PAGE native Loading Buffer, β-mercaptoetanol was replaced by water.)

Table 2.15 – SDS-PAGE and Zymogram gels' recipes. Acrylamide is on w/v percentage; The number of gels these recipes makes considered 0.75mm thick gels.

	SDS-PAGE Resolving Gel (12.5% Acrylamide; 2 gels)	*SDS-PAGE Stacking Gel (4% Acrylamide; 2 gels)*	*Zymogram Resolving Gel (10% Acrylamide; 1 gel)*	*Zymogram Staking Gel (4% Acrylamide; 1 gel)*
ddH_2O (MilliQ for zymograms)	3.4mL	1.8mL	1.856mL	1.5mL
1.5 M Tris-HCl pH 8.8	2.48mL	-	1.25mL	-
0.5 M Tris-HCl pH 6.8	-	750µL	-	625µL
30% Acrylamide	4.1mL	400µL	1.688mL	350µL
10% SDS (w/v)	100µL	30µL	-	25µL
10% APS (w/v)	50µL	15µL	93.75µL	37.5µL
Substrate cells (100mg/mL)	-	-	100µL	-
TEMED	5µL	3µL	12.5µL	5 µL

2.5 Reagents and Materials

Miniprep KIT: Wizard Plus SV Minipreps DNA Purification Systems (Promega Corporation, reference: A1465)

Clean-Up KIT: Wizard SV Gel and PCR Clean-Up System (Promega Corporation, reference: A9285)

3. Results

3.1. Analysis of the NCTC 8325 *saouhsc_00773* and its surrounding regions in different *S. aureus* strains

In order to start the study of NCTC 8325 *saouhsc_00773*, an analysis of its DNA sequence in different *S. aureus* strains was performed: COL, NEWMAN, USA300 FPR3757 and MW2. All the genetic sequences are available in the NCBI's Nucleotide Database, GeneBank. The amino acid sequence analysis was preformed using the NCTC 8325 genome information while the laboratorial work used the NCTC 8325-4 strain. In all *S. aureus* strains, the gene that corresponds to the NCTC 8325 *saouhsc_00773* was identified and shown to encode a LysM-domain-containing protein.

S. aureus NCTC 8325 *saouhsc_00773* is composed of 840 nucleotides that codify a 279 amino acids protein. The gene starts with a 72 nucleotide sequence that encodes a signal peptide (24 amino acids). This sequence is followed by a 135 nucleotide sequence that encodes the LysM domain (45 amino acids) and a 366-nucleotide sequence that encodes the CHAP domain (122 amino acids) that is positioned at the C-terminal end of the protein (Figure 3.1, line 4).

The amino acid sequence of NCTC 8325 *saouhsc_00773* was compared with its corresponding protein of others *S. aureus* strains: *sacol0820* from COL, *nwmn_rs04100* from NEWMAN, *sausa300_rs03985* from USA300 FPR3757 and *nwmn_0724* from MW2. The comparison of the predicted proteins (Figure 3.1), as well as the DNA encoding sequences, show that this gene is conserved in all strains except MW2. Single nucleotide mutations, which result in different amino acids at the 4th position (signal peptide) and at 118th and 142th positions (linker between the LysM and CHAP domains), were observed.

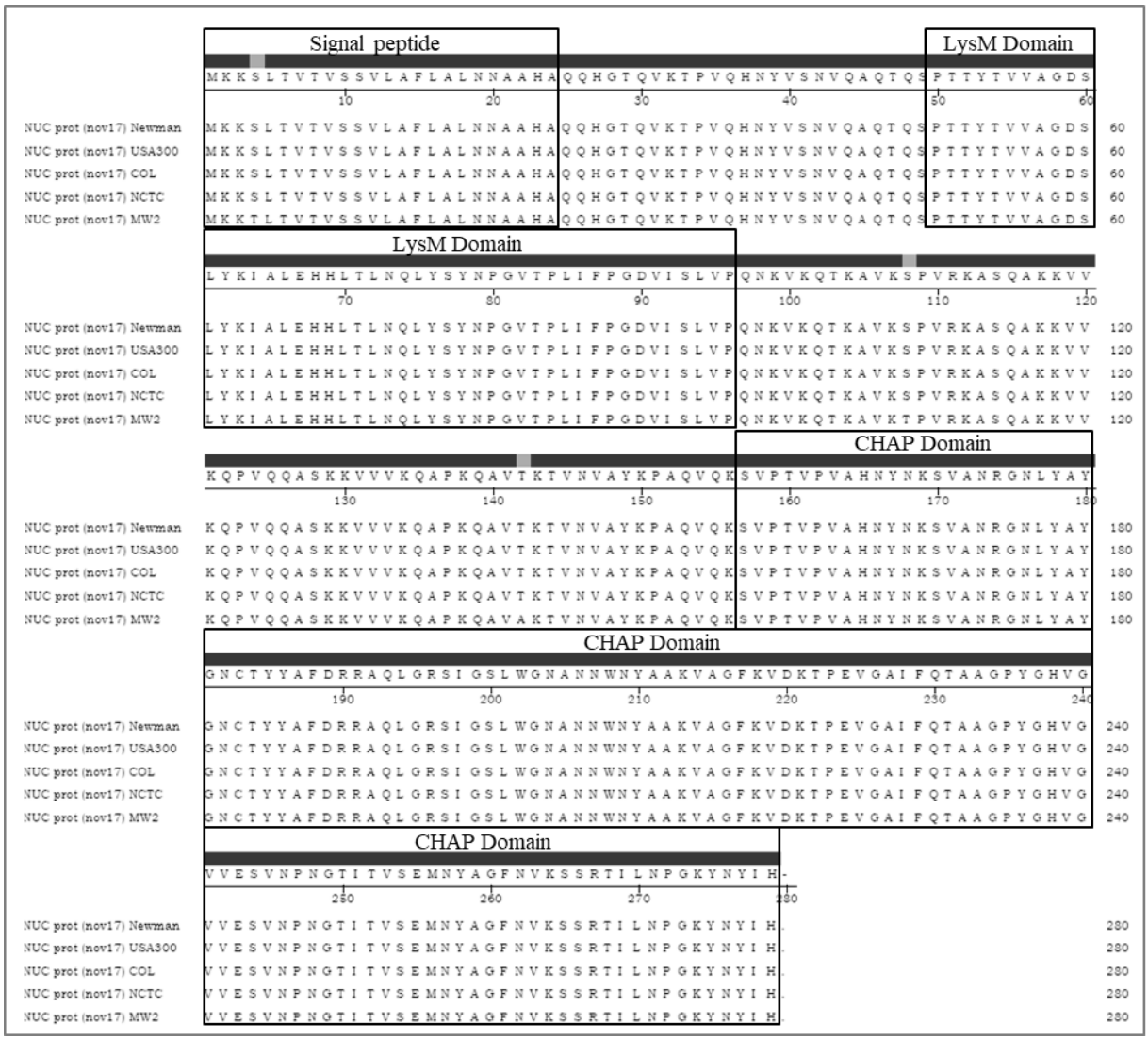

Figure 3.1 – Comparison of the NCTC 8325's SAOUHSC_00773 protein with the analogues from several *S. aureus* strains: SACOL0820 from COL, NWMN_RS04100 from NEWMAN, SAUSA300_RS03985 from USA300 FPR3757 and NWMN_0724 from MW2. The signal peptide, the LysM domain and the CHAP domain are identified.

3.2. Comparison of the NCTC 8325-4 SAOUHSC_00773 protein domains with other *S. aureus* known autolysin and putative protein

3.2.1. Comparison of LysM and CHAP domains from SAOUHSC_00773 with those present in Sle1 and SAOUHSC_00671 proteins

After the identification of the LysM and CHAP domains present in the SAOUHSC_00773 (773) protein, I compared their sequence with equivalent domains that are found in SAOUHSC_00671 (671) and Sle1 proteins. Sle1's LysM_2 and LysM_3 present in Sle1 show the most similarity with only a difference in one residue. The most similar to them is SAOUHSC_00773's LysM domain (3rd line in Panel A of Figure 3.2) and after it, the third Sle1's LysM domain (LysM_1). SAOUHSC_00671'

domains (1st and 2nd lines in Panel A of Figure 3.2) appear very different from the others but similar with each other.

