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Departamento de Química

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Bart Devreese, professor catedrático da Universidade de Gent e da Doutora Ana Cristina Esteves, professora auxiliar convidada (a tempo parcial) do Departamento de Biologia da Universidade de Aveiro.

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Palavras-chave HipAB, toxina-antitoxina, *Shewanella oneidensis* MR 1, *cross linking*, espectometria de massa.

Resumo

A resistência bacteriana é um ponto importante de estudo em investigação relacionado com o ambiente e a saúde pública, devido à ineficiência dos antibióticos, que resulta de uma subpopulação de células bacterianas que entram num chamado estado dormente. Diversos estudos indicam que os sistemas toxina-antitoxina estão envolvidos nessa persistência bacteriana. Um exemplo de um sistema toxina-antitoxina é o complexo HipAB, em que a HipA e a sua respetiva antitoxina estão intimamente relacionadas. HipB é muito instável e propenso à degradação por proteases (por exemplo: Lon protease), deixando a HipA a exercer o seu efeito tóxico nas células, Para estudar as conformações e interações da HipAB na *Shewanella oneidensis*, foi necessário sob expressar e purificar as proteínas e promover as reações de *cross linking* que foram analisadas por espectrometria de massa. A reações de *cross linking* foram realizadas usando o BS_3 (Bis[sulfosuccinimidyl] suberate), um *cross linker in solution*, testado a diferentes concentrações (0.5mM, 1mM, 2mM) para encontrar as condições de reação mais eficientes. Pelas análises de SDS-PAGE foi possível concluir que a melhor concentração de *cross linker* foi a mais alta testada. Para além disso, foram feitas algumas previsões de possíveis sítios de ligação, intra- ou intermolecular, no entanto só foi possível identificar uma das interações entre a HipA(143) com a HipA(264).

Keywords HipAB, toxin-antitoxin, *Shewanella oneidensis* MR 1, cross linking, mass spectometry.

Abstract

Bacterial resistance is a major issue in research related to environmental and public health, due to the ineffectiveness of antibiotics, resulting from a subpopulation of bacterial cells entering a dormant state. Multiple studies revealed that toxin-antitoxin systems are involved in such bacterial persistence. An example of such a toxin-antitoxin system is the HipAB complex, where HipA, and its cognate antitoxin are intimately related. HipB is very unstable and it is prone to degradation by proteases (e.g. Lon protease), leaving HipA exerting its toxic effect on cells. To study the conformations and interactions from HipAB in *Shewanella oneidensis*, it was necessary to overexpress and purify the proteins and to perform cross linking reactions which were analysed by mass spectrometry. The cross linking reactions were performed with BS3 (Bis[sulfosuccinimidyl] suberate), an in solution cross linker, tested at different concentrations (0.5mM, 1mM, 2mM) to find more efficient reaction conditions. By SDS-PAGE analysis it was possible to conclude that the best cross linker concentration is the highest tested. Furthermore, some predictions from possible intra- and intermolecular binding sites were made, unfortunately it was only possible to identify an interaction between HipA(143) and HipA(264).

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List of Abbreviations

ACN – Acetonitrile

APS – Ammonium persulfate

ATP – Adenosine-5'-triphosphate

BS_3 – Bis[sulfosuccinimidyl] suberate

Cb – Carbenicillin

CDK – Cyclin-dependent kinase

ddH2O - double-distilled water

DNA - Deoxyribonucleic acid

DSS – Disuccinimidyl suberate

EDTA – Ethylenediamine tetraacetic acid

EF-Tu – Elongation factor thermo unstable

EPS – Extracellular polymeric substance

ESI – Electrospray ionization

FT-ICR – Fourier transform ion cyclotron resonance

GDP - Guanosine diphosphate

GTP - Guanosine-5'-triphosphate

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HipA – High persistent A

HipB – High persistent B

HPLC – High performance liquid chromatography

IMAC – Immobilized metal affinity chromatography

IPTG – Isopropyl β-D-1-thiogalactopyranoside

LB – Lysogeny broth

m/z – mass/charge

MALDI – Matrix-assisted laser desorption/ionization

MS – Mass spectrometry

MS/MS – Tandem mass spectrometry

Ni-NTA – Nickel-nitrilotriacetic acid

SD200 – Superdex 200

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC – Size-exclusion chromatography

TA – Toxin-antitoxin

TEMED – Tetramethylethylenediamine

1. Introduction

Bacterial antibiotic resistance and tolerance play an important role in bacterial survival upon antibacterial treatment being a major issue in research related to public health. One mechanism by which bacteria defend themselves against antibiotics is a transition towards a dormant state called persistence, by which, cellular metabolism and growth are slowed. Recent studies led to the concept that toxin-antitoxin (TA) complexes are one of the systems involved in the regulation of bacterial persistence (Schumacher et al., 2009). In *Escherichia coli*, a *HipA* gene encodes a toxin protein of approximately 433 amino acid residues (Schumacher et al., 2009). HipA is a high persistence toxin that is neutralized by its cognate antitoxin, HipB. Release of HipB leads to activation of HipA that negatively regulates protein translation. Curiously, during a transposon mutagenesis screen for proteins involved in biofilm formation, a HipA homologue in the metal reducing organism *Shewanella oneidensis* MR-1 was observed (Theunissen et al., 2010). This study aimed to analyse the interactions of this HipA homologue with its antitoxin and make a structural comparison with the *E. coli* complex.

1.1. Biofilms formation in bacterial persistence

In their natural environment the majority of bacteria exist as a complex and organized ecosystem, called biofilm, rather than as individual planktonic cells. Biofilms are formed when bacterial cells attach themselves to a biotic or abiotic surface (Costerton et al., 1999). The bacterial cells have specific mechanisms to attach to a surface, and to develop the biofilm structure, as well for decoupling from the biofilm. It is suggested that biofilm is a single entity that has control over the individual member to yield the activities that are necessary for colony survival (Wolcott et al., 2008).

A biofilm has a “mushroom-like” shape that usually consists of several microcolonies of cells which are separated from each other by a network of water channels (Stoodley et al., 2002). The water channels allow the uptake of nutrients and the removal of metabolites, but are also used for intercellular communication by means of signalling molecules that are secreted. The

cells form a matrix and produce an extracellular polymeric substance (EPS) that gives the biofilm strength and protection (Stoodley et al., 2002). Therefore, a biofilm is not a homogeneous structure and the actual structure will depend on many parameters, such as the microbial community composition, the nutrient stock and the physicochemical properties of the environment.

1.2. Persister Cells

Antibiotics play a major role in the treatment of infectious diseases, but their effectiveness is highly compromised by the rise of antibacterial resistance. An antibacterial agent can kill bacteria by inducing the formation of corrupted products which lead to cell death (Figure 1) (Lewis, 2010). However, this can be prevented by antibiotic resistance or tolerance from the bacteria to the antibacterial agent. Within a population, particularly in a biofilm, it has been demonstrated that a subgroup of cells, called persister cells, tend to survive antibiotic treatment. When a bacterial population is treated with a single antibiotic, only regular cells die, whereas persisters can survive. Persister cells are not mutant cells but dormant cells that are formed stochastically (Yamaguchi et al., 2011). The antibiotic tolerance results from a change in sensitivity to elicitors (Ayres and Schneider, 2012), meaning that the antibiotic has no longer effect on the cells. Being antibiotic tolerant is quite different from being antibiotic resistant. Antibiotic resistance results from a random or stress-related mutation in the bacterial genome or it can result from horizontal gene transfer, and is permanent or hereditary, since this change occurs at the gene level. When exposed to antibiotics, the mutant bacteria are able to survive.

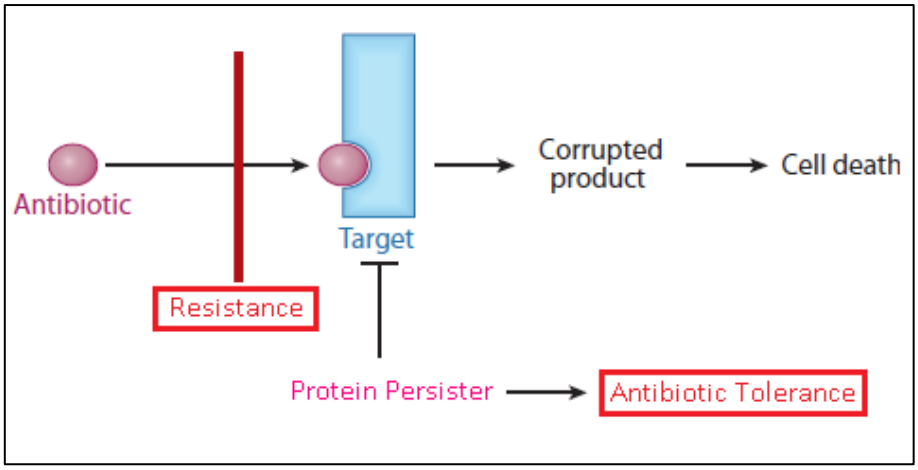

Figure 1 - Antibiotic resistance and antibiotic persistence scheme (image adapted from Lewis, 2010).

This phenomenon is more pronounced in a biofilm environment (Yamaguchi et al., 2011). Regular cells within a biofilm are likely to be killed, but the matrix protects the persister cells. When the concentration of antibiotic decreases, the persisters repopulate the biofilm (Figure 2).

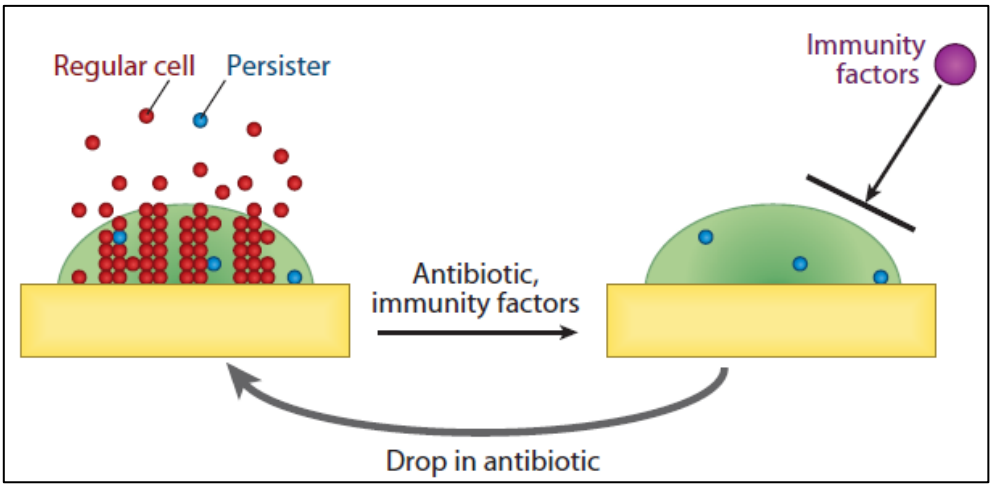

Figure 2 - Biofilm formation on a surface including persister cells and regular cells. High doses of antibiotic applied kill the regular cells. The matrix from the biofilm protects the persister cells and cause the relapse of the infection (Lewis, 2010).

1.3. Toxin-Antitoxin Systems

The development of persistence is partially controlled by so-called toxin-antitoxin systems. It is thought that toxins may promote cellular adaptation by slowing down cell growth, inhibiting or leading to cell death as a result of physical and chemical stress (Van Melderen, 2010, Van Melderen and De Bast, 2009). A toxin-antitoxin system generally consists of two components: a stable toxin and a labile antitoxin (Van Melderen, 2010). The antitoxin can be translated into an mRNA or a protein, which is mainly what defines the toxin-antitoxin type systems. The antitoxin instability is typically due to the less ordered structure which makes it more susceptible to degradation.

1.3.1. Toxin-Antitoxin system Types

Toxin-antitoxin (TA) systems can be classified into three different types (type I, II and III), depending on the antitoxin nature and/or mode of action. Antitoxins can be either RNA molecules (type I and III) or proteins (type II). RNA antitoxins interfere with the toxin function by forming an RNA-protein complex (Yamaguchi et al., 2011). On the other hand, the protein antitoxin interacts with its toxin, neutralizing its toxic effect. Type I and II systems were discovered on plasmids in the 80s (Jaffe et al., 1985, Ogura and Hiraga, 1983) and in chromosomes in the 90s (Masuda et al., 1993). In this work, we will focus on type II systems. Typically, they are organized in an operon that consists of a gene encoding for a small toxin, with an approximated size of 400 amino acid residues that is preceded by a gene encoding for its antitoxin (Figure 3).

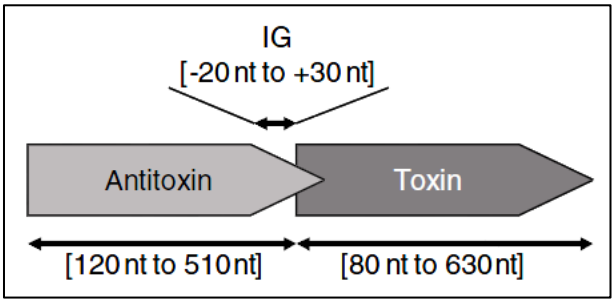

Figure 3 - Typical TA system *loci* organization, with the antitoxin preceding the toxin, their sizes and distance profiling are schematically represented (image from Sevin and Barloy-Hubler, 2007).

1.3.1.1. Type II toxin-antitoxin system types

Type II TA systems have been the most studied systems (Van Melderen, 2010). It is shown that: (a) TA systems are organized in operons with the antitoxin gene being upstream of the toxin gene, which are usually overlapping by a few bases (Figure 3) (Yamaguchi and Inouye, 2009, Sevin and Barloy-Hubler, 2007); (b) the toxin and its antitoxin form a stable TA complex to block the function of the toxin; (c) the antitoxin is more labile than the toxin so in order to block the toxic effect from the toxin it needs to be continuously produced; (d) antitoxins are degraded by stress-induced proteases releasing toxins from the TA complexes in the cell, which leads to growth inhibition or cell death (Christensen et al., 2004). The toxin and antitoxin have different lifetimes. The toxin is highly resistant to proteases, whereas the antitoxin can be degraded by a specific protease belonging to the Lon family of ATP-dependent proteases (Figure 4) (Yamaguchi et al., 2011).

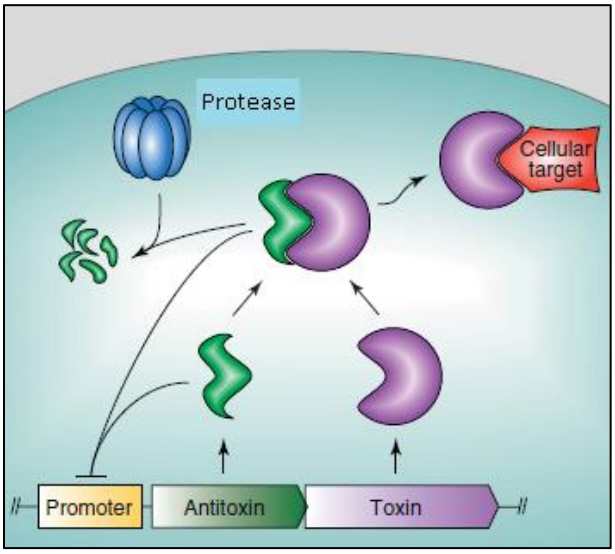

Figure 4 - Toxin-antitoxin system scheme. The antitoxin neutralizes the toxin. However, the antitoxin is very labile and unstable and can be degraded in the presence of proteases allowing the toxin to have its toxic effect in its different cellular targets (image from (Williams and Hergenrother, 2012).

1.3.2. Toxin-Antitoxin systems in chromosomes

Type II systems are quite common in bacterial chromosomes and are often found in multiple copies with the genome (Makarova et al., 2009). Chromosome-encoded TA systems are thought

to confer an evolutionary advantage on the bacterial population as they enter in the regulatory network of the host cell. There are no systematic studies on how chromosomal TA systems appeared however, for type II TA systems is quite clear that they have entered host cells by horizontal gene transfer (Pandey and Gerdes, 2005). Latest studies have shown that chromosomal type II TA systems have some similarities to the plasmid-encoded TA systems, as they can be involved in the stabilization of genomic fragments or in integrative processes of conjugative elements (Leplae et al., 2011).

1.3.3. HipAB and persistence in *Escherichia coli*

The HipAB operon is constituted of 2 genes, *HipB* and *HipA* (Figure 4). HipAB is a typical type II TA system. The operon encodes for two proteins with 10kDa and 51kDa, HipB and HipA respectively (Schumacher et al., 2009) playing an important role in persistence formation. The persistence formation occurs when HipA is overexpressed (Falla and Chopra, 1998). However, HipA can be neutralized by HipB, acting as a neutralizer and a transcriptional repressor of HipAB operon (Shanbhag et al., 1994).

The *HipA* gene is associated to different phenotypes, such as the toxic wild-type HipA and the high persistent or cold sensitivity of HipA7. The HipA7 mutant contains a higher frequency ability of 10^{-2} persistent cells when compared to the wild-type. It was shown that HipA7 is nontoxic, and that it is the result of a double mutation (G22S and D291A) (Korch et al., 2003). In addition, in the absence of HipB, it was shown that HipA7 expression ensures a high persistence phenotype, leading to the conclusion that HipB is not essential for this phenotype. Persistence and toxicity might, therefore, be independent: the HipA7 mutant seems to be less efficient in inhibiting macromolecule synthesis as compared to the wild-type HipA. The *HipA7* allele is possibly responsible for the tolerance to several antibiotics and other stressful situations (Korch et al., 2003, Van Melderen and De Bast, 2009).

1.3.3.1. HipB

Neutralization of the HipA toxin by the HipB antitoxin differs from other TA systems. HipB has no known homology with other TA systems antitoxins. Normally, protein antitoxins have a C-terminal extension which will only bind to its cognate toxin (Hansen et al., 2012). *HipB* encodes for a small DNA-binding protein with a helix-turn-helix DNA-binding motif functioning as a regulator of the *HipAB* operon by binding to a consensus sequence $TATCCN_8GGATA$ (being N any nucleotide) (Hansen et al., 2012, Korch et al., 2003).

1.3.3.2. HipA

HipA is a serine/threonine kinase that phosphorylates its targets, being a member of the superfamily of phosphatidylinositol 3/4-kinases (Correia et al., 2006, Schumacher et al., 2012, Schumacher et al., 2009). *E. coli* HipA has structural homology with CDK2/cyclin A kinase (Schumacher et al., 2009). The structural homology between HipA and CDK2 is highest in the C-terminal region that contains CDK2 catalytic residues, suggesting that HipA functions as a protein kinase, as reported (Correia et al., 2006). A key element in the function of this protein is the autophosphorylation of serine 150 by ATP, which plays a major role in the regulatory mechanism of HipA (Schumacher et al., 2012). The finding of the autophosphorylation position was possible using a mutated HipA in Asp309→Gln309 (D309Q), to compare the ATP binding affinity with HipA (Schumacher et al., 2009). Indeed, the D309Q mutation abolishes persistence, suggesting that the kinase function of HipA is a key element, resulting in the phosphorylation of one or more target proteins. In *E. coli*, the target of HipA appears to be EF-Tu, the Elongation Factor Thermo unstable (Correia et al., 2006).

