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Structural and functional insights of sortases and their interactions with antivirulence compounds

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ABSTRACT

Sortase proteins play a crucial role as integral membrane proteins in anchoring bacterial surface proteins by recognizing them through a Cell-Wall Sorting (CWS) motif and cleaving them at specific sites before initiating pilus assembly. Both sortases and their substrate proteins are major virulence factors in numerous Gram-positive pathogens, making them attractive targets for antimicrobial intervention. Recognizing the significance of virulence proteins, a comprehensive exploration of their structural and functional characteristics is essential to enhance our understanding of pilus assembly in diverse Gram-positive bacteria. Therefore, this review article discusses the structural features of different classes of sortases and pilin proteins, primarily serving as substrates for sortase-assembled pili. Moreover, it thoroughly examines the molecular-level interactions between sortases and their inhibitors, providing insights from both structural and functional perspectives. In essence, this review article will provide a contemporary and complete understanding of both sortase pathways and various strategies to target them effectively to counteract the virulence.

1. Introduction

Sortases play a crucial role in pathogenesis of Gram-positive bacteria and are considered to be an important virulence factor (Alharthi et al., 2021; Telford et al., 2006). Conserved in all Gram-positive bacteria, sortases are cysteine transpeptidases responsible for pilus assembly in majority of pathogenic bacteria such as *Streptococcus pyogenes* (*S. pyogenes*), *Enterococcus faecalis* (*E. faecalis*), *Streptococcus pneumoniae* (*S. pneumoniae*), *Corynebacterium diphtheriae* (*C. diphtheriae*) and *Staphylococcus aureus* (*S. aureus*). Sortases are grouped into six classes and those are SrtA (Housekeeping activity), SrtB (Heme-iron scavenging activity), SrtC (Pili polymerization activity), SrtD& E (Recruitment of pilin subunits), and SrtF (likely to perform housekeeping activity) (Barnett et al., 2004; Hendrickx et al., 2011; Kandaswamy et al., 2013; Kline et al., 2009; Raz and Fischetti, 2008). However, not all bacterial species contain sortases from each class as their presence varies among different organisms. For instance, *E. faecalis* is reported to have two classes of sortases, one being a housekeeping sortase (SrtA) and the other being pili polymerizing sortase (SrtC) while *S. pyogenes* is known to possess an additional novel class of sortase (SrtC2) that anchors surface protein containing QVPTGV motif to the cell wall (Barnett et al., 2004; Kline et al., 2009; Raz and Fischetti, 2008). Furthermore, *S. pneumoniae* possesses three SrtC classes, namely SrtC1, SrtC2, and SrtC3 each with distinct functions (LeMieux et al., 2008).

Given the importance of sortases in covalent anchoring of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs), a thorough understanding of the sortase pathway is very crucial from a structural biology standpoint (Abujubara et al., 2023; Nuttee Suree, Liew et al., 2009). Furthermore, sortases are responsible for bacterial virulence, interactions with host immune cells, biofilm formation, and development of disease outcomes such as septic endocarditis, pneumonia, septic arthritis, mastitis, and gastrointestinal infection (Abujubara et al., 2023; Cho et al., 2022; Govindarajan and Kandaswamy, 2022; Govindarajan et al., 2022; Kudryavtsev et al., 2021;

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Marraffini et al., 2006; Shanmugasundarasamy et al., 2022). In addition, sortase substrates (e.g., Pilin subunits) possess the ability to bind to fibronectin, a high-molecular weight glycoprotein found on human tissues and leads to bacterial colonization and disease progression (Oh et al., 2006).

Owing to their virulence nature, sortases are considered as an attractive target for the development of a new class of antibacterial drugs and inhibiting them could reduce virulence and disease outcomes very significantly (Alharthi et al., 2021; Cascioferro et al., 2015; Kudryavtsev et al., 2021). Therefore, this article critically reviews on structural and functional aspects of various classes of sortases along with their substrate proteins. Additionally, subtle nuances on the pilus assembly mechanism across various Gram-positive bacteria were thoroughly discussed. Furthermore, this review article provides a comprehensive understanding on various sortase specific inhibitors (e.g., Diarylacrylonitriles, Rhodanines, Morpholinobenzoate, and Dihydro-β-carboline derivatives) and their mode of action in attenuating the virulence machinery without killing the bacteria (Maresso et al., 2007; Oh et al., 2004; Nuttee Suree, Yi et al., 2009). In summary, this article provides complete understanding on recent findings with respect to sortase assembly mechanism and various strategies to combat virulence and pathogenesis.

2. Sortases of Gram-positive bacteria

2.1. Sortase classes and their structural features

Sortases are a family of transpeptidases found in Gram-positive bacteria, play a crucial role in anchoring secreted proteins to the bacterial cell surface. Substrate proteins of sortases possess a conserved C-terminal Cell Wall Sorting Signal (CWSS), as well as a recognition motif followed by a hydrophobic domain and a positively charged tail (Paterson and Mitchell, 2004). Most Gram-positive bacterial species contain several sortase enzymes, which can be categorized into six distinct classes (A to F) based on their amino acid sequences and substrate specificity (Dramsi et al., 2005). For instance, Sortase B (SrtB) in *S. aureus* recognizes the NPQTN motif, whereas in *S. pyogenes* SrtB recognizes proteins with a LPXTG motif (Table 2) (Gaspar et al., 2005). The LPXTG sorting motif is cleaved to form a substrate enzyme intermediate (Clancy et al., 2010). All classes of sortases feature a conserved His-Cys-Arg catalytic active site and an eight stranded β barrel structure (Ilangovan et al., 2001). The structure of each class of sortase enzyme varies mainly in the β6/β7 loop, β7/β8 loop and the regions before and after the active site region (Table 1) (Ilangovan et al., 2001). Unique structural features of sortase A (of *B. anthracis)* include N-terminal extension, presence of a second groove that can interact with substrates in *S. pyogenes* and in *Streptococcus agalactiae* (*S. agalactiae*) (Khare et al., 2011; Race et al., 2009; Weiner et al., 2010). The β6/β7 loop of SrtB is a crucial structural element that recognizes sorting signal of substrates (Jacobitz et al., 2014). Class C enzymes have a distinctive lid structure firmly attached within the active site through interactions with essential catalytic residues. Specifically, a sulfur-aromatic interaction between Cys in the active site region and an aromatic residue on the lid ensures its secure anchoring within the active site (Manzano et al., 2009). Therefore, this section discusses distinct structural features of various classes of sortases.

2.1.1. Sortase A

Sortase A (SrtA), commonly referred to as housekeeping sortase, serves as cysteine transpeptidases anchoring a diverse array of proteins with distinct functions to the cell wall and are involved in the pili assembly making them a crucial component during infection and pathogenesis (Comfort and Clubb, 2004). SrtA is predominantly identified in bacteria with low GC content, such as *B. anthracis*, *Bacillus subtilis* (*B. subtilis*), *Listeria monocytogenes* (*L. monocytogenes*), *S. pneumoniae,* and *S. pyogenes* (Susmitha et al., 2021).

