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Sortase mediated ligation.

Lligament mediat per sortases.

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Nada en este mundo debe ser temido... solo entendido. Ahora es el momento de comprender más, para que podamos temer menos.

Marie Curie

Aquesta memòria no hagués estat possible sense la dedicació, saviesa i ajuda del meu tutor Dr. Miquel Pons.

Gràcies al suport, força i paciència de la meva família durant aquests quatre anys de viatge que han desembocat en aquest treball. M'agradaria dedicar-lo especialment a la meva mare; la dona més forta, intel·ligent, valenta i pacient que m'ha acompanyat en tot moment i ha sabut donar-me l'empenta quan més ho he necessitat. Per abocar-me tanta tranquil·litat, no rendir-te mai i estimar-me incondicionalment; gràcies mama, sense tu tot el que he aconseguit no hagués sigut possible.

REPORT

CONTENTS

1. SUMMARY

Sortases are transpeptidases (enzymes) that covalently anchor proteins to the peptidoglycan of Gram-positive bacteria's cell wall. They recognise and cleave a pentapeptide motif within the cell wall sorting signal (CWSS) of their target substrates. Afterwards, they covalently link it to a N-terminal amino group or lysine side chain forming a peptide or isopeptide bond, respectively; resulting in their attachment to the bacteria cell wall. The most studied and characterized sortase is class A sortase from *Staphylococcus aureus* (Sa-SrtA). This sortase cleave the C-terminal LPXTG (with X being any aminoacid) pentapeptide motif of their target substrate between their Thr-Gly bond. Then, a cross-linked oligoglycine called lipid-II is covalently linked via peptide bond to the protein substrate. Finally the resulting substrate-lipid-II is anchored to the peptidoglycan of the bacteria cell.

Sortases have a specific His-Cys-Arg catalytic triad in their active site. If this triad is properly charged, sortases are able to catalyse a cysteine transpeptidase mechanism that enable them to develop their physiological functions; anchoring surface proteins to the bacteria cell wall, assembling and polymerization of pilin subunits and anchoring iron acquisition proteins to the cell envelope are some examples. Although there are different classes of sortases (A-F), all of them perform their catalysis following the similar mechanism.

Sortases play an important role in infections and pathogenesis as most of the proteins they bind to the cell wall are virulent. It has been emphasized the finding of sortase inhibitors in order to avoid the infection course of these enzymes.

In addition, this transpeptidase reaction called Sortase Mediated Ligation (SML) has led to many applications involving anchoring proteins of interest, cell to cell ligations, immunologic treatments and reengineering of protein structures, as well as industrial applications.

Keywords: Sortases, transpeptidase, peptidoglycan, Gram-positive, CWSS, LPXTG, lipid-II

2. RESUM

Les sortases són transpeptidases (enzims) que uneixen covalentment proteïnes al peptidoglicà de la paret cel·lular dels bacteris Gram-positius. Aquestes reconeixen i separen el motiu pentapeptídic dins de la Senyal d'Inserció a la Paret Cel·lular (SIPC) dels seus substrats diana. A continuació, els uneixen covalentment a un grup amino N-terminal o a una cadena lateral de lisina formant un enllaç peptídic o isopeptídic, respectivament; resultant en el seu acoblament en la paret cel·lular. La sortasa més estudiada i caracteritzada és la sortasa de classe A del *Staphylococcus aureus* (Sa-SrtA). Aquesta sortasa talla el motiu pentapeptídic C-terminal LPXTG (sent X qualsevol aminoàcid) del seu substrat diana entre l'enllaç Thr-Gly. Seguidament, una oligoglicina entrecreuada anomenada lípid-II s'uneix covalentment mitjançant l'enllaç peptídic al substrat proteic. Finalment, el substrat-lípid-II resultant, s'ancora al peptidoglicà de la cèl·lula bacteriana.

Les sortases tenen una tríada catalítica de His-Cys-Arg específica en el seu lloc actiu. Si aquesta tríada està carregada correctament, les sortases són capaces de catalitzar un mecanisme de cisteïna transpeptidasa que els permet desenvolupar les seves funcions fisiològiques. L'ancoratge de proteïnes superficials a la paret cel·lular dels bacteris, el muntatge i la polimerització de subunitats de pili i les proteïnes d'adquisició de ferro a l'embolcall cel·lular són alguns dels exemples. Tot i que hi ha diferents classes de sortases (A-F), totes elles realitzen la seva catàlisi seguint un mecanisme similar.

Les sortases tenen un paper important en les infeccions i la patogènesi ja que la majoria de les proteïnes que s'uneixen a la paret cel·lular són virulentes. S'ha prioritzat la recerca d'inhibidors de sortases per tal d'evitar el procés d'infecció d'aquests enzims.

A més, aquesta reacció transpeptídica anomenada Lligació Mediada per Sortases (LMS), ha permès el desenvolupament de moltes aplicacions que inclouen l'ancoratge de proteïnes d'interès, els lligaments entre cèl·lules, els tractaments immunològics i la reenginyeria d'estructures proteiques, així com aplicacions industrials.

Paraules clau: Sortases, transpeptidasa, peptidoglicà, Gram-positiva, LPXTG, lípid-II

3. INTRODUCTION

The bacterium cell wall forms an envelope outside the cell membrane that protects and gives rigidity to the cell surface and controls the interaction with their surroundings. The basic structure of the cell wall is a network formed by long sugar chains, an alternate sequence of N-acetyl-glucosamine (NAG) and N-acetylmuramic (NAM) bonded through β-1,4 links, crosslinked by peptide bridges that have a fixed part containing D-aminoacids and a species-specific bridge that, in the case of *Staphylococcus aureus*, is a pentaglycine chain. This composition receives the name of peptidoglycan (1).

Despite the cell wall change among species, its structure allows to classify bacterium cells in two types: Gram-positive and Gram-negative. The main difference between them is on the cell wall composition: Gram-positive has a wide cell wall that surrounds the periplasmic space and the plasma membrane and Gram-negative, on the other side, contains an outer membrane and two periplasmic spaces that surrounds the cell wall. Besides, its peptidoglycan's cell wall is much thinner (2).

Sortases are transpeptidases that covalently link proteins to the peptidoglycan's cell wall. They are mostly found in Gram-positive bacterium and, in a lesser extent, in Gram-negative. One of the reasons of this preference is because of peptidoglycan's availability in Gram-positive bacteria. In Gram-positive cells, the secretory pathway that bacteria displays to export its proteins outside of the cell and link them to the peptidoglycan afterwards is more favoured than in the Gram-negatives; due to its bigger outer peptidoglycan layer and the absence of the outer membrane.

Once sortases have linked the proteins to the peptidoglycan, the cell is capable to interact with the environment in different ways. "Sorting" or "Sortagging" are the words used to describe this anchoring function that sortases display between proteins and the cell wall.

The proteins linked by sortases are obtained in the bacteria cytoplasm, carried through the cytoplasm membrane and covalently bonded to the peptidoglycan of the cell wall. This proteins can be recognized by sortase enzymes because they contain a cell wall sorting signal (CWSS) in their C-terminus formed by a pentapeptide recognition motif, a hydrophobic zone and a cationic tail constituted by lysine and arginine residues (3). Sortases develop a transpeptidation reaction in order to link this protein substrate to an amino nucleophile group. All sortases contain a catalytic triad formed by His, Cys and Arg where cysteine plays an essential role (4).

Sortases have some structural and functional traits in common that enable them to play a similar role in their interactions with the proteins that they link to the cell wall. One example is the recognition of the LPXTG-like pentapeptide in their protein substrate, where the Thr is almost invariable among target proteins. This significant characteristic with a properly charged His-Cys-Arg triad allows sortases to start their transpeptidation reaction properly.

The activity of sortase enzyme was discovered in the early 1990s by Schneewind and his co-workers (5). Among the great variety of sortases, the most studied and best known is sortase A of *Staphylococcus aureus* (Sa-SrtA), which in 1999 was the first sortase isolated (6).

The proteins on the cell surface of bacteria are mainly virulence factors and many of them are linked to the cell wall by sortases, hence its essential role in pathogenesis. Sortases inhibitors can be used in order to avoid the virulence function.

This powerful linking enzyme has led to many promising applications. Sortases function has been tested in many fields in which they can be displayed as antibacterial targets, anti-infective agents and microbiological tools. They can be also re-engineered to take advantage of their transpeptidase activity to, for example, re-engineer antibodies. Sortases present a great potential for novel therapeutics and have also been studied to play significant roles in industrial applications.

4. CLASSIFICATION OF SORTASES

4.1. GENERAL FEATURES

Sortases were divided and classified extensively throw the years. The most significant properties to highlight are sortase's cell wall sorting signal (CWSS), their physiological and biological behaviour and their structure. If these characteristics are taken as a criteria, sortases can be divided in four major classes, A to D, and two less-common classes, E and F:

- **Sortase A (SrtA):** SrtA enzymes are known as "housekeeping" sortases. They are mostly found in a large number of Gram-positive bacteria and are responsible for the covalently linking of many different proteins to the peptidoglycan cell wall.
- **Sortase B (SrtB):** These type of sortase enzymes play a role in iron homeostasis by anchoring iron acquisition proteins to the cell envelope. Their main function is the linking of a unique substrate called IsdC. This substrate is expressed by SrtB in conditions where iron is scarce. Unlike sortase A, class B sortases are only presented in a few bacteria.
- **Sortase C (SrtC):** They are responsible for the pilus assembly of pili subunits to the cell wall envelope. They display this polymerization in some Gram-positive bacteria.
- **Sortase D (SrtD):** They are responsible to the sporulation in Gram-positive bacteria.
- **Sortase E (SrtE) and F (SrtF):** Class E and F sortases are the newest discovered. It is thought that they play a role on pili polymerization even though their functions still to be determined. Interestingly, they are mostly found in Actinobacteria.

In the following table, it can be appreciated sortases' classification according to their substrate recognition motif, their Guanine-Cytosine ratio (GC rate) and the group of bacteria in which they are mostly found.

Sortase class	Substrate recognition motif	Substrates	Main function	GC rate	Species
A	LPXTG	Surface proteins	Cell wall anchoring	Low	Many Gram-positive bacteria
B	NP(Q/K)TN	Haem transport factor	Pilus polymerization, Haem uptake	Low	Gram-positive Bacilli and cocci, Listeria, *S. aureus*
C	(I/L)(P/A)XTG*	Pilin proteins	Pilus polymerization	Low and High	Gram-positive bacteria like Actinomyces, corynebacteria, Enterococci, Streptococci
D	LPNTA	Endospore envelope proteins	Spore formation	-	Gram-positive bacteria like Bacilus species
E	LAXTG	Pili	Pilus attachment	High	Few Gram-positive bacteria
F	-	-	-	-	Actinobacteria

*Also QVPTG

Table 1. Classification of Sortases (7, 8)

Despite this classification, sortases can also work together to carry out specific functions on the cell surface. Sortases will be further explained depending of their functional characteristics, main substrates and general aspects as it follows.

4.1.1. SrtA

Type A sortases were the first to be discovered and the most studied ones, especially the Sa-SrtA of S. *aureus*. They are known as the “housekeeping” sortases since their main function is to anchor different types of proteins to the cell wall. They link the widest variety of proteins among all types of sortases. It is caused by the genes that encode type A sortases are not close to the genes that encode their target substrates (9).