The alignment of the SAOUHSC_00773, SAOUHSC_00671 and Sle1's CHAP domains (Panel B of Figure 3.2) show that Sle1 and 671 have the same size domain (125-amino acid length) but the sequences only align partially (from the 45th to the 125th position).

SAOUHSC_00773 and Sle1' CHAP domains align intermittently over the sequence. Since the CHAP is the catalytic domain of these proteins, these discrepancies between Sle1 and SAOUHSC_00773 may indicate a difference in the protein possible activity in the capability of digesting PGN.

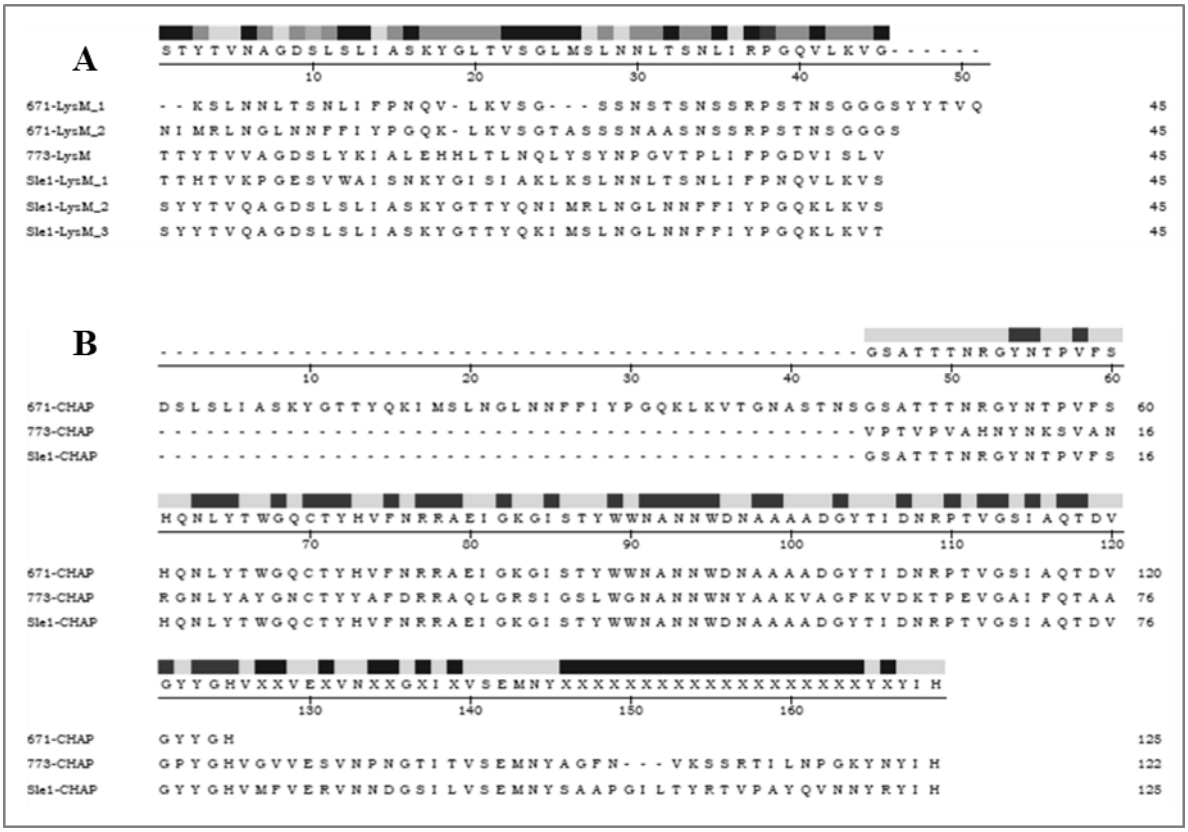

Figure 3.2 – Comparison of the functional domains of the NCTC 8325 SAOUHSC_00773 protein with the same strain Sle1 protein and SAOUHSC_00671 protein. **Panel A**: Comparison of the proteins LysM domains. **Panel B**: Comparison of the proteins CHAP domains.

3.2.2. Comparison of the NCTC 8325 *saouhsc_00773* surrounding regions with another *S. aureus* strains.

In order to understand the *S. aureus* NCTC 8325 SAOUHSC_00773 gene placement, a revision of its surrounding regions was made. The 2kb upstream and downstream regions of NCTC 8325 *saouhsc_00773* were compared with the correspondent regions of *sacol0820* from COL, *nwmn_rs04100* from NEWMAN, *sausa300_rs03985* from USA300 FPR3757 and *nwmn_0724* from MW2. After the alignment of these regions, no differences in the DNA sequence were found.

In NCTC 8325 *saouhsc_00773*' upstream region was found a putative peptide chain release factor 2, confirmed and identified in COL, USA300 and MW2 as *prfB*. In the downstream region was found a conserved hypothetical protein and another protein identified as an excinuclease ABC, B subunit in all five strains.

3.3. Construction of NCTC 8325-4 *S. aureus* mutant strains unable to produce *saouhsc_00773*

In order to obtain a NCTC 8325-4 stain unable to produce the SAOUHSC_00773 protein, a knock-out strain, lacking the gene was produced. A method that uses the ability of the bacteria to allow genetic recombination through crossing-over events with foreign similar-sequence-DNA was employed. Therefore, a pMAD derivative plasmid that carried the upstream and downstream regions of *saouhsc_00773* was constructed and named pMAD del 773. To maintain the integrity of the gene-surrounding-regions in the final *S. aureus* mutant strain, the start and stop codons of *saouhsc_00773* were kept in the cloned fragments.

3.3.1. Choosing the positive DH5α pMAD del 773 colonies and confirming the created pMAD del 773 plasmid's sequence

In order to have a bacteria able to host and replicate the desired plasmid, the ligation product for the construction of pMAD del 773 was transformed to *E.coli* DH5α competent cells that are able to receive and replicate foreign DNA and easy to work in Laboratory conditions. To select the colonies that received the pMAD del 773 plasmid, colony screening PCR was performed using "*pMAD I*" as the forward primer and "*pMAD II*" as the reverse primer. Screened *E. coli* DH5α pMAD del 773 colonies revealing the right size amplification (2398pb) were selected. The ligation product was used in the PCR as the positive control and to confirm the occurrence of the insertion of the UP+DOWN fragment in the plasmid; pMAD DNA was used as the negative control. The resulting agarose gel electrophoresis is presented in Figure 3.3. Lane 1, in Figure 3.1 reveals a positive-result-screening to pMAD del 773 and therefore, the corresponding colony was selected to continue the experiments.

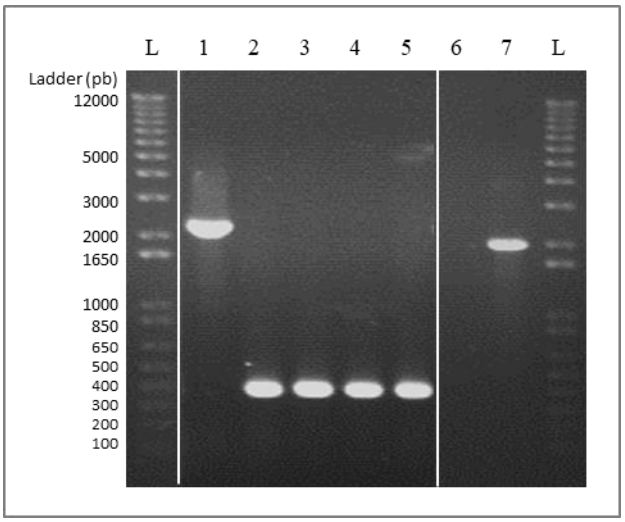

Figure 3.3 – Screening of DH5α pMAD del 773 colonies by agarose gel electrophoresis analysis of DNA fragments amplified by PCR. PCR fragments amplified from DNA from several colonies are shown in the different lanes. Lane 1 – colony PCR resulting in a band with the expected size (2398pb) for the insert cloned in pMAD del 773; Lanes 2 to 4 – colony PCR resulting in bands corresponding to an empty plasmid (no insert – 428bp); Lane 5 – amplified fragment from colony transformed with an empty pMAD plasmid (negative control); lane 6 – PCR carried out with no template DNA (negative control); lane 7 – fragment used as insert in the cloning procedure into pMAD plasmid; Lanes L – DNA ladders with band sizes (in base pairs).