In a typical sorting reaction, SrtA catalyzes the cleavage of surface proteins containing a sorting signal and LPXTG motif. All substrate proteins of SrtA contain recognition motif comprising Leu-Pro-any-Thr-Gly (LPXTG, X being any amino acid) and are destined for cell wall attachment, such motif specific sorting is a distinctive feature of SrtA (Clancy et al., 2010). The cleavage specifically occurs between the Gly and Thr residues within the LPXTG motif, which results in the formation of a covalent intermediate. In this intermediate, the active site Cys residue of SrtA temporarily attaches to the Thr residue. The sortase specific cleavage is followed by transpeptidation, facilitating the creation of an amide bond between the amino-group of cell-wall peptidoglycan and the carboxyl-group of Thr. This step ensures the covalent attachment of the cleaved protein to cell wall components (Dramsi et al., 2005; Hendrickx et al., 2011; Paterson and Mitchell, 2004).

The SrtA structure is characterized by an elliptical β-barrel comprising eight strands, which was unveiled by examining the Sa-SrtA (*S. aureus* SrtA) structure using NMR spectroscopy (Ilangovan et al., 2001). This β-barrel is shaped by the interaction of two predominantly antiparallel β-sheets. The β-sheets are organized with the following strand arrangement: β1, β2, β5, and β6 form single sheet, and β3, β4, β7, and β8 complete the barrel structure. A 3_{10} helix is positioned between β2 and β3, and a short α-helix is found between β4 and β5. Whereas the β6–β7 loop demonstrates flexibility. Notably, a hydrophobic groove is a prominent feature of the structure, constructed on a single side of the barrel by β4 and β7 strands, along with β2–β3, β3–β4, β6–β7, and β7–β8 loops. Within this groove, the catalytic residues, Arg197, Cys184, and His120 are precisely situated at the termination of strands β4 and β7 and the commencement of strand β8 (Ilangovan et al., 2001; Nuttee Suree, Liew et al., 2009). These structures exhibit broad consistency across Class A sortases found in various species such as *B. anthracis*, *S. agalactiae*, and *S. pyogenes*. While preserving the central β-barrel and surrounding helices identified in Sa-SrtA, certain variations are evident, including extra short helices and species-specific extensions at both the N- and C-terminals. Nonetheless, the conformation of loops, particularly those forming the hydrophobic groove, remains generally conserved (Khare et al., 2011; Race et al., 2009; Zong et al., 2004).

2.1.2. Sortase B

Group B sortases are commonly identified in Gram-positive bacteria characterized by low GC content, such as Staphylococcus spp., Clostridium spp., Listeria spp., and Bacillus spp., (Bradshaw et al., 2015). It plays a role in promoting heme-iron scavenging activity, facilitating cross-linking with heme near the cell membrane through the signal sequence NP(Q/K)TN. SrtB is a polypeptide composed of 246 amino acids with N-terminal membrane anchor and an active site located within the TLXTC motif (Mazmanian et al., 2002). In contrast to SrtA, which serves as an anchor for 20 distinct polypeptides, SrtB demonstrates specificity for only one substrate, namely, IsdC, synthesized in its precursor form (Marraffini and Schneewind, 2005). In *B. anthracis* and *S. aureus*, SrtB is involved in heme uptake function (Schneewind and Missiakas, 2014). Whereas, in *S. pyogenes* it is known to be involved in pilus assembly which is generally mediated by SrtC (Tamai et al., 2017).

The acquisition of iron is an essential process for bacterial pathogens, vital for energy generation and DNA replication. In *S. aureus*, the iron-regulated surface determinant (Isd) system coordinates heme acquisition. Comprising ten genes distributed among five operons: *isdA*, *isdB*, *isdCDEFsrtBisdG*, *isdH*, and *orfXisdI*, the Isd system encodes SrtB responsible for anchoring the iron acquisition protein IsdC to the cell wall (Clancy et al., 2010; Hammer and Skaar, 2011; Mazmanian et al., 2002) (Fig. 1A). SrtA plays a crucial role in anchoring IsdA, IsdB, and IsdH to the cell wall which then act as a receptor for hemoprotein ligands like hemoglobin and hemoglobin-haptoglobin, respectively (Dryla et al., 2003; Torres et al., 2006). In an IsdC-dependent manner, heme-iron is liberated from the hemoprotein and transported across the cell wall. Heme is transferred from IsdB or IsdH to cell surface-located IsdA (Muryoi et al., 2008). IsdA then facilitates the transfer of heme to

Table 1
Diversity in structural and functional aspects of sortase classes across various Gram-positive bacteria.

Species	Number of Sortases	Sortases Class and Functions	Substrate proteins	Structural features.	References
E. faecalis	2	SrtA- housekeeping Sortase SrtC- pilin protein polymerization	EbpA, B, and C	SrtA lacks crystallography data. Predictions for the active sites, including Leu20, Gly25, Arg62, Asn42, Thr64, and Asn15, were made using CASTp. Crystal structure of SrtC is not available.	(Hendrickx et al., 2009; Sivaramakrishnan et al., 2019)
B. anthracis	4	SrtA- housekeeping sortase SrtB- iron acquisition BaSrtC- role in sporulation SrtD- pilin polymerization	IsdC, IsdK, Hal, BasA to G (*B. anthracis* surface protein), BcpA	SrtA, SrtB and SrtC have a common eight-stranded β-barrel structure. SrtA: Active site residues include His126, Cys187, and Arg196 are present in β6, β7 strand and the β6/β7 loop. SrtB: Active site residues include His140, Cys233, and Asp234 are located on the β3, β4, β7, and β8 strands. BaSrtC: Active site residues include His116, Cys173, and Arg185 located in the cleft formed by β4, β7, and β8 strands. SrtD: Structure is similar to SrtA.	(Balderas et al., 2012; Budzik et al., 2008; Fouet, 2009; Marraffini and Schneewind, 2006; Robson et al., 2012; Weiner et al., 2010; Zhang et al., 2004)
B. subtilis	2	YhcS- anchoring of the substrate YhcR to the cell wall. YwpE- function not yet identified	YhcR (sorting signal- LPDTS) and YfkN (sorting signal- LPDTA)	Crystal structures of YhcS and YwpE are not available.	(Liew et al., 2012; Nguyen et al., 2011)
S. aureus	2	SrtA- housekeeping sortase SrtB- iron acquisition	SpA, FnbpA, FnbpB, ClfA, ClfB	SrtA: Active site residues include His120 in the β6 strand, Cys184 in the β7 strand, Arg197 in the β8 strand. SrtB: Active site residue, including Cys223, is present at the end of the β7 strand.	(Marraffini et al., 2004; Mazmanian et al., 2001; Zong et al., 2004; Zong et al., 2004).
S. Pyogenes	4	SrtA- housekeeping sortase SrtB- pilus assembly, anchoring of T6 protein SrtC1- pilus assembly SrtC2- anchoring of surface proteins with QVPTGV motif	M protein, G-related α2-macroglobulin-binding protein (GRAB), protein F, and ScpA	SrtA: Active site residues include Cys208, His142, and Arg216. SrtB: Active site residues include Cys221, His126, His127 and Arg229 Crystal structures of SrtC1 and SrtC2 are not available.	(Barnett et al., 2004; Barnett and Scott, 2002; Kang et al., 2011; Manetti et al., 2007; Race et al., 2009).
B. cereus	4	SrtA, SrtB- anchoring of pili to the bacterial envelope SrtC- pilus assembly in vegetative cells SrtD- pili polymerization	BcpA and BcpB	Crystal structures of SrtA, SrtB, SrtC and SrtD are not available.	(Budzik et al., 2007)
C. diphtheriae	6	SrtA- polymerization and anchoring of SpaA-type pilus fibers SrtB/SrtC- assembly of SpaD-type pilus fibers SrtD/SrtE- assembly of SpaH-type pilus fibers SrtF- anchoring of SpaA-type pilus to the cell wall	SpaA, B, C, D, E, and F	SrtA: Active site residues include Cys222, His160, and Arg231. Crystal structures of SrtB, SrtC, SrtD, SrtE and SrtF are not available.	(Gaspar and Ton-That, 2006; Swaminathan et al., 2007; Swierczynski and Ton-That, 2006; Ton-That and Schneewind, 2003).
S. pneumoniae	7	SrtA-anchoring of formed pilus to the cell wall. SrtB- anchoring of pilus to cell wall. SrtC-1, SrtC-2, SrtC-3 - assembly of P1 structure. SrtG1, SrtG2- assembly of P2 structure.	P1 pilus subunits- RrgA, RrgB, and RrgC P2 pilus subunits- PitA, PitB	SrtC1: Active site residues include Cys193 at the C-terminus of the β7 strand, His131 is located within the loop following the β4 strand, and Arg202 is positioned at the N-terminus of the β8 strand. SrtC2: Structure similar to SrtC1. SrtC3: Active site residues include Cys206, Arg215, and His144. Crystal structures of SrtA, SrtB, SrtD, SrtG1 and SrtG2 are not available.	(Izoré et al., 2010; Manzano et al., 2008; Miao et al., 2023; Neiers et al., 2009; Zähner et al., 2010).