One of the main characteristics of sortase A is the recognizing of the substrate pentapeptide at the carboxyl terminus of surface proteins. This pentapeptide is formed by Leucine-Proline-X-

Threonine-Glycine with X being any amino acid, named as the LPXTG motif. Sortase identify this sequence within the substrate protein, cutting the T-G peptide bond and creating a new bond between T and the N-terminal glycine of a G5 motif located in the cell-wall building bloc present in another substrate. This exchange of peptide bonds is known as a transpeptidase reaction. The product of this reaction is a surface protein that is covalently linked to G5 motif and is then incorporated into the peptidoglycan cell wall envelope. The reaction and the mechanism will be explained in detail further below.

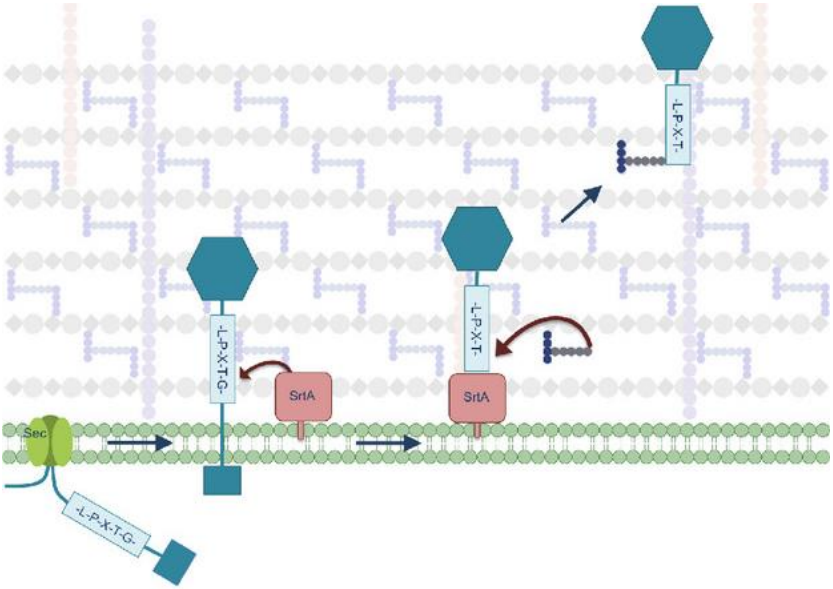

Figure 1: Example of SrtA-mediated anchoring of surface proteins to the cell envelope. Reprinted with permission (Bradshaw et al., ref. 8).

4.1.2. SrtB

Class B sortases have been identified in a lesser extent than SrtA. As class A, the archetypal sortase correspond to S. *aureus*, named Sa-SrtB. Although SrtB share significant primary sequence homology among them, members of this group can have radically distinct functions.

This kind of enzymes may attach haemoproteins to the cell wall into some pathogenic microbes (10) and even assemble pili like class C sortases, such as those in S. *pyogenes* (11).

This fundamental difference highlights the limits of assigning function strictly based on primary sequence homology and emphasizes the need for experimental work to find sortase function.

Class B enzymes identify a CWSS that varies slightly between species. They recognize an unusual NP(Q/K)(T/S)(N/G/S)(D/A) sorting signal, even though NPQTN is the principal motif and the corresponding S. *aureus* pentapeptide. This motif differs markedly from the canonical LPXTG motif recognized by class A enzymes (8).

If Sa-SrtB is taken as a reference, it can be seen that it plays a key role in the regulation of haem (heme) uptake into cells. Sa-SrtB is part of an iron-regulated locus called iron-responsive surface determiner (isd), which includes the gene encoding of Sa-SrtB. IsdC is thought to facilitate haem capture from human haemoglobin by relaying haem from upstream haem-receptors to a membrane transporter complex that imports haem into the cell. Upstream of the isd locus promoter is a binding site (Fur box) for the ferrichrome uptake repressor (Fur), which controls the transcription of isd genes based on the extracellular iron concentration. For example, when S. *aureus* grows in the presence of excess extracellular iron, it abolishes the expression of SrtB. So Sa-SrtB, unlike Sa-SrtA, and its substrate IsdC, are only expressed in iron deplete conditions (12).

Once the fur gen has been deleted, the proteins coded for it by the isd locus (IsdC, IsdA, IsdB and IsdH) have CWSS motifs that can be cleaved by either SrtA or SrtB. Haem translocation in S. *aureus* requires both SrtA and SrtB because they recognize and anchor different surface proteins required for uptake. Interestingly, class A and B enzymes in S. *aureus* usually attach their substrates to different sites within the cell wall. Sa-SrtA attaches its substrates to surface exposed sites that are heavily cross-linked and Sa-SrtB attaches IsdC to a buried site within the peptidoglycan that is not heavily cross-linked (12). This process will be further explained.

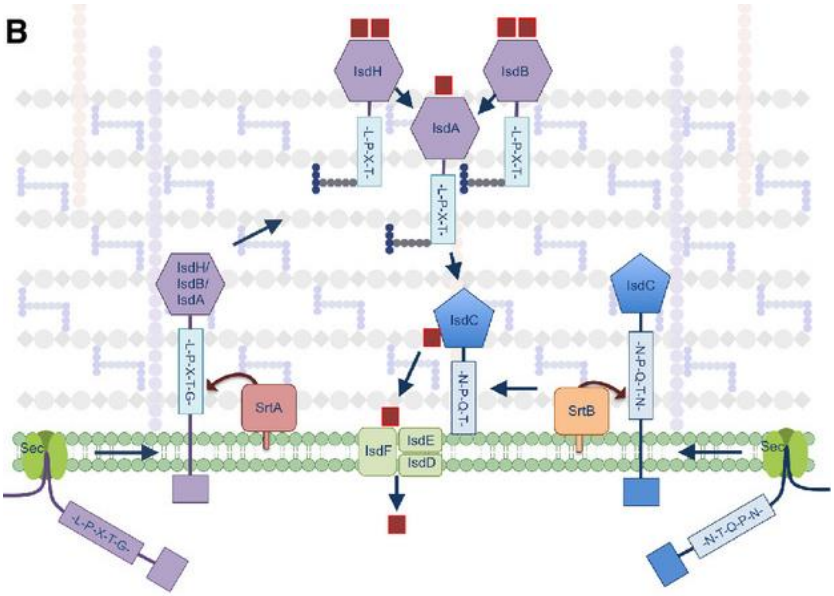

Figure 2: SrtB-mediated assembly of the isd iron-acquisition system in S. *aureus.* Reprinted with permission (Bradshaw et al., ref. 8).

4.1.3. SrtC

The main function of these sortases is to construct and assemble pili using multiple pilin subunits in order to promote microbial adhesion and biofilm formation.

Pilin-specific sortases recognize multiple pilin proteins both as substrates for cleavage of the LPXTG-like motif and as nucleophiles. Pilin-specific class C sortases, like class B sortases, are often located in pilin gene clusters with their substrates and often contain a C-terminal hydrophobic domain not found in sortases from other classes (13).

The pilus assembly process extend pili 0,2-3 μm from the cell surface and is constructed in a two-stage process, in spite of this process and mechanism varies between species as a result of differences in composition and structure of pili. First, one or more class C enzymes form the long thin shaft of the pilus by linking together pilin subunits via isopeptide bonds. Secondly, the base of the pilus subunits is anchored covalently to the cell wall by a housekeeping sortase or a class C enzyme itself (14). There are some pilus assembly situations where the assistance of

housekeeping sortase is needed and others where is not, it depends on the studied cell. Pilus assembly will be further detailed.

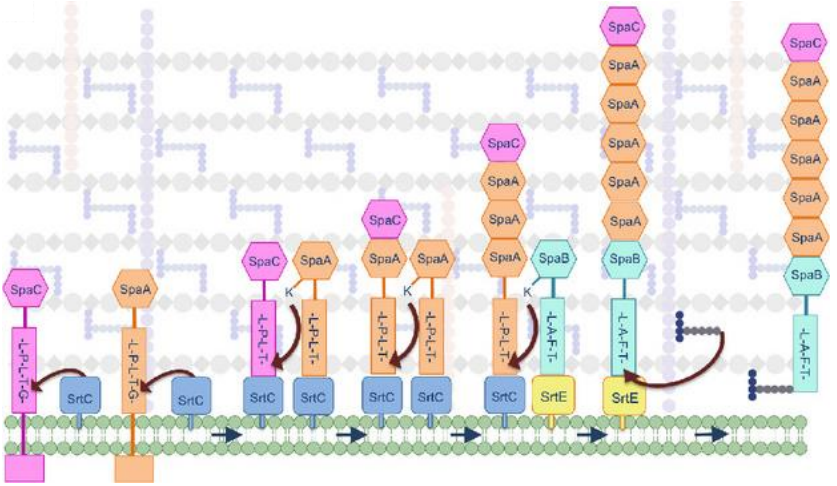

Figure 3: Example of pilus assembly using SrtC with assistance from other housekeeping sortases. Reprinted with permission (Bradshaw et al., ref. 8).

4.1.4. SrtD

Class D sortases, unlike others, are predominantly found in bacilli cells. In fact, the typical archetype SrtD sortase is the SrtD of *Bacilus anthracis*, sometimes referred as "SrtC". This microbe encodes a single class D enzyme called Ba-SrtC. SrtD are not extensively characterized but it is known that they play sort of a housekeeping role, similar to Class A sortases. The usual recognition motif for a SrtD is LPNTA (8).

Sortase class D enzymes are also involved in spore-forming microorganisms. Some experiments have shown that SrtD are expressed during sporulation. In Bacillus and Streptomyces species, SrtD enzymes recognize and cleave the sorting signals of select substrates to immobilize their anchored products in the cell wall envelope during spore formation (15). Besides, the genes encoding Class D sortases in other Bacillus species are often next to genes encoding substrates that also have LPNTA sorting signals. Another sign that indicates that this class of sortases play a role in spore formation.

Ba-SrtC joins two proteins that contain a LPNTA motif to the cell wall, BasH and BasI, from distinct structures. BasH is attached to the forespore and BaSI to the diaminopimelic acid moiety of the predivisional cells' peptidoglycan (15).

4.1.5. SrtE

SrtE and SrtF have recently been studied as less information is known about them; in fact, only SrtA to SrtD were described in the past years. SrtE and SrtF were mostly described as Class D or subfamily-5 sortases; however, nowadays they are described separately.

Unfortunately, there is not much information regarding their functions. Nevertheless, the only one that has been characterized experimentally is a high GC content class E enzyme inside of bacteria called C. diphtheria (Cd-SrtF). This bacteria uses SrtE as their housekeeping sortases as well as for assembling pili to the cell wall (16). Instead of class B, C and D sortases, class E does not have their encoding genes located in the same loci as their substrates (9), although they share limited primary sequence homology to other sortases, especially SrtA. In fact, comparative genome analyses suggest that class E enzymes recognize an LAXTG sorting signal, instead of the canonical LPXTG motif recognized by class A enzymes (9).

4.1.6. SrtF

Class F sortases are the most recently characterized. However, considering that 57 enzymes of this family are presented in 20 bacterial species, they are mostly found in Actinobacteria and their main function still to be determined. Yet, it has been studied the possibility of them playing essential roles in sorting surface proteins (14).