Plasmid DNA from the positive colony was extracted using the Promega Miniprep KIT and digested with the NEB restriction enzyme *Hind*III (conditions at Table 2.4) to confirm that the cloned fragment is indeed positioned correctly. *Hind*III cuts the plasmid in three places in either plasmid, as shown in Panel C of Figure 3.4. In the empty pMAD plasmid there is a restriction site for *Hind*III in the MCS that was "replaced" for the one in the cloned fragment, originating a difference in two bands (Panel A of Figure 3.4). However, in the agarose gel electrophoresis, there is only visible one difference between the digestion bands' pattern, as shown in the Panel B of Figure 3.4. Since the resultant size fragments were the expected, the region that was introduced into the plasmid pMAD del 773 12+ (colony number 12, well 1 in Figure 3.3) was sequenced (at STABvida using Sanger sequencing technique). The sequencing showed a point mutation (A→C) at the position 6363 of the *in silico* constructed plasmid, that correspond to the 135th nucleotide of the UP region cloned to pMAD. Since this mutation does not affect the knock-out process and will not stay in the bacterial chromosome, this plasmid was used in the follow-up experiments. From this point, this plasmid will be referred as pMAD del 773.

Expected Digestion Fragments'size of pMAD del 773 with HindIII	*Expected Digestion Fragments'size of pMAD with HindIII*
7783 bp	7193 bp
2866 bp	**1521** bp
952 bp	952 bp

A

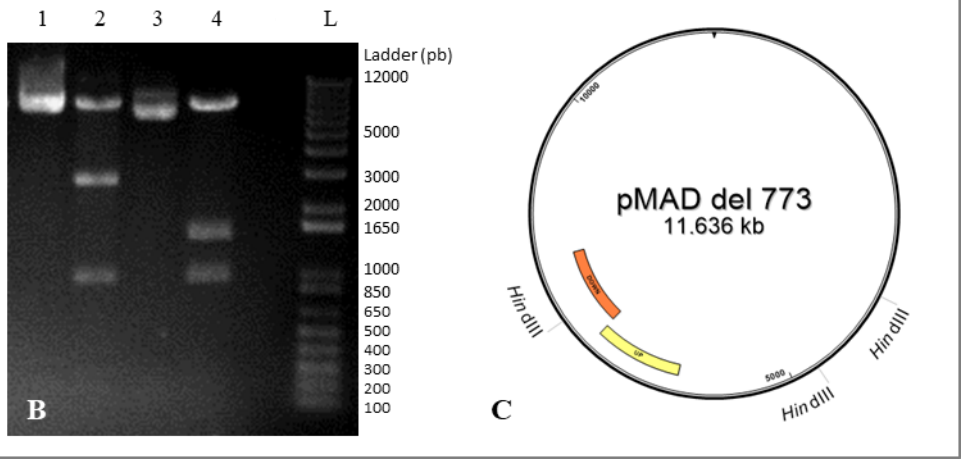

Figure 3.4 – Panel A: Expected digestion fragments' size of pMAD and pMAD del 773 with *Hind*III. In bold are presented the fragments' size that allows to differentiate between pMAD del 773 and pMAD. **Panel B: pMAD del 773 digestive pattern confirmation by agarose gel electrophoresis analysis of DNA fragments digested with *Hind*III.** DNA fragments resulting from digestion with *Hind*III restriction enzyme are shown in the different lanes. Lane 1 – undigested DNA from pMAD del 773; Lane 2 – digested DNA from pMAD del 773 showing DNA fragments with the expected sizes for the created plasmid; Lane 3 – undigested DNA from pMAD; Lane 4 – digested DNA from pMAD showing DNA fragments with the expected sizes for an empty plasmid; Lane L – DNA ladders with band sizes (in base pairs) on the right. **Panel C: *In silico* constructed pMAD del 773 plasmid's expected *Hind*III restriction enzyme recognition sites.**

3.3.2. NCTC 8325-4 Δ773 mutant construct

Competent cells of *S. aureus* RN4220, an *S. aureus* strain that can be transformed through electroporation, were transformed with pMAD del 773 in order to obtain an *S. aureus* strain that carried the plasmid not integrated into the bacterial chromosome. With the resulting colonies, through a transduction protocol, 80α phage lysate with pMAD del 773 was obtained and used to infect the *S. aureus* NCTC 8325-4 strain so that the plasmid could be transferred and the NCTC 8325-4 pMAD del 773 strain produced. In the Transduction protocol, the strain RN4220 was used to propagate/multiply the original sample of 80α phage, the constructed strain RN4220 pMAD del 773 was used as the donor strain and NCTC 8325-4 was used was the receiving strain.

In order to delete *saouhsc_00773* from the *S. aureus* genome, the NCTC 8325-4 pMAD del 773 strain was then used in the Integration and Excision Protocol that allow the selection of strains that have undergone through different crossing-over events to produce the NCTC 8325-4 Δ773 mutant. At the end of the Integration step of this protocol, the integration of pMAD del 773 was verified (the NCTC

8325-4::pMAD del 773 strains) by Colony Screening PCR. The direction of the integration (if it had occurred through the cloned upstream DNA fragment (UP) or through the cloned downstream DNA fragment (DOWN) was screened using two different pairs of primers: (1) "*UP FW EcoRI*" as the forward primer and "*pMAD II*" as the reverse primer to search for integration through the downstream DNA fragment (2964bp amplification); (2) "*pMAD I*" as the forward primer and "*DOWN RV BamHI*" as the reverse primer to search integration through the upstream DNA fragment (3123bp amplification). DNA from NCTC 8325-4 (no amplification) and from pMAD del 773 (2130bp amplification when searching for Down integration and 2289bp amplification when searching for Up integration) were used as the negative controls in the CS-PCR.

Ideally, this protocol would be continued with a UP integrated strain and a DOWN integrated strain, but only clean Down integrated mutants were obtained, as shown in Lanes 1 and 2 in Figure 3.5. These two Down integrated mutants were named 1D and 2D respectively and used to proceed for the Excision part of the protocol.

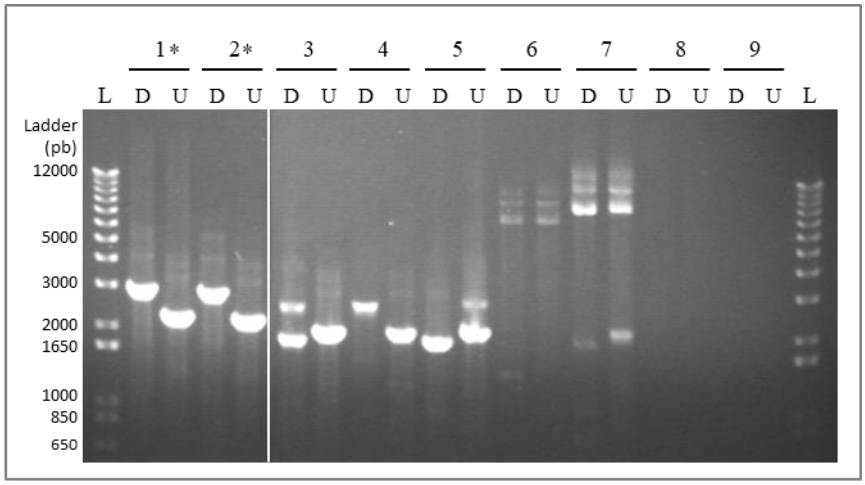

Figure 3.5 – Screening of NCTC 8325-4::pMAD del 773 mutants by agarose gel electrophoresis analysis of DNA fragments amplified by PCR. PCR fragments amplified from DNA extracted from several colonies are shown in the different lanes. Lanes D represent CS-PCR searching for integration via DOWN. Lanes U represent CS-PCR searching for integration via UP. Lanes 1, 2 and 4 **–** PCR resulting from DNA extracted from several colonies showing positive results for integration via DOWN and negative results for integration via UP; Lane 3 – PCR resulting from DNA extracted from a colony showing a mixture of positive and negative results for integration via DOWN and negative results for integration via UP; Lane 5 – PCR resulting from DNA extracted from a colony showing negative results for integration via DOWN and a mixture of positive and negative results for integration via UP; Lane 6 – PCR resulting from pMAD plasmid DNA (negative control); Lane 7 – PCR resulting from pMAD del 773 plasmid DNA (negative control); Lane 8 – PCR resulting from NCTC 8325-4 DNA (negative control); Lane 9 - PCR carried out with no template DNA (negative control); Lanes L **–** DNA ladders with band sizes (in base pairs); * **–** Chosen colonies to proceed for the Excision part of the protocol.