1.3.3.3. EF-Tu

HipA acts on the EF-Tu activity by phosphorylation of Thr382. EF-Tu is a prokaryotic elongation factor, which, in its non-phosphorylated form, catalyses the formation of a tertiary complex with aminoacyl-tRNA and GTP, and is a key mediator in the translation process. EF-Tu belongs to the superfamily of guanosine trifosfatases (GTPases) (Van Melderen and De Bast, 2009). EF-Tu is

an essential GTP-binding elongation factor that mediates the delivery and entry of charged tRNA molecules to the ribosomal A site (Hayes and Van Melderen, 2011). In the Thr382 phosphorylated state, the GDP-bound conformation favours the elimination of the aminoacyl-tRNA bound. This bond elimination occurs due to a conformational change to an open form, which does not allow the binding to the ribosome obstructing translation (Schumacher et al., 2009).

1.4. *Shewanella oneidensis* Metal Reducing 1

The species *Shewanella oneidensis* Metal Reducing 1 belongs to the phylum Proteobacteria, class γ-Proteobacteria, order *Alteromonadales* and the family *Shewanellaceae. S. oneidensis* MR-1 stands for "metal reducing", meaning that it can use metals as a terminal electron acceptor, a special feature of this bacterium (Bagge et al., 2001). The *Shewanellaceae* family encloses approximately 30 *Shewanella* species, which can be found in soil and in sediment environments; it has also been described as a pathogen in humans and animals (Venkateswaran et al., 1999). *Shewanella* species are gram-negative, capable of surviving and proliferating in both aerobic and anaerobic conditions, and exhibit a strong flagellar motility (Theunissen et al., 2010). This organism has a high ability to spread and form biofilms, which can make it highly persistent in the environment. Theunissen et al. (2010) found during a transposon mutagenesis screen for proteins involved in biofilm formation a HipA homologue in the metal reducing organism *S. oneidensis* MR-1. HipA (Figure 5) and HipB (Figure 6) show respectively 29.9% and 20% sequence identity with the *E. coli* proteins. However, the number of amino acid residues differs from *E. coli* to *S. oneidensis*, with HipA shorter and HipB longer in *S. oneidensis*.

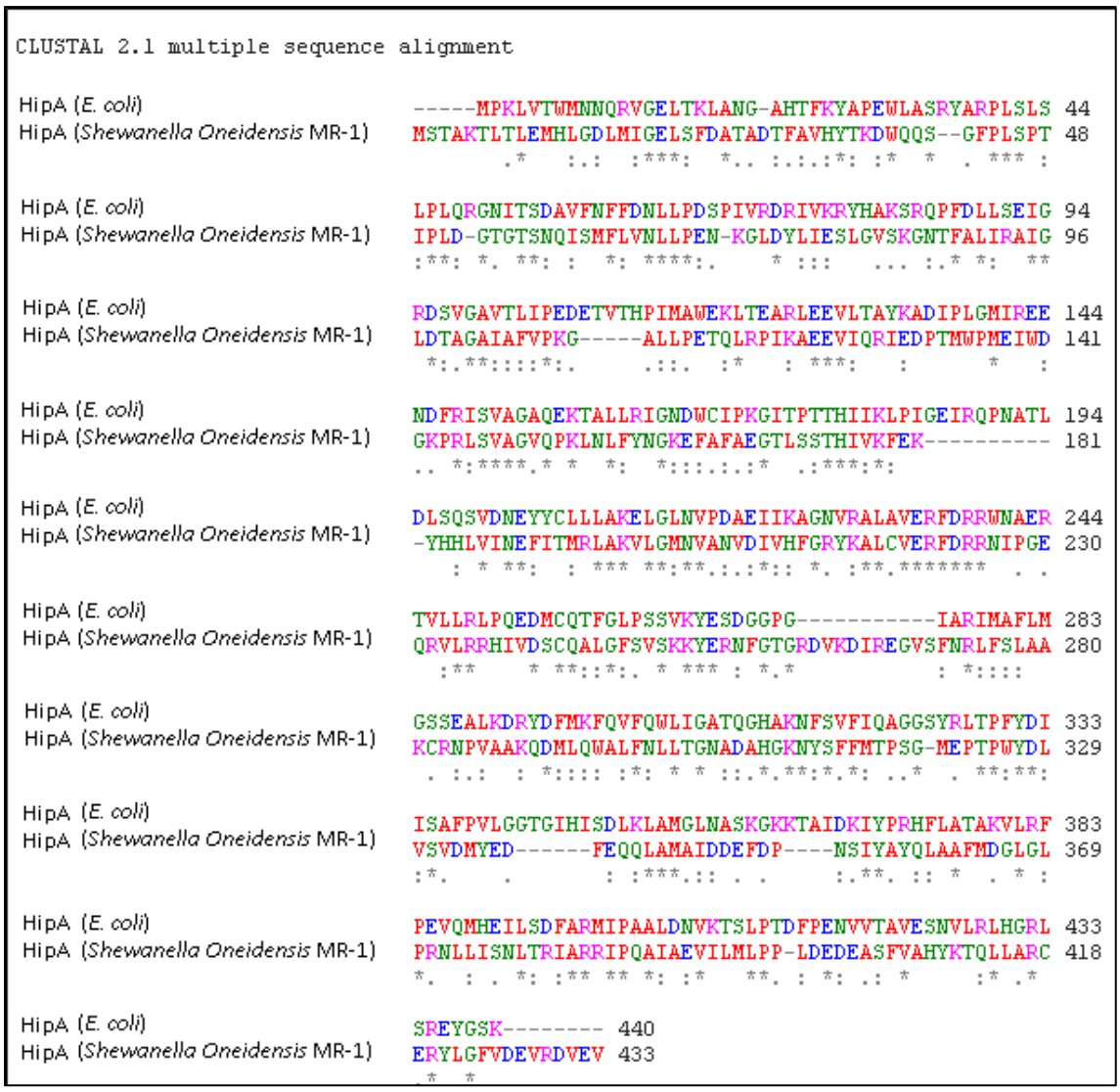

Figure 5 - Sequence alignment from HipA in *E. coli* with HipA in *S. oneidensis*. The signs : and . under the sequences note the different amino acids in the sequences, * stands for the same amino acids.

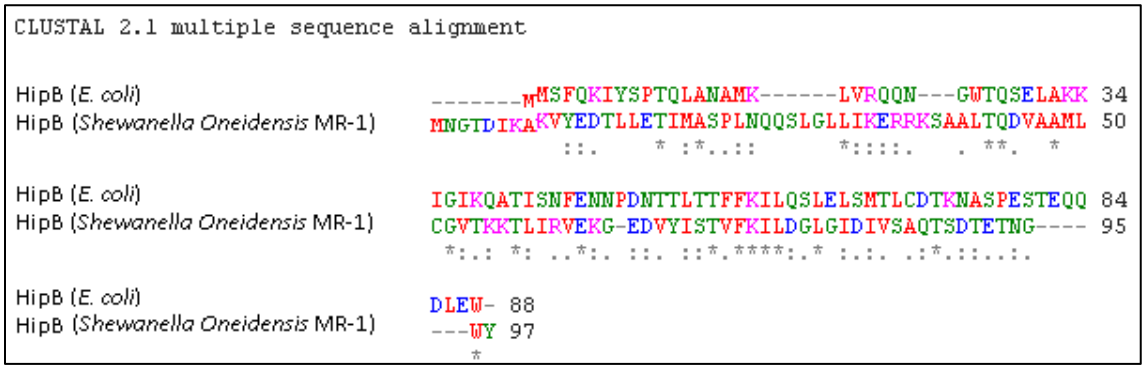

Figure 6 - Sequence alignment from HipB in *E. coli* with HipB in *S. oneidensis* MR-1. The signs : and . under the sequences note the different amino acids in the sequences, * stands for the same amino acids.

Cross-linking studies have shown that, in solution, HipB forms a dimer (Shanbhag et al., 1994). As referred before, HipB binds to HipA and is also a DNA-binding protein. Structural studies revealed that HipB in its dimer form interacts with DNA through major groove contacts as well as with two HipA molecules. HipA-HipB bound to DNA causes it to bend at an angle of 70˚ which is induced by HipB (Figure 7). This deflection ensures that the *N*-terminal region of HipA can bind to HipB subunit and that the C-terminal region of HipA is capable of binding to a different HipB subunit (Schumacher et al., 2009). HipB makes use of noncontiguous residues to bind to HipA well-ordered domains and not to loops. When HipA is bound to HipB it is neutralized and cannot exert its toxic effect, by phosphorylating EF-Tu and interrupting translation of proteins. HipA's interaction with HipB happens in normal conditions; however, the toxic effect can be activated due to the presence of certain proteases, able to degrade HipB. Therefore, if HipA disengages from the DNA-bound tetramer structure it can exert its toxic effect.

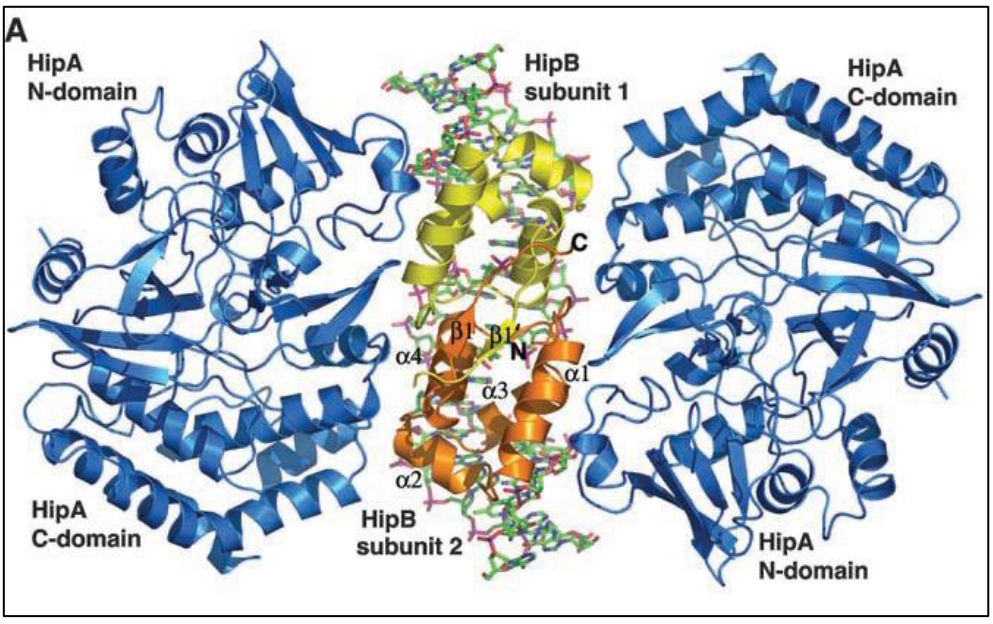

Figure 7 - HipA-HipB-DNA complex. (A) HipA-HipB-DNA operator complex, two HipA are represented in blue and HipB dimer is in yellow and orange, the DNA is represented in sticks (Schumacher et al., 2009).

1.5. Experimental approaches

A typical workflow for the study of proteins and their interactions usually involves several steps, beginning with the overexpression of proteins, additional analyses of the proteins' profile through mass spectrometry (MS) followed by the protein purification by chromatographic separation (Figure 8). This study made use of three proteins: HipA, HipB and mutant HipA. The mutant HipA, Asp306→Gln306 (D306Q) was selected as this abolishes the activity in *E. coli*, allowing to compare the different behaviours from a persistent and non-persistent bacteria.

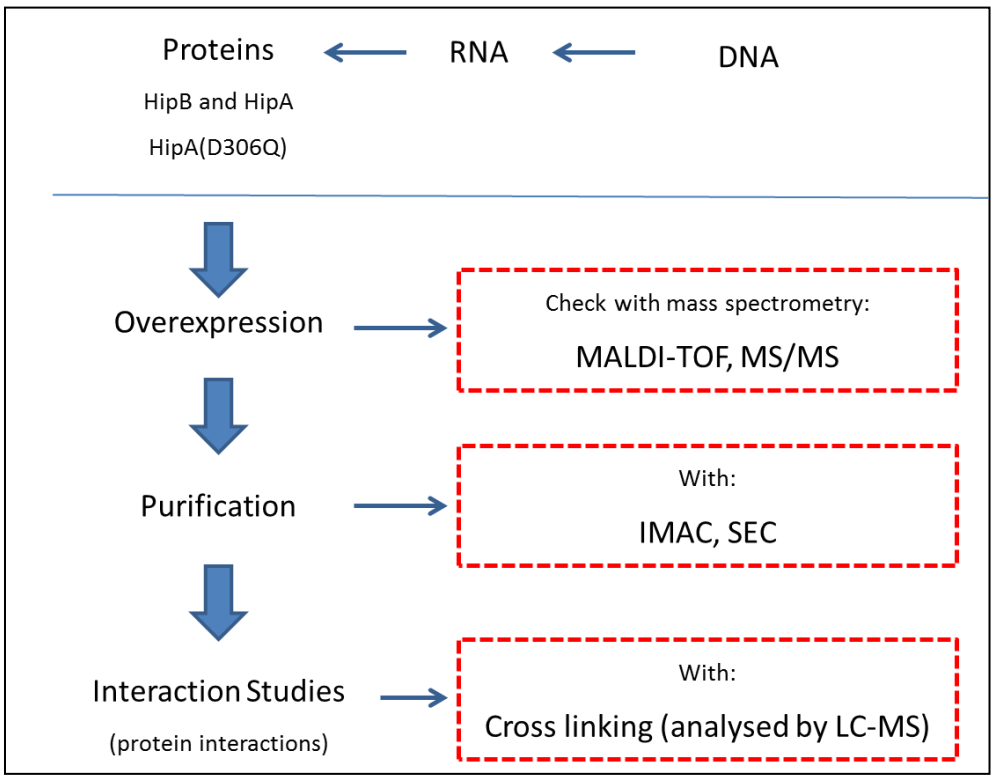

Figure 8 - Workflow used for the analysis of HipA, HipB and HipA (D306Q).

1.5.1. Protein overexpression and purification techniques

- **Immobilized metal affinity chromatography (IMAC)**

Immobilized metal affinity chromatography allows purifying a protein by its affinity to the resin. Often, when cloned, proteins are tagged with histidine. Histidine displays a high affinity to

metals. Nickel-nitrilotriacetic acid (Ni-NTA) resin has a high affinity to the 6-histidine-tagged proteins, allowing the specific purification of His-tagged proteins.

- **Size-exclusion Chromatography**

Size-exclusion chromatography (SEC) allows the purification of proteins according to their hydrodynamic volume, which is for globular proteins related to their size. The first compounds to be eluted are larger molecules. The Superdex 200 is a separation resin based on highly cross-linked agarose beads covalently to which dextran molecules are bound. The gel filtration properties of the Superdex matrix are led mainly by the dextran chains.

1.5.2. LC-MS analysis

- **High Performance Liquid Chromatography (HPLC)**

Liquid chromatography (LC) is a separation technique based on the distribution of the components of a mixture between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). In HPLC, typically the stationary phase consists of silica particles of a smaller size than the ones used in medium pressure chromatography, leading to increased resolution, but requiring the use of high pressure systems. HPLC is characterised by its high capacity, efficiency, selectivity, and resolution and can be easily automated.

To optimize the separation of different compounds, recent studies often couple LC with detection by mass spectrometry.

1.5.2.1. Mass Spectrometry

Mass spectrometry (MS) was the main method used for the analysis and monitoring the HipA and HipB in these studies.

A mass spectrometer, in general, is based on four main components: a sample injector, an ion source, an analyser and a detector (Figure 9); data obtained are processed using an electronic

support, with mass spectra as an output. In more detail, in the ion source the molecules of the sample are ionized becoming positively or negatively charged. The ions are then separated by its mass/charge (m/z) ratio in the analyser and are detected by the detection system.

In a mass spectrum, the x axis stands for the m/z, value of the ion and in the y axis is the relative abundance represented, using the main abundant ion intensity for normalization.

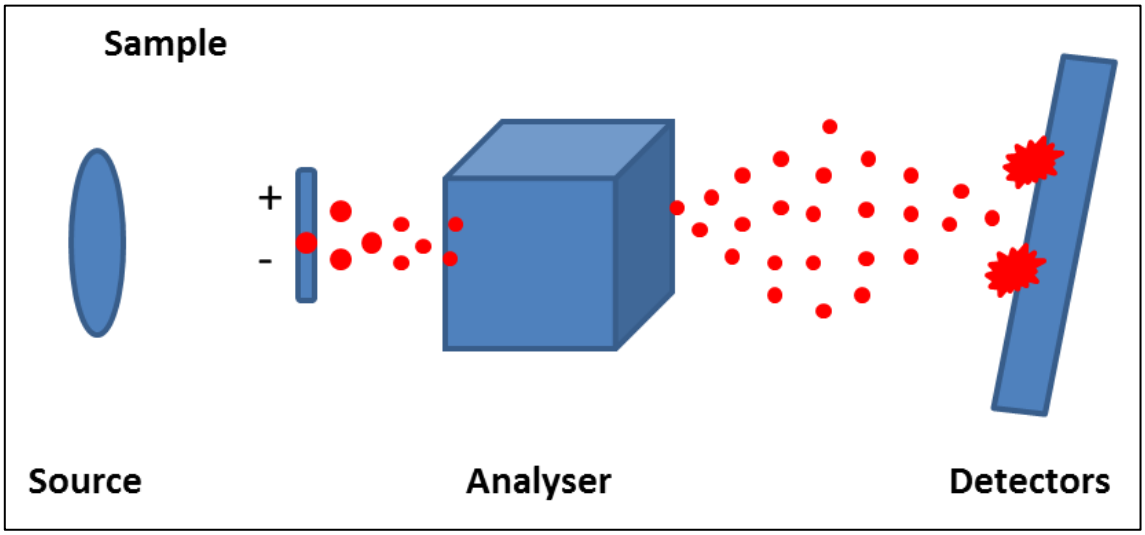

Figure 9 - Mass spectrometer components scheme.

In this study the main ion sources used were the electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI).

1.5.2.2. Ionization sources used in this project

- **Electrospray Ionization (ESI)**

For electrospray ionisation, the sample is solubilised in a solvent, which is normally more polar than the analyte. Thereafter, the solution is injected to the source with a needle which is inserted in a metallic capillary with a continuous flux. To make the solution flow out of the capillary a potential difference is applied, leading to the formation of a Taylor's cone. From his

cone, charged droplets are released containing a number of dissolved analyte molecules. The released droplets are dried with a non-charged gas, like nitrogen, which induces solvent evaporation and leads to a decrease on particle size (Figure 10). As the particles get smaller, they explode due to charge repulsions, originating in smaller droplets. This process occurs several times until there is no solvent present in the solution and only the analyte ions remain (El-Aneed et al., 2009). ESI has been widely used in the analysis of biomolecules; its great success in the analysis of proteins is due to its ability of producing multiple charges allowing the analysis of compounds with a higher molecular weight with a standard mass spectrometer.