4.2. SORTASE STRUCTURES

It has seen sortases' classification according to their general and functional characteristics. Now, they will be detailed based on their structure. It should be considered that they are constructed in general terms. Thus, the same sortase can be part of a class depending on its structure or to another regarding its function. Even it have been seen structural and / or functional differences between two sortases of the same class in different bacteria. This inconvenience has given rise to many misunderstandings and discrepancies between authors. One of the reasons is that this classification stills changing within the years.

4.2.1. SrtA

Sa-SrtA structure is composed by a central eight-stranded β-barrel flanked by loops, one α-helix and one 3_{10} helix. The β-barrel is formed by two anti-parallel β-sheets, each one formed by four strands: β1, β2, β5, β6 from one and β3, β4, β7, β8 from the other (17).

Strands β2 and β3 flank a 3_{10} helix, while β4 and β5 form a short α-helix between. The high-flexible β6–β7 loop is a preformed binding pocked adjacent to the cysteine active site. The LPXTG sorting signal binds in this pocked which contain a short helix inside, common in the structure of all SrtA. The base of this pocket is constituted by residues in strands β4 and β7, and by β6/β7, β3/β4 and β2/H2 loops. The His-Cys-Arg triad that forms the catalytic residue is placed between β4 and β7 end strand and the start of strand β8 (17, 18). The active-site is the sulfhydryl form of cysteine, which act as a nucleophile enabling the resultant thiolate in order to cleave the peptide bond between threonine and glycine in LPXTG peptides.

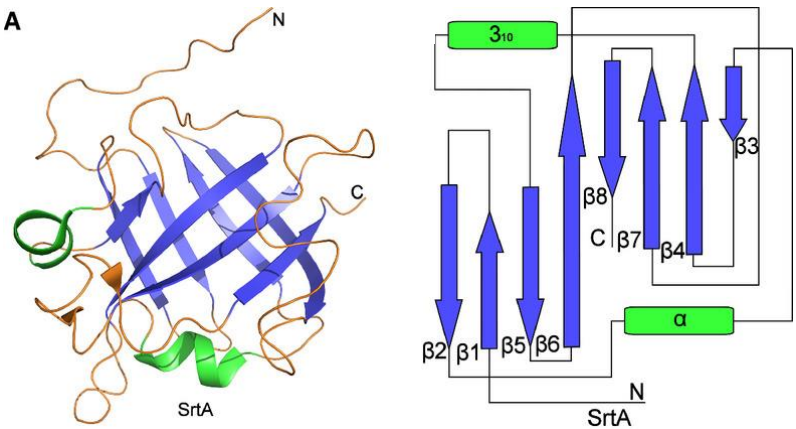

Figure 4: Typical Sa-SrtA structure. Reprinted with permission (Bradshaw et al., ref. 8).

4.2.2. SrtB

Sa-SrtB possesses almost the same structure as Sa-SrtA. Sa-SrtB has a core β-barrel structure like Sa-SrtA, with more peripheral helices. If they are deeply compared, two main differences are shown: the catalytic domain in Sa-SrtB has more helices in the N-terminal direction and β6/β7 loop contains an extra α-helix than Sa-SrtA. This extra elongation of the β6/β7 loop is involved in recognizing the NPQTN sorting signal. Also, a significant difference that affect the accessibility of the substrate is the N-terminal extension in class B sortase. This extension locate the two termini on the same protein position (19).

Other B sortases, that surprisingly posses' similar tertiary structure as SrtBs' anchoring proteins, are involved in pilus assembling as the ones in S. *pyogenes* (Spy-SrtB). The main difference is the N-terminal helical bundle length, because in S. *pyogenes* is shorter (11).

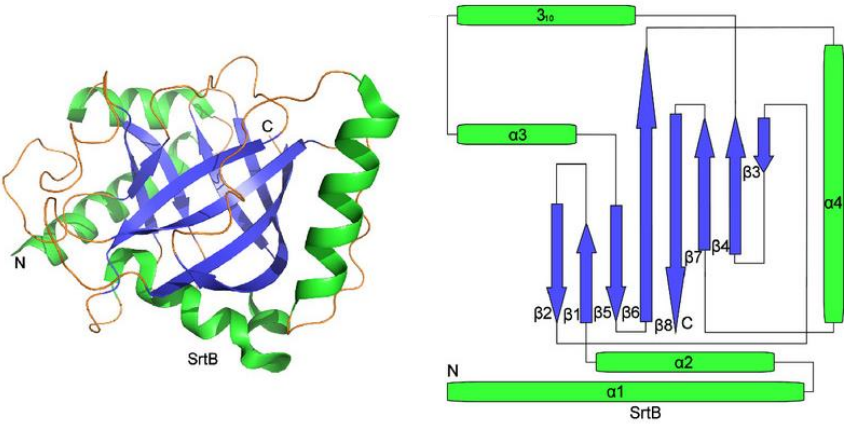

Figure 5: Typical Sa-SrtB structure. Reprinted with permission (Bradshaw et al., ref. 8).

4.2.3. SrtC

The most-studied SrtC sortase structure is from S. *pneumoniae*, which is involved in pilus biogenesis. It has some structural similarities in common with SrtA and SrtB; their core is formed by an eight-stranded β-barrel and is protected by helices. However, the main difference is the N-terminal loop. This loop forms a flexible "lid" that blocks the active site holding the enzyme in an inactive state when there is not any substrate around. Nevertheless, the enzyme may become activated again when the lid leaves its block form, enabling the formation of the

enzyme-substrate thioacyl intermediate with the link of the sorting signal. Therefore, it is deducible that the substrate specificity is controlled by surface recognition coupled to lid opening, unlike non-pilus sortases (20). The lid contains a DP(F/W/Y) motif and is anchored to the active site by two ways: on one hand, by a salt bridge between the lid aspartate and the arginine residue in the His-Cys-Arg triad of the active site and, on the other hand, by sulfur–aromatic interactions between the active site cysteine and aromatic residues in the lid. Also, SrtC possesses a C-terminal nonpolar group incrusted in the membrane (21).

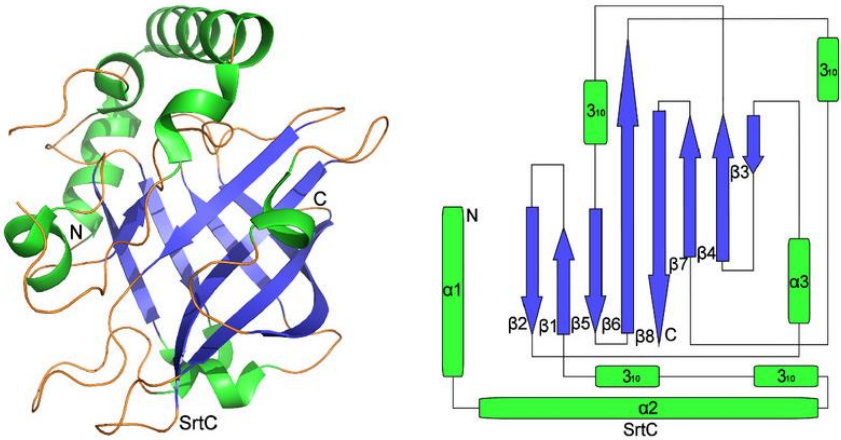

Figure 6: Typical S. *pneumoniae* SrtC structure. Reprinted with permission (Bradshaw et al., ref. 8).

4.2.4. SrtD

The best characterized structure of SrtD belongs to B. *anthracis* sortases (Ba-SrtD). This type shows much similarities with SrtA sortases; as its catalytic domain adopts the eight-standed β-barrel fold and it has the 3_{10} helix within the β6/β7 loop (yet the loop conformations have some variations). Other differences between sortase classes are the elongated β6/β7 loop, as SrtB, and the “lid”, as SrtC (22).

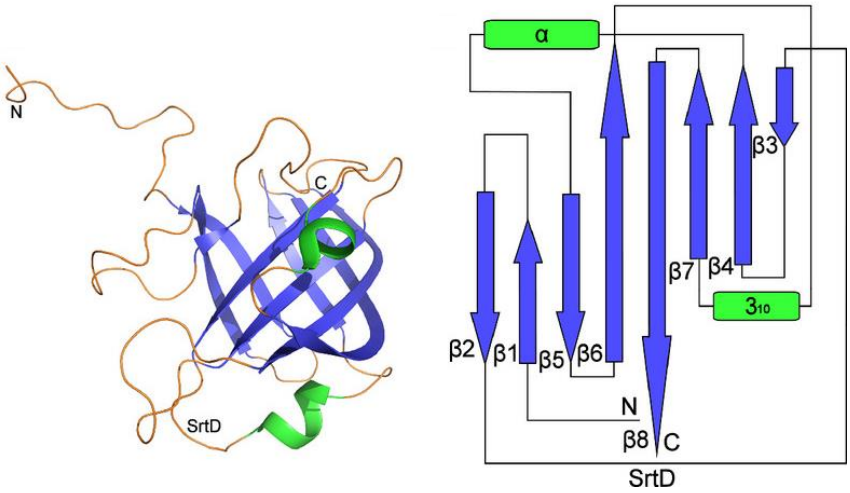

Figure 7: Typical Ba-SrtD structure. Reprinted with permission (Bradshaw et al., ref. 8).

Taking Sa-SrtA archetype as reference, several features are generally conserved among sortases and some are not. The most important four are depicted in the following table:

Common components	Uncommon components
Core eight-stranded β-barrel	Number and length of helices
His-Cys-Arg triad in the C-terminal domain	β6/β7 loop
N-terminal signal sequence	β7/β8 loop
Non-polar segment of amino acids	The N-terminal and the C-terminal segments on each side of the catalytic domain

Table 2. (22)

Interestingly, the N-terminal sequence from target substrate proteins instigate their translocation through the bacterial membrane (23). In addition, the C-terminus hydrophobic domain and the positively charged tail of the sorting signal promote the maintaining of the translocated protein in the plasma membrane side until sortases recognize the substrate and perform the catalysis (5).

The main common components that is indispensable for sortases' performance is the His-Cys-Arg catalytic triad. This triad is directly involved in the transpeptidation reaction controlling each step of it. Prior to this, sortases identify the LPXTG-like sorting signal of their protein substrate. However, it is important to highlight the pentapeptide composition of the motif as

threonine residue is common in all sortases sorting signal (despite the pentapeptide recognition motif varies among them). One of the reasons is the high specificity to the arginine catalytic residue. The LPXTG-like sorting signal is recognized by sortase to posteriorly cleave it between Thr and Gly. Interestingly, Thr performs a two hydrogen bond with Arg in a Thr-in conformation, which indicates that Thr is inside the active site. Chan et al. (18), Jacobitz et al. (19) and Sure et al. (17) studied this Thr-in conformer in SaSrtA, SaSrtB and BaSrtA with their respective SaSrtA-LPXT*, SaSrtB-NPQT* and BaSrtA-LPAT* sortase-substrate complexes (T* is referred as a Threonine moiety which possesses a sulfhydryl group instead of a carbonyl carbon) . Jacobitz et al. (19) reported that this Thr-in conformation enables arginine from the active site to be next to the formed thioacyl bond between sortase and the substrate. Besides, this orientation permits arginine's guanidine group to neutralise negative oxyanion's intermediates charges formed along transpeptidation, leading to the stabilization of the two tetrahedral oxyanion intermediates.