After the Excision step, Colony Screening PCR was used again to search for colonies lacking *saouhsc_00773*, the NCTC 8325-4 Δ773 mutants. To do so, "*UP FW EcoRI*" was used as the forward primer and "*DOWN RV BamHI*" as the reverse primer; the knock-out mutants (the ones missing the gene) would have a smaller size amplification (2021bp *vs* 2855bp) comparing to the original strain as

presented in Lanes A4, A7, B1, B3 and B5 of Figure 3.6. The first four NCTC 8325-4 Δ773 obtained mutants were kept and named NCTC 8325-4 Δ773 1D.20, 1D.23, 2D.16 and 2D.18 respectively.

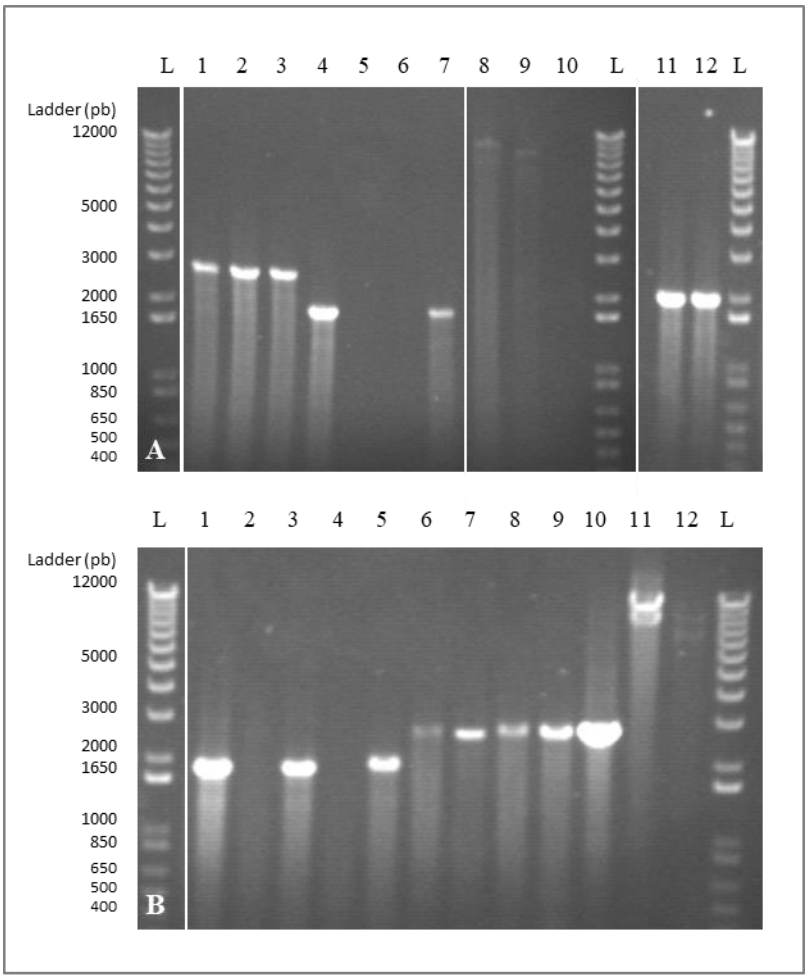

Figure 3.6 – **Panel A: Screening of NCTC 8325-4 Δ773 mutants derivate from the Down integrate NCTC 8325-4::pMAD del 773 (colony 1D obtained in the Integration) by agarose gel electrophoresis analysis of DNA fragments amplified by PCR.** PCR fragments amplified from DNA extracted from several colonies are shown in the different lanes. Lanes 1 to 3, 5 and 6 – PCR for NCTC 8325-4 Δ773 DNA resulting in bands (or with no amplification) corresponding to a non-deleted gene colony; Lanes 4 (colony 1D.20), 7 (colony 1D.23), 11 and 12 – PCR for NCTC 8325-4 Δ773 DNA resulting in bands corresponding to a deleted gene colony; Lane 8 – PCR resulting from NCTC 8325-4::pMAD del 773 (1D) DNA (negative control); Lane 9 – PCR resulting from NCTC 8325-4 DNA (negative control); Lane 10 **–** PCR carried out with no template DNA (negative control); Lanes L **–** DNA ladders with band sizes (in base pairs).

Panel B: Screening of NCTC 8325-4 Δ773 mutants derivate from the Down integrate NCTC 8325-4::pMAD del 773 (colony 2D obtained in the Integration) by agarose gel electrophoresis analysis of DNA fragments amplified by PCR. PCR fragments amplified from DNA extracted from several colonies are shown in the different lanes. Lanes 1 (colony 2D.16), 3 (colony 2D.18) and 5 – PCR for NCTC 8325-4 Δ773 DNA resulting in bands corresponding to a deleted gene colony; Lanes 2, 4 and 6 to 8 – PCR for NCTC 8325-4 Δ773 DNA resulting in bands (or with no amplification) corresponding to a non-deleted gene colony; Lane 9 – PCR resulting from NCTC 8325-4::pMAD del 773 (2D) DNA (negative control); Lane 10 – PCR resulting from NCTC 8325-4 DNA (negative control); Lane 11 – NCTC 8325-4::pMAD del 773 (2D) DNA; Lane 12 – NCTC 8325-4 DNA; Lanes L – DNA ladders with band sizes (in base pairs).

3.3.3. NCTC 8325-4 Δ773 mutant characterization

The four selected NCTC 8325-4 Δ773 mutant strains were used in a bacterial growth assay. The cultures growth rate was assayed by measuring the optical density at 600nm (OD_{600}) of a culture incubated at 37ºC and enquire whether the deletion of *saouhsc_00773* had any impact on the propagation of *S. aureus* bacteria. Figure 3.7 shows that all the constructed knock-out mutants' growth rate is similar to the NCTC 8325-4 *wt* strain.

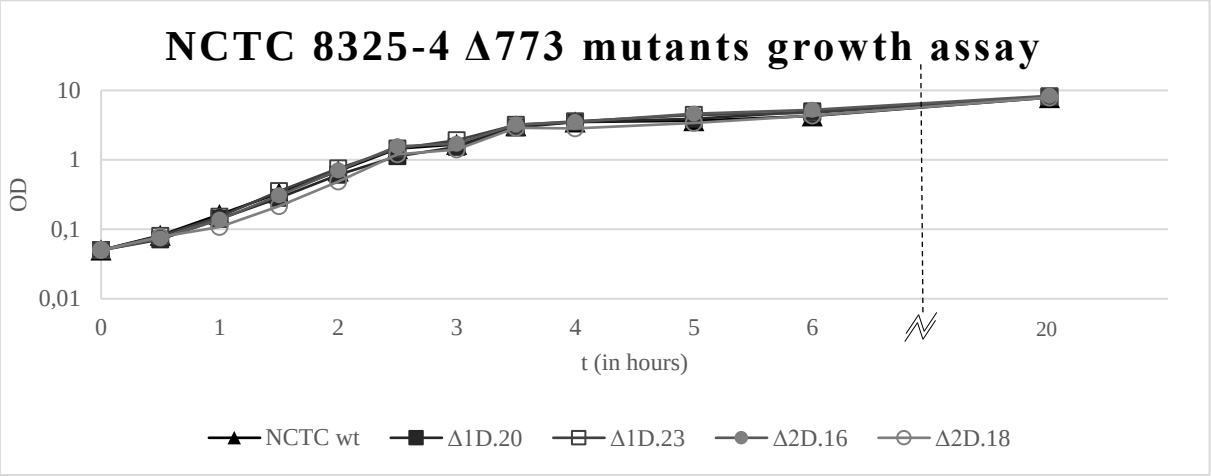

Figure 3.7 – **Comparison of several produced NCTC 8325-4 Δ773 mutants growth with NCTC 8325-4 (*wt*).**

To examine possible differences in the production of PGN hydrolases, a Zymogram gel was done. In this analysis, I compared the constructed NCTC 8325-4 Δ773 2D.16 mutant strain with the parental NCTC 8325-4 and with other NCTC 8325-4 mutant strains that lack PGN hydrolases (Atl and Sle1) or express mutated Atl proteins.

It was possible to identify in Figure 3.8 several bands from PGN hydrolases already described in the literature: Sle1, Atl and also from Atl subunits AM and GL. However, the constructed *saouhsc_00773* null mutant – NCTC 8325-4 Δ773, Lane 7 in Figure 3.8 – did not present differences from the *wt* in the bands' digestion patterns.