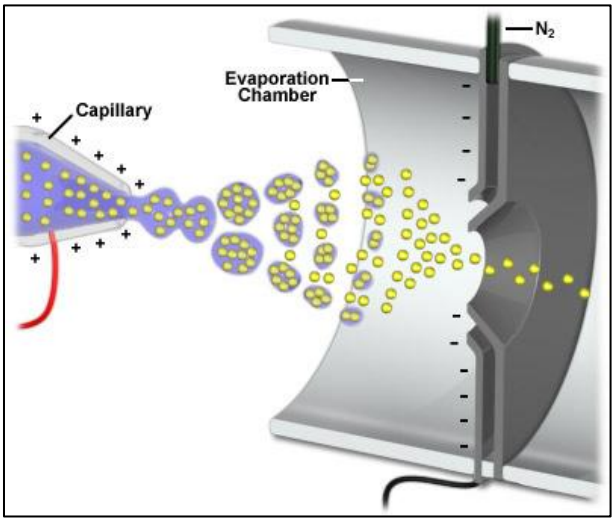

Figure 10 - Schematic representation of ionization by ESI (image from www.magnet.fsu.edu/).

- **Matrix-assisted laser desorption ionization (MALDI)**

In MALDI, ions are desorbed from a solid phase. A sample is first dissolved in a suitable solvent and afterwards an excessive amount of an appropriate matrix is added to the sample. In this study, the matrix used was α-cyano-4-hydroxycinnamic (α-cyano). After on MALDI plate and drying, the plate is loaded into the mass spectrometer and irradiated with a laser beam. Energy is absorbed by the crystals and dissipated, with the result that protonated or deprotonated sample molecules and the matrix pass to the gas phase, resulting mainly in mono-charged molecules (Figure 9) (El-Aneed et al., 2009).

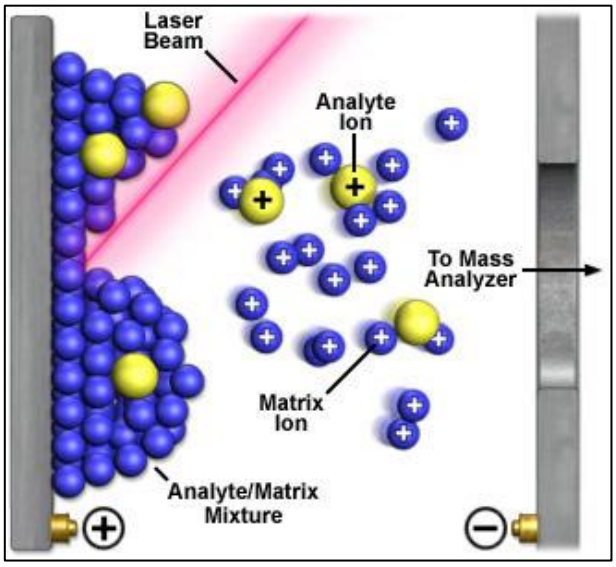

Figure 11 - Schematic representation of ionization by MALDI (image fromwww.magnet.fsu.edu/).

The ions that are produced are usually analysed by TOF analysers, and more recently by TOF-TOF geometry allowing tandem mass experiments and providing additional structural information, with high resolution and accuracy.

1.5.2.3. Mass Analysers used in this project

- **Linear Ion Trap**

A linear ion trap results from the application of a specific electric field on a quadrupole which traps the ions produced by the ionization source. At the end of the quadrupole, a voltage gradient is applied so that the ions can leave the ion trap by an increasing m/z value.

- **Time of flight (TOF) analyser**

The TOF analyser determines the time that the ions take to reach the detector. Therefore, ions are accelerated by an electric field. When the ions are accelerated their velocities are inversely

proportional to the square root of their mass, so the lighter ions reach higher speeds and arrive at the detector before the ions with higher mass.

- **Fourier transform ion cyclotron resonance (FT-ICR)**

The Fourier transform ion cyclotron resonance is based on the determination of the cyclotron frequency of the ions in a fixed magnetic field. The ions are injected into the cell perpendicular to the magnetic field (Marshall et al., 1998). The applied magnetic field causes the ions to oscillate in the z direction of the magnetic field, causing the cyclotron motion. The ion movement has a frequency associated to the ion m/z. It is determined by a set of electrodes through which a current is formed by the ion movement in the magnet. The resulting signal is called free induction decay (FID), that consists of a superposition of sine waves. Data is processed by a Fourier transform enabling to determine the m/z of the ions (Marshall et al., 1998). Among other mass spectrometry analysers, FT-ICR has the best resolving power and mass measurement accuracy (Marshall et al., 1998).

- **Tandem mass spectrometry**

Tandem mass spectrometry allows generating a specific ion fragments from the analyte. It consists of selecting and isolating a specific m/z value (precursor ion) that is submitted to dissociation. Consequently, ion fragments originating from the "parent" ion are produced. In order to achieve this specific fragmentation, several analysers are coupled in series, so that the ion isolation is performed by the first analyser, followed by fragmentation in the collision cell and the fragmented ions are separated based on their m/z value by the last analyser. However, some instruments (linear ion trap and FT-ICR) that have only one analyser are also able to perform multiple MS (MS^n), making these efficient instruments in the structural identification of several molecules including peptides. The linear ion trap allows the study of different ion fragmentations. It allows to retain the ion fragments to study in the ion trap and doing an MS^n (with n>2, representing the number of generations of ions to be analysed) denominated tandem mass spectrometry (El-Aneed et al., 2009).

Tandem mass spectrometry has an increased relevance when it is necessary to know more information about the biomolecule under study. Using this technology it is possible to generate information about the structure of the molecules.

1.5.3. Protein cross linking techniques

Different molecules can have different affinities to each other and can establish covalent or ionic bonds. There are different cross linkers that can be added to promote the reaction between different compounds. When choosing the cross linker it is important to know whether the protein is soluble or not and what sort of linking reactions will be promoted. The most common cross linkers are DSS (Disuccinimidyl suberate) and BS_3 (Bis[sulfosuccinimidyl] suberate). The BS_3 reacts with primary amino groups (-NH_2) to form stable amide bonds releasing *N*-hydroxysuccinimide. HipA and HipB proteins have several primary amine groups in the side chain of lysine (K) residues and in the *N*-terminal of each peptide or protein. A common technique used to study cross linking is by LC-MS, where the ionization source is ESI and the analyser is a FT-ICR. The cross linker will add to a peptide or protein 158Da, resultant from the cross linking reaction. We aimed to use the study of the HipAB interactions using cross linking techniques to compare the complex formed in *S. oneidensis* with the known structure from *E. coli*.

1.5.4. Phosphorylation studies

In cells, phosphorylation is mainly mediated by ATP (Schumacher et al., 2009). HipA is a toxin whose effects depend on its phosphorylation level; it is also responsible for the phosphorylation of other molecules, such as EF-Tu, as it was explained in section 1.3.3.3. So it became important to understand what happens to phosphorylated and non-phosphorylated HipA. To phosphorylate HipA *in vitro*, similarly to phosphorylation *in vivo*, ATP and the cofactor Mg^{2+} were added. On the other hand, to obtain the non-phosphorylated HipA *in vitro*, a phosphatase is added to the protein in order to remove all the phosphate groups binding HipA. The

availability of phosphorylated and non-phosphorylated HipA are important to study the mechanism of persistence and the conformational alterations.

2. Aim of the research project

The overall objective of this study is to understand the difference between structure and function of *S. oneidensis* MR-1 HipA, HipB and HipA(D306Q) compared to the known *E. coli* TA structure. In order to achieve this objective, several specific tasks will be carried out:

- Overexpression of the three proteins: HipA, HipB and HipA (D306Q).

- Purification of the overexpressed proteins;

- Analysis of the protein interactions. In order to study protein interactions, a cross linking experiment was performed followed by the analysis by SDS-PAGE, digestion, extraction and analysis by LC-MS. The cross linker used, BS_3, has an average reaction time of 30 to 45 minutes and the aim was to optimize the reaction conditions and to find the ideal final concentration of BS_3, in order to identify some of the different molecules formed after the cross linking reactions, for the phosphorylated and non-phosphorylated HipA, HipB and for the complex HipAB.

Overall, it is the intention of this study to understand the mechanisms of action of the toxin-antitoxin system in *S. oneidensis*.

3. Results and discussion

3.1. Protein Overexpression

A *E. coli* strain containing a pET15b vector for overexpression of HipA and HipB was available. The cloned genes, *HipA* and *HipB*, were fused with an *N*-terminal His-tag (Figure 12). Due to the high affinity of histidine to immobilized metals, such as nickel, the *N*-terminal His-tag is often used for protein affinity purification. For HipA, HipB and HipA(D306Q) protein expression, cells were cultured in 1L LB medium supplemented with 250mg/mL carbenicillin and incubated at 37 °C shaking at 200 rpm until the optical density (O.D.)(λ=600nm) = 0.6 was reached. The antibiotic used to select the bacteria was carbenicillin since the vector used contains a resistance marker to this antibiotic (Figure 12). During the incubation time the O. D. at 600nm was monitored. When the bacterial cells growth was maximum, IPTG was added to induce protein overexpression. After the addition of IPTG, an increase in optical density measured at 600 nm (Friedrich et al., 1995) was observed.

The ability of IPTG to mimic allolactose makes it important for use in overexpression experiments. Allolactose is a disaccharide similar to lactose. Lactose acts as an inducer of the *Lac* operon which will cause the cell to produce β-galactosidase and consume the lactose. After the addition of the IPTG, the *Lac* repressor will not bind anymore to the Lac operon, and the RNA polymerase is transcribed. Once the T7 RNA polymerase protein is expressed, it binds to the T7 promoter sequence upstream of the gene of interest on the plasmid insert and transcribes it (Clark et al. 2012). 3 hours after adding the IPTG, the protein of interest will be overexpressed in the cell, therefore it can be extracted for further studies. Expression was monitored every 60 minutes by collection of a 1 mL aliquot to be later analyzed by SDS-PAGE.

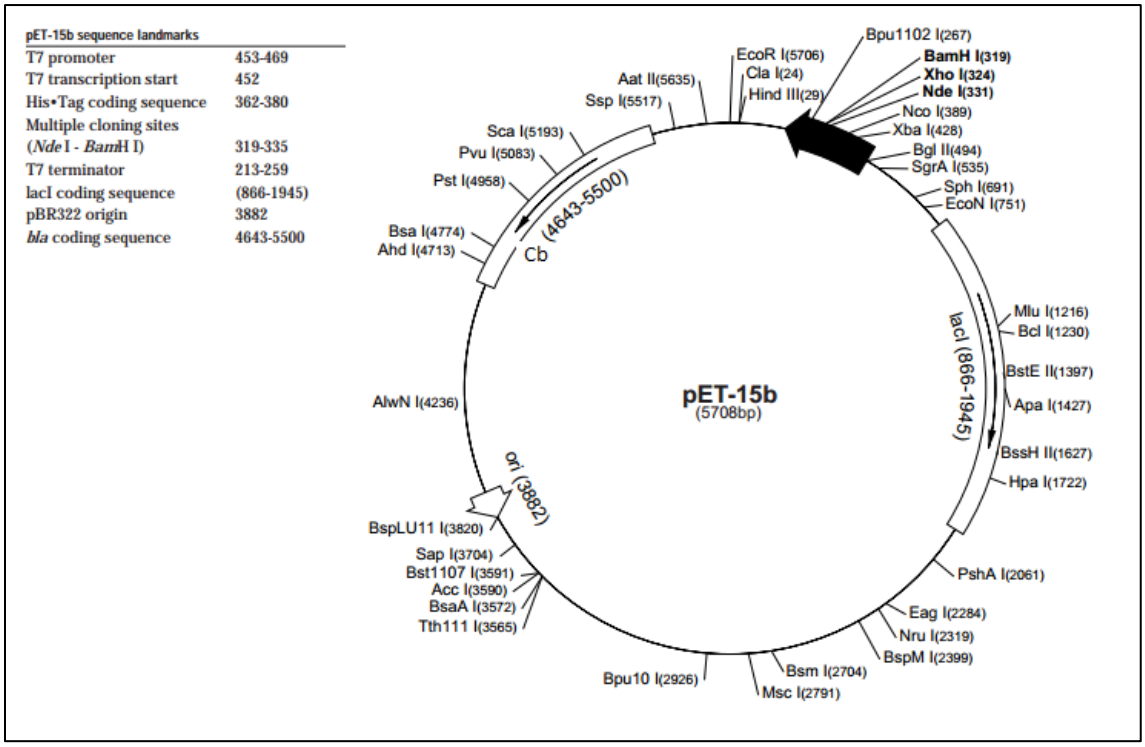

Figure 12 – Schematic representation of the pET-15b vector. The pET-15b vector carries an N-terminal His-Tag sequence (362-380) followed by a thrombin site and three cloning sites. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 e expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. Cb (4643-5500) is referent to the carbenicillin vector resistance gene. Cb is a bacteriolytic antibiotic belonging to the carboxypenicillin subgroup of the penicillins (image from www.aidsreagent.org/pdfs/pet15b.pdf).

3.1.1. HipA, HipB and HipA(D306Q) overexpression – analysis by SDS-PAGE

Each sample withdrawn before and after the addiction of IPTG was analyzed by SDS-PAGE (Figure 13).

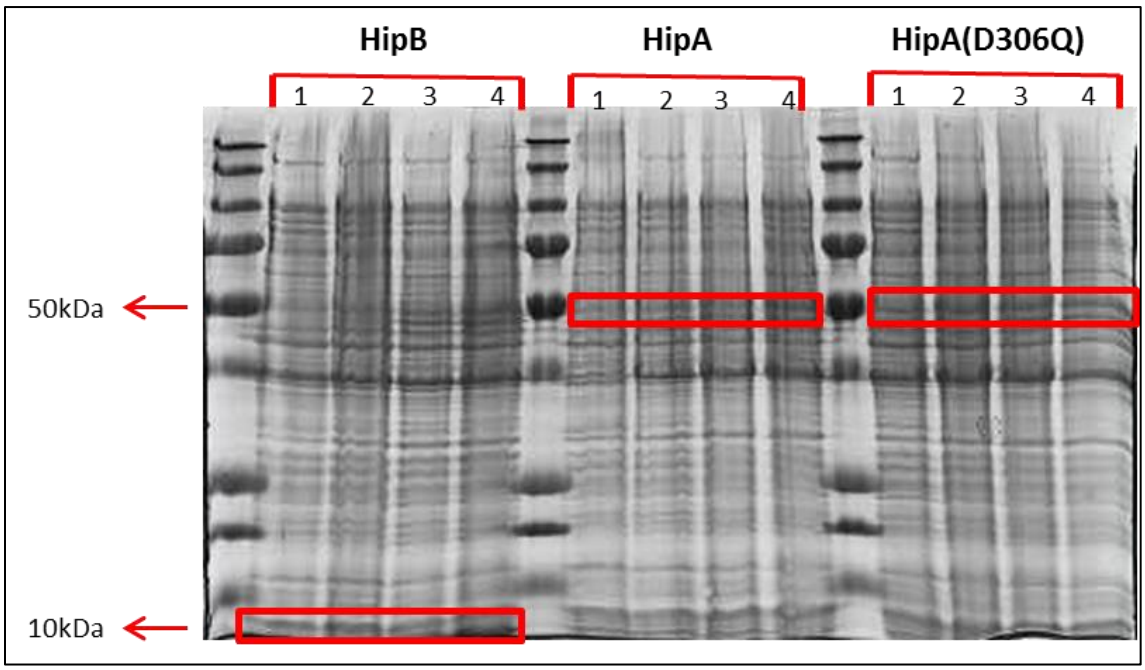

Figure 13 - SDS-PAGE showing the overexpression of HipB, HipA and HipA(D306Q). Under each protein name are the numbered lanes. Lane 1 represents the aliquot collected before the IPTG addition; lane 2 represents the aliquot collected 1h after the IPTG addition; lane 3 represents the aliquot collected 2h after the IPTG addition; lane 4 represents the aliquot collected 3h after the IPTG addition. The red rectangles highlight the overexpressed protein bands selected for MALDI-TOF identification. The bands were subjected to in gel, where the proteins were digested and the peptides extracted for analyse.

The bands that indicated (Figure 13) were cut and *in gel* digestion was performed using trypsin, a proteolytic enzyme commonly used. Trypsin cleaves the peptide chain mainly at the carboxyl side of the amino acids: lysine (K) and arginine (R), except when either is followed by proline (P). Trypsin is a robust enzyme and produces peptides with advantageous properties like length, charge and fragmentation behavior. The resulting peptides were analyzed using MALDI-TOF. From the MALDI-TOF spectrum (Figure 14), a Mascot database MS search for protein identification was performed. Protein identification was based on MS data by matching at least five peptide masses and/or MS/MS data, identifying no less than two unique peptides, respectively.

HipB was not detected, most probably due to low concentration. HipB is a relatively small 13 kDa and unstable protein, which could explain that it was not possible to find the protein

neither in the SDS-PAGE nor in the MALDI-TOF analyses. Another possibility to explain the non-detection of HipB can be due to the detection methods, the Coomassie blue G-250 (~30ng) is not sensitive enough to detect the HipB bands. One solution to improve could be to use a more sensitive detection method, for example the Silver staining (~5-10ng) this is less compatible with the MS analysis.

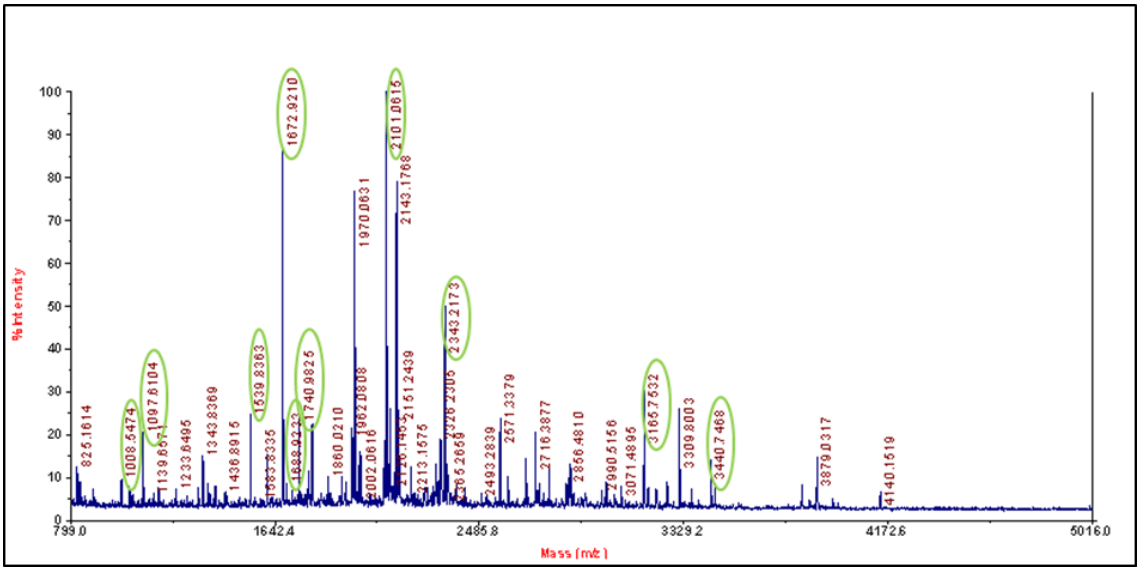

Figure 14 - MALDI-TOF peptide mass fingerprint from a tryptic digest of the 50kDa band displayed in Figure 13, HipA samples. The green circles indicate peaks of which the m/z value corresponds to tryptic fragments of HipA.