5. MECHANISM

5.1. SORTING MECHANISM

In this section, the properties and mechanisms of the cell wall sorting by *Staphylococcus aureus* sortase (SrtA) is studied. The first step of the catalysed reaction is carried out by SrtA (a thiol transpeptidase that develop a cell wall anchoring reaction) when a surface protein is linked to the sortase between the Thr and Gly of its LPXTG motif. However, in order to begin the transpeptidase reaction, the His-Cys-Arg triad must be properly charged. More specifically, histidine has to be found in its imidazolium form and cysteine in its thiolate form to carry on the acid-base and nucleophilic attack procedures. Yet, pka values of Cys and His in sortase catalytic triad are 9,4 and 6,2, respectively (24). In other words, at physiological pH 7, cysteine is protonated and histidine deprotonated. Besides, there is not a suitable pH where Cys is in its thiolate form and His in its imidazolium form. As a result of these event, Frankel et al. (24) proposed many models to explain how this reaction could be carried. Finally, they provide a reverse protonation mechanism in which a small fraction (0,06%) of Cys-His-Arg triad is

capable, at pH 7, to develop catalysis. This model cannot be explained with pH-rate profiles because at pH 7 the majority of Cys would be protonated and the majority of His deprotonated. Although, at this pH, there is a minority of Cys deprotonated and His protonated, meaning, their reverse protonation form. Reverse protonation states are also related to pH and pka values as normal protonated states. Interestingly, the fraction of overlapped region of reverse protonated Cys and His correspond to an estimated $10^{-\Delta pKa}$ value, the 0,06% at physiological pH previously mentioned. Surprisingly, the catalytic triad activity stills being high with kcat/Km of $>10^5$ M^{-1} s^{-1} (24).

After outlining this point, the transpeptidation reaction can be followed by these steps (22, 24):

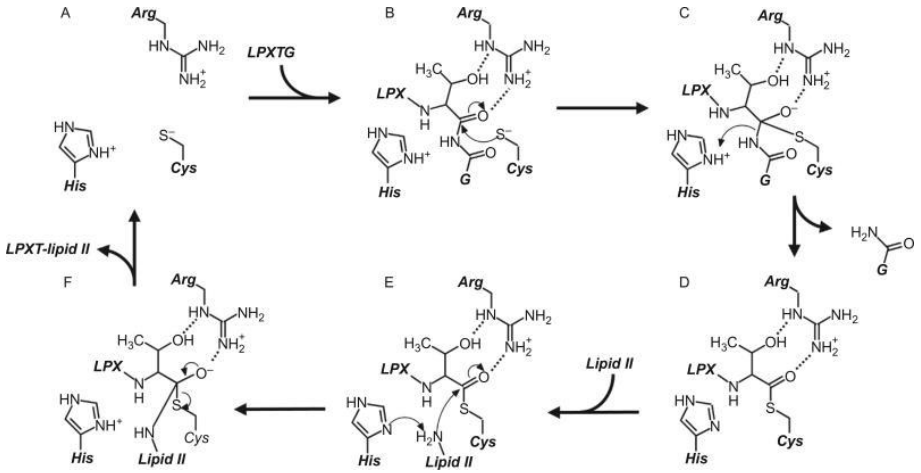

Figure 8: The current model of the molecular mechanism of the Sa-SrtA enzyme. (Jacobitz et al., ref. 22, copyright).

A: When the catalytic site is properly charged with histidine in its imidazolium form and cysteine in its thiolate form, the reaction can be displayed. The triad His-Cys-Arg within the active site of the sortase is active and available to interact with the LPXTG motif of the substrate.

B: The cysteine thiolate of the sortase act as a nucleophile attacking the carbonyl carbon of the Thr residue of the LPXTG motif. The two electrons of the carbonyl carbon of the amide bond

can be relocated to the oxygen for resonance during the nucleophilic attack of thiolate group. The two nitrogen of the arginine residue form two hydrogen bonds with the oxygens of the Thr, allowing to maintain the structure of Thr fixed and properly oriented. This two hydrogen bonds also enable that carbonyl carbon become more electrophile and more suitable to receive the attack.

C: Afterwards, the first tetrahedral intermediate is formed between thiolate active cysteine and threonine of the substrate motif, constituting a sortase-substrate intermediate. The tetrahedral oxyanion intermediate is stabilized by the hydrogen bonds of arginine.

D: Then, imidazolium histidine protonate the amino living group of Gly residue. This step occurs quickly and leads to the formation of a thioester acyl bond that joins the substrate with the Cys active site of sortase. Histidine now is on its neutral form.

E: As it follows, the N-terminal amine nitrogen of the second substrate, which is a pentaglycine cross-bridge of peptidoglycan called lipid II, nucleophylically attacks the carbonyl carbon of the thioacyl bond while His deprotonates it. This role that His plays, permits to recover its imidazolium form and make lipid II amino group more nucleophilic. This constitute the second tetrahedral intermediate between the lipid II, the protein substrate and the cysteine thiolate of sortase.

F: This new tetrahedral intermediate collapses and forms a peptide (amide) bond between the substrate threonine and lipid II. Thiolate cysteine of sortase active site is then released and recovered. The process is also stabilized by the two hydrogen bonds of arginine. Finally, the linked substrate-lipid II is released and ready to be incorporated to the cell wall.

These whole mechanism proves the important role and specificity that Arg and Thr play. From then on, sortases are able to catalyse two main functions: cell wall anchoring and pilus assembly. When sortases link proteins to the peptidoglycan cell wall, they join protein substrates to N-amino terminal of lipid II pentaglycine tail; whereas sortases that display pilus assembly join pilin subunits to N-amino lysine from another pilin protein.

Nevertheless, it is worth to mention that the previous detailed process does not always happens. In fact, the sortases catalysis is regulated by a “ping-pong” mechanism (25).

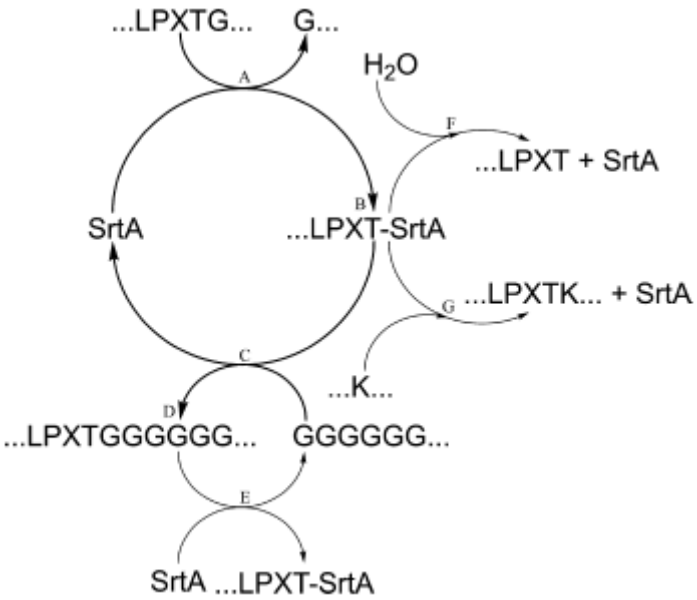

Figure 9: SrtA "ping-pong" mechanism. Reprinted with permission (Bradshaw et al., ref. 8).

"Ping-pong" mechanism is at is follows (where A-D is the principal pathway, Figure 8 and 9) (8, 25):

A, B: SrtA nucleophylically attacks the CWSS, forming an acyl-enzyme complex. The SrtA is bound between Thr and Gly of the LPXTG motif and Gly residue is released.

C, D: The pentaglycine peptidoglycan of lipid-II is linked via peptide bond to LPXT substrate motif and the resulting substrate-lipid-II complex is released (LPXTGGGGG…). The product at the end of D phase would be the pertinent if the catalytic site was properly charged and if the reaction follow its course without inconvenient.

However, the next alternative steps could happen (Figure 9):

E: In case of an excess of lipid-II, SrtA would attack the LPXTGGGG… product, reforming the acyl-enzyme LPXT-SrtA complex in step B. Since SrtA cleaves LPXTG motif between Thr and Gly, the resulting product between substrate-polyglycine and SrtA would release the cross-linked product and generate the SrtA-substrate complex. This reaction depends on the reagents and products concentration to avoid an excess of those.

F: In the absence of a specific nucleophile, water can act as it performing a slow hydrolysis of peptides while is cleaving the thioacyl bond between threonine substrate and cysteine sortase. This leads to the regenerating of free sortase (LPXT + SrtA). So, when substrate concentration is low, this reaction is accomplished instead of transpeptidation.

G: If there is another suitable substrate instead of pentaglycine lipid-II, the LPXT-SrtA adduct can be attacked by this new nucleophile, forming an alternative product.

The pathway (A-D) starts when the sortase recognizes the motif of a target protein. As it is known, the active site of the enzyme must be charged with cysteine in its thiolate form and histidine in its imidazolium form. However, it represents a problem when sortases work in isolation conditions because less than 1% of the SrtA have properly charged the active site. Only a few sortases can achieve it and that is the reason why catalysis can occur very slowly (26).

Huang et al. (25) discovered, with the support of kinetic parameters, that when a nucleophile is not available the LPETG motif is hydrolysed by sortase. Both reactions, transpeptidation and hydrolysis, have an acyl-enzyme intermediate in common. Yet, each path has a distinct rate-limiting step: "the formation of the acyl-enzyme in transpeptidation and the hydrolysis of the same acyl-enzyme in the hydrolysis reaction". Huang et al. (25) also proved that diglycine is specific to the nucleophile binding site of Sa-SrtA and the "lengthening of the polyglycine acceptor nucleophile beyond diglycine does not further enhance the binding and catalysis". Studies proportioned by Frankel et al. (27) showed that "ping-pong" mechanism only fits the experimental results if the hydrolysis of the acyl-enzyme is involved.

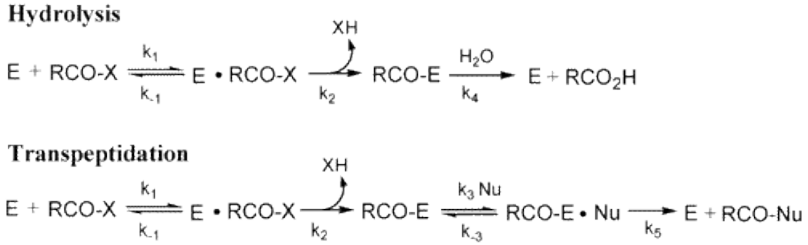

Figure 10: Transpeptidation and hydrolysis in "ping-pong" mechanism of Sortases. Reprinted (adapted) with permission from (Race et al., ref 29). Copyright (2009) American Chemical Society.

5.2. ROLE OF METAL IONS

It was reported the influence that some metal ions have in the activity of sortases' sorting reaction. Naik et al. (28) studied the role that Ca^{+2} plays in Sa-SrtA. Calcium binds to a pocked between β3/β4 and β6/β7 loop near to the active site. Ca^{+2}-in and Ca^{+2}-out has an allosteric control to the sortase activity modulating the structure and dynamics of the pocked. Therefore, Ca^{+2} ion facilitates the affinity to the sorting signal and allows to increase the speed of the substrate recognition. It was observed orders of milli- to microsecond Ca^{2+} dependent dynamics. In fact, there is a direct relation between the Ca^{+2} and the rate of the substrate binding. Naik et al. (28) used enzyme kinetic and detailed NMR nitrogen-15 measurements for evidencing that ion binding allosterically controls the movement of the β6/β7 loop active site. This β6/β7 loop would fluctuate between a closed shape stabilized by Ca^{+2} and an open shape that removes the substrates by contacting residues from the active site. Ca^{+2} dependent activity could be an explanation of the high host colonization in human tissues because it is known that there is an elevated concentrations of this ion.