Table 2
Sortase classification and their substrate recognition motifs.

Sortase type	Substrate	Signal Motif	Function of sortase	Species	References
A	Surface protein (e.g., WapA, Pac, SspA, Bas A to G)	LPXTG	Anchors surface proteins	*S. aureus, B. anthracis, S. pneumoniae, Streptococcus mutans, Lactobacillus acidophilus, Enterobacter faecalis, C. diphtheriae, Streptococcus gordonii*	(Call, Goh, Selle, Klaenhammer, & O'Flaherty, 2015; Davies et al., 2009; Gaspar et al., 2005; Kemp et al., 2007; Kharat and Tomasz, 2003; Lee and Boran, 2003; Novick, 2000; Ton-That et al., 1999).
B	Heme binding protein (e.g., IsdC)	NP(Q/K)(T/S)(N/G/S)(D/A)	Iron uptake and regulation	*S. aureus, B. anthracis, L. monocytogenes, B. cereus, C. perfringens, C. diphtheriae, Streptococcus suis, S. pyogenes*	(Barnett and Scott, 2002; Boekhorst et al., 2005; Budzik et al., 2007; Lu et al., 2011; Mandlik et al., 2007; Maresso et al., 2006; Mariscotti et al., 2009; Mazmanian et al., 2003)
C	Pilin subunits (e.g., SpaA, D, H, BasI and H)	(I/L)(P/A)XTG	Pilin polymerase	*S. pneumoniae, Actinomyces oris* (*A. oris*), *B. cereus, Lactobacillus lactis, S. suis, S. agalactiae*	(Budzik et al., 2007; Comfort and Clubb, 2004; Dieye et al., 2010; Dramsi et al., 2006; Manzano et al., 2008; Osaki et al., 2002; Robson et al., 2012; Wu et al., 2012).
D	Endospore coat protein (e.g., BcpA, BcpB)	LPNTA	Spore formation	*B. anthracis, C. perfringens, B. cereus, C. diphtheriae, S. suis*	(Mandlik et al., 2007; Marraffini and Schneewind, 2006; Osaki et al., 2002; Suryadinata et al., 2015).
E	Chaplins (e.g., ChpA-D)	LAXTG	Aerial hyphae development, surface protein attachment, anchoring of pilus	*S. coelicolor, A. oris, C. diphtheriae, Corynebacterium glutamicum, Streptomyces avermitilis*	(Chang et al., 2019; Claessen et al., 2003; Das et al., 2017; Duong, 2015; Duong et al., 2012; Kattke et al., 2016; Susmitha et al., 2019)
F	Base pilin (e.g., SpaB)	LPXTG	Anchors protein substrates to the cell wall	Actinobacteria, *P. acnes, C. diphtheriae*	(Di Girolamo et al., 2019; Ramirez et al., 2020; Swaminathan et al., 2007).

IsdC, mediating its passage across the cell wall (Liu et al., 2008; Zhu et al., 2008). Alternatively, IsdH and IsdB may directly transfer heme to IsdC or IsdE (Muryoi et al., 2008). The IsdDEF, an ABC transporter, includes IsdD, a permease; IsdE, a lipoprotein binding heme; and IsdF, an ATPase. This transporter facilitates the transfer of heme from IsdC to IsdE, eventually leading to its transportation into the cytoplasm (Fig. 1B). IsdC is buried deep within the cell and is linked to mature peptidoglycan of bacterial cell wall by action of SrtB, which recognizes its NPQTN motif (Schneewind and Missiakas, 2014; Skaar and Schneewind, 2004). The IsdC C-terminal motif NPQTN undergoes cleavage between Thr and Asn (Marraffini and Schneewind, 2005).

Unlike SrtA, SrtB has more α helices in its secondary structures. While the β barrel structure and active site configuration remains the same between the two. The β6/β7 loop is present in a closed conformation as it is not activated by Ca^{2+}. Additionally, the loop is longer along with the presence of an α helix. SrtB features polar surfaces that facilitates recognition of more polar sorting signals like NPQTN compared to LPXTG in SrtA (Clancy et al., 2010; Jacobitz et al., 2017). SrtB mainly differs from SrtA in having two short α-helices at the N terminal (1 and 2) and a long-helix which is present near the active site (Marraffini and Schneewind, 2005). SrtB has a unique fold of two structural motifs, having one long and three short β strands (Zhang et al., 2004). Proline at the second position in the motif aids in substrate recognition in SrtB (Mariscotti, García-del Portillo and Pucciarelli, 2009).