The 50kDa band corresponds to HipA. There were 13 matched peptides (table 1) which corresponds to a protein sequence coverage of 36%. For the mutant HipA(D306Q), 16 peaks matched HipA(D306Q) tryptic peptides. As shown in Figure 15, the mutated amino acid appears in the peptide with 2356Da. The change from protein HipA wild type to the mutant HipA(D306Q), results in an increase of 13Da as expected, since the molecular weight (Mw) of the aspartic acid residue is 115.09Da and the Mw of glutamine residue it is 128.13Da. The peak resultant from this mutation is highlighted in Figure 15, which is the spectrum referent to the HipA(D306Q) sample analysis.

Table 1 - HipA matched peptides with the starting and ending position, the observed molecular relative mass (Mr), the expected Mr, the calculated Mr and the peptide sequence. The peptide sequence with the amino acid that was after mutated is highlighted.

Start – End (Peptide sequence)	**Observed (Mr)**	**Expected (Mr)**	**Calculated (Mr)**	**Peptide Sequence**
6 – 36	3440.8450	3439.8377	3439.6680	K.TLTLEMHLGDLMIGELSFDATADTFAVHYTK.D
129 – 145	2102.1235	2100.1163	2099.9808	R.IEDPTMWPMWPMEIWDGKPR.L
129 – 145	2133.1152	2132.1080	2131.9707	R.IEDPTMWPMEIWDGKPR.L + 2 Oxidation (M)
179 - 194	2093.2007	2092.1934	2092.0564	K.FEKYHHLVINEFITMR.L + Oxidation (M)
182 – 194	1672.9846	1671.9773	1671.8555	K.YHHLVINEFITMR.L
182 – 194	1688.9480	1687.9407	1687.8504	K.YHHLVINEFITMR.L + Oxidation (M)
198 – 213	1741.0435	1740.0362	1739.9141	K.VLGMNVANVDIVHFGR.Y
275 – 283	1008.5783	1007.5710	1007.5586	R.LFSLAAKCR.N
290 – 310	**2343.2776**	**2342.2703**	**2342.1447**	K.QDMLQWALFNLLTGNA**D**AHGK.N
372 – 380	1043.6876	1042.6803	1042.6135	R.NLLISNLTR.I
372 - 384	1539.8794	1538.8721	1538.9368	R.NLLISNLTRIARR.I
284 – 411	3165.8252	3164.8179	3164.6580	R.RIPQAIAEVILMLPPLDEDEASFVAHYK.T
421 - 429	1097.6497	1096.6424	1096.5553	R.YLGFVDEVR.D

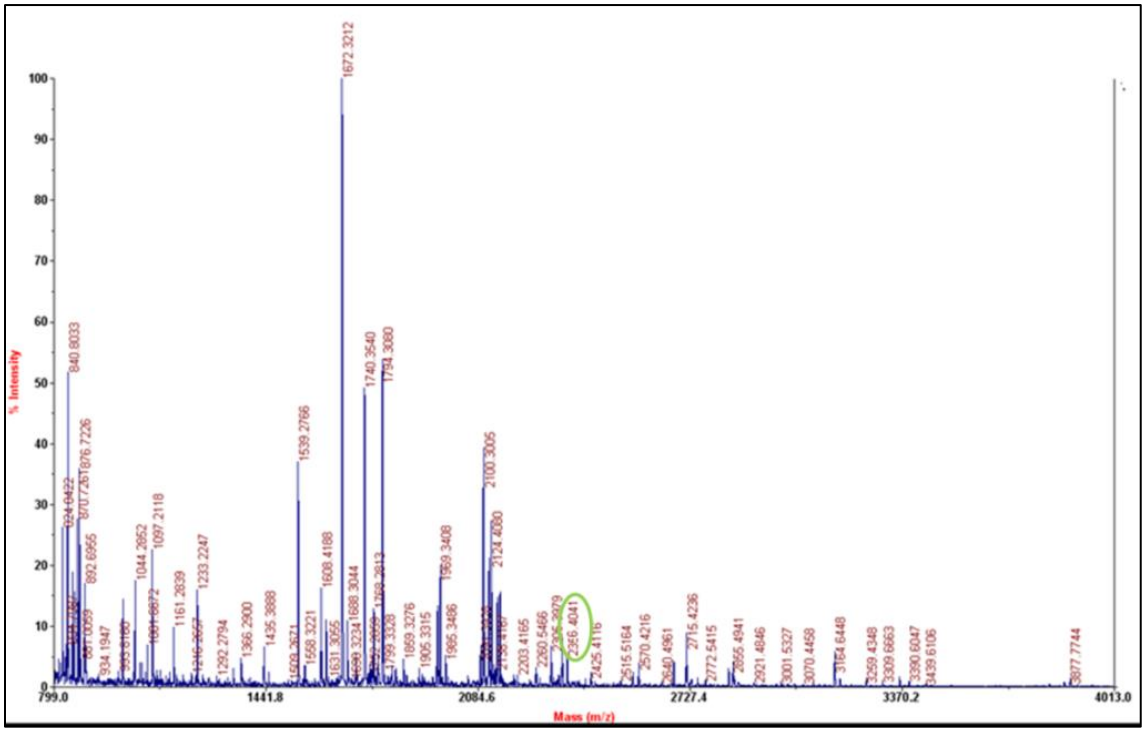

Figure 15 - MALDI-TOF peptide mass fingerprint from a tryptic digest of the 50kDa band displayed in Figure 13, HipA(D306Q) samples. The green circle indicate peaks of which the m/z value corresponds to tryptic fragments of HipA.

3.1.2. HipA, HipB and HipA(D306Q) purification analyses

In order to understand the mechanism and function of a specific protein, this proteins need to be purified. Similarly to studies made with in *E. coli* (Hansen et al., 2012) TA system, for further studies of *S. oneidensis* TA system, HipA, HipB and HipA(D306Q) were overexpressed and purified. As mentioned in section 4.4.1, the proteins for these studies were engineered with a His-tag, therefore the immobilized metal affinity chromatography was performed in order to separate the His-tagged proteins (Gräslund et al., 2008).

3.1.2.1. HipA purification analyses – IMAC

The Ni-NTA columns can be used to separate the His-tagged proteins but can also bind proteins that contain histidine clusters. In these cases applying a small amount of imidazole during the

purification before applying the imidazole gradient can help to control off-target binding (Gräslund et al., 2008).

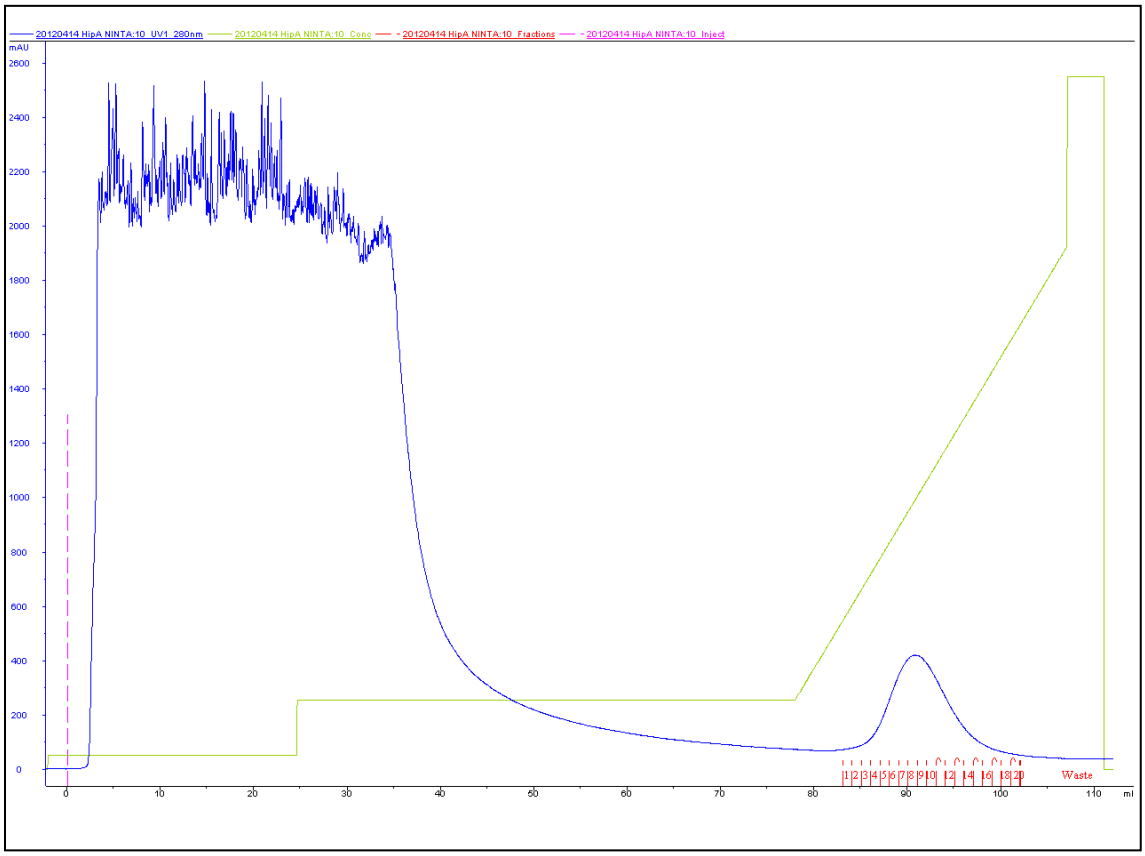

Figure 16 - HipA purification using the Ni-NTA column by IMAC. The protein is eluted when an imidazole gradient is applied. The green line represents the concentration of imidazole in the buffer, the blue line represents the sample absorption at 280 nm, and the red lines are referent to the collected fractions.

3.1.2.2. HipA purification analyses - SEC

To complement the IMAC separation, a SEC purification was performed. The SEC purification allows a more specific purification since separates mainly by the size of the proteins. The purified protein from the IMAC was concentrated to a final volume of 2 mL using Vivaspin ultracentrifugation. SEC resolution can increase if the volume of injected protein is smaller, being important to concentrate the protein to a smaller volume. If the injection volume is

greater than 2mL generally the peak width will be bigger reducing the separation resolution (Hong et al., 2012).

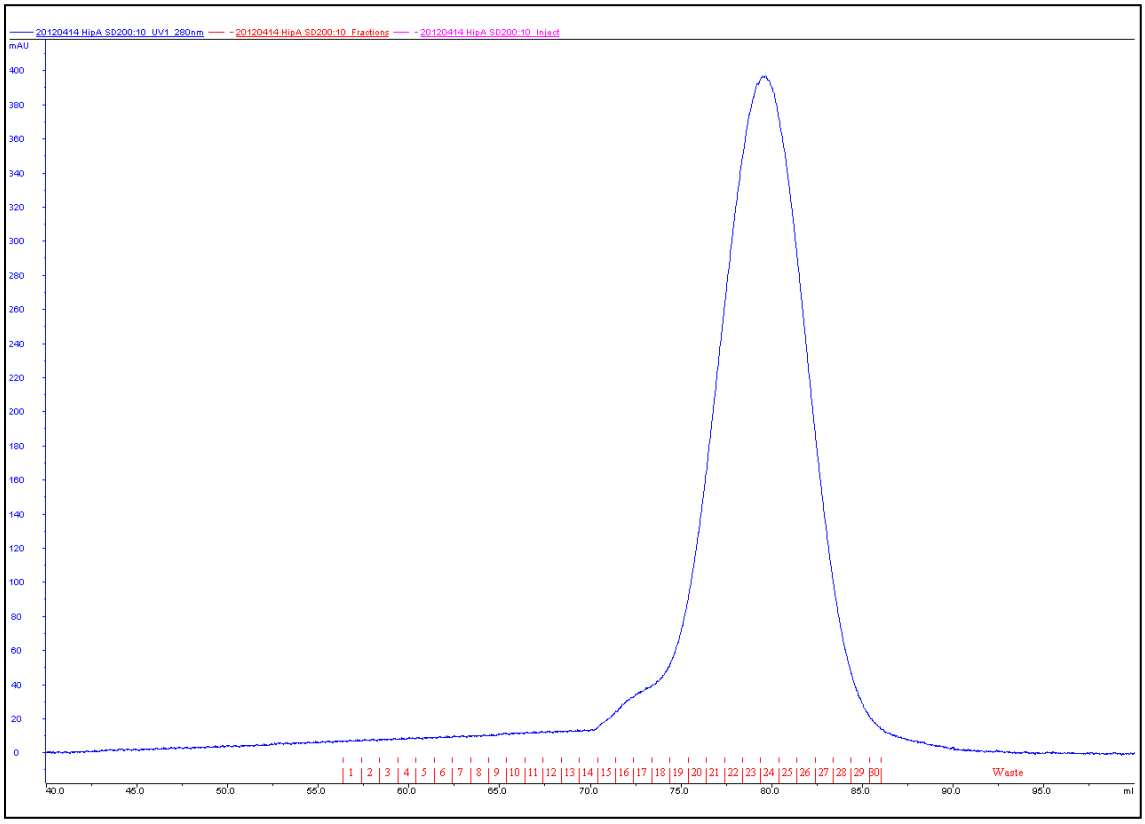

Figure 17 - HipA purification using the SD200 column by SEC. The protein is eluted at approximately 75mL. The blue line is the absorbance at 280nm, the red lines are the collected fractions.

After injection of 2mL of protein, a sharp peak in SEC purification was obtained (Figure 17), resulting in a good resolution chromatogram which allows a clear view of the protein from the main fractions collected and analyzed by SDS-PAGE. In order to confirm the purity of HipA, an SDS-PAGE was performed using the fractions 22-24 (Figure 18), which are corresponding to the highest absorbance from the SEC purification.

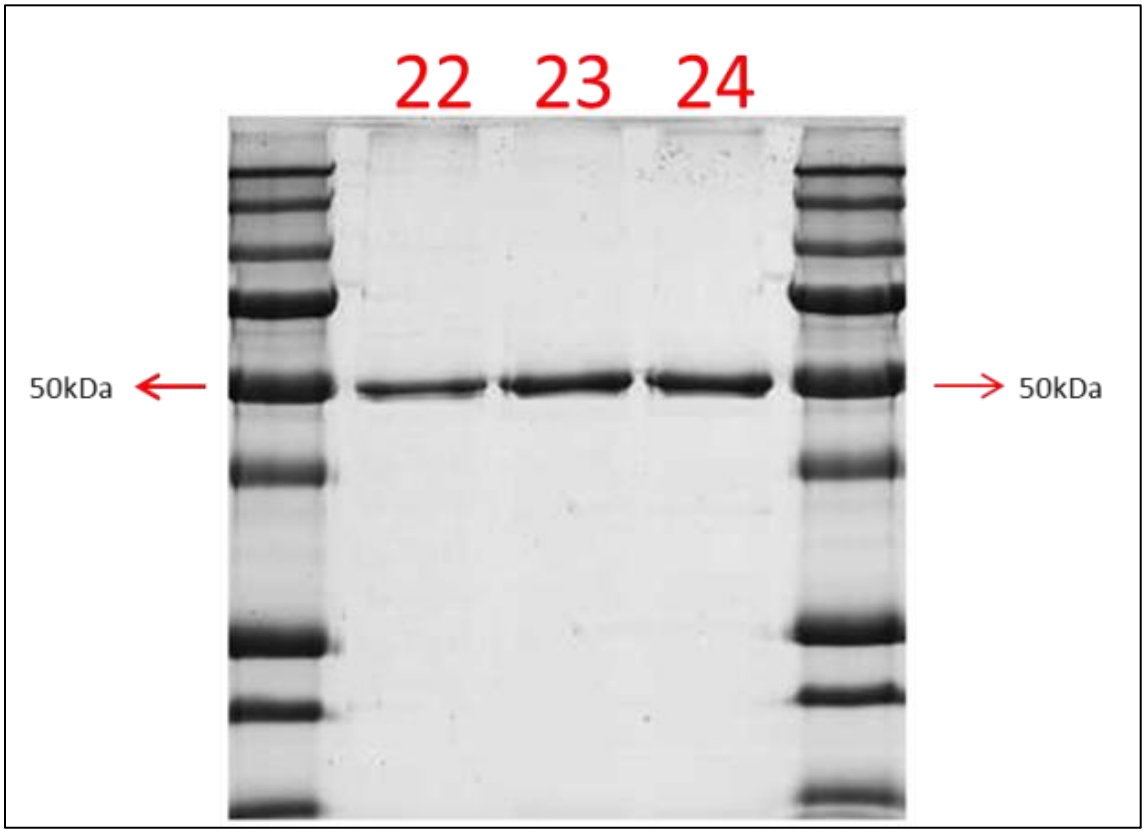

Figure 18 - HipA purification fraction analysis by SDS-PAGE.

3.1.2.3. Generation of phosphorylated HipA (pHipA)

HipA is a toxin whose effects depend on its phosphorylation level, being also responsible for the phosphorylation of other molecules, such as EF-Tu studied in *E. coli* (Schumacher et al., 2009), as explained in section 1.3.3.3. For that reason it became important to study the phosphorylated and non-phosphorylated HipA. Similar studies were performed in *E. coli*, mainly to understand the role of autophosphorylation in HipA (Hansen et al., 2012). So, after purifying HipA with Ni-NTA, the protein was separated in two aliquots. One aliquot was treated with ATP-Mg^{2+} and the other aliquot was used for SEC purification, this allowed us to obtain the phosphorylated HipA (Figure 19) and HipA. The addition of ATP-Mg^{2+} will result in complete phosphorylation of HipA (Schumacher et al., 2009). Proteins are biomolecules that differ greatly in size from salts and other small molecules, so high molecular substances are excluded from

the medium and elute first. The HipA will phosphorylate forming the higher molecular substance from the medium and thus eluting first, being separated from other phosphate salts. The smaller molecules with lower molecular weight enter the pores from the medium Sephadex, eluting later from the Hiprep 26/10 desalting column.