Llangovan et al. (4) tested two sortase enzymes ($SrtA_{\Delta N}$ and $SrtA_{\Delta N59}$), with similar activities, with some common metal ions found in human body (Ca^{+2}, Mn^{+2}, Mg^{+2}, Fe^{+2}, Zn^{+2}, Cd^{+2}, Co^{+2} and K^{+}). They confirmed that Sa-SrtA activity increases with the presence of Ca^{+2}, as expected, as well as with Mn^{+2} and Mg^{+2} ions. In fact, $SrtA_{\Delta N}$ and $SrtA_{\Delta N59}$ activities rose 8-fold times with the addition of 2 mM of Ca^{+2}. Mn^{+2} and Mg^{+2} also improves enzymes activity but to a lesser extent; whereas Fe^{+2}, Zn^{+2}, Cd^{+2}, Co^{+2} and K^{+} did not.

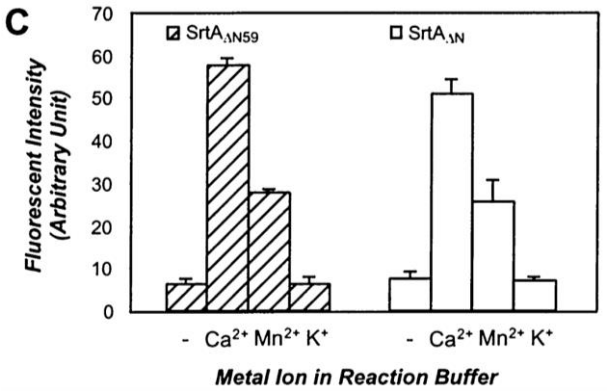

Figure 11: Increasing in activity of $SrtA_{\Delta N}$ and $SrtA_{\Delta N59}$ according to the metal ion. (Ilangovan et al., ref 4. Copyright (2001) National Academy of Sciences.)

Further NMR and biochemical studies revealed that Ca^{+2} binding site, located in an acidic surface formed by β3/β4 and loops, is formed and coordinated by side chains of Glu105, Glu108, Asp112, Glu171 and Asn114, identified as calcium-binding residues (4, 28). This was confirmed by Naik et al. (28) through alanine mutations. Interestingly, Sa-SrtA is the single sortase that has this Ca^{+2} dependence in its activity. One of the reasons is the possession of the β3/β4-β6/β7 pocket where calcium can allosterically interact. As a matter of fact, there are cases where the presence of Ca^{+2} does not act the same way with the sortase and even decrease their activity, as $SrtA_{\Delta 81}$ from *Streptococcus pyogenes*. The reason is that the residue Glu-105 replace the position of the binding pocked (29).

6. PHYSIOLOGICAL ROLES OF SORTASES

As it has seen, sortases mostly develop two distinct functions in bacteria: the attaching of proteins and the attacking of pilus to the cell wall. Both reactions are mechanistically alike and work on proteins that have a five-residue motif within the cell wall sorting signal. Sortases function as cysteine transpeptidases anchoring proteins with a cell wall sorting signal to an amino group from the cell wall (15). These proteins contain a Sec-dependent secreted N-terminal peptide and a COOH-terminal CWSS which is formed by a LPXTG motif, a hydrophobic chain composed by amino acids and a terminus of positively charged residues (30). Sortases catalyse this transpeptidation reaction in order to join this LPXTG-like sorting signal within their polypeptide substrate to the cell wall or to other pilin subunits. In some cases, sortases are capable to carry out both functions: joining proteins to the cell wall and polymerizing pili. Either processes are displayed on the extracellular membrane, where is on enzyme and its substrate are coupled (21).

6.1. SORTASE MEDIATED CELL WALL ANCHORING

The most studied sortase, Sa-SrtA, catalyses the covalent attachment of surface proteins to the S. *aureus* peptidoglycan cell wall. The sorting reaction begins with the exportation of a full-length precursor protein, which contains an amino terminal peptide group, from the cytoplasm to the cell wall. This precursor is synthesised in bacteria's cytoplasm and later become protein

substrates for sortases' cleavage. Protein substrate molecules are translocated across the cell wall through a secretory pathway. Lipid-II, before being used as second substrate for sortase, is generated in cytoplasm and modified in cytoplasmic membrane by penicillin proteins. This modification tether a cross-linked peptidoglycan (principal cell wall component) to the lipid-II. Afterwards, the cross-linked peptidoglycan act as nucleophile in sorting reaction and is further incorporated into the peptidoglycan cell wall (31).

The cell wall anchoring is based on the following transpeptidation steps (13, 22, 31, 32):

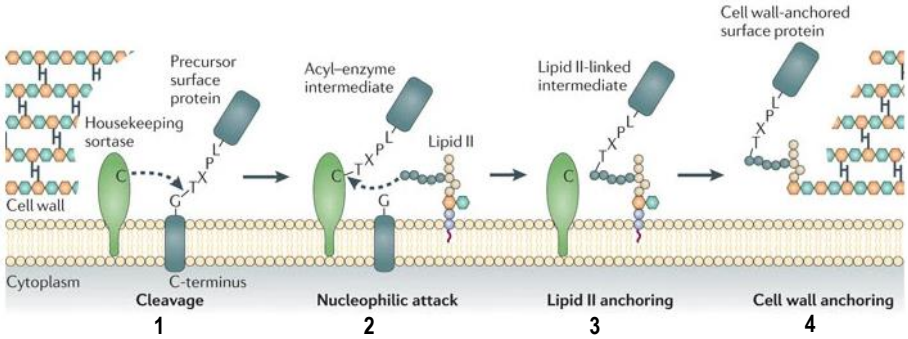

Figure 12: Sortase mediated cell-wall anchoring. Reprinted with permission (Hendrickx et al., ref. 13).

1) The reaction starts with the recognition of the LPXTG motif within the CWSS by the cysteine residue of the SrtA active site and the following nucleophilic attack to the carbonyl carbon of the substrate's threonine.

2) The previous step generates a tetrahedral intermediate that rapidly forms a LPXT-SrtA thioacyl bond, where Sa-SrtA cysteine is covalently linked to its protein substrate via acyl-enzyme bond.

3) Then, Sa-SrtA identifies the second substrate lipid-II (the cell wall precursor) and the nucleophilic attack of the N-terminal glycine from lipid-II to this thioacyl intermediate is produced. This step forms a tetrahedral intermediate between the amino group of glycine and the thioester bond of the acyl-enzyme.

4) This tetrahedral intermediate collapses and forms a peptide bond between the protein and the lipid II. After, transglycosylation and transpeptidation reactions of the cell wall will incorporate this complex into the peptidoglycan.

6.2. SORTASE MEDIATED PILUS ASSEMBLY

Pili are filamentous structures found on the surface of bacteria cells. Their main function is the development and facilitation of the bacterial adhesion. The pilus anchoring to the cell wall is performed by sortases called pilin polymerases. They construct pili by the polymerization of pilin protein subunits. Pilus formation is a biphasic process that needs pilin polymerases for the assembling and housekeeping sortases to link them to the peptidoglycan cell wall afterwards. However, this process can be displayed only by dedicated sortases as SrtC or by a Class C with housekeeping sortases help. Both examples can be performed in *Corynebacterium diptheriae*, which expresses three distinct pili: SpaABC, SpaDEF, and SpaHIG (Spa: Sortase-mediated pilus assembly) (16).

The transpeptidation steps in pilus assembly are similar to Sa-SrtA cell wall anchoring reaction. A sortase-substrate thioacyl intermediate is produced but the recognized substrate is a pilin protein. And, instead of using a lipid II glycine as a nucleophile to attach proteins to the cell wall, a lysine amino group located within a protein pilin subunit is used as a secondary substrate to attack the sortase–substrate thioacyl intermediate (13, 25). The Lysine residue within the pilin motif from another pilin protein carries out a nucleophilic attack to the thioacyl intermediate and a new isopeptide bond is formed between both proteins, linking the pilin subunits. This process is repeated as long as the final pilus tail is.

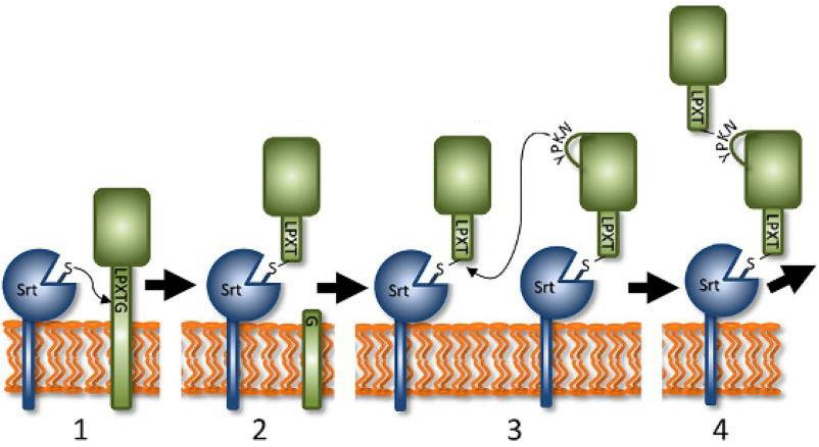

Figure 13: Pilus assembly process. (Jacobitz et al., ref. 22, copyright).

However, pilus assembly varies between species, principally in their pilin subunits. If the following table (Figure 14) is observed, it is possible to differentiate some pilin subunits used by many kinds of bacteria.