2.1.3. Sortase C

The Group C sortases play a crucial role in the polymerization of pili, catalyzing transpeptidation and establishing a strong covalent interaction with these fibrous structures. SrtC, an enzyme identified in certain bacteria, primarily Gram-positive bacteria with either low GC content (e.g., Bacilli spp., Streptococci spp., and Clostridia spp.) or those with high GC content (e.g., Corynebacterium spp.). SrtC is instrumental in building pili composed of several pilin subunits, facilitating microbial attachment and biofilm formation (Dramsi et al., 2005; Spirig et al., 2011).

While the assembly process of pili varies across species, a common two-step mechanism is prevalent. Initially, SrtC covalently binds pilin subunits, forming the pilus shaft. Subsequently, SrtA secures the pilus into the cell wall (Bradshaw et al., 2015). SrtC facilitates the polymerization of pilin through a transpeptidation reaction, creating lysine–isopeptide bonds to connect pilin proteins and form pili (Manzano et al., 2009). SrtA then identifies and cleaves specific sorting signals to anchor them to the cell wall (Bhat et al., 2021). The pilin proteins are attached to the cell wall as the amino group of the precursor reacts with the intermediate. Ultimately, the enzyme is released, completing the attachment process (Paterson and Mitchell, 2004). The interplay between pilus-specific sortase and housekeeping sortase enzymes contribute to both pilus length and the attaching of surface proteins to the cell wall, playing an important role in cell–cell interactions and thereby contributing to pathogenesis (Chang et al., 2019).

The assembly of SpaA, SpaD, and SpaH-type pili in *C. diphtheriae* is exclusively facilitated by various classes of SrtC (Ton-That and Schneewind, 2003). Furthermore, specific pilus systems, which consist of only two subunits without basal pilin subunits, such as the ones observed in *Bacillus cereus* (*B. cereus*) and the FCT-1-type pili of *S. pyogenes*, are also orchestrated by Class C sortases (Budzik et al., 2007). The α3-helices form a flexible lid with an elongated N-terminal region that extends over the catalytic pocket, playing a crucial role in substrate recognition and maintaining sortase stability in Gram-positive bacteria (Khare et al., 2011; Manzano et al., 2009). Findings about SrtC structures reveal minor shifts or electron density variations that are crucial in determining the lid's configuration. When in a closed state, the enzyme is inactive as the lid obstructs the sorting signal binding site. Activation occurs when the lid undergoes a partial shift, enabling the sorting signal to bind and form the enzyme–substrate thioacyl intermediate (Khare et al., 2011).

2.1.4. Sortase D

Group D sortases have been identified in Streptococcus spp. and Bacillus spp., with their structures have been characterized in both *B. anthracis* and *Clostridium perfringens* (*C. perfringens*) to date. In *B. anthracis,* Sortase D (SrtD) specifically recognizes substrates with the LPNTA sorting signal and anchor them to Lipid II in the cell wall (Marraffini and Schneewind, 2006, 2007). Despite limited knowledge about class D sortases, they are known to contribute to spore formation under oxygen-limiting conditions (Marraffini and Schneewind, 2006, 2007).

B. cereus pili are composed of two subunits: the primary pilin BcpA, which is distributed along the entire length of the pilus, and the secondary pilin BcpB, situated at the fiber tip (Budzik et al., 2007). SrtD cleaves the LPXTG motif of BcpA between Thr and Gly. Subsequently, it creates an amide bond with the ε-amino group of Lys in the YPKN motif of neighboring BcpA subunits (Budzik et al., 2008). Although the BcpB

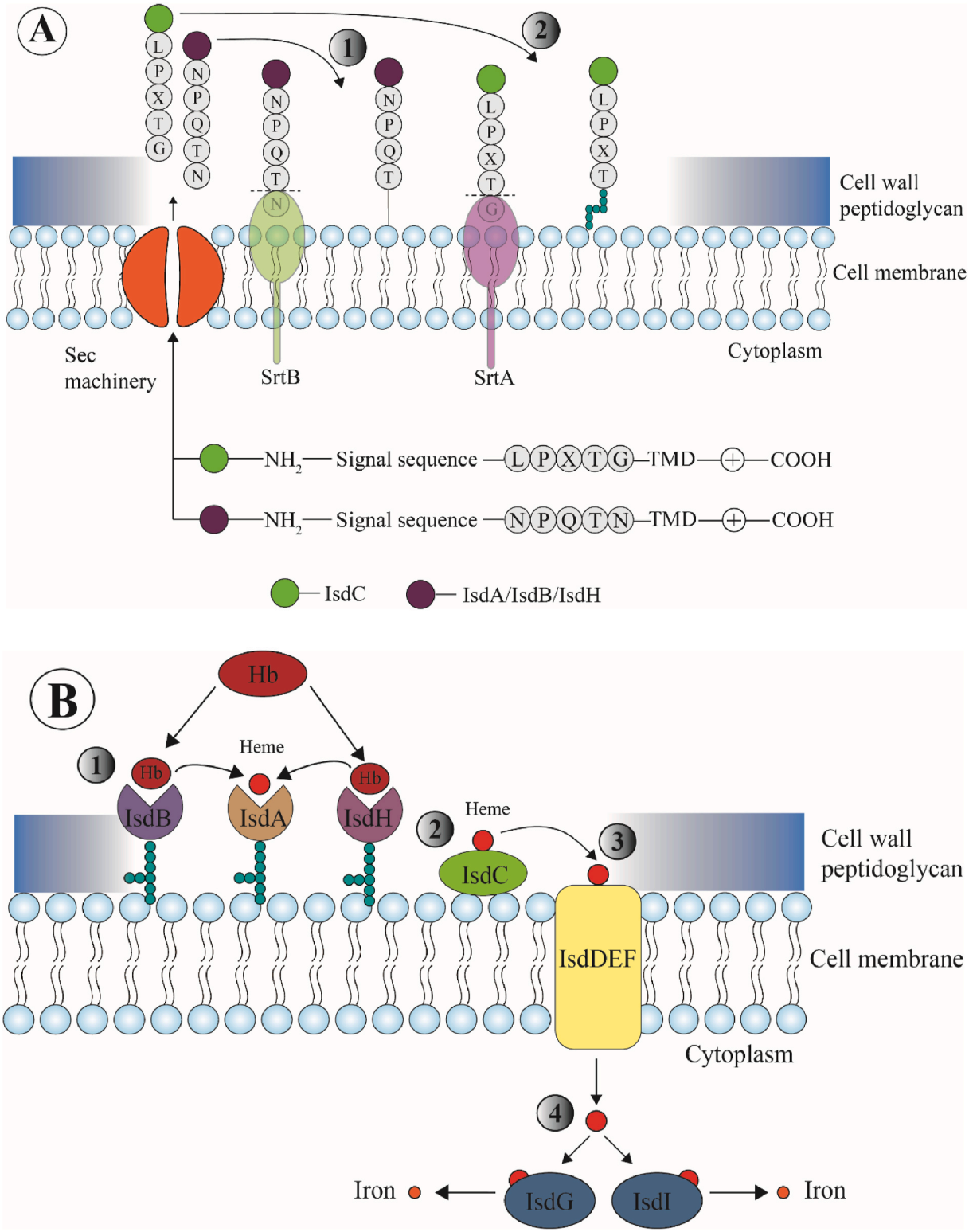

Fig. 1. (A) Signal cleavage pattern and motif recognition in *S. aureus*- (1) SrtB is responsible for recognizing and anchoring the substrate IsdC, which contains the NPQTN motif. IsdC is directly attached to the peptidoglycan layer without lipid II, (2) SrtA anchors IsdA/B/H, each of which possesses an LPXTG motif. **(B) Role of SrtA and SrtB in IsdC mediated iron uptake in *S. aureus*:** Under iron deprived conditions IsdC is primarily involved in iron uptake. (1) IsdB and IsdH act as receptors for substrates like haemoglobin, (2) After binding to IsdB/H, heme is detached from haemoglobin and passed onto IsdA. Heme can be passed from to IsdC via IsdA or directly from IsdB or IsdC, (3) IsdDEF acts as a permease system, transporting heme into the cytoplasm, (4) Heme oxygenases, IsdG and IsdI degrade heme to release iron.