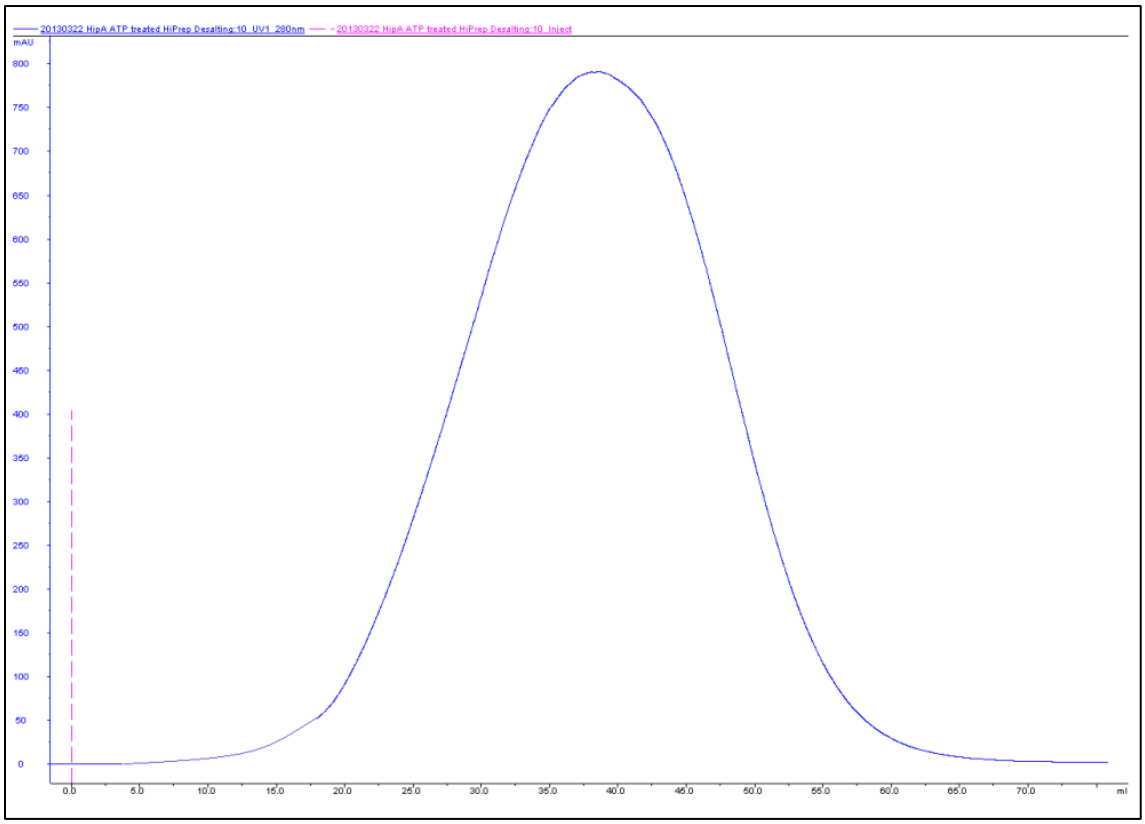

Figure 19 - pHipA desalting separation after treatment with ATP and Mg2+ using a Hiprep 26/10 desalting column. The blue line is the absorbance at 280nm.

The phosphorylated HipA was used for cross linking studies, to understand the alterations in the protein conformation and the protein-protein interactions after cross linking.

3.1.2.4. Generation of non-phosphorylated HipA (npHipA)

One aliquot of pure protein was used to perform the non-phosphorylated studies. In the aliquot, phosphatase was added which will cleave any phosphate groups bound to HipA. Similarly to the phosphorylated HipA, the non-phosphorylated HipA was also separated with a Hiprep 26/10 desalting column, to separate the protein from the phosphate salts and other smaller molecules (Figure 20).

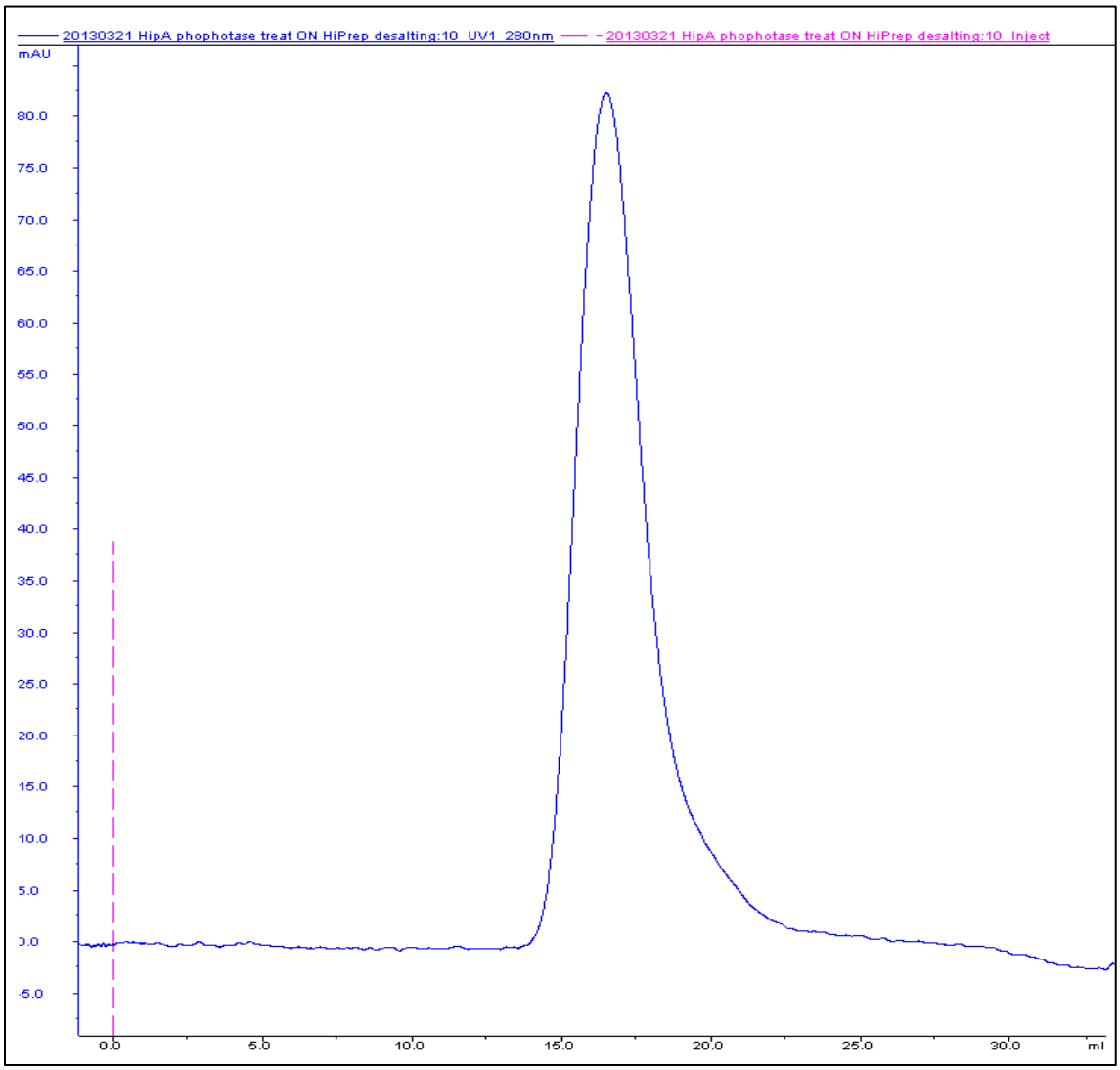

Figure 20 - npHipA desalting separation after treatment with phosphatase using a Hiprep 26/10 desalting column. The blue line is the absorbance at 280nm.

3.1.2.5. HipB purification – IMAC and SEC

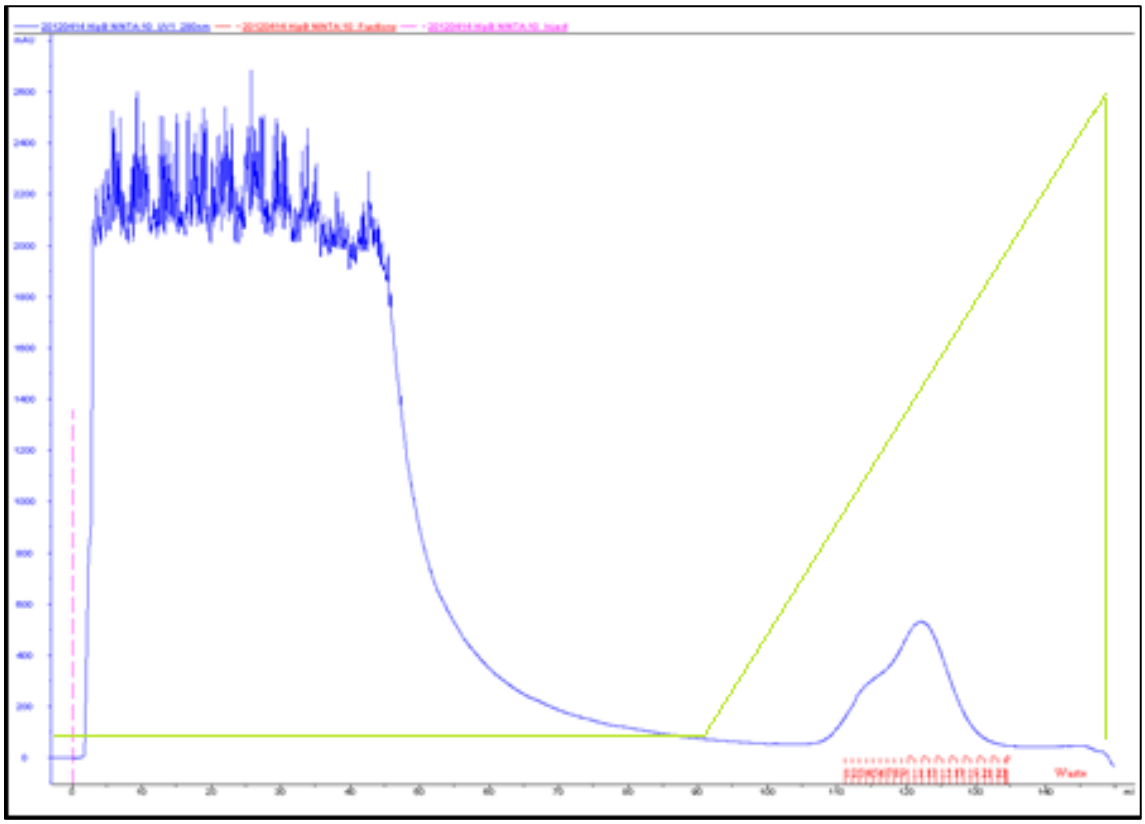

Figure 21 - HipB purification using the Ni-NTA column by the IMAC principles. The protein is eluted when a imidazole gradient is applied. The green line represents the concentration of imidazole in the buffer; the blue line represents the sample absorption at 280nm.

The HipB protein is more stable as a dimer, having two binding sites, one for HipA and one for DNA (Schumacher et al., 2009). However, for the binding to HipA which occurs at the *N*-terminal, the His-tag needs to be cleaved. The His-tag can be cleaved with thrombin (Gräslund et al., 2008). To study the interactions of HipB as a dimer and as a complex with HipA, after IMAC purification thrombin was added and the cleavage was performed overnight. After the cleavage HipB was separated by SEC (Figure 22).

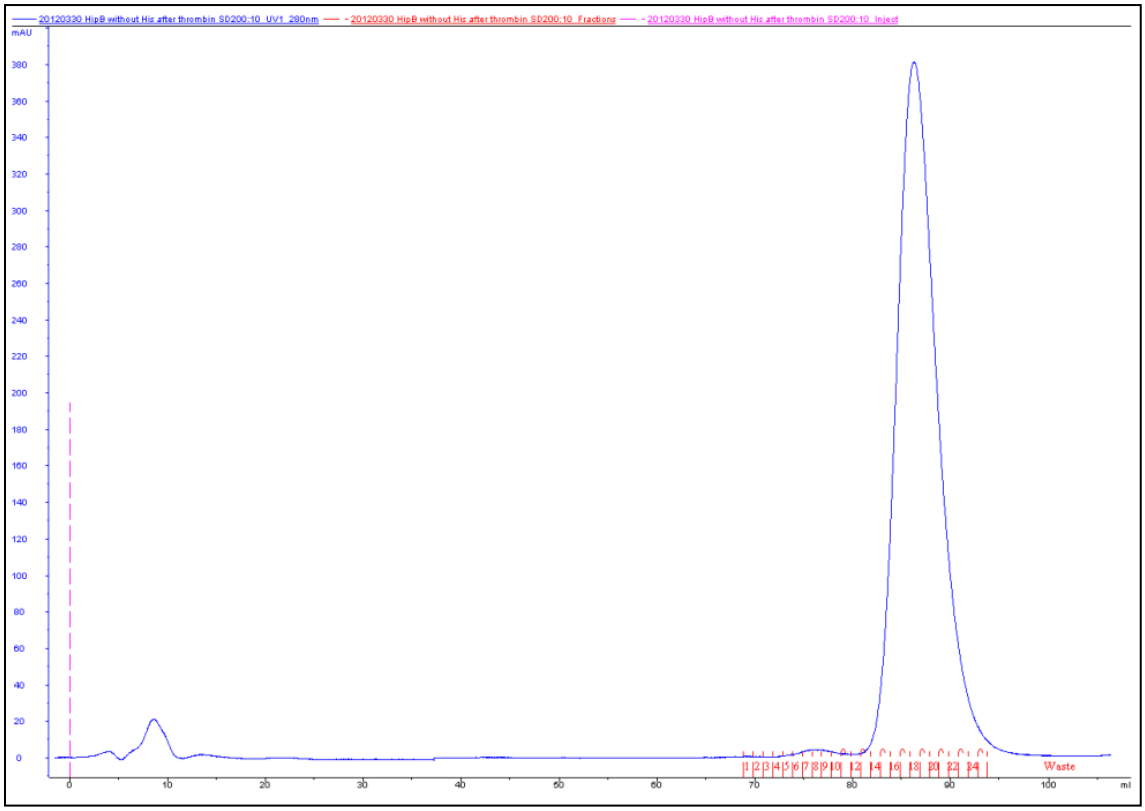

Figure 22 - HipB purification using the SD200 column by the SEC principles. The protein is eluted around 85mL. The blue line is the absorbance at 280nm.

3.1.2.6. HipA(D306Q) purification analyses

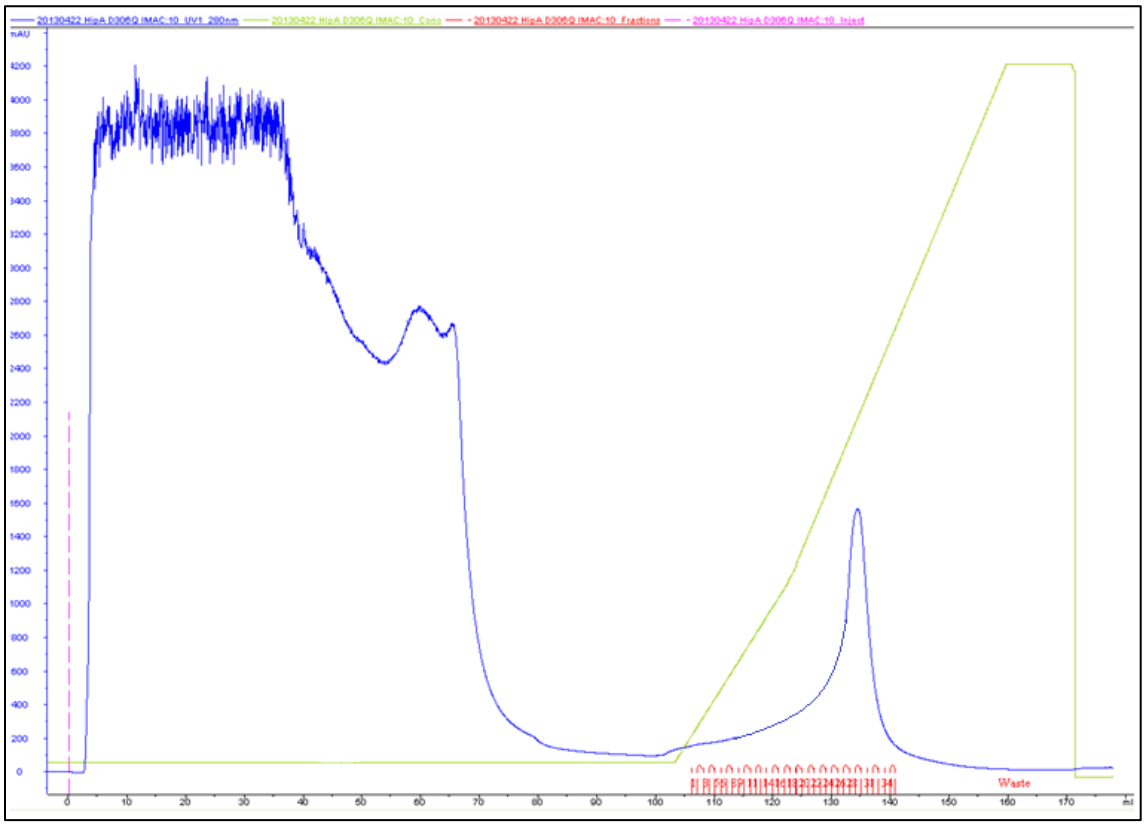

Figure 23 - HipA(D306Q) purification using the Ni-NTA column by IMAC. The protein is eluted when an imidazole gradient is applied. The green line represents the concentration of imidazole in the buffer; the blue line represents the sample absorption at 280 nm, and the red lines are referent to the collected fractions.

As studied in *E. coli* the mutant HipA(D309Q) is not high persistent, in contrast to the wild type HipA. The HipA and the HipA(D396Q) allow the study and comparison from the ATP binding, using isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR) (Schumacher et al., 2009). So the same procedures were adopted for HipA(D306Q) purification, was purified by SEC (Figure 24). However, further studies were carried on with the HipA(D306Q) by the PhD student Yurong Wen.

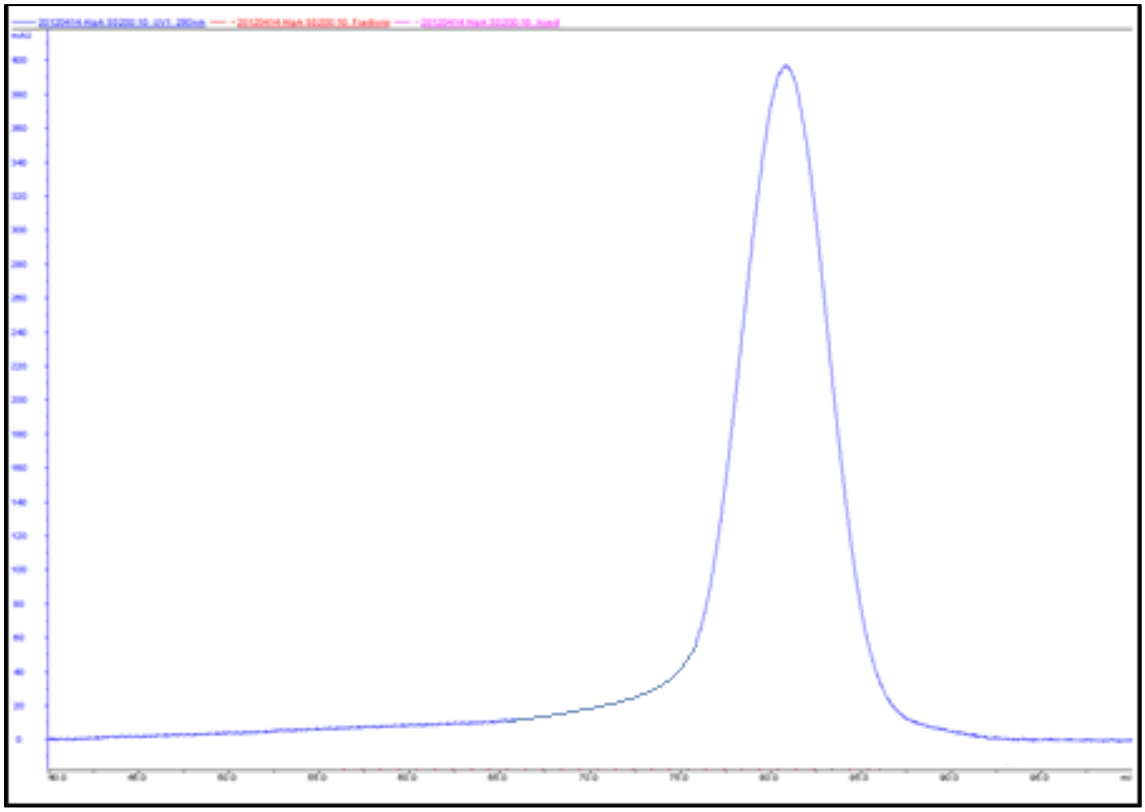

Figure 24 - HipA(D306Q) purification using the SD200 column by SEC. The protein is eluted around 75mL. The blue line is the absorbance at 280nm.