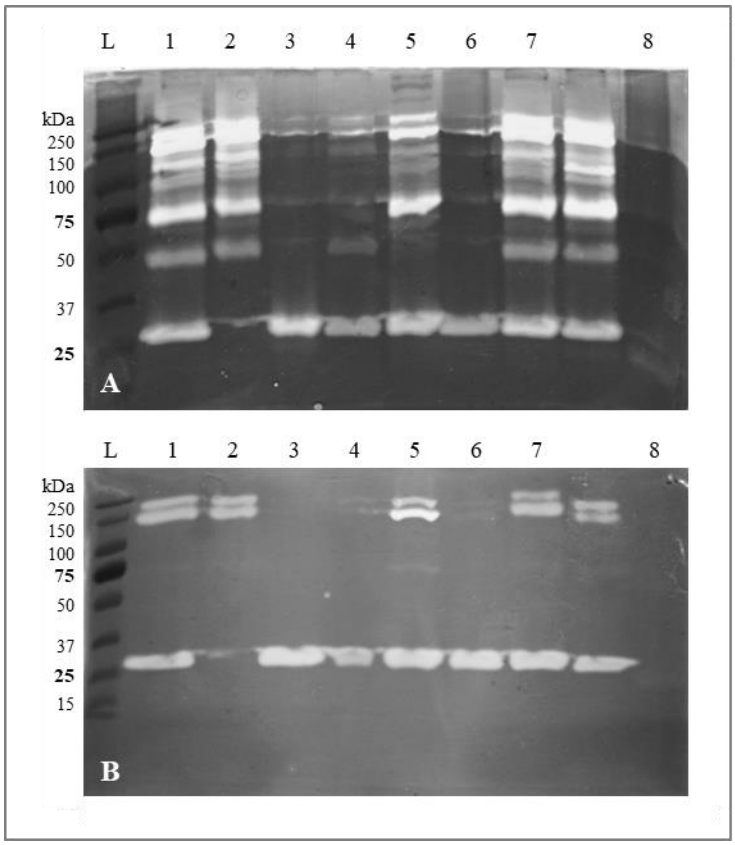

Figure 3.8 – **Analysis of several NCTC 8325-4 strains' crude autolytic extracts by Zymography gel to compare patterns of PGN degradation in different protein renaturation conditions. Panel A:** Zymogram gel incubated in 1x Renaturation Buffer; **Panel B:** Zymogram gel incubated in 10x Renaturation Buffer; Lane 1 – NCTC 8325-4 *wt*; Lane 2 – NCTC 8325-4 ΔSle1; Lane 3 – NCTC 8325-4 ΔAtl; Lane 4 – NCTC 8325-4 AM^- (the Atl's amidase domain is inactivated); Lane 5 – NCTC 8325-4 GL^- (the Atl's glucosaminidase domain is inactivated); 6 – NCTC 8325-4 AM^-GL^- (both Atl's amidase and glucosaminidase domains are inactivated); 7 – created NCTC 8325-4 Δ773 2D.16 mutant; 8 – purified protein. Lanes L – protein ladder with band sizes (in kDa) on the left.

3.4. Purification and characterization of the *S. aureus* SAOUHSC_00773 protein

To be able to express and purify the SAOUHSC_00773 protein, a plasmid with *saouhsc_00773* had to be created. After analysis of the gene sequence, an N-terminal Signal Peptide was identified. In the fragment that was cloned into the into the MCS of the pET21a plasmid, downstream to an IPTG-inducible promoter, the Signal Peptide part was replaced by a histidine-tail tag to allow the easy purification of the PGN hydrolase.

3.4.1. Choosing the positive DH5α pET21a 773 colonies

In order to have a bacteria able to host and replicate the desired plasmid, the ligation product that aimed the construction of this expression plasmid – pET21a 773 – was transformed to *E. coli* DH5α competent cells, a strain capable to efficiently receive and replicate foreign DNA.

To identify the colonies of transformants that carried the intended plasmids, a colony screening PCR was performed using commercial pET21a's primers "*T7 promoter primer*" and "*T7 terminator*

primer". The ligation product that was carried out to make the pET21a 773 plasmid was used in the PCR as the positive control and the empty pET21a plasmid was used as the negative control.

Screened DH5α pET21a 773 colonies revealing amplification with the expected size (986pb) were selected. The resulting agarose gel electrophoresis is presented in Figure 3.9. Lane 1, in Figure 3.9 reveals a positive-result-screening to pET21a 773 and therefore was selected to continue the experiments.

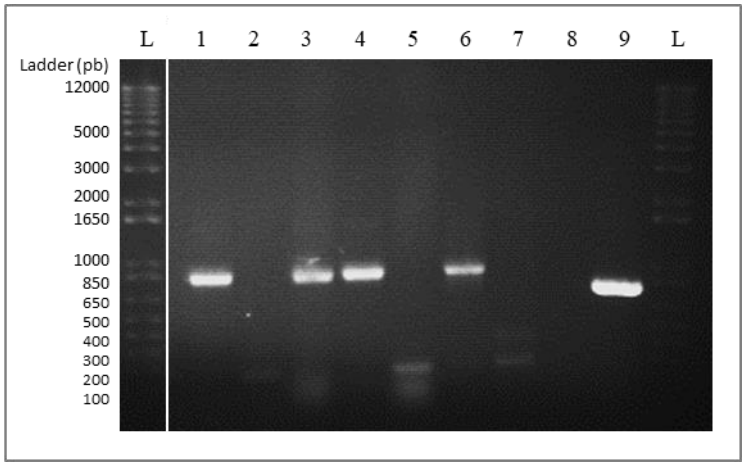

Figure 3.9 – Screening of DH5α pET21a 773 colonies by agarose gel electrophoresis analysis of DNA fragments amplified by PCR. PCR fragments amplified from DNA from several colonies are shown in the different lanes. Lane 1, 3 and 4 – colony PCR resulting in a band with the expected size (986pb) for the insert cloned in pET21a 773; Lanes 2 and 5 – colony PCR resulting in bands corresponding to an empty plasmid (no insert – 259pb amplification); Lane 6 – amplified fragment from the ligation product used to construct pET21a 773 plasmid; Lane 7 – amplified fragment from empty pET21a plasmid (negative control); Lane 8 – PCR carried out with no template DNA (negative control); Lane 9 – fragment used as insert in the cloning procedure into pET21a plasmid; Lane L – DNA ladder with band sizes (in base pairs) on the left.

Plasmid DNA from the chosen positive colony (named DH5α pET21a 773) was extracted using the Promega Miniprep KIT and digested with the NEB restriction enzyme *Nae*I (conditions at Table 2.4) to confirm that the cloned fragment is indeed positioned correctly. *Nae*I cuts both, pET21a and pET21a 773, in four places all from the pET21a original plasmid, as shown in Panel C of Figure 3.10 originating four distinct size fragments. So, in the agarose gel electrophoresis, the only difference between the bands' pattern (Panel B of Figure 3.10) is the band from the plasmid region that received the cloned gene, as shown in bold the Panel A of Figure 3.10. Since the resultant size fragments were the expected, plasmid DNA from pET21 773 10+ (colony number 10, Lane 1 in Figure 3.9) was sequenced (at STABvida using Sanger sequencing technique) using commercial pET21a's primers "*T7 promoter primer*" and "*T7 terminator primer*". The sequencing result was consistent with the *in silico* construct of the plasmid, revealing no mutations on the promoter region nor in the inserted gene. From this point, this plasmid will be referred only as pET21a 773.