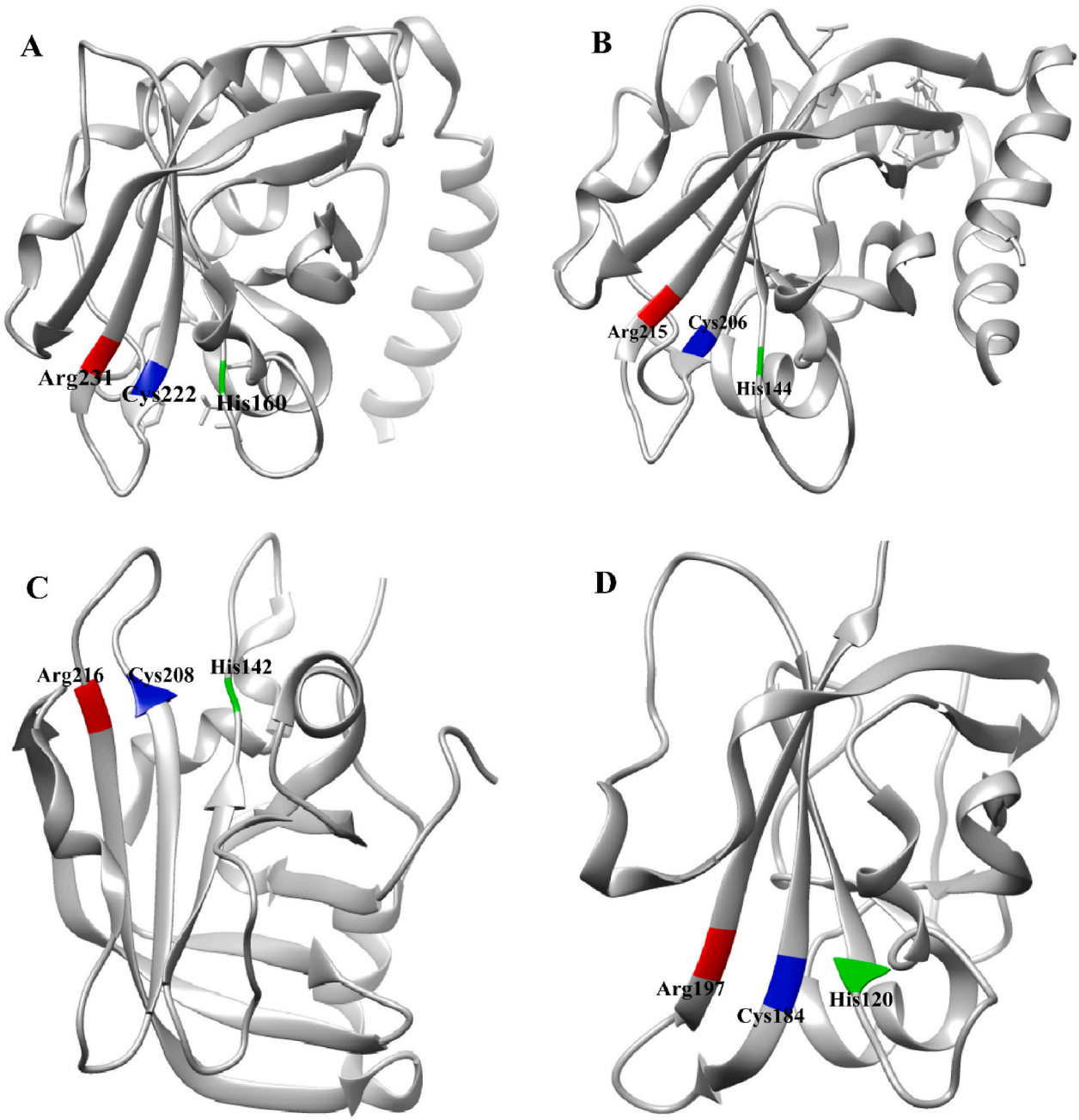

Fig. 2. (A) Structure of *C. diphtheriae* SrtA (PDB ID: 5K9A)- The active sites, His160 on the β6 strand highlighted in green, Cys222 on the β7/β8 loop highlighted in blue, and Arg231 on the β9 strand highlighted in red. **(B) Structure of *S. pneumoniae* SrtC3 (PDB ID: 2W1K)-** The active site components Cys206 (blue), Arg215 (in red), and His144 (green) are highlighted. **(C) Structure of *S. pyogenes* SrtA (PDB ID: 3FN5)-** The catalytic triad Cys208 (blue), Arg216 (red), and His142 (green) are highlighted. **(D) Structure of *S. aureus* SrtA (PDB ID: 1T2P)-** The active site components consisting of His120 located on the β6 strand (green), Cys184 situated on the β7 strand (blue), and Arg197 positioned on the β8 strand (red) are highlighted. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

precursor does not contain the YPKN pilin motif, it does exhibit an N-terminal signal peptide and a C-terminal IPNTG sorting signal (Budzik et al., 2007; Budzik et al., 2009). SrtD cleaves the IPNTG signal of BcpB, amide-linking its C-terminal Thr to the YPKN motif present in BcpA, placing BcpB at the pili tip (Budzik et al., 2009). On the contrary, SrtA is also involved in the process, cleaving BcpA precursors and attaching them to the side chain amino group within lipid II. This action terminates fiber elongation and immobilizes BcpA pili within the cell wall (Budzik et al., 2008).

C. perfringens SrtD (CpSrtD) is expected to adopt a monomeric form, featuring a conserved eight β-strand β-barrel structure observed in all other sortases. Similar to other sortase enzymes, it also displays a conserved active site on the surface, featuring a catalytic Cys171 within the β7 strand, Arg178 in the β8 strand, and His109 positioned in the β3–β4 loop. Notably, CpSrtD exhibits N-terminal α-helices, a characteristic that is lacking in the structure of *B. anthracis* SrtD. This observation is very significant as the earlier observations had only identified long N-terminal α-helices in class B and C sortases (Suryadinata et al., 2015).