3.2. Cross linking studies

Bis[sulfosuccinimidyl] suberate (BS_3) is a homobifunctional *N*-hydroxysuccinimide ester (NHS ester) and is water soluble. BS_3 reacts with primary amino groups ($-NH_2$) to form stable amide bonds releasing *N*-hydroxysuccinimide, at a pH range from 7 to 9. HipA and HipB proteins have several primary amine groups in the side chain of lysine (K) residues and in the *N*-terminal of each peptide. The BS_3 cross linker has to be prepared immediately before use and is soluble only up to 100mM.

In *E. coli* the main studies focused in the study and characterization of HipA, HipB and its interaction, no further studies as cross linking studies are known for HipAB. However, structural analysis helped to understand how HipA interacts with HipB, in *E. coli*, which was then found out recently to be similar in *S. oneidensis* by Yurong Wen. Another major finding in *E. coli*

studies is the autophosphorylation of HipA which was also carried on by PhD Yurong Wen in *S. oneidensis*, being again similar.

Cross linking studies were performed to further find out which were the interactions between proteins, the phosphorylated and non-phosphorylated, HipA and HipB. Validation experiments were performed to understand which was the best cross linker concentration.

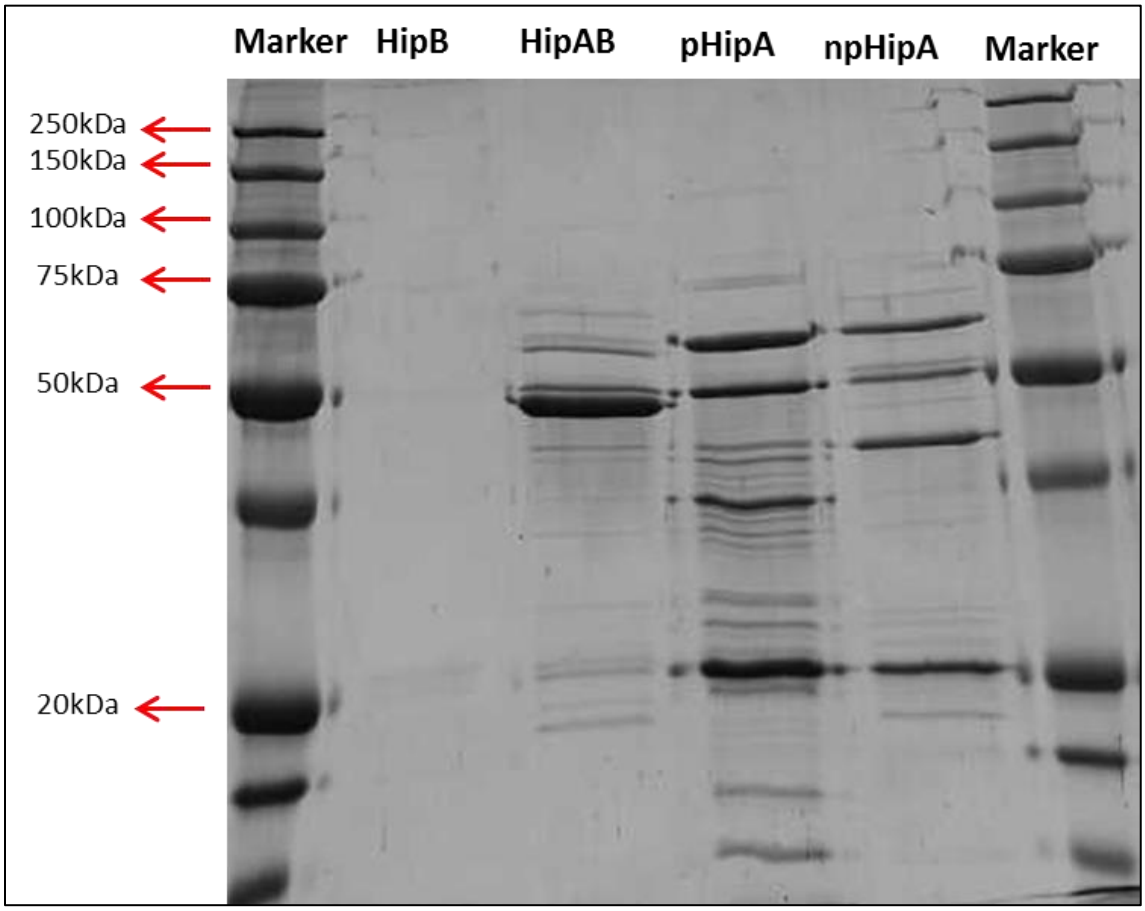

Figure 25 - SDS-PAGE showing the four controls of HipB, HipAB, pHipA and npHipA.

Analysing the SDS-PAGE control gel (Figure 25) shows that the 50kDa bands corresponding to HipA, a 51kDa protein. However, as previously explained in section 3.1.1, HipB is difficult to find in SDS-PAGE analysis and again was not found. Therefore, for further studies a MS analysis should be performed to allow to detect the presence of certain proteins.

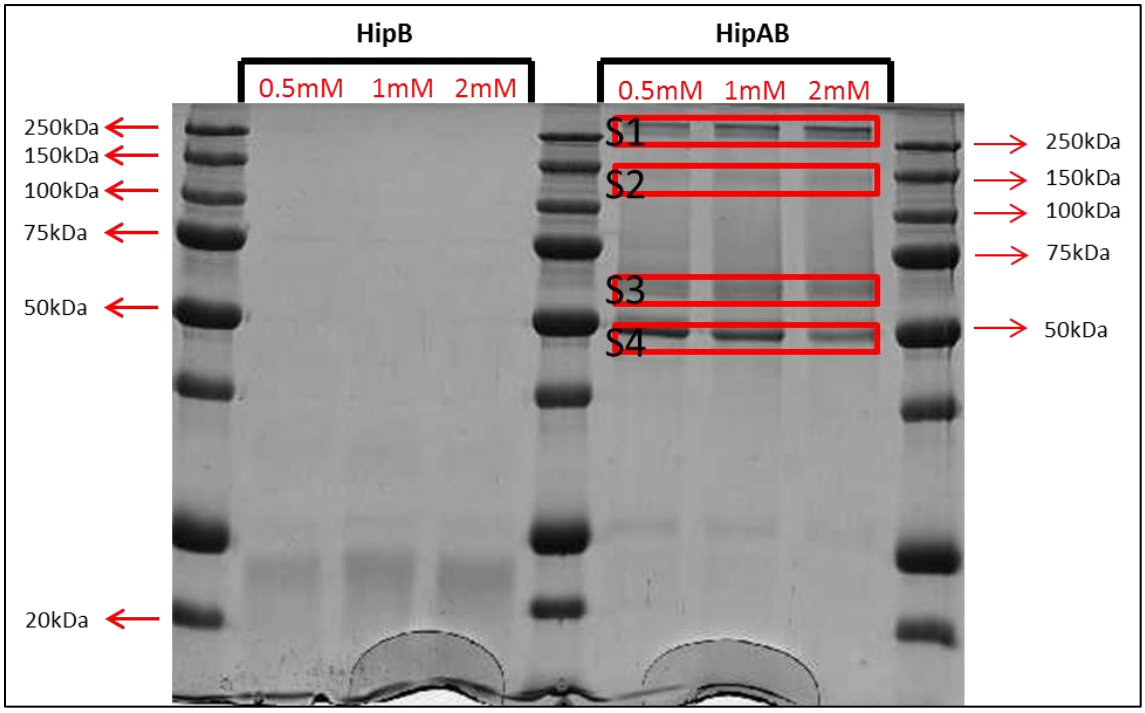

Figure 26 - SDS-PAGE showing the cross linking reactions of HipB and HipAB. Under each protein name are the cross linker, BS3, concentrations used. The bands numbered S1-S4 were further analysed by MS.

We performed a cross linking experiment with HipB. However, analysis of the SDS-PAGE pattern (Figure 26) did not allow to conclude about HipB multimerization. This lack of reaction can be related with the small size of the protein without the His-tag, around 10kDa or the used protease inhibitor may not be efficient in HipB case. The protease inhibitor may be critical to maintaining yield, structure and function.

The HipAB cross linking reaction, using different BS_3 concentrations, different products could be observed (Figure 26). The more HipA that reacts, the more effective the cross linker will be, leading to the conclusion that the 2mM cross linker is more efficient that the other two cross linker concentrations, 0.5mM and 1mM.

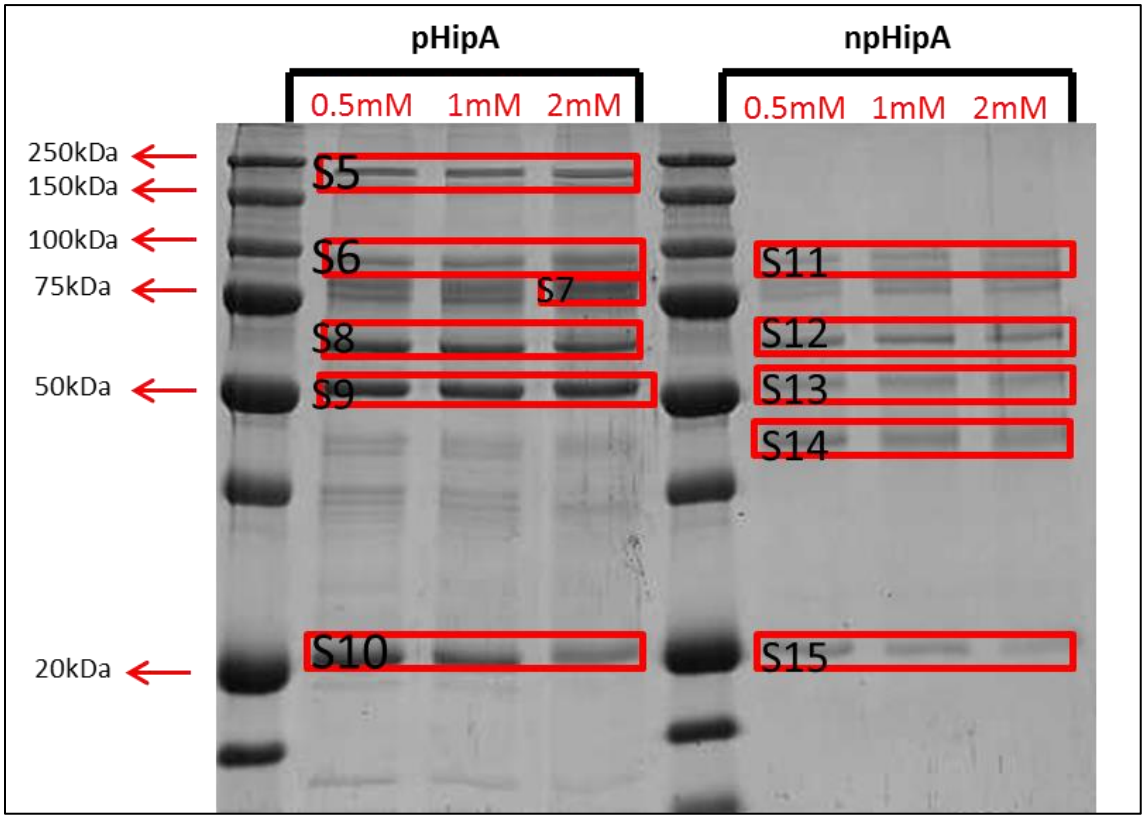

Figure 27 - SDS-PAGE showing the cross linking reactions of HipB and HipAB. Under each protein name are the cross linker, BS3, concentrations used. The cut bands are numbered S5-S15.

The HipA and HipB have 24 and 7 lysine residues, respectively. The cross link reactions the bounding between the lysine residues, intermolecular or intramolecular. Where the cross linker binds there will be an increase of 158Da. There were some predictions carried using PyMOL, to analyse some possible cross linked insights on HipB (Figure 28), HipA (Figure 29) and HipAB (Figure 30).

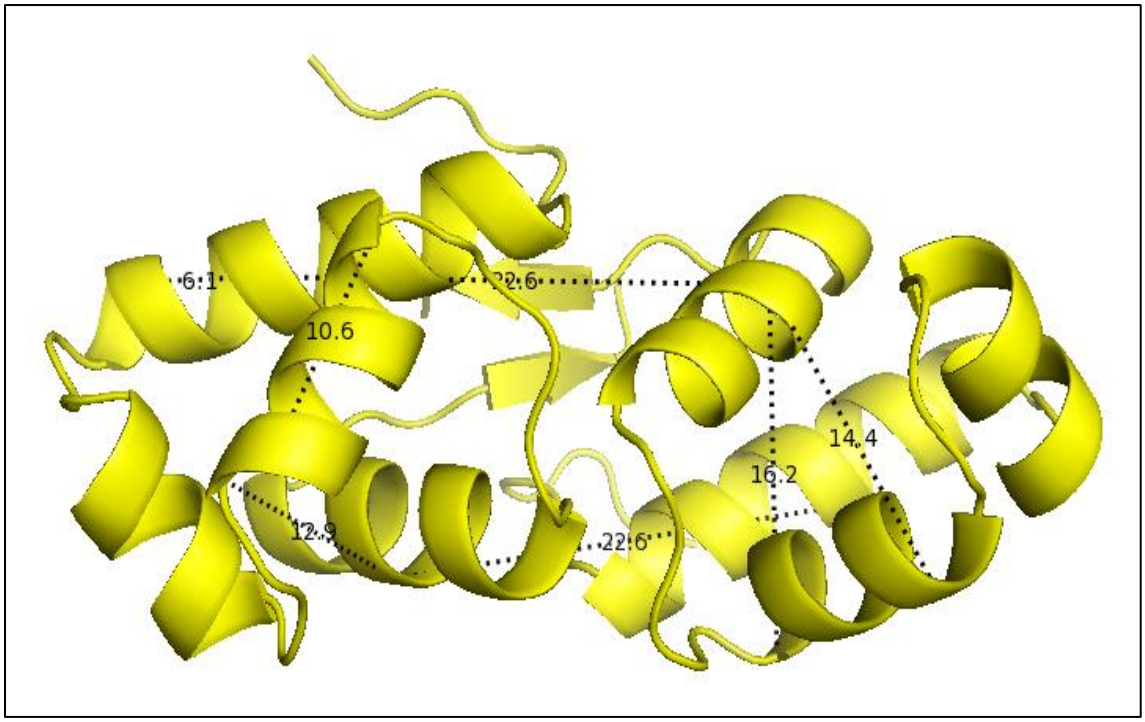

Figure 28 - HipB dimer with some predicted intramolecular cross linking bonds. Dashed black lines indicate the calculated distance (Å) between the two Cα Lysines.

Within HipB 7 predicted binding Lysines are at a distance smaller than 30Å, so it is possible that the cross linking reaction occurs, since the calculated distances range from 6.1-22.6Å.

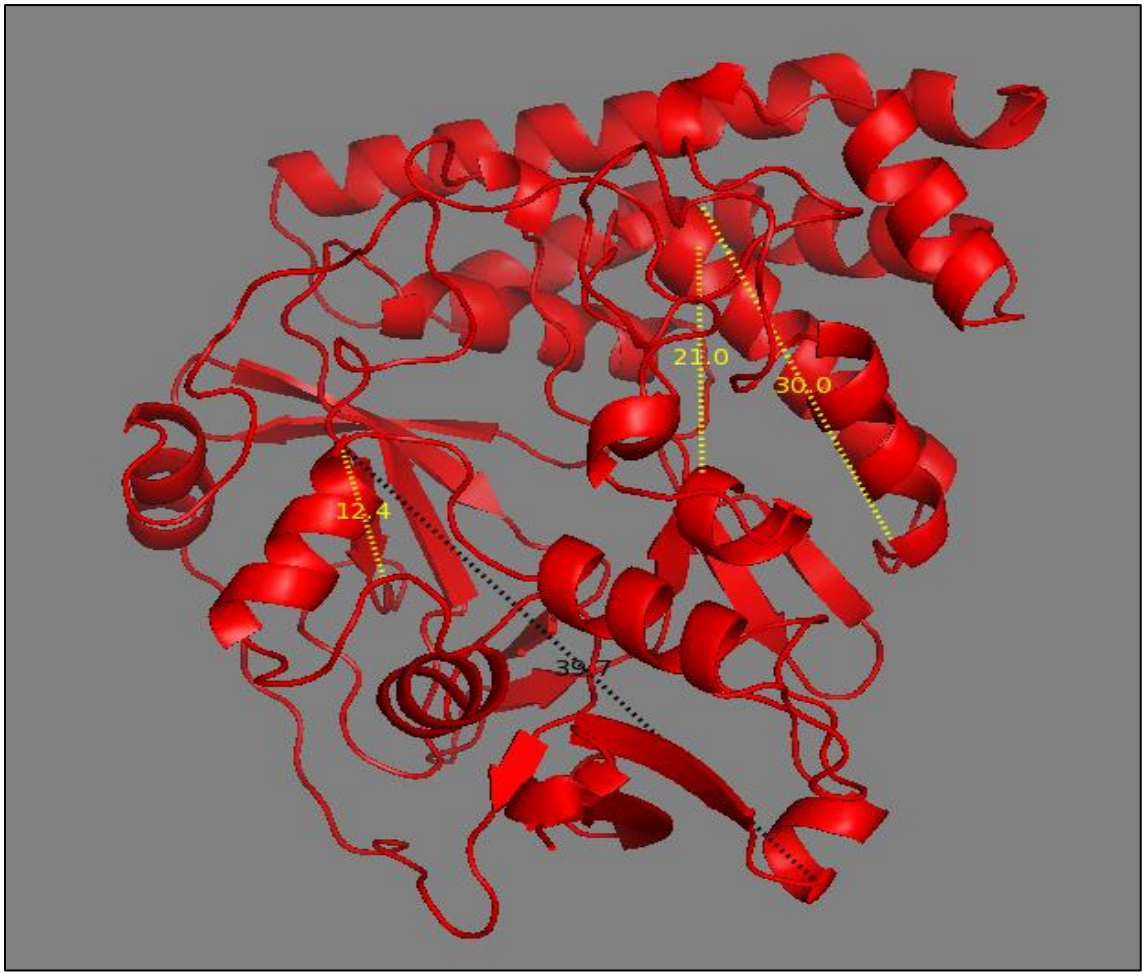

Figure 29 - HipA with some predicted intramolecular cross linking bonds. Dashed black lines indicate the calculated distance (Å) between the two Cα Lysines. The black dashed line refers to one predicted bond which has fewer possibilities to occur because it is bigger than 30Å, the maximum distance to have a bond between different Cα Lysine residues.

Within HipA 3 predicted binding Lysines are at a distance smaller than 30Å, so it is possible that the cross linking reaction occurs, since the calculated distances range from 12.4-30Å.