	C. diphtheriae	*S. pyogenes*	*S. agalactiae*	*S. pneumoniae*	*E. faecalis*	*B. cereus*
Major subunit	SpaA [46]	T3M3 [66] T6M6 [62]	GBS80PI-1 [61] GBS59PI-2a [63]	RrgB [91,94,99,]	EbpC [73]	BcpA [68]
Minor tip subunit	SpaC [46]	CpaM3 [66] CpaM6 [62]		RrgC [74,91]	EbpA [73]	BcpB [68]
Interspersed on fiber	SpaB [46]	Orf130M1 [62]	GBS104PI-1 [63] GBS67PI-2a [63]	RrgA [71,91,94,99] RrgC [91,94]	EbpA [73] EbpB [73]	
Surface associated		CpaM6 [62] Orf130M1 [62]	GBS104PI-1 [63] GBS67PI-2a [63]	RrgA [91]	EbpA [73]	BcpB [68]
Assembly factor	SrtA [46]	SrtC2M3 [65]		SrtB/C-1 [74,91,99] SrtC/C-2 [74,91,99] SrtC/C-3 [74,91,99]	SrtC/Bps [73]	SrtD [68]
Putatitve chaperone		SipA2M3 [66]				
Base associated terminator	SpaB [77]		GBS150PI-1 [78]			
Cell wall attachment	SrtF [76]		SrtA [93]			SrtA [68]

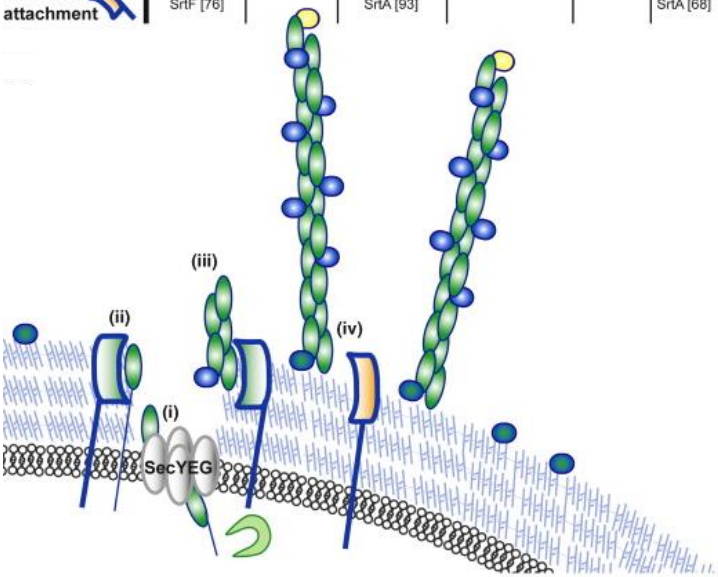

Figure 14: pilus subunits for Gram-positive bacteria. Reprinted with permission (Kline et al., ref. 33).

These bacteria develop a similar process in their pilus assembly (figure 14).

First, pilin subunits are transported across the cell membrane by the secretory system (i). Then, those subunits are linked to the cell membrane by a transmembrane domain within the CWSS (ii). After, LPXTG is recognized and cleaved by sortase enzymes (iii) and, finally, the pilus fiber is built and anchored to the cell wall (iv) (33).

Corynebacterium diphtheriae manage three pilin subunits: major subunit SpaA, minor tip subunit SpaC and interspersed subunit SpaB. The three of them are generated in cytoplasm as precursors with their sorting motifs in C-terminus position and a nucleophile N-terminal amino group (16). Then, they are translocated to cell wall via secretory pathway. Pilus assembly in C. *diphteriae* starts with a biphasic process on the exoplasmic region of the cytoplasmic membrane and is performed by the upcoming steps (13, 14, 16):

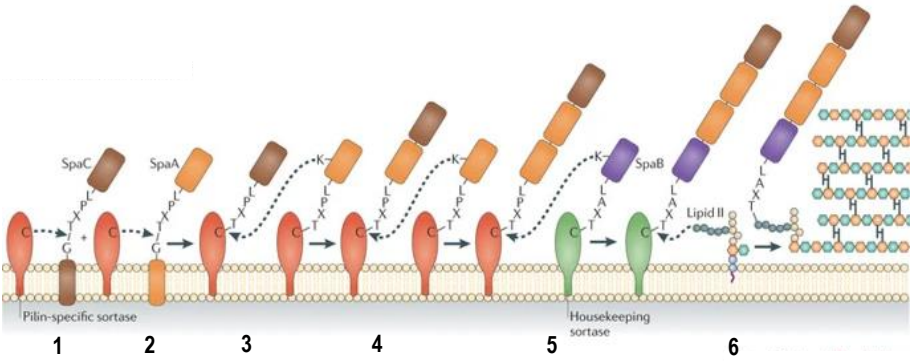

Figure 15: *C. diphteriae* pilus assembly. Reprinted with permission (Hendrickx et al., ref. 13).

1) The reaction is initiated by the formation of the Sortace C and the nucleophilic attack by sortase C cysteine to the T-G bond of the SpaC LPXTG motif. This reaction cleave the LPXTG motif of the SpaC tip and generate a SrtC-SpaC complex via thioacyl acyl-enzyme bond.

2) In a similar way, SrtC enzyme identifies the SpaA major pilin subunit and forms another acyl-enzyme bond between SrtC and SpaA through the LPXTG sorting motif. Some SpaA subunits do not construct this bond in order to allow further polymerizations.

3) Afterwards, the SrtC-SpaC complex is broken due to the nucleophilic attack of the free amino group from the lysine of the YPKN motif within SpaA of the previously formed SrtC-SpA. Interestingly, the N-terminal nucleophilic amine that performs the linkage does not belongs to the principal amino lysine chain, but it does to the side-chain of K. That is why this step results in a SpaA-SpaC complex linked through an isopeptide bond, instead of a peptide bond.

4) SpaA major pilin subunits are anchored to the building-up chain following the same method as step 3. SpaC always stays in the top of the chain.

5) Finally, the pilus subunits chain is nucleophylically attacked by the Lys of the SpaB subunit. However, SpaB is linked to a housekeeping sortase, usually SrtE (notice that the sorting signal motif is LAXTG instead of LPXTG); so, when SpaB is incorporated to the chain, the housekeeping sortase is now the bearer of the chain.

6) Due to this fact, the housekeeping sortase is nucleophylically attacked by lipid-II in order to make a peptide bond between the pilus chain and lipid-II. Then, pilus chain is incorporated to the peptidoglycan cell wall by lipid-II.

As an end, pilus length is determined by the number of SpaA subunits available and linked to the chain. Finally, the resulting anchored pilus chain is composed by repetitions of major pilin subunits SpaA with SpaC in the top of the chain and SpaB in the basis.

Bacilus cerecus pilus biogenesis, for instance, perform its pilus chain similarly as C. *diphteriae*. Although, it uses Bcpa and BcpB as pilin subunits instead of SpaA and SpaC as C. *diphteriae*. The acyl-enzyme intermediate is generated by the cleavage of the sorting signals of these two pilin subunits at the Thr of the LPXTG motif. Then, the isopeptide bond is formed between BcpB and BcpA (or BcpA-BcpA) by the nucleophilic attack of the Lys in the YPKN motif of BcpA and the pilus assembly is finished when the nucleophilic attack of lipid II within the acyl intermediate formed by sortase C and BcpA transfers the pilus to the cell wall (13).

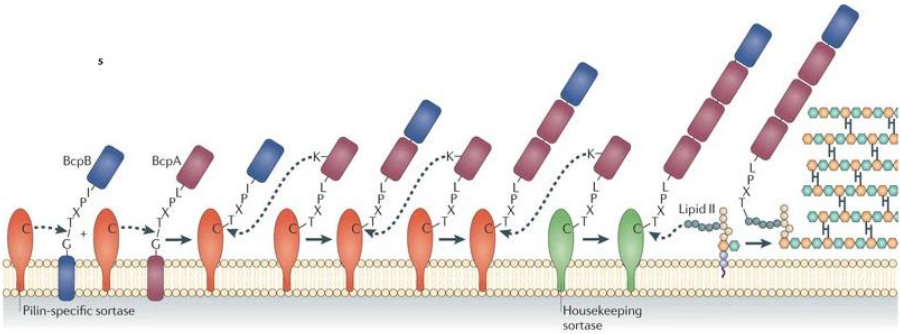

Figure 16: B. *cereus* pilus assembly. Reprinted with permission (Hendrickx et al., ref. 13).

As previously showed in figure 14, other bacteria develop pilus biogenesis. Each of them contains at least one associated class C sortase enzyme, responsible to the building using pilus subunits, mostly major pilin ones, to finally construct a pili chain with a common range of 0,3 to 3 μm length . Nevertheless, minor pilus subunits are not always incorporated into the base of the chain. In fact, they can be found at the base, the top or following a patron along the fibber, interspersed between major pilin subunits. Interestingly, the lysine that nucleophylically attacks the pilus chain can be part of an YPKN motif within major pilus subunits, like SpaA in C. *diphteriae* and BcpA in B. *cereus*; or be found as a free Lys plus a different motif, as in S. *pyogenes*. The same rule can be applied in interspersed pilus subunits like SpaB in C. *diphteriae* (13, 33).

6.3. SORTASE MEDIATED IRON ACQUISITION

Iron acquisition in S. *aureus* is a unique demonstration of sortases working together to create a specific uptake system. SrtA recognizes and anchors IsdA, IsdB and IsdH, which have LPXTG motifs and are haem binding proteins. Whereas, SrtB anchors IsdC, which possesses a C-terminal NPQTN motif (34). Mazmanian et al. (12) proposed that the SrtA anchored proteins are receptors for haemoproteins (such as haemoglobin) and IsdC is considered to be involved in translocation of haem through the cell wall.

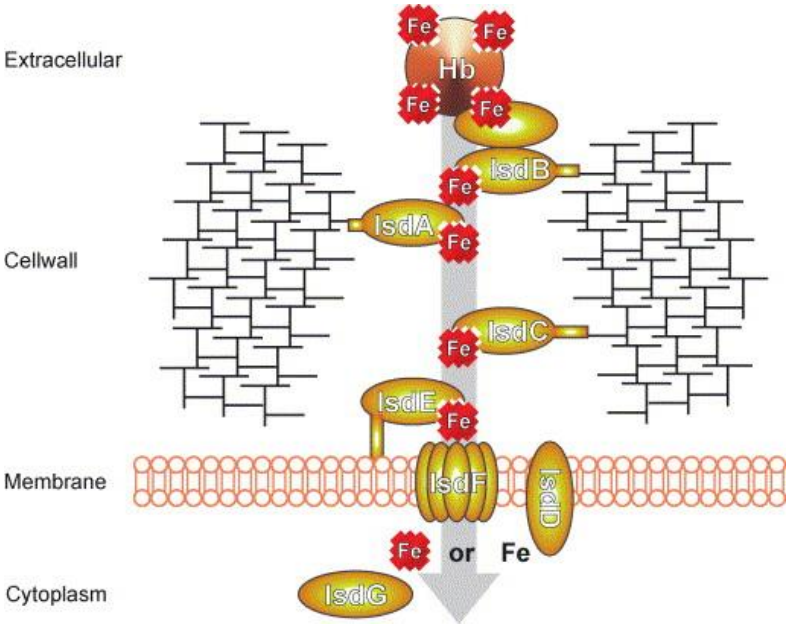

Figure 17: S. *aureus* iron acquisition. Reprinted with permission (Ton-That et al., ref. 31).

This process is initiated by SrtA anchoring haem binding proteins, IsdA, IsdB and IsdH to the cell wall; and SrtB anchoring IsdC. SrtB recognizes and cleaves the NPGTN motif of IsdC, between the threonine and asparagine residues, and catalyses the transfer of IsdC deep into the peptidoglycan. SrtA, however, recognizes and cleaves the LPXTG motif of IsdH, IsdB and IsdA, between the threonine and glycine residues (Figure 2)

First, heme-iron is extracted from IsdA and IsdB surface detectors, then is transported to the IsdC determiner and then to IsdD, IsdE and IsdF membrane translocators. When heme has crossed the membrane and is into the cytoplasm, IsdG cleaves the tetrapyrrol ring of heme and releases iron (31).

Iron acquisition is significant for bacterial pathogenesis, as iron is a vital cofactor in the course of bacterial growth. Bacteria need iron for aerobic respiration and DNA replication, among others. In human infections, this iron can be achieved in the environment or can be stolen from tissues (12). Thus, in order to prevent bacterial growth, the concentration of free iron in human and animal tissues is low, and bacteria have learned how to acquire iron from host cells. This provide evidences that Class B sortases are involved in acquisition and are beneficial to pathogenesis.