2.1.5. Sortase E

Actinobacteria, commonly present in terrestrial and aquatic

environments, employ Class E sortases (SrtE) to covalently anchor substrates with LAXTG sorting signal. SrtE1 and SrtE2 from *Streptomyces coelicolor* (*S. coelicolor*) are the extensively studied Class E enzymes (Elliot et al., 2003; Nuttee Suree, Liew et al., 2009). The structural characterization of SrtE1's catalytic domain reveals a sequence of strands and loops, including β1, H1, β2, H2, β3, β4, H3, β5, β6, H4, β7, and β8. The active site histidine residue (His251) resides within an extended segment between β4 and β5. Positioned at the end of the β7 strand, the cysteine residue (Cys320) is present. Additionally, the β8 strand harbors active site arginine residue (Arg329). SrtE1 displays distinctive active site loops (β3/β4 and β6/β7), distinguishing it from other classes of sortases. Structural comparisons highlight the unique arrangement of the active site loops β3/β4 and β6/β7 in SrtE1, potentially contributing to the binding site containing the LAXTG sorting signal. Interestingly, the β3/β4 loop in SrtE1 is marginally more extended compared to other sortase enzymes, like those in class A, and features a conserved tyrosine residue (Kattke et al., 2016). The unique substrate specificity identified in SrtE1 seems to be a defining trait of class E enzymes. Genome comparisons indicate their probable role in attaching proteins to the cell, with these proteins carrying sorting signals characterized by an alanine residue at position P3 (Comfort and Clubb, 2004).

2.1.6. Sortase F

Sortase F (SrtF) identified as the sole transpeptidase in *Propionibacterium acnes* (*P. acnes*), functions similarly to SrtA. Presumed to be the primary housekeeping sortase, it attaches potential substrates to the cell wall (Di Girolamo et al., 2019). In *C. diphtheriae,* SrtA facilitates pilus polymerization, while SrtF ensures cell wall anchoring by incorporating SpaB at the pilus base (Swaminathan et al., 2007). The precise function of SrtF in various organisms remains unclear, and its structural characteristics are yet to be determined.

2.2. Structural characteristics of pilin subunits as sortase substrates

In the intricate assembly of Gram-positive pili, individual protein subunits, known as pilins, are organized in pilus operons and carry unique sortase recognition motifs. These pili exhibit a distinctive structural pattern, featuring a backbone pilin that forms the pilus shaft, along with two or three minor pilins which form the base of the pilus. Pilin subunits undergo cohesive linking facilitated by a pilus-specific sortase, often identified as SrtC. These specialized sortases recognize unique sorting motifs near the C-terminal end of each pilin, orchestrating the incorporation of pilins into a polymeric structure through covalent intermolecular isopeptide bonds. In the final stages, SrtA facilitates the formation of a covalent bond between the basal subunit and the cell wall peptidoglycan, ensuring a stable anchoring of the fully assembled pilus. This section explores the structural aspects of some of the well-studied sortase specific pilin substrates.

2.2.1. Spy0128

Spy0128 is a pilin protein consisting of 340 amino acid residues, featuring a distinctive sorting recognition motif GVPTG, found between residues 308 and 312. Spy0128 protein subunits often display an elongated configuration with two domains, measuring 98 Å in length and a width of 20–30 Å. Both domains showcase irregular β-structures, representing modified adaptations of the immunoglobulin fold. Structurally, the N-terminal domain, spanning residues 18 to 171, constructs a β-sandwich. However, the C-terminal domain, covering residues 173 to 307, incorporates 11 β strands. Its central structure forms a β sandwich, with a five-stranded β sheet tightly packed against a four-stranded β sheet. Additionally, the protein has two intramolecular isopeptide bonds, strategically contributing to stability. These bonds arise from the covalent interaction between the side chains of Lys and Asn, with Lys36-Asn168 forming the linkage in the N-domain and Lys179-Asn303 forming the linkage in the C-domain. The positioning of these isopeptide linkages immediately preceding the interdomain junction and the sortase recognition motif favours the fusion of leading and trailing ends of β strands in a coherent fashion (Kang et al., 2007).

2.2.2. SpaA

In *C. diphtheriae*, SpaA forms the pilus shaft, comprising three domains: the M-domain (middle domain), C-domain (C-terminal domain), and N-domain (N-terminal domain). The crystal structure of SpaA molecules exhibit a stacked arrangement, with the C-domain preceding the N-domain, resembling assembled pili. SpaA adopts a conformation featuring three consecutive Ig-type domains, resulting in an elongated structure measuring 105 Å in length. The M and C-domains are stabilized by intramolecular isopeptide bonds, while the N-domain lacks such isopeptide bonds. Specifically, the isopeptide bond in the M-domain is formed between Lys199 and Asn321, and in the C-domain, it is formed between Lys363 and Asn482 (Fig. 3). Additionally, an E-box motif containing Glu446 plays a crucial role in incorporating minor subunits SpaB and SpaC. The structural integrity of SpaA is reinforced by a metal binding site in the M-domain and a disulfide bond in the C-domain. The presence of Asp241 in M-domain or Glu446 in the C-domain is crucial for bond formation (Kang et al., 2009).

2.2.3. RrgA

RrgA in *S. pneumoniae,* an extensive 893-residue protein, is characterized by a conserved LPXTG-like sorting signal at its C-terminal end. Its intricate architecture encompasses four domains (D1-D4), contributing to an elongated structure spanning approximately 195 Å in length and 70 Å in width. The integration of elements from both N-terminal and C-terminal regions occurs in domains D1 and D2. Notably, D3 is intricately inserted into D2, and the combination of D2/D3 is further inserted into D1, facilitated by short linkers that suggest potential domain flexibility (Izoré et al., 2010).

D1 resembles an IgG domain and features five β-strands formed from residues 47–133 at the N-terminal end. The final strand, linked to D4, originates from C-terminal residues 723–731. Simultaneously, D2, comprising N-terminal residues 134–220 and C-terminal residues 588–722, exhibits a 11-β-stranded sandwich configuration (Izoré et al., 2010). Also, it resembles the B domain of the collagen-binding adhesin Cna from *S. aureus* (Deivanayagam et al., 2000). D2 and the major domain, D3 are linked via a short β-hairpin structure. Preceding the C-terminal CWSS sequence, domain D4 is separated from D3 by a stretched linker region composed of 13-residues. Similar to D1, D4 also resembles an IgG domain, consisting solely of C-terminal residues 736–859. Within RrgA, two vital intramolecular isopeptide bonds exist-one in the D2 domain (Lys191-Asn695) and another in D4 (Lys742-Asn854) which play a key role in structure stability (Izoré et al., 2010) (Fig. 4A). The sequential alignment of domains D1, D2, and D4, with D3 situated at the end, suggests a role in extending the ECM-recognition D3 domain outside the pilus fiber to reach out for the host cell (Izoré et al., 2010).