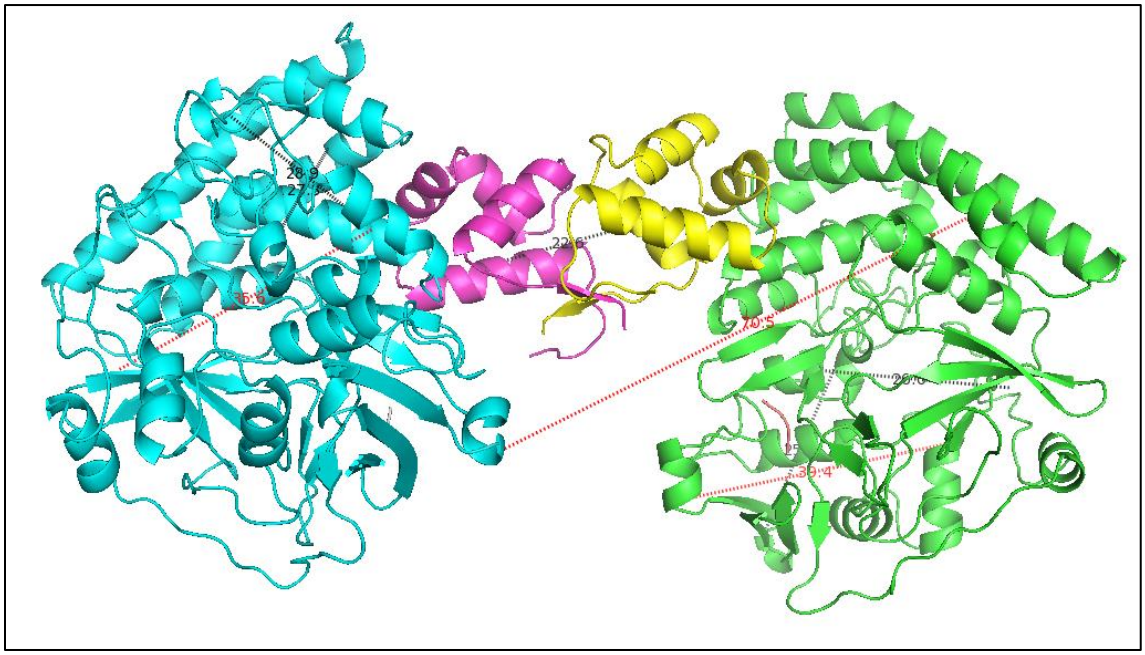

Figure 30 - HipA with some predicted intramolecular cross linking bonds. Dashed black lines indicate the calculated distance (Å) between the two Cα Lysines. The black dashed line refers to one predicted bond which has fewer possibilities to occur because it is bigger than 30Å, the maximum distance to have a bond between different Cα Lysine residues.

Within HipAB 5 predicted binding Lysines are at a distance smaller than 30Å, so it is possible that the cross linking reaction occurs, since the calculated distances range from 20.6-28.9Å.

The SDS-PAGE pattern (Figures 26 and 27) shows some evidence for cross linking reactions from the HipAB, pHipA and npHipA bands. We observed that the band corresponding to HipA was still present. However, as the cross linker concentration was increasing these bands became less intense, leading to the conclusion that the higher cross linker concentration allows efficient cross linking. There was a strong evidence of cross linking in the different samples, as determined by the SDS-PAGE analysis. After the *in gel* digestion and LC-MS analysis it was possible to detect evidence of toxin-antitoxin in the samples as well as other proteins. However, in some bands we observed some *E. coli* proteins, indicating that the proteins purifications were not complete.

3.2.1. pLink Search

To understand which intermolecular and intramolecular interactions exist in the proteins, pHipA, npHipA and HipAB, we used software package for data analysis of cross linked proteins coupled with mass-spectrometry analysis, pLink. pLink is a software that uses MS/MS data and the protein sequences as input (table 2) and outputs the spectra with the best score (Figure 31), identifying the cross linked peptides as well as the peptides with the cross linker. pLink is associated with a pBuild software which gives the report identification. In the report identification it is specified which is the proteins interact and which are the lysine positions.

Table 2 – Proteins identified by Mascot Daemon search in NCBI databases. pLink input information was the name of the protein and FASTA sequence. Table describes which is the organism referent to each protein and in which samples the protein was present (Figures 25 and 26).

Protein	Organism	Sample Bands	FASTA Sequence
HipA	*Shewanella oneidensis*	S1, S2, S3,S4, S5, S13, S14,S15	MSTAKTLTLEMHLGDLMIGELSFDATADTFAVHYTKDWQQSGFPLSPT IPLDGTGTSNQISMFLVLLPENKGLDYLIESLGVSKGNTFALIRAIGLDTA GAIAFVPKGALLPETQLRPIKAEEVIQRIEDPTMWPMEIWDGKPRLSVA GVQPKLNLFYNGKEFAFAEGTLSSTHIVKFEKYHHLVINEFITMRLAKVL GMNVANVDIVHFGRYKALCVERFDRRNIPGEQRVLRRHIVDSCQALGF SVSKKYERNFGTGRDVKDIREGVSFNRLFSLAAKCRNPVAAKQDMLQ WALFNLLTGNADAHGKNYSFFMTPSGMEPTPWYDLVSVDMYEDFEQ QLAMAIDDEFDPNSIYAYQLAAFMDGLGLPRNLLISNLTRIARRIPQAIA EVILMLPPLDEDEASFVAHYKTQLLARCERYLGFVDEVRDVEV
HipB	*Shewanella oneidensis*	S1, S2, S3,S4	MNGTDIKAKVYEDTLLETIMASPLNQQSLGLLIKERRKSAALTQDVAAM LCGVTKKTLIRVEKGEDVYISTVFKILDGLGIDIVSAQTSDTETNGWY
Galactitol-1-phosphate dehydrogenase	*Escherichia coli*	S5	MKSVVNDTDGIVRVAESVIPEIKHQDEVRVKIASSGLCGSDLPRIFKNGA HYYPITLGHEFSGYIDAVGSGVDDLHPGDAVACVPLLPCFTCPECLKGFY SQCAKYDFIGSRRDGGFAEYIVVKRKNVFALPTDMPIEDGAFIEPITVGL HAFHLAQGCENKNVIIIGAGTIGLLAIQCAVALGAKSVTAIDISSEKLALA KSFGAMQTFNSSEMSAPQMQSVLRELRFNQLILETAGVPQTVELAVEI AGPHAQLALVGTLHQDLHLTSATFGKILRKELTVIGSWMNYSSPWPGQ EWETASRLLTERKLSLEPLIAHRGSFESFAQAVRDIARNAMPGKVLLIP
Trehalose-6-phosphate	*Escherichia*	S6, S8, S11, S12	MTNLPHWWQNGVIYQIYPKSFQDTTGSGTGDLRGVIQRLDYLHKLGV DAIWLTPFYISPQVDNGYDVANYTAIDPIYGTLDDFDELVTQAKSRGIRII LDMVFNHTSTQHAWFREALNKESPYRQFYIWRDGEPETPPNNWRSK

hydrolase	*coli*		FGGSAWRWHAESEQYYLHLFAPEQADLNWENPAVRAELKKVCEFWA DRGVDGLRLDVVNLISKDPRFPEDLDGDGRRFYTDGPRAHEFLHEMN RDVFTPRGLMTVGEMSSTSLEHCQRYAALTGSELSMTFNFHHLKVDYP GGEKWTLAKPDFVALKTLFRHWQQGMHNVAWNALFWCNHDQPRI VSRFGDEGEYRVPAAKMLAMVLHGMQGTPYIYQGEEIGMTNPHFTRI TDYRDVESLNMFAELRNDGRDANELLAILASKSRDNSRTPMQWSNGD NAGFTAGEPWIGLGDNYQQINVEAALADDSSVFYTYQKLIALRKQEAIL TWGNYQDLLPNSPVLWCYRREWKGQTLLVIANLSREIQPWQPGQMR GNWQLVMHNYEEASPQPCAMTLRPFEAVWWLQK
Thioredoxin	*Escherichia coli*	S6, S11, S15	MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADE YQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVASATKVGALSKG QLKEFLDANLA
Glutamyl-tRNA synthase	*Escherichia coli*	S9, S13	MKIKTRFAPSPTGYLHVGGARTALYSWLFARNHGGEFVLRIEDTDLERS TPEAIEAIMDGMNWLSLEWDEGPYFQTKRFDRYNAVIDQMLEEGTAY KCYCSKERLEALREEQMAKGEKPRYDGRCRHSHEHHADDEPCVVRFA NPQEGSVVFDDQIRGPIEFSNQELDDLIIRRTDGSPTYNFCVVVDDWD MEITHVIRGEDHINNTPRQINILKALKAPVPVYAHVSMINGDDGKKLSK RHGAVSVMQYRDDGYLPEALLNYLVRLGWSHGDQEIFTREEMIKYFTL NAVSKSASAFNTDKLLWLNHHYINALPPEYVATHLQWHIEQENIDTRN GPQLADLVKLLGERCKTLKEMAQSCRYFYEDFAEFDADAAKKHLRPVA RQPLEVVRDKLAAITDWTAENVHHAIQATADELEVGMGKVGMPLRV AVTGAGQSPALDVTVHAIGKTRSIERINKALAFIAERENQQ
GTP-cyclohydrogenase-1	*Escherichia coli*	S7	MPSLSKEAALVHEALVARGLETPLRPPVHEMDNETRKSLIAGHMTEIM QLLNLDLADDSLMETPHRIAKMYVDEIFSGLDYANFPKITLIENKMKVD EMVTVRDITLTSTCEHHFVTIDGKATVAYIPKDSVIGLSKINRIVQFFAQR PQVQERLTQQILIALQTLLGTNNVAVSIDAVHYCVKARGIRDATSATTTT SLGGLFKSSQNTRHEFLRAVRHHN
FKBP	*Escherichia coli*	S10	MKVAKDLVVSLAYQVRTEDGVLVDESPVSAPLDYLHGHGSLISGLETAL EGHEVGDKFDVAVGANDAYGQYDENLVQRVPKDVFMGVDELQVGM RFLAETDQGPVPVEITAVEDDHVVVDGNHMLAGQNLKFNVEVVAIRE ATEEELAHGHVHGAHDHHHDHDHDGCCGGHGHDHGHEHGGEGCC GGKGNGGCGCH

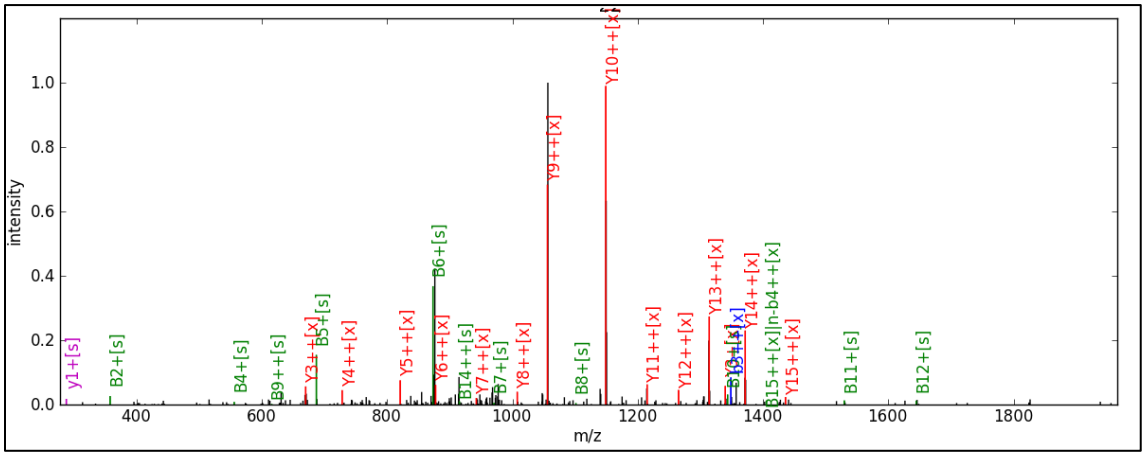

Figure 31 - Spectra labeling output resulting from pLink search. Different MS/MS comparisons which resulted in this spectrum. Different peptides (Y, B) cross linked (x) or with the cross linker (s).

The pLink search was followed by a spectrum labelling (Figure 31) and an identification report (table 3) which allows to conclude about the protein and the lysine residue that cross linked.

Table 3 - Cross linked positions of each sample with the score and binding proteins with lysine positions.

Samples	Score	Proteins (positions)
S1	**$1.27e^{-002}$**	**HipA(143) – HipA(264)**
S5	$9.74e^{-001}$	Glutamyl-tRNA synthase(1) – FKBP(1)
S6	$8.95e^{-001}$	HipA(281) – FKBP(1)
S7	$2.30e^{-001}$	Trehalose-6-phosphate hydrolase(144) – HipA(252)
S7	$7.94e^{-001}$	Glutamyl-tRNA synthase(352) – Thioredoxin(1)
S8	$4.31e^{-001}$	Galactitol-1-phosphate dehydrogenase(2) – Thioredoxin(1)
S10	$8.63e^{-001}$	HipA(264) – Glutamyl-tRNA synthase(378)
S14	$1.46e^{-001}$	Glutamyl-tRNA synthase(4) – Glutamyl-tRNA synthase(352)
S14	$1.93e^{-001}$	HipA(281) – FKBP(1)

The identification report from S1 had a score of $1.27e^{-002}$ that is relatively high, meaning the analysed peptides were significantly with that cross linked positions. S1 compared with the other samples, S5, S6, S7, S8, S10, S14, being the probability of cross linking reduced, which is indicating possible false positives.

Therefore, was just possible to identify one intramolecular binding from HipA(143) and HipA(264) shown in Figure 32.

Figure 32 - HipA with one intramolecular cross linking bond. Dashed white line with the value that is the calculated distance (Å) between the two Cα Lysines.

4. Conclusion

The HipAB complex is deeply studied in *E. coli*, but little is known of its structure and function in *S. oneidensis*. Both bacteria are good models to study the phenomenon of persistence, being the subject of on-going research. In this study, the main aim was to analyse and study protein interactions, overcoming the technical limitations. To better understand this aim some predictions of conformational alterations were made to give a better overview of the different interactions, stressing that may be due to those alterations that the main functions of the proteins are altered.

Mimicking what happens in bacterial cells, cross linking reactions were performed, using BS_3 an in solution cross linker. BS_3 can be used in different concentrations, being more or less efficient in cross linking, therefore three different concentrations were used 0.5mM, 1mM and 2mM. After the data analysis we concluded that the better cross linking efficiency was obtained with a higher concentration. Furthermore, analysis of the cross linking reactions, with LC-MS followed by Mascot Daemon research using NCBI database, allowed to confirm the presence of the HipA and HipB in the different samples. However for better results some improvements are suggested avoiding the increase of temperature to denature the sample for SDS-PAGE, larger volume injection in the LC-MS study, to have a larger response for MS analysis. In addition, LC-MS data was used for analysis with pLink. This allows to identify where the cross linking reactions happen (which protein, which lysine residue). After identification of the lysine residue involved in cross linking, it is possible to calculate the distance between the bonds, using PyMOL. Therefore, this analysis allowed to confirm the bond from HipA(143) with HipA(264) with a distance of 17.8Å.

For further studies, my suggestion is to repeat the protocol with the improvements and additionally apply using native mass spectrometry, which can give a better overview from the complexes resulting from the cross linking reactions.

5. Materials and Methods

5.1. Chemicals

The following reagents were obtained from Sigma-Aldrich: carbenicillin (Cb), disodium phosphate, sodium chloride, imidazole, HEPES, isopropyl β-D-1-thiogalactopyranoside (IPTG) and glycerol. The reagents for SDS-PAGE (SDS, APS, TEMED) were obtained: from Bio-RAD. All the solvents used were obtained from VWR and were HPLC grade. All solutions were prepared and filtered using ultra-purified water (Millipore) from Milli-Q system.

5.2. Instrumental

MALDI-TOF analyses were carried out on a 4800 Plus MALDI-TOF analyser (AB SCIEX). SDS-PAGE analyses were performed using a Mini-Protean 3 cell electrophoresis system from Bio-Rad.

Protein purification was carried out on an AKTA Purifier (GE Healthcare). A nickel-nitrilotriacetic acid (Ni-NTA) superflow cartridge from Qiagen and a Superdex 200 10/300 GL column (GE Healthcare) were used.

For His-tagged protein purification, the Ni-NTA column was used due to its characteristics: deliver of high yields of high-purity protein, up to 50mg/mL and its high stability, which means that they are compatible with a wide range of buffer components, including strong denaturants, detergents and reducing agents. The affinity of Ni^{2+} ions is independent on the protein structure (native or denatured) (http://www.qiagen.com/Products/Catalog/Sample-Technologies/Protein-Sample-Technologies/Purification-Kits-and-Resins/Ni-NTA-Superflow-Cartridges#orderinginformation, 2013).

The SD200 column is a high performance gel filtration column. This is a pre-packed glass column intended for use in gel filtration of proteins, peptides and other biomolecules according to size. The gel matrix is composed of Superdex, which is based on highly cross-linked agarose beads covalently bonded to dextran molecules. The gel filtration properties of the superdex matrix are governed mainly by the dextran chains. The SD200 10/300 GL column has a mean bead size of

13 µm and the fractionation range for the globular proteins is 3 000 – 600 000 Da (http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/pt/GELifeSciences/products/AlternativeProductStructure_17414/17517501, 2012).

5.3. Software

MALDI-TOF samples were analysed using the 4000 Series Explorer software with available databases tools (Mascot from Matrix Science). The Mascot database software package makes the search in the NCBI database. Another software package used was pLink, a programme which allows the study of the cross linking positions with resource to MS/MS and protein sequence data.

5.4. Methods

5.4.1. Expression of HipA/HipB

Each gene (*HipA*, *HipB and HipA(D306Q)*) was cloned into *BamHI* and *NdeI* cloning sites of pET15b vector and fused with an *N*-terminal His-tag. The vector was then introduced into *E. coli* C43 (DE3) competent cells, by electroporation. Cells were cultured in 1% LB (Lysogeny Broth) medium supplemented with carbenicillin 0.04% (v/v) and incubated at 37ºC for 3h at 200rpm. At the optical density (O. D.) at 600nm for HipA (and the mutant HipA(D306Q)) and HipB was 0.7 and 0.6 respectively. When the O. D. 0.7 and 0.6 was reached, isopropyl β-D-1-thiogalactopyranoside (final concentration 1mM, IPTG) was added and the culture was allowed to grow for 3 hours at 37ºC. The culture was centrifuged at 10 000rpm for 5 minutes at 4°C (Beckman Coulter Avanti J30 I centrifuge). The supernatant generated was discarded and the pellet was either stored at -20°C or used for purification.

5.4.1.1. Protein Overexpression

For evaluation of *HipA* and *HipB* overexpression, the O.D. was measured as described above and 1mM IPTG was added to the samples. In order to control the growth of the bacterial cells, 1mL of sample was taken and the O.D. measured every 60 minutes for the following 3 hours. Samples (1mL) were centrifuged at 25 000rpm for 7 minutes at 4°C using a Beckman Coulter Avanti J30 I centrifuge. The supernatant was discarded and the protein pellet was resuspended in ultra-pure water and analysed by SDS-PAGE. Overexpressed protein bands were cut and digested overnight with 0.1µg/µL trypsin. The samples were then analysed by mass spectrometry (MALDI-TOF).

5.4.1.1.1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-Page was performed according to instructions of the manufacturer (www.bio-rad.com, 2000). Bellow a detailed description of the procedure is made.