Expected Digestion Fragment size of pET21a 773 with NaeI	*Expected Digestion Fragment size of pET21a with NaeI*
3180 bp	3180 bp
1588 bp	1588 bp
1241 bp	**515** bp
160 bp	160 bp

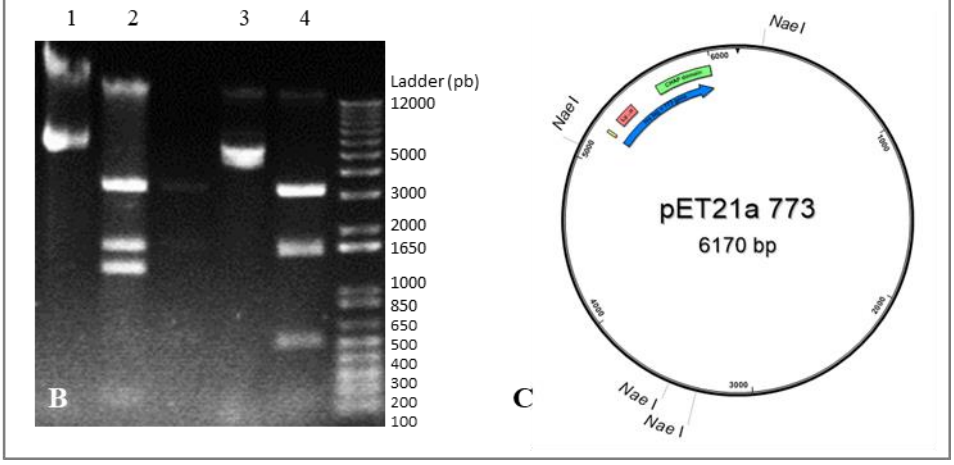

Figure 3.10 – Panel A: Expected digestion fragments' size of pET21a and pET21a 773 with *Nae*I. In bold are presented the fragments' sizes that are different between pET21a 773 and pET21a. **Panel B: pET21a 773 digestive pattern confirmation by agarose gel electrophoresis analysis of DNA fragments digested with *Nae*I.** DNA fragments resulting from digestion with *Nae*I restriction enzyme are shown in the different lanes. Lane 1 – undigested DNA from pET21a 773; Lane 2 – digested DNA from pET21a 773 showing DNA fragments with the expected sizes for the created plasmid; Lane 3 – undigested DNA from pET21a; Lane 4 – digested DNA from pET21a showing DNA fragments with the expected sizes for an empty plasmid; Lane L – DNA ladders with band sizes (in base pairs) on the right. **Panel C: *In silico* constructed pMAD del 773 plasmid's expected *Nae*I restriction enzyme recognition sites.**

3.4.2. Protein Induction and Purification

The confirmed pET21a 773 plasmid was transformed to BL21(DE3) competent cells in order to enable the protein production as this strain produces a T7 RNA polymerase when IPTG is added to the culture. This polymerase is needed to express genes that are under the influence of a T7 promoter, such as the one present in the pET21a plasmid were *saouhsc_00773* is cloned.

Cells were propagated in LB media and at an OD_{600} of 0.4 the culture was divided and half of it received IPTG to induce the cloned protein expression. Samples were collected before the addition of IPTG and 3 and 16 hours after the addition of IPTG. The resulting SDS-PAGE gel electrophoresis is presented in the Figure 3.11. In the same Figure, the Lane 2+ shows that an extra band appears at approximate 29kDa (28.9kDa), the expected size for the cloned protein, confirming its production. In a second rehearsal, the culture growth rate was followed by the OD_{600}. As shown in Figure 3.12, the IPTG-induced half part of the culture has no difference from the negative control were IPTG was not added, suggesting that neither the protein nor the IPTG impacts the culture growth rate.

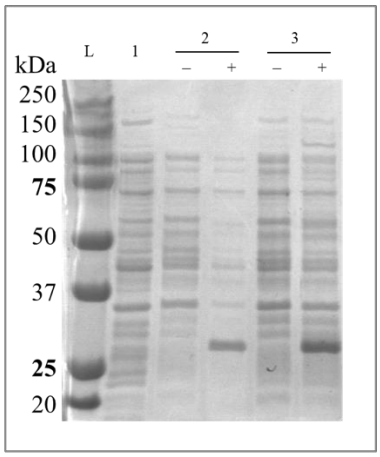

Figure 3.11 – **Analysis of the ability of BL21DE3 pET21a 773 strain to produce SAOUHSC_00773 by SDS-PAGE gel electrophoresis analysis.** Culture samples from a BL21DE3 pET21a 773 strain are shown in the different lanes. Lane 1 – sample taken before protein induction with IPTG (time 0h); Lane 2 – sample taken of a 3h culture: non-induced (–) and IPTG-induced (+); Lane 3 – sample taken of an O/N culture: non-induced (–) and IPTG-induced (+); Lanes 2+ and 3+ show the presence of an extra band (in comparison with non-induced culture at the equivalent time) of approximate 29kDa, the induced protein expected size. Lanes L – protein ladder with band sizes (in kDa) on the left. SDS-PAGE LB was used and the samples were boiled.

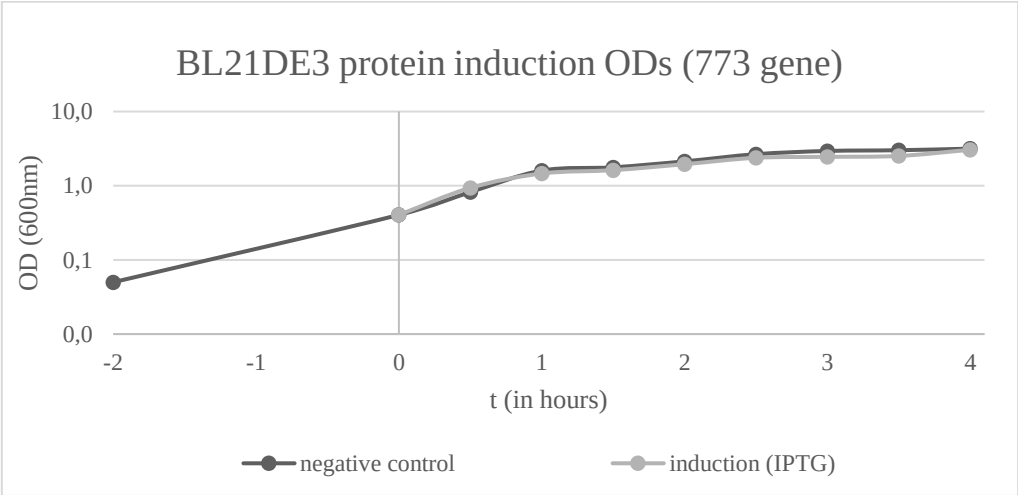

Figure 3.12 – **Growth rate comparison of BL21(DE3) pET21a 773 induced (with IPTG) culture with non-induced culture.** t0 represents the moment of the induction.

After confirmation of the production of a protein with the desired size, a culture of a larger volume, with a 3h induction, was done and the protein was purified. Since a new transformation of BL21(DE3) cells was done, the induction of protein expression was confirmed again before the lysis step of the purification protocol, as shown in Figure 3.13. Several samples were collected during the protocol and an SDS-PAGE gel performed to follow the protein purification. As shown through Lanes 5 to 8 of Figure 3.14, the amount of protein in the Resin's washed supernatants diminishes, what implies that the protein is binding to the Resin as it's supposed to. This protocol was successful and the isolated

protein was run in another SDS-PAGE gel in different conditions: native to desaturated (Figure 3.14, Lanes 9 to 12) to confirm that the protein doesn't present different migration patterns, as it only has one Cysteine residue and therefore does not make disulphide bridges. As expected, no significant changes were observed. The final purified protein was a 2.3µg/µL concentration accordingly to a Nanodrop software and using 1x PBS as the blank.

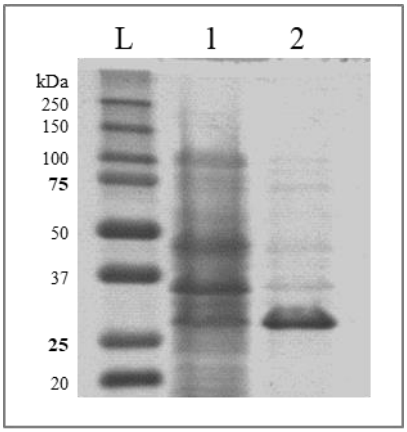

Figure 3.13 – **Screening of BL21DE3 pET21a 773 colony by SDS-PAGE gel electrophoresis analysis for protein induction.** Culture samples from a scale-up BL21DE3 pET21a 773 colony are shown in the different lanes. Lane 1 – sample taken before protein induction with IPTG (time 0h); Lane 2 – sample taken of the 3h IPTG-induced culture; Lane 2 shows the presence of an extra band of approximate 29kDa, the induced protein expected size. Lane L – protein ladder with band sizes (in kDa) on the left. SDS-PAGE LB was used and the samples were boiled.