2.2.4. RrgB

RrgB's structure encompasses four domains. Three domains (D1, D2, and D4) are aligned linearly, while D3 is laterally positioned relative to D2 (Fig. 4B). Each of the four domains exhibit β-barrel folds, complemented by extra β-strands and one or two helices decorating a side of the β-barrel in D1 and D4. Within the D1 domain, the amino acid Lys183 establishes a covalent link with the IPQTG motif of an adjoining RrgB, thereby facilitating the polymerization of RrgB. The study reveals the existence of four intramolecular isopeptide bonds, distributed among the D1, D2, D3, and D4 domains. In D1 (residues 29–188), the isopeptide bond is established by Lys41 and Asn184, stabilized by Glu143. The isopeptide bonds in D3 (residues 330–432) and D4 (residues 447–627) are formed by Lys349-Asn428 and Lys453-Asn62, respectively, receiving stabilization from residues Glu405 and Glu577. In the D2 domain (residues 189–329), the intramolecular linkage

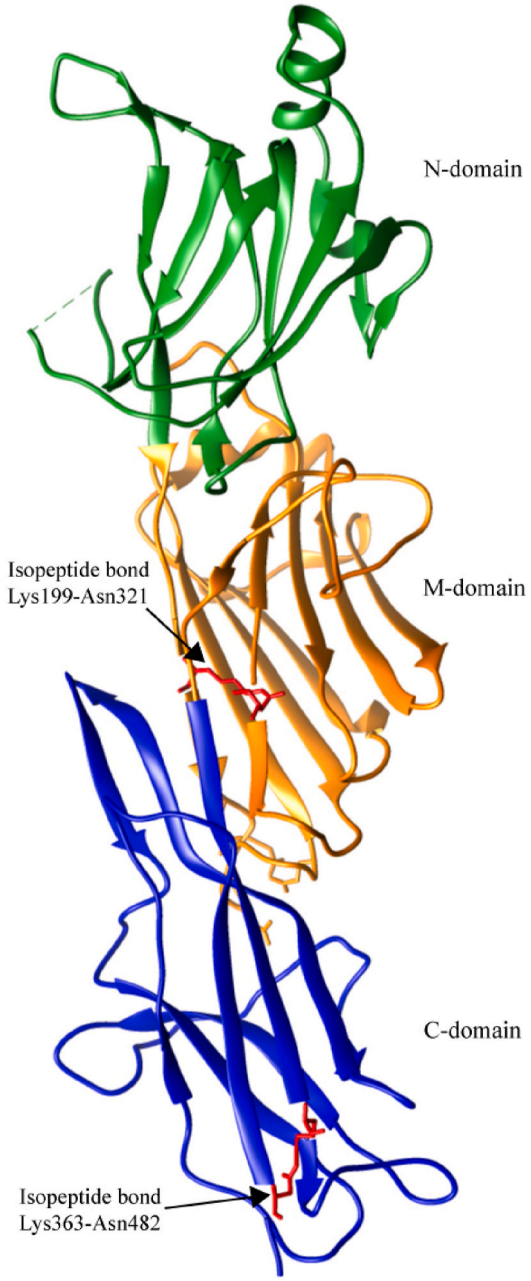

Fig. 3. Structure of SpaA subunit (PDB ID: 3HR6)- It comprises M (green), N (orange), and C (blue) domains. The intramolecular isopeptide bonds within domains M and C are highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Lys193–Asn318) is associated with Asp241. The IPQTG signal motif is present in the region 628–632 (El Mortaji et al., 2012; Spraggon et al., 2010).

2.2.5. RrgC

In *S. pneumoniae*, the pilin protein RrgC is composed of 393 residues, featuring three domains (D1, D2, and D3), a distinct sortase recognition motif (VPDTG), and a transmembrane anchoring domain at the C-terminal. The structure resembles a curved, bent rod, with approximate dimensions of 110 × 40 × 25 Å. D1 spans residues 25–147, adopting a β-sandwich structure with exposed alpha-helices. On the other hand, D2 (residues 148–255) and D3 (residues 256–359) form antiparallel β-sheets (Fig. 4C). Flexible regions connect these domains. The core β-barrel in both D2 and D3 is stabilized by two intramolecular isopeptide bonds, contributing to enhanced thermal resistance and protecting against proteolysis. Specifically, in D2, the isopeptide bond forms between Lys155 and Asn252, while in D3, it forms between Lys264 and Asn354 (Shaik et al., 2014).

2.3. Structural and functional aspects of species specific sortases

Sortases of Gram-positive bacteria have a dual function: they can either covalently attach surface proteins to the cell wall or assemble pilin proteins to form a matured pili on microbial surface that enhance bacterial adhesion to host tissue (Scott and Barnett, 2006; Scott and Zähner, 2006). In pathogenic Gram-positive bacteria, sortases often act as crucial virulence factors by anchoring cell wall proteins that significantly contribute to disease outcomes such as soft tissue infections, sepsis, osteomyelitis, pneumonia, and endocarditis (Kadirvelu et al., 2024; Zhang et al., 2014). These cell wall-anchored proteins serve as adhesins, invasins, and disruptors of the host's innate or specific immune defenses, enhancing bacterial adhesion, and nutrient acquisition (Cascioferro et al., 2014; Jacobitz et al., 2017). The function of sortases varies across the Gram-positive organisms. For instance in *E. faecalis,* SrtA anchors pili to the cell wall, while SrtC is responsible for the polymerization of pili subunits (Choo et al., 2023; Nielsen et al., 2013). In *S. aureus* SrtB is involved in iron uptake (Schneewind and Missiakas, 2014). Therefore, this section illuminates the role of various sortase class of enzymes present in well-studied Gram-positive bacteria such as *C. diphtheriae, S. pneumoniae, E. faecalis, S. pyogenes, B. anthracis,* and *S. aureus* (Table 1).

2.3.1. Corynebacterium diphtheriae

C. diphtheriae, the agent responsible for respiratory and cutaneous diphtheria, relies on pili for initial attachment to the host and the establishment of infections (Ott et al., 2022). The bacterium assembles three different types of pili structures, SpaABC (Spa-sortase mediated pilus assembly), SpaDEF and SpaHIG each encoded by distinct gene clusters. In the SpaA-type pilus, there are three subunits: SpaA, the predominant pilin protein that extends along the length of the pilus shaft; SpaB, present at the base; and SpaC, located at the pilus tip. Conversely, the SpaD-type pilus consists of three subunits- SpaD and SpaE forming the pilus shaft and SpaF comprising the pilus tip (Gaspar and Ton-That, 2006). The organism has six distinct sortase genes: *srtA, srtB*, *srtC*, *srtD*, *srtE*, and *srtF* which play a pivotal role in processing these precursors, facilitating their transformation into functional polymeric structure (Ton-That and Schneewind, 2003). Pilus-specific SrtA is associated with both the polymerization and anchoring of the SpaA-type pilus, it catalyzes assembly through repetitive, irreversible transpeptidation reactions, forming covalent links via isopeptide bonds (Ton-That and Schneewind, 2003). Whereas SrtF aids in attaching SpaA-type pili and pilin monomers to the cell wall, without participating in pilin polymerization alongside with SrtA. In the case of the SpaD-type pilus, both SrtB and SrtC play a major role in polymerization by recognizing the LPMTG motif of SpaD, SrtB is crucial for the incorporation of SpaE, with the sorting motif LALTG (Khare and VL Narayana, 2017; Necchi et al., 2011). Furthermore, SrtD and SrtE are involved in anchoring the SpaH-type pilus (Gaspar and Ton-That, 2006; Swaminathan et al., 2007). In the assembly of both SpaA and SpaD type of pili, major subunits (SpaA or SpaD) play a crucial role, while minor subunits (SpaB, SpaC, SpaE, SpaF) are not essential (Gaspar and Ton-That, 2006; Swierczynski and Ton-That, 2006; Ton-That and Schneewind, 2003).