1. **Resolving gel**

A 15% (w/v) polyacrylamide gel was prepared using 10mL Tris-HCl pH 8.8, 10mL ProtoGel 30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1 ratio), 100µL 20% SDS and 10µL N,N,N'N'-tetramethylethylene diamine (TEMED) made up to 15mL with double distillated water (ddH_2O). The polymerization was initiated by the addition of 200µL 0.1mg/mL ammonium persulphate (APS). The solution was gently poured into the space between the two glass plates to about 3cm from the top of the short glass plate. To ensure a uniform interface between the stacking and resolving gels, about 1mL ddH_2O was gently overlaid on top and set for 1 hour at room temperature.

2. **Stacking gel**

A 6% (w/v) polyacrylamide gel was prepared using 676µL 1M Tris-HCl pH 6.8, 867µL ProtoGel 30% (w/v) acrylamide/methylene bisacrylamide solution (37.5:1 ratio), 75µL 20% SDS, 7.5µL

N,N,N'N'-tetramethylethylene diamine (TEMED) made up to 10.5mL with ddH2O. The polymerization was initiated by the addition of 150µL 0.1mg/mL APS.

The stacking gel was immediately added on top of the resolving gel and the appropriate comb (10 wells; 5mm x 1mm or 15 wells; 3 mm x 1mm) positioned. The stacking gel was allowed to polymerize for 30 min.

After polymerization, the gel plates were removed from the cast and fitted in the cassette. The gel cassette was introduced into the buffer reservoir tank, and the tank was filled with electrode buffer [25mM Tris-HCl, pH 8.2, 250mM glycine and 0.1% (w/v) SDS] to cover the cassette.

3. Sample preparation

For preparation of protein samples, 10µL 6x SDS-PAGE loading dye buffer (360mM Tris-HCl, pH 6.8, 60% (v/v) glycerol, 12% (w/v) SDS and 0.06% (w/v) bromophenol blue) and 1µL 1M DTT were made up to 30µL with ddH_2O. The mixture was then heated for 5 minutes at 95°C and further centrifuged at 14,000*g* for 15s to collect any condensed vapour and to sediment any insoluble materials.

4. SDS-PAGE electrophoresis

The gel was electrophoresed at 130volts. Once the bromophenol blue dye front reached the bottom of the gel, the run was stopped and the gel was stained with Coomassie Brilliant Blue G250 overnight and destained with 30% methanol.

5.4.1.1.2. *In-gel* digestion and peptides extraction for MALDI-TOF analyses

1. Destaining of the gel

After destaining the gel, the bands with the corresponding molecular weight of HipA (and the muntant HipA(D306Q)) and HipB were cut from the gel and placed into a 1.5mL vial in small slices. The gel pieces were washed with 150µL of 50% ACN/200mM ammonium bicarbonate to each vial and incubated for 20min at 30ºC. The gel bands were vortexed and centrifuged at 14

000 rpm for 3 minutes, 4ºC (Eppendorf centrifuge 5415R), to remove unpolymerized acrylamide and remaining Coomassie. The destaining procedure was performed two more times with 120µL and 100µL of 50% ACN/200mM ammonium bicarbonate solution as described above. The destained gel pieces were dried in a speedvac for 10 minutes.

2. *In-gel* Digestion

In-gel gel digestion was performed by adding trypsin in 50mM ammonia bicarbonate, to each sample (0.002µg/µL final concentration). Reaction was kept on ice for 45 minutes, to allow the gel pieces to rehydrate. Forty µL of 50mM ammonium bicarbonate were added to each piece and the bands were incubated overnight at a 37ºC.

3. Extraction of Peptides

The digested gel bands were centrifuged at 14 000rpm, 4ºC for 30 seconds (Eppendorf centrifuge 5415R), and the supernatants transferred to new 0.5mL vials. Peptides were extracted by adding 40µL of 60% ACN/0.1% HCOOH to each gel piece and incubated at 30ºC for 20 minutes. The samples were then agitated in a vortex for 3 minutes, centrifuged and combined with the supernatant to the corresponding 0.5mL vial. The extraction step was repeated once, by adding 20µL of 60% ACN/0.1%HCOOH. The peptide solution in the 0.5mL vial was dried in a speedvac for approximately 6 hours.

4. MALDI-TOF sample and matrix preparation

The dried peptides were dissolved in 10µL of 0.1% HCOOH and 0.8µL of this solution were spotted on the MALDI-TOF plate. The plate was allowed to dry for a few minutes. The matrix was prepared by dissolving α-cyano-4-hydroxycinnamic acid in 50% ACN, 0.1% TFA (5mg/mL). The matrix (0.8 µL) was spotted on the plate on top of the spotted peptides and allowed to dry.

α-cyano-4-hydroxycinnamic acid matrix was chosen for analysing this proteins and peptides, because it is particularly good for molecules smaller than 10kDa.

5.4.1.2. Purification of HipA/HipB

The protein pellet, from the point 1.4.1., was thawed and resuspended in buffer (25mM Na_2HPO_4, 500mM NaCl), with a free EDTA using a ratio of 1g pellet/25mL extraction buffer. For purification, the cell walls were lysed mechanically using sonication. During sonication, high intensity ultrasound is used to break the cell walls and release the soluble cytosolic recombinant protein. The protein suspension was sonicated for 4 minutes with amplitude of 20%, in a cycle of 1 second pulse, and 1 second break, using a Branson 200 and 400 watt sonicator. The lysate was centrifuged at 25 000 rpm for 30 min at 4 °C (Beckman Coulter Avanti J30 I centrifuge). The supernatant, containing the target protein, was filtered (0.22µL membrane filters) and purified.

The affinity chromatography is based on IMAC using a Ni-NTA resin. The resin was equilibrated in 25mM Na_2HPO_4, 500mM NaCl, pH of 7.5. The sample was injected into the column and eluted with a gradient of 0-100% of 500mM imidazole in equilibration buffer (pH 7.5) was used. The imidazole competes with the metal from the resin allowing the protein to be eluted from the column. After having the protein separated by affinity, size-exclusion chromatography was performed. In order to use HipB for the cross linking experiments it was necessary to cleave the His-tag terminal with thrombin, overnight (Zhu et al., 2010). After the cleavage overnight from the His-tag, HipB was separated a second time by IMAC to have the purified protein.

Size-exclusion chromatography was carried out with a Superdex 200 (GE Healthcare) resin. The buffer used was 10mM HEPES, 300mM NaCl, 5% glycerol at pH 7.5. A concentrated sample from the previous purification (2mL) was applied to the column and eluted with 10mM HEPES, 50mM NaCl at a pH of 7.5. The HipA is 51kDa and HipB is 10kDa, so the predicted elution volume of these proteins were determined based on the elution volumes of a calibration kit on HiLoad 16/60 Superdex 200 pg column (Figure 12 and table 1).

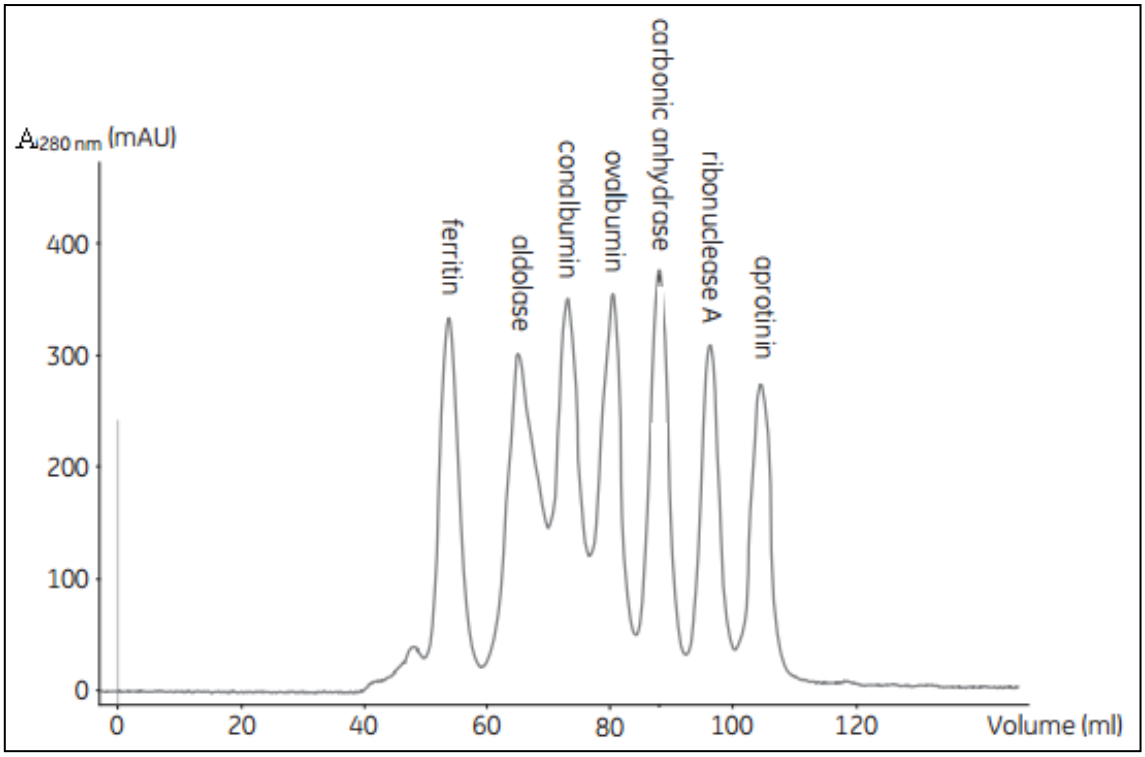

Figure 33 - Elution profile of the protein calibration kit on HiLoad 16/600 Superdex 200 pg column. Elution volumes were determined at peak maximum height (Healthcare, 2006).

Table 4 - The content of Gel Filtration Calibration kit LMW (low molecular weight) (Healthcare, 2006).

Protein	**Molecular weight (kDa)**
Aprotinin	6500
Ribonuclease A	13 700
Carbonic anhydrase	29 000
Ovalbumin	44 000
Conalbumin	75 000

By comparing the molecular weight and the elution volume it was possible to determine that, at the conditions used, HipA had an elution volume of approximated 80mL and HipB of

approximated 100mL. After purification an SDS-PAGE analysis was performed to see the purified proteins.

5.4.1.3. Phosphorylation studies

5.4.1.3.1. Phosphorylated HipA

The phosphorylation studies were carried out using the purified HipA protein. HipA was autophosphorylated overnight with a final concentration of 1mM ATP, pH 7.5. The HipA phosphorylates forming a higher molecular molecule from the medium and smaller molecules with lower molecular weight which are separated by Hiprep 26/10 desalting column, where the small molecules enter the pores from the medium Sephadex, eluting later from the thus eluting first the phosphorylated HipA.

5.4.1.3.2. Non-phosphorylated HipA

The non-phosphorylated HipA was obtained by treating the HipA with 1mM of lambda protein phosphatase, from BioLabs, overnight. The separation of the non-phosphorylated protein was also done with a Hiprep 26/10 desalting column.

5.4.1.4. Protein cross linking studies

For the cross linking experiments the proteins used were the phosphorylated HipA, the non-phosphorylated HipA, HipB and the complex HipAB. The cross linker was added in excess to the protein samples. Two mg of BS_3 were dissolved in water. After dissolving BS_3 the solution was diluted into a final concentration of 100mM. For samples that had a protein concentration lower than 5mg/mL, a 20 to 50-fold molar excess of BS_3 was used. The final concentrations of BS_3 for the different proteins used were: 0mM (control), 0.50; 1.00; 2.00mM. This was performed to check which was the ideal concentration. Reactions were carried out at room temperature for 35 minutes. The reaction was stopped by adding 50mM Tris left to incubate 15 minutes at room temperature. The 15 different protein samples were analysed by SDS-PAGE as

described in 4.4.1.1.1. Expressed proteins bands were cut and digested overnight with 0.1µg/µL trypsin as described in 4.4.1.1.2. without the point 4 because the samples were then analysed by mass spectrometry (LC-ESI-FT-ICR).

1. **LC-ESI-FT-ICR sample preparation**

LC-MS was performed according to Chesini (Chesini et al., 2011). The dried peptides were dissolved in 50µL of 0.1% formic acid. For the LC-MS experiments the buffer used was 0.1% of formic acid and were injected 5µL of each sample was analysed. Peptides were first separated on an Agilent 1200 chromatographic system (Agilent, Santa Clara, CA, USA) and on-line measured on a LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific, Wal-tham, MA, USA). The samples were first loaded and desalted on a (5mm x 0.3mm) Zorbax 300SB-C18 trapping column at a 4µl/min flow rate using a 2% (v/v) acetonitrile, 0.1% formic acid buffer, and then separated on a (150mm x 75µm) Zorbax 300SB-C18 analytical column (Agilent) by a 50min linear gradient ranging from 2% (v/v) to 80% (v/v) acetonitrile, 0.1% formic acid at a 0.3µl/min flow rate. The LC-effluent was directly coupled to a Triversa NanoMate ESI source (Advion, Ithaca, NY, USA), working in nano-LC mode and equipped with a D-chip whereon a 1.7kV voltage was supplied. During the LC-separation the FT-ICR mass analyser acquired MS scans at 100,000 resolution, the 3 most intense precursor peptides for each MS scan were automatically selected and fragmented by the LTQ ion trap mass analyser.

- **Mass spectrometry analysis**

The MS and MS/MS data were used for research in Mascot Daemon, where the research was made using NCBI database.

- **pLink research**

The pLink research was made using the MS/MS data and the protein sequence information, outputting the cross linked peptides in a pBuild software support and a spectrum identification from the MS/MS matching.

6. References

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7. Attachments

7.1. Expression of HipA/HipB

- Carbenicillin (Cb) - Antibiotic

C = 250mM

m = 2.5g

10mL MQ water

- Use 40µL/100mL

(save 2mL in eppendorfs at -20ºC)

- IPTG (Isopropyl β-D-1-thiogalactopyranoside)

m = 2,38g IPTG (freeze)

10mL MQ water and dissolve

1. **Preculture**

LB	Cb	Proteins
20mL	5µL	20µL or use from colonies (with a tip)
40mL	10µL	40µL or use from colonies (with a tip)
100mL	25µL	100mL or use from colonies (with a tip)
200mL	50µL	20mL or use from colonies (with a tip)

LB and Cb are in a dilution of (1:4)

GROW OVER NIGHT at 37ºC (for maximum 60h!)

2. **Culture**

LB	Cb	Preculture		IPTG (-20ºC)
50mL	20µL	0,5mL		50µL

1L	400µL	10mL		1mL
2L	800µL	20mL		2mL

WAIT 3H measure OD (next step) and ADD IPTG (dilution of 1:100)!

3. **Optic Density at 600nm**

- Before adding IPTG:

  1mL around 0.6 for HipB and 0.7 for HipA

- After adding IPTG:

Time	Quantity	OD
1H	1mL	Should increase
2H	1mL	
3H	1mL	

7.2. Protein Overexpression

1. **Samples Preparation for SDS-PAGE**

1mL is collected before adding IPTG and after adding IPTG, 1H/1H

- Centrifuge samples for 5mins – separate pallet
- Resuspend supernatant with MQ water:
  - A = 2,00 add 100µL
  - A = 1,00 add 50µL
- Take 10µL from the sample and add 10µL loading buffer to the resuspended samples
- Leave 5mins at 95ºC
- 20µL sample and 15µL marker
- Run gel for 45mins-1h30
- Add Coomassie brilliant blue R-250, leave overnight
- Distain the gel with 30% methanol

2. **In-gel digestion of proteins (from SDS-PAGE):**

- Always were gloves!!
- Always keep solutions closed to prevent contamination of dust.

Day 1

a. Wash (rinse) the gel with water to remove dust particles
b. Cut out the protein of interest and transfer it into an 1,5mL vial in small slashes (for a protein band use a scalpel, for a 2-PAGE protein spot use a spot picker)
c. Wash the gel pieces by adding 150µL of 50% ACN/200mM ammonia bicarbonate to vial and incubate for 20min at 30ºC. Vortex and centrifuge (to remove unpolymerized acrylamide and other contaminants, such as Coomassie, which may give a higher background during MS measurement)

TIP: see the final volume that is needed and add for example: 1mL ACN and 1mL of ammonia bicarbonate (NH_4HCO_3)

d. Do the wash step in c. until the coomassie dye has disappeared
e. Dry gelpieces in speedvac (they will turn smaller and white)
f. Add 10µL diluted trypsin (50 x dilution of stock = 0,1µg/µL trypsin in 50mM ammonia bicarbonate)

TIP: first prepare the ammonia bicarbonate, for example, for a total volume of 2mL, use 250mL of 400mM with 1750 of MQ water.

Dilute 1µL of trypsin (keep trypsin in ice) in 49µL of your 50mM ammonia bicarbonate

g. Keep your solution on ice for 45mins
h. Add 40µL of 50mM ammonia bicarbonate (gel pieces need to be submerged)
i. Digestion overnight at 37ºC bath

Day 2

a. Centrifuge the tube and transfer supernatants (solution containing peptides) to newly labeled 0,5mL vial
b. Extract the peptides by adding 40µL of 60% ACN/0,1% HCOOH to the gel piece and incubate at 30ºC for 20min (the amount can be increased if the gel piece isn't entirely covered in liquid)

TIP: For a final volume of 1mL pipe 600µL of ACN and 400µL of HCOOH

c. Vortex for 3min, centrifuge and combine the supernatant to the corresponding 0,5mL vial (which already contains peptides)
d. Repeat the extraction step, by adding 20µL of 60% ACN/0,1%HCOOH
e. Dry the peptide solution in the speedvac
f. Dissolve the peptides in 10µL of 0,1% HCOOH (if you will not analyse the peptides immediately, better not dissolve them before storing at -20ºC)

3. **Preparing the matrix:**

a. Always set the correct matrix in the acquisition method (α-cyano, DHB)
b. 5mg/mL matrix, 50% ACN, 0,1% TFA

TIP: Total volume – 1mL which is 1000µL so is 500µL of ACN, 1µL of TFA and 499µL of MQ water

If sample doesn't dry fast enough (this will lead to bad crystallization) use 70% ACN.

7.3. Purification of HipA/HipB

1. **Immobilized Metal Affinity Chromatography**

Buffer 1 (500mL) at pH = 7.5:

25mM Na_2HPO_4

Mw = 141.96g/mol

m = 3.54g

500mM NaCl

Mw = 58.443g/mol

m = 29.2215g

Buffer 2 (200mL) at pH = 7.5:

25mM Na_2HPO_4

Mw = 141.96g/mol

m = 3.549g

500mM NaCl

Mw = 58.443g/mol

m = 29.2215g

500mM Imidazole

Mw = 68.077g/mol

m = 6.80775g

7.4. Cross linking studies

Samples	Protein Quantity	Cross Linker (25mM)			Tris (1M)
		0.5mM	1mM	2mM	
Phosphorylated HipA	50µL	1µL	2µL	4µL	50µL
Non-phosphorylated HipA	50µL	1µL	2µL	4µL	50µL
HipB	50µL	1µL	2µL	4µL	50µL

HipAB	50µL	1µL	2µL	4µL	50µL

- BS_3 preparation:

  Dissolve 2mg in 140µL of water

- Gel preparation:

  25µL of sample + 10µL loading buffer