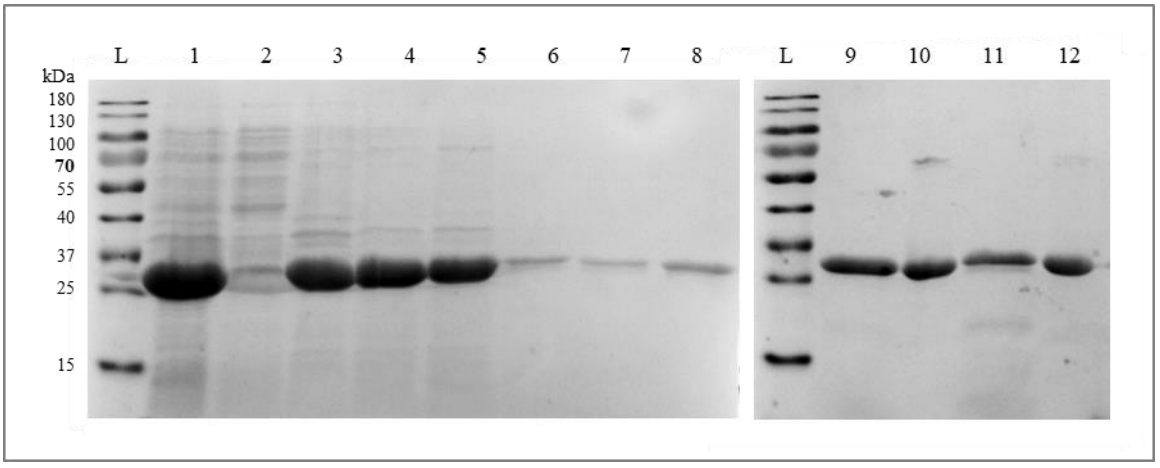

Figure 3.14 – **Analysis of the presence of the SAOUHSC_00773 protein after different steps of the purification protocol by SDS-PAGE gel electrophoresis analysis.** Samples collected along a protein purification from an IPTG-induced BL21DE3 pET21a 773 culture are shown in the different lanes. Lane 1 – sample taken after French Press lysis; Lane 2 – sample taken before French Press lysis; Lane 3 – sample of the pellet non dissolved in the 8M urea solution; Lane 4 – sample of the dissolved pellet (supernatant) in the 8M urea solution; Lane 5 – sample of the supernatant after the Talon resin; Lane 6 – sample of the supernatant after wash solution; Lane 7 – sample of the supernatant after 5mM imidazole wash solution; Lane 8 – sample of the supernatant after 10mM imidazole wash solution. Samples from lanes 1 to 8 were boiled and SDS-PAGE LB was used. Lanes 9 to 12 – final purified protein in different denaturation phases: Lane 9 – denatured 773 purified protein (SDS-PAGE LB + boil); 10 – semi-denatured 773 purified protein (SDS-PAGE native LB + boil); 11 – semi-denatured 773 purified protein (SDS-PAGE LB, no boil); 12 – native 773 purified protein (SDS-PAGE native LB, no boil). Lanes L – protein ladders with band sizes (in kDa).

3.4.3. Testing of the activity of the purified SAOUHSC_00773 Protein

After having purified a significant amount of protein (approximately 11.5mg), and in order to test its activity, the protein was run in a Zymogram gel. If the purified protein was active, we would be able to detect clear regions in the gel due to lysis of the cells present. In Figure 3.15, Panel A there is no evidence of PGN degradation by the purified SAOUHSC_00773 protein. However, when we increased the concentration of salts (panels B and C in Figure 3.15), we were able to observe a degradation band at approximately 29kDa (pointed by the white arrows), the expected size of the purified protein. The action of the NCTC 8325 *wt* autolytic extracts, used as a positive control of the presence of PGN hydrolases, diminished with the increase of salt concentration (Panel A to C in Figure 3.15). This result suggests that the overall autolytic extract enzymes loss their activity when the salt concentration is increased (Panel A gel was incubated in 1x Renaturation Buffer, in supposedly ideal conditions, while Panel B gel was incubated in 5x Renaturation Buffer and Panel C gel in 10x Renaturation Buffer).

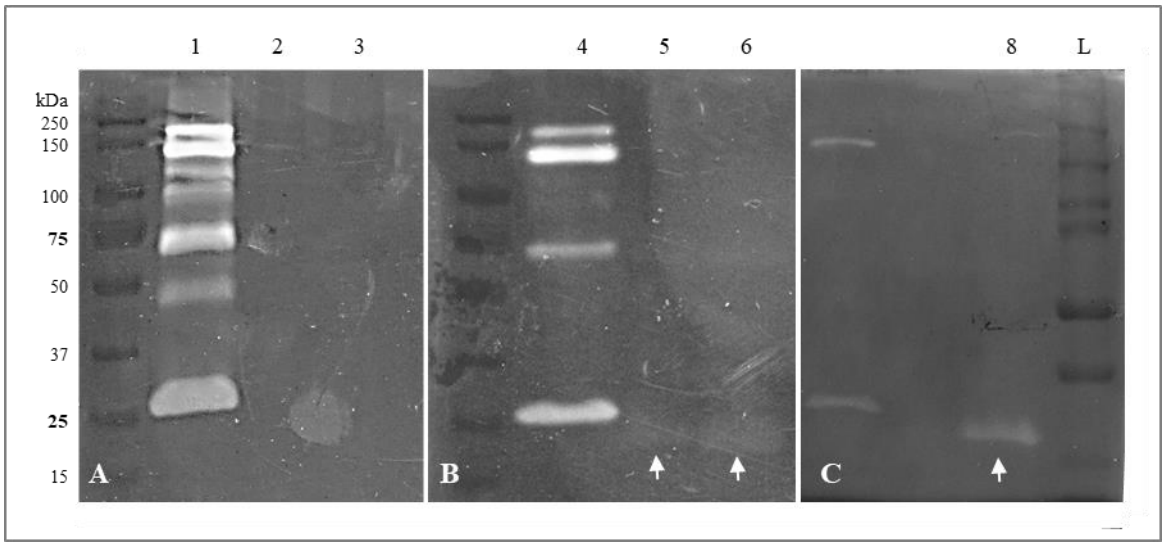

Figure 3.15 – **Analysis of the purified SAOUHSC_00773 protein and NCTC 8325-4 crude autolytic extracts by Zymography gel to compare patterns of PGN degradation in different protein renaturation conditions. Panel A:** Zymogram gel incubated in 1x Renaturation Buffer; **Panel B:** Zymogram gel incubated in 5x Renaturation Buffer; **Panel C:** Zymogram gel incubated in 10x Renaturation Buffer; Lanes 1, 4 and 7 – NCTC 8325-4 *wt* crude autolytic extracts; Lanes 2, 5 and 8 – purified 773 protein (5µg); Lane 3 and 6 – purified 773 protein (15µg); Lanes L – protein ladders with band sizes (in kDa). The arrows point to the purified SAOUHSC_00773 protein digestion band located at approximately 29kDa, the purified protein expected size.

The purified SAOUHSC_00773 protein was also incubated with purified PGN to check PGN's degradation by the reduction of the PGN's pellet size and confirm the previous Zymogram result. A picture of the final pellet is presented in Figure 3.16. In the negative control (Lane 1), the resultant pellet has a compact form, while in the digestion with the purified protein (Lane 3) it's smaller and lumpy, showing, hoverer, that the isolated protein presents activity in PGN degradation.

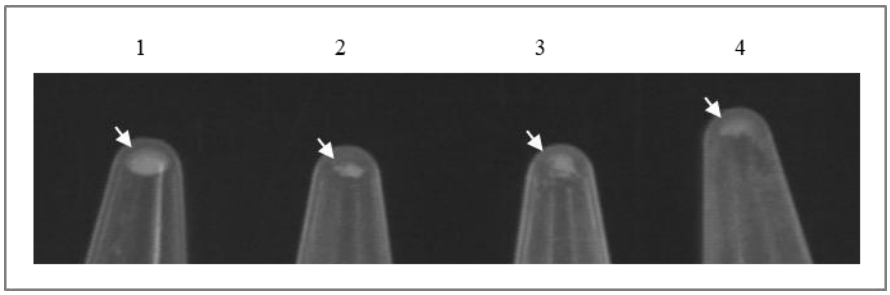

Figure 3.16 – Analysis of the purified PGN digestion ability of the SAOUHSC_00773 purified protein previously produced and comparison with known PGN-degradative enzymes. Lane 1 – PGN incubation carried with no enzyme (negative control); Lane 2 – PGN incubation with mutanolysin. As expected, reduction in the pellet size is visible.; Lane 3 – PGN incubation with the purified 773 protein. It's visible the diminishing of the pellet size in comparison with the control (1), what confirms the activity of 773 in PGN degradation.; Lane 4 – PGN incubation with LytA. As expected, reduction in the pellet size is visible.