7. SORTASE INHIBITORS

Sortases in pathogenic bacteria are frequently determinant virulence factors, as many of the proteins that they face present important roles in infection course. Nutrient attainment, host cell adhesion, and avoidance and abolition of the immune response are some examples. For instance, it is known that SrtA is an essential virulence factor in S. *aureus*. In fact, S. *aureus* mutants lacking sortases do not process and display surface proteins and are defective in settling infections (35). Mazmanian et al. (35) explains that "the genome of S. *aureus* encodes at least 10 different surface proteins bearing C-terminal sorting signals with an LPXTG motif and many of them interact with various human tissues, serum proteins, or polypeptides of the extracellular matrix". Mazmanian et al. (35) give the example of the protein A. This protein binds to the immunoglobulins clumping factors as ClfA and ClfB to fibrinogen in order to promote the bacterial adhesion to vascular and endocardic lesions, among others. That is why a significant amount of effort was put to discover sortase inhibitors that could act as anti-infective agents. Sortase A inhibitors may decrease bacterial virulence by blocking key processes in biofilm formation, the host cell entry, avoidance and suppression of the immune response and even procurement of essential nutrients.

As sortases are cysteine transpeptidases, a significant focus of the study was the abrogation of the catalytic cysteine function through irreversible inhibitors. One of the first inhibitors of this class was MTSET (2-(Trimethylammonium)-ethyl-methanethiosulfonate), a thiol protease inhibitor that reacts irreversibility with sortase A and sortase B cys (36). In 2004, Zong et al. (36) tested the class B sortase, $SrtB_{\Delta N30}$, in vitro with MTSET and reported that the 2-(trimethylammonium) ethyl thiol group of MTSET covalently links to the thiol group of the catalytic Cys with binding constants of 20 μM.

In addition, several authors opted to develop strategic substrate analogues with LPXTG-like motifs that simulates the sorting signal pentapeptides of substrates. In 2002, Christopher et al. (37) synthetized "novel substrate-derived inhibitors against the *Staphylococcus aureus* cysteine protease-transpeptidase": Cbz(benzyloxycarbonyl)-Leu-Pro-Ala-Thr-CHN2 (I) and Cbz-Leu-Pro-Ala-Thr-CH2Cl (II). Both inhibitors were generated via solid-phase following the Fmoc amino acid synthesis strategy. The only difference between substrate-derived inhibitors and the real LPXTG motif is the replacement of the Thr-Gly scissile bond by a diazoketone (–COCHN2) (I) and a chloromethyl ketone (–COCH2Cl) (II). Christopher et al. (37), in order to facilitate the

synthesis, used alanine as the X amino acid since it was small and did not have a nucleophilic side chain that needed to be protected. Both inhibitors were tested in front of a $SrtA_{\Delta N}$ sortase and the calculated kinetic constants were successfully significant, with ki/Ki values of 2.2 ± 0.2 x 104 $M^{-1}{\cdot}min^{-1}$ (I) and 5.3 ± 0.6 x 104 $M^{-1}{\cdot}min^{-1}$ (II). They explained that "the greater effectiveness of the chloromethane inhibitor can be attributed to the chemical reactivity of the electrophilic groupings of both inhibitors towards thiol nucleophiles" (37).

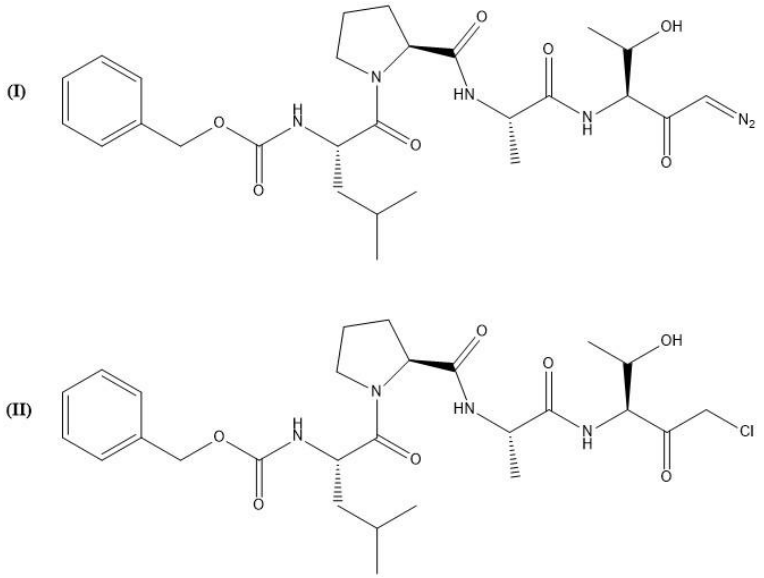

Figure 18: Molecular structures of Cbz-Leu-Pro-Ala-ThrCHN2 (I) and Cbz-Leu-Pro-Ala-Thr-CH2Cl (II) inhibitors. Genered by ChemDraw.

Many authors developed and studied techniques and inhibitors to discover anti-infective agents that could avoid the bacterial resistance. Raimondi et al. (38), in 2019, designed and synthesized novel pyrrolomycins that showed a good inhibitory response towards SrtA in a IC_{50} range of 130-300 μM. The best of them extraordinarily intervenes with biofilm formation, which is a sign that many molecules were anchored by sortases at the surface of the cells. In 2014, Zhang et al. (39) identified compounds, such as 3-(4-pyridinyl)-6-(2-sodiumsulfonatephenyl) [1,2,4]triazo-lo[3,4b][1,3,4]thiadiazole (with an IC_{50} of 9.3 μM) that frustrate sortase activity blocking sortase-catalysed cleavage and transpeptidation reactions inhibiting the incorporation of surface proteins into the staphylococcal envelope, in both vitro and vivo. Dafina et al. (40), in 2017, based their study in a new potential SrtA inhibitors that contained a 2-phenylthiazole

moiety and prevent biofilm formation at very low concentrations. Especially the ones into E. *faecalis*, with values between 2-16 μ g/mL. Zhulenkovs et al. (41) centred their study in a novel class of benzisothiazolinone-based compounds: (N ′ -(2-(3-oxobenzo[d]isothiazol-2(3H)-yl)acetyl)adamantine-1-carbohydrazide) and (N ′ -(3-(3-oxobenzo[d]isothiazol-2(3H)-yl)propanoyl)adamantane-1-carbohydrazide), that, with an IC_{50} between 3,39-7,06 μM, covalently binds the side chains of the cysteine active site and inhibits the linkage with the substrate with a reduced cytotoxicity (2014).

These are some examples of many studies that were carried out for the treatment of the virulent functions that sortases present. Fortunately, there are many ways to prevent the sortases anchoring of pathogenic proteins to host cell walls and many promising molecules were tested.

However, there are some conditions that have to be considered before being treated with anti-sortase therapies. Firstly, the organism has to include a sortase in its system, which also anchors virulence proteins to the cell wall. Secondly, the inhibition treatment must avoid the functional presentation of these proteins instead of non-pathogenic ones. Thirdly, it is important to consider the possible bacteria resistance to antibacterial drugs, besides plausible side effects in the target organism.

8. APPLICATIONS

As discussed, sortases are enzymes that anchors surface proteins with the LPXTG-like recognition sequence to oligoglycine units of the cell wall of Gram-positive bacteria. This transpeptidase activity makes SrtA attractive for various purposes and encourage researchers to study multiple in vivo and in vitro sortase-mediated ligations (SML). This technic presents high versatility and allows the anchoring of proteins, peptides and pili using other enzymatic methods. The applications are manifold: from ligation to artificial peptides substrates to the cell wall till protein engineering.

In this memorandum, it is given a summary about sortase applications in different research fields. SrtA can ligate proteins to another biomolecules, synthetic or natural, to a polymer or a

surface. The only substrate requirement is a C-terminal LPXTG-like motif and a N-terminal glycine sequence.

Due to sortase-mediated ligation was extensively studied through years, there are a lot of different and novel applications that include plenty of disciplines: modifications of the cell surface of bacteria, modification and ligation of proteins with biomolecules (DNA, lipids and carbohydrates) and synthetic molecules, protein sorting to polymers, retention of proteins in planar and particle surfaces, modification of living cells, synthesis of challenging protein structures, as well as the linkage between two artificial compounds (42). Currently, the most-wanted research fields involves therapeutic and industrial applications. Despite, it will only be given an especial overview to current investigations in fields involving cell wall anchoring, therapeutic and industrial applications.

8.1. CELL SURFACE MODIFICATIONS

As sortase A can anchor surface proteins on Gram-positive bacteria's cell wall, it was investigated the sorting of target proteins in the laboratory. Nelson et al. (43) reported, in 2010, the first example of cell wall engineering of pathogenic Gram-positive bacteria. The study was focused in the re-engineering of Sa-SrtA to contain non-native small molecules. They covalently incorporated functional molecules like fluorescein, biotin and azide to the S. *aureus* cell wall. The Sa-SrtA enzymes attach these non-native molecules identifying their LPETG motif and covalently cleave them to the peptidoglycan.

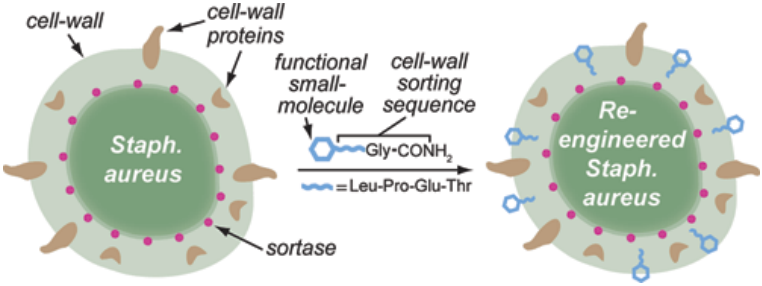

Figure 19: Sorting of small molecules to the S. *aureus* cell wall. Reprinted (adapted) with permission from (Nelson et al., ref. 43). Copyright (2010) American Chemical Society.

After that, there was a lot of researching about bacteria cells re-engineering and their sorting applications. In 2011, Nguyen et al. (44) constructed two putative sortases, YhcS and YwpE, that recognized two surface proteins, YhcR and YfkN, in *Bacillus subtilis*. They discovered that

displaying these two surface proteins mostly depended of the presence of YhcS and not YwpE. After further experiments fusing the sortases with α-amylase enzymes, they found out that YhcS can anchor YhcR to the peptidoglycan and realized the plenty of different recombinant proteins could be anchored through this sorting method. Similarly, Willson et al. (45) studied the anchor of recombinant enzymes to the *Clostridium acetobutylicum* envelope by Clostridium's sortases.

As well as using native sortases of Gram-positive bacteria cells to anchor target proteins, also non-native sortases can be added into a foreign bacteria through sorting. In 2013, Tomita et al. (46) reported the incorporation and ligation between an unnatural poly(ethylene glycol)(PEG) - lipid and a target protein. They explained that the addition of direct proteins was easily manageable, faster and reliable than protein expression through gene transfer because it would save problems of uncontrollable processes such as transformation, folding and intercellular trafficking.