Sortase activation initiates pilus formation by creating a SrtA-SpaA intermediate, the amine groups of lysine (K190) from another SpaA pilin initiate an attack on the thioacyl linkage (T494) in the intermediate, forming a K190-T494 isopeptide bond. Successive reactions catalyzed by sortase covalently link SpaA, SpaB, and SpaC subunits resulting in pilus formation (Swaminathan et al., 2007; McConnell et al., 2021; Sue et al., 2023). The addition of SpaB to the pilus base serves as a

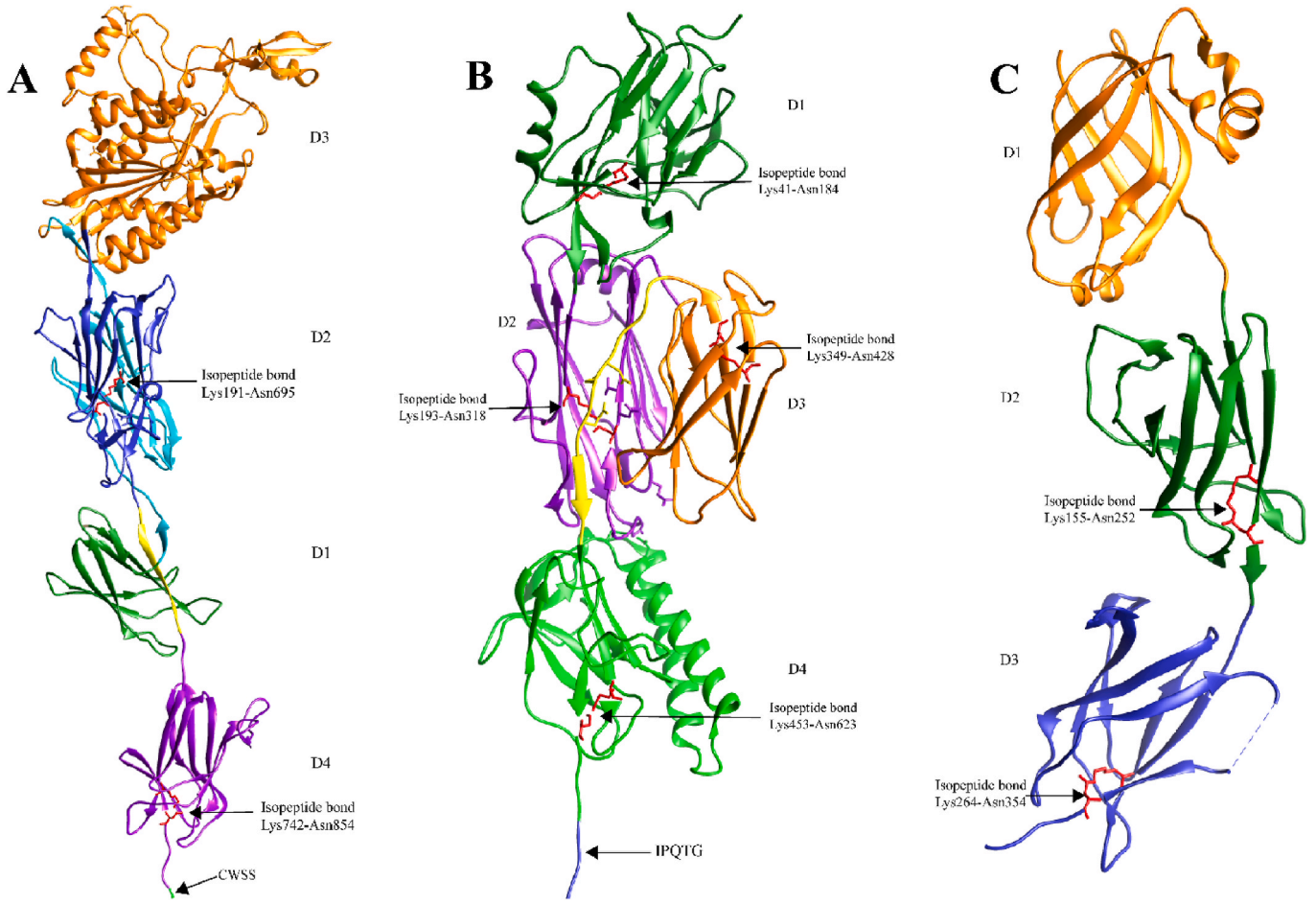

Fig. 4. (A) Structure of RrgA subunit (PDB ID: 2WW8)- It comprises four domains D1 (dark green), D2 (blue), D3 (orange), and D4 (purple). The isopeptide bonds in domains D2 and D4 are highlighted in red. CWSS is present in the C-terminal end highlighted in green. **(B) Structure of RrgB subunit (PDB ID: 2Y1V)-** It consists of four domains: D1 (dark green), D2 (purple), D3 (orange), and D4 (green), along with an IPQTG motif in the C-terminal. The isopeptide bonds in domains D1, D2, and D3 are highlighted in red. **(C) Structure of RrgC subunit (PDB ID: 4OQ1)-** It comprises three domains: D1 (orange), D2 (dark green), and D3 (blue). The isopeptide bonds in domains D2 and D3 are highlighted in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

signaling event for the attachment of the pilus to the cell wall through the activity of the SrtF (Swaminathan et al., 2007). SrtA forms a SpaA-x-SpaA ("-x-'' represents isopeptide bond) crosslink by recognizing the LPLTG motif in another SpaA pilin. Likewise, SpaA-x-SpaC crosslink incorporates the tip pilin. Pilus polymerization concludes as SrtA identifies LAFTG in SpaB, catalyzing the formation of a SpaB-x-SpaA crosslink resulting in pilus formation (Fig. 5A) (Swaminathan et al., 2007; Ramirez et al., 2020; Sue et al., 2023).

Within the SrtA, the catalytic triad crucial for pilus polymerization is composed of Cys222, His160, and Arg231 (Fig. 2A). The overall structure adheres to the typical sortase fold, featuring an eight-stranded β-barrel core and seven α-helices. An additional three α-helices form a lid at the N-terminal, covering the active site. The H1 helix facilitates homodimerization, and H2 and H3, connected by a loop with a DPW lid motif, interact with the