4. Discussion

The objective of this work was to produce an *S. aureus* NCTC 8325-4 strain with the *saouhsc_00773* deleted from its chromosome in order to determine this gene role in the concealment of bacteria to host PGN receptors. An additional objective included the purification of the SAOUHSC_00773 protein, the determination of its role in the degradation of PGN and the sub-cellular sites in the bacterial cell surface that are targeted by this protein and also the comparison of the processes used by a bacterial PGN hydrolase and a host PGN receptor to bind PGN within the bacterial cell surface.

The tasks that accompanies these objectives were four: (1) to create an *S. aureus* NCTC 8325-4 strain that lacked *saouhsc_00773*; (2) to construct a plasmid that allowed the expression of *saouhsc_00773*, and its derivative linked to GFP that would permit the co-visualization with mCherry-PGRP-SA, a fluorescent PGN host receptor; (3) to purify the *S. aureus* SAOUHSC_00773 protein and to determine its ability to digest purified PGN; (4) to determine the sub-cellular sites of the bacterial cell surface of S. aureus that are targeted by the SAOUHSC_00773 hydrolase through fluorescence microscopy.

The *S. aureus* NCTC 8325-4 strain that lacks *saouhsc_00773* was successfully accomplished. Deletion of *saouhsc_00773* by double crossing-over from the NCTC 8325-4 genome was possible using pMAD. Similar to what has been described with *S. aureus Sle1* mutants, the absence of the protein did not notoriously impair the propagation of cells. Analysis by Zymography gel was also performed to see if any band disappeared in strains that had *saouhsc_00773* deleted. There were no detectable differences between the constructed NCTC 8325-4 Δ773 strain and the parental strain. This may have occurred because the protein may not be essential to the bacteria, or because it may work in synergy with another enzyme. In this last case, we would only see an impact in the propagation of cells with the deletion of both genes. Another hypothesis is that the SAOUHSC_00773 protein may not be expressed under laboratory conditions. Also, the enzyme shows activity in non-standard conditions that are not attendant in the autolytic extracts protocol so the production of SAOUHSC_00773 wouldn't happen when the autolytic extracts are generated.

The construction of a plasmid with a recombinant *saouhsc_00773* (the gene fragment cloned to the expression plasmid lacked the original signal peptide and has instead a histidine-tail) was also successfully accomplished, as well as the purification of the recombinant protein and it activity testing.

Due to the lack of time, a GFP derivate of the expression plasmid was not obtained, what made impossible to its co-visualization with mCherry-PGRP-SA and the determination of the protein localization in the bacterial cell surface.

The expression of *saouhsc_00773* was obtained by cloning the gene fragment into pET21a. After the induction of the expression with IPTG, it was verified that *saouhsc_00773* from NCTC 8325-4, when cloned in a vector and induced, is capable to be transcribed in a 28.9kDa protein. At the final of

the purification protocol, 11.5mg of protein at a concentration of 2.3μg/μL was obtained. In the *E. coli* strain used for this induction, the expression of the protein did not impair the bacteria growth.

The purified SAOUHSC_00773 protein did not show visible activity in a regular Zymography gel, interestingly when incubated with Renaturation Buffer at higher salt concentrations (5x and 10x – Figure 3.15, Panels B and C; pointed by the white arrows), the purified protein seemed to show activity while other Atl subunits don't. The SAOUHSC_00773's band is also not visible in the *wt* autolytic extract in Zymography (Figure 3.8). That may be due to the overpower of the SAOUHSC_00773's band by Sle1's band (32kDa in size and the original SAOUHSC_00773 protein has 30.3 kDa), but most likely due to the enzyme's need of a co-factor, only be expressed in *vivo* or in other conditions and will not be produced in enough quantity, since in the same Figure, Sle1 null mutant strain also does not present a band with the original SAOUHSC_00773 protein size (30.3 kDa). However, the protein incubation with purified PGN confirmed the Zymography results once the SAOUHSC_00773 purified protein showed visibly that has PGN degradation activity and is, therefore, an enzyme.

Two other pET21a derivate plasmids, containing only the first portion of *saouhsc_00773* (what included the LysM domain) and other containing the second portion of *saouhsc_00773* (the CHAP domain) were constructed and its sequence confirmed. Its goal was to see if the protein showed activity with only one of the domains (LysM or CHAP). However, due to the lack of time to proceed the experiments, these plasmids were not utilised, and therefore are not presents in this report.

Since isn't already clear the condition here the enzyme was its optimal activity, others growth conditions need to be tested changing settings such as media, osmotic stress and temperature. A double null mutant with Sle1 or SAOUHSC_00671 could also be constructed to test any synergetic effect between the enzymes.

Future work would also pass for complete the tasks that were not accomplished: the construction of an expression plasmid of a fluorescent derivate of the protein, to localize the protein in the cell surface and the co-visualize it with mCherry-PGRP-SA, a fluorescent PGN host receptor.

The results obtained for this new enzyme are promising, although other studies are required in order to fully unravel the function of this *S. aureus* PGN hydrolase. Is still yet to clarify if SAOUHSC_00773 activity in higher salt concentration is just an artefact or if this enzyme only appears in stress conditions.

5. References

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6. Appendix 1

Sequence from NCTC 8325-4 saouhsc_00773 cloned for pET21a.

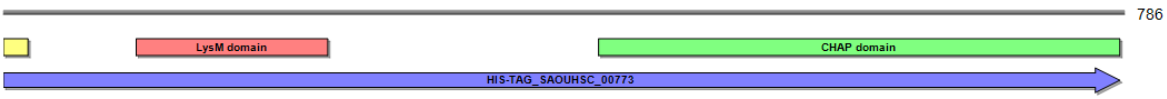

Figure 6.1 – Scheme of the sequence from NCTC 8325-4 *saouhsc_00773* cloned into pET21a to construct the expression plasmid. At yellow is presented the histidine-tail that replaced the signal peptide; At pink is the LysM domain; At green is the CHAP domain.

5’atggCTAGC**CATCACCATCACCATCAC**CAACAACATGGCACACAAGTAAAAACA
CCTGTTCAACATAATTATGTATCAAATGTTCAAGCACAAACGCAATCA**CCGACAACTTAT**
ACAGTAGTTGCTGGCGATTCATTATATAAGATTGCTTTAGAGCATCACTTAACGTTG
AATCAATTATATTCATACAATCCTGGTGTAACACCTTTAATTTTTCCTGGTGACGTGA
TTTCACTTGTGCCTCAAAATAAAGTGAAACAAACTAAAGCGGTTAAATCACCAGTAAGA
AAAGCAAGCCAAGCTAAAAAGGTAGTAAAACAACCTGTACAACAAGCATCTAAAAAAGT
AGTAGTTAAGCAAGCACCTAAGCAAGCAGTAACTAAGACAGTTAATGTAGCATACAAAC
CTGCTCAAGTACAAAAATCA**GTACCAACTGTACCTGTTGCACATAACTACAATAAATC**
AGTTGCTAACAGAGGAAACTTATATGCTTATGGAAACTGCACATATTATGCTTTCGA
TCGTCGTGCACAATTAGGTAGAAGTATAGGAAGTTTATGGGGCAATGCAAATAACTG
GAATTACGCAGCAAAAGTTGCAGGATTTAAAGTAGATAAAACACCAGAAGTTGGCG
CTATTTTCCAAACAGCTGCTGGTCCATATGGACATGTTGGTGTTGTTGAATCTGTAA
ACCCTAATGGAACAATTACTGTTTCTGAAATGAACTATGCTGGATTTAATGTTAAAT
CTTCAAGAACAATTTTAAATCCAGGAAAATATAATTACATCCACTAA3’

Figure 6.2 – Sequence from the gene expressed through the pET21a 773 plasmid constructed with *saouhsc_00773* from NCTC 8325-4 . At yellow is presented the histidine-tail that replaced the signal peptide; At pink is the LysM domain; At green is the CHAP domain; At capitals is the gene sequence cloned to the expression plasmid.