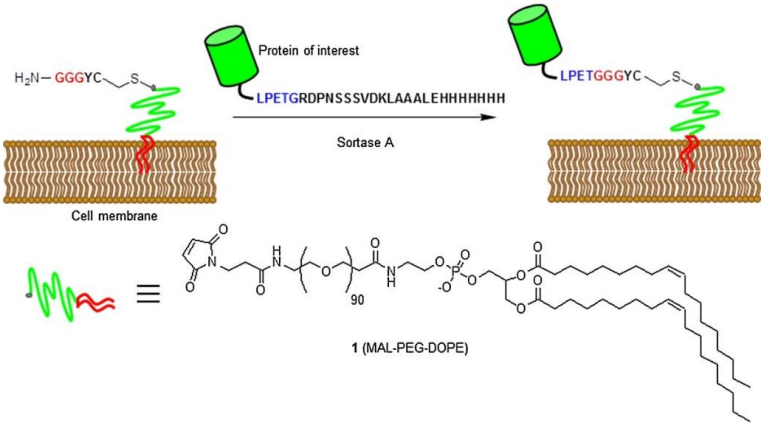

Figure 20: Sorting of an unnatural EGFP-LPETG protein to a GGG-PEG-lipid by Sa-SrtA (46). Reprinted with permission (Tomita et al., ref. 46).

First, an enhanced green fluorescent protein (EGFP) with the LPETG motif was introduced on the surface of HeLa cells, then the poly(ethylene glycol)(PEG) - lipid was ligated to a GGGYC peptide and incorporated into the cell. Finally, the EGFP-LPETG and the Sa-SrtA were displayed and started the sorting reaction. The experiment was successful because the fluorescence of the EGFP was observed on the cell membrane. The experiment was carried out both in vitro and in situ.

Later, Tomita et al. (46) used this method for modulating interactions of cancerous cells and induce their phagocytosis via dentritic cells.

SML was also tested two years ago to study and identify interactions between receptors and ligands bringing closer and connecting cells in vivo between them. Pasqual et al. (47) realized the essential benefits of interactions between different cell types, such as "multiple biological processes, including immunity, embryonic development and neuronal signalling". The method was tested in living mice and was called LIPSTIC ("Labelling Immune Partnerships by Sortagging Intercellular Contacts"); in other words, a "proximity-dependent labelling across cell–cell interfaces using the *Staphylococcus aureus* transpeptidase sortase A (SrtA)". However, SML was used at the same time than ligand-receptor interaction. The first cell was equipped with SrtA and the ligand, the second with the G5 residue plus the ligand. Furthermore, to ensure that the cell approaching occurred by the ligand-receptor affinity instead of SrtA-oligoglycine, they used a re-engineer SrtA with 13-fold lower affinity than the common SrtA sortase. The interactions were studied between T cells and dentritic cells. The SML interaction can be detected by flow cytometry, which enable the identification and following of the ligand-receptor connection.

8.2. THERAPEUTIC APPLICATIONS

As a result of sortases treating a huge array of virulence factors, it is understandable that the researching focus is on identifying these factors targeting key sortases and following their process. If some sortases can be inhibited, they would lose their virulence function and plenty of bacterial infections can be avoided. Besides inhibition field, there is an important research area based on antibodies' modification. One of the most recent studies was given by Gébleux et al. (48). They reported the anti-cancer therapy based on antibody-drug conjugates (ADCs). ADCs used a specific antibody linked to small-molecule cytotoxic payloads for targeting cancer cells and remove their tumour activity. They used the Sa-SrtA transpeptidation reaction to link immunoglobulins with a C-terminal LPETG motif to a suitable pentaglycine toxin, the method was called SMAC™ (sortase-mediated antibody conjugation). They explained that "C-terminal conjugation has no impact on antigen binding as it is distant from the complementarity determining regions (CDRs) that drive antigen recognition". Therefore, once the antibody was tagged with a LPETG motif, the toxin was modified with a GGGGG-peptide and the sortase A was added, the reaction began.

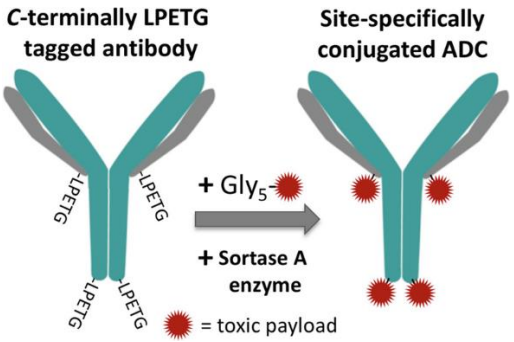

Figure 21: SMAC™ representation. Reprinted with permission (Gébleux et al., ref. 48).

SMAC™ started with the nucleophilic attack of the C_{184} thiol group of SrtA to the T-G peptide bond of the LPETG sorting signal to form a thioacyl intermediate. Then, the glycine of the motif is released and the Gly_5-Toxin nucleophylically attacks the carbonyl carbon of the thioacyl bond to link via peptide bond the antibody with the toxin in a resulting $LPETG_5$-Toxin.

This novel ADCs technique promises a great deal against cancer due to their potentially anti-tumour drugs applications and it was used previously for many authors such as Beerli et al. (49) and Rashidian et al. (50) with remarkable results.

In addition, Pishesha et al. (51) engineered erythrocytes in combination with sortases to prevent autoimmune diseases. They "exploited the non-inflammatory natural means of clearance of red blood cells (RBCs), in combination with sortase-mediated RBC surface modification to display disease-associated autoantigens as RBCs' own antigens". This technique gave a powerful treatment against prophylaxis, sclerosis and diabetes. They attached disease-associated autoantigens peptides through SML to erythrocytes in order to induce an antigen-specific tolerance.

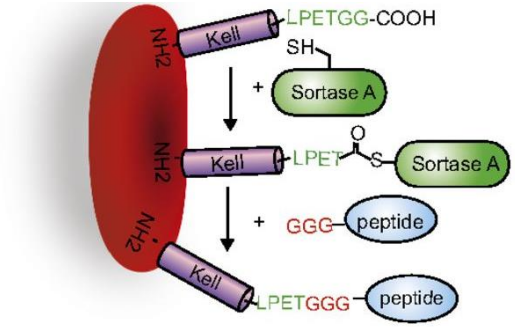

Figure 22: Sortase linked with GGG-antigens peptides. (Pishesha et al., ref 51. Copyright (2017) National Academy of Sciences).

8.3. INDUSTRIAL APPLICATIONS

The sorting technique allows to anchor proteins, biomolecules and lipids in a specific way. It is not surprising that this powerful method was deeply investigated in industrial applications fields, besides therapeutic targets, due to its high specificity and performance. When SML is focused in achieving a successful specific role in a process, it can be done on a large scale in industrial applications. That is why sortase and substrate engineering for specific functions is conceivably a vast field.

This has led to develop mutant sortases with extremely increased activity. Chen et al. (52) improved the catalytic activity of Sa-SrtA about 140 times more than wild-type sortase. They used yeast display, enzyme-mediated bioconjugation and fluorescence-activated cell sorting to achieve this target. The sorting target was recombinant LPETG-tagged human CD154, a type of proteins expressed on T cells. Deng et al. (53) demonstrated as well the total chemical synthesis of a sortase A, more precisely the sortase $A_{\Delta N59}$. Deng et al. (53) explained that this artificial sortase was built from the ligation of four synthetic peptide segments. This article reflects the possibility to synthesize unnatural sortases originated by engineering methods, a vast versatile opportunity.

Moreover, Heck et al. (54) reported the Sa-SrtA covalent link of green-fluorescent proteins (GFPuv) on a solid support made by triglycine-modified polystyrene. They followed the process using a Real-time flow cytometry (RT-FCM) that enabled the detailed and qualitative analysis of this enzymatic reaction. This analytical tool permitted the real-time continuous monitoring of dynamic-enzymatic and non-enzymatic reactions directly on microparticle surfaces.

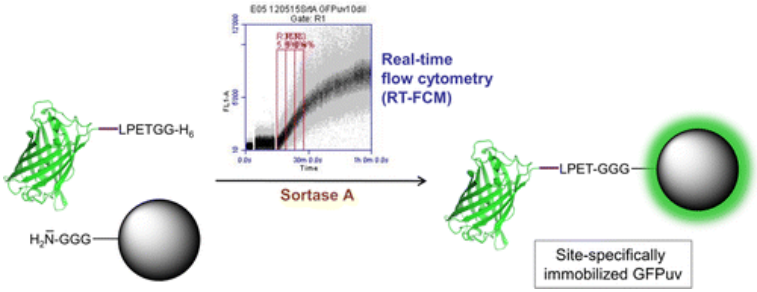

Figure 23: Immobilization of GFPuv on a solid support by SrtA. Reprinted (adapted) with permission from (Heck et al., ref. 54). Copyright (2014) American Chemical Society.

The solid support anchored by Sa-SrtA is a valuable technique that must be used in industrial applications due to its easy control and specificity.

Similarly, Yang et al. (55) immobilized Sa-SrtA on chitosan macro-particles using glutaraldehyde as a crosslinking agent. This technique was tested by peptide-peptide sorting with a successfully 80% conversion yield. Moreover, despite SrtA was immobilized, it was remarkably reused for five cycles without losing its activity. The chitosan-immobilized SrtA method is a low-cost, biocompatible and good option as it has a vast potential for industrial applications.

These reports, nevertheless, represent a little portion of the performance carried out with respect to SML. Sorting applications may confirm to become a formidable and flexible molecular biology tool.

9. CONCLUSIONS

Sortases are a novel and promising discovery since Schneewind et al. found them in 1990s. They act modifying surface proteins by recognizing and cleaving the T-G bond of their carboxyl-terminal LPXTG-like sorting signal in order to link another substrates to the bacteria peptidoglycan cell wall. Although there are many different sortases, common traits as threonine substrate motif and His-Cys-Arg catalytic triad of sortase enable to develop SML with similar mechanisms. This mechanism let several physiological roles among sortases. For instance, the archetypical sortase A of *Staphylococcus aureus* plays a key role in anchoring surface proteins with a LPXTG motif to oligoglycines, such as lipid II, to the peptidoglycan of the cell wall. Moreover, sortases can also anchor pilin subunits and perform iron acquisition proteins to the cell envelope.

The cell wall sorting signal, consisted by a LPXTG motif followed by a segment of hydrophobic amino acids and a tail composed primarily of positively charged residues, is imperative in the transpeptidation process. Furthermore, the properly charged His-Cys-Arg triad of the catalytic active site plays a key role in the process.

In addition, there is an important dependence of the Ca^{+2} against the rate activity. It was seen how Ca^{+2}, Mg^{+2}, Mn^{+2} remarkably increased the speed of the reaction and the importance of sortase inhibition so as to avoid its virulence activity.

The applications are manifold, such as: protein modification, synthesis of protein–polymer conjugates and protein immobilization, among others. On one hand, sortase-mediated ligation (SML) enables promising results in the synthesis of antibody conjugates and their potentially anti-tumour drugs. On the other hand, the high specificity and versatility of sortases can be used to establish some industrial applications as well as particular couplings that other enzymes cannot afford.

Nowadays, the main spot is on the evolution of new engineered sortases with other substrate recognitions, distancing from the typical LPXTG motif and attaching other nucleophiles than N-terminal Gly. These would end up with more efficient sortases that recognize a wider range of substrates enabling more coupling reactions and applications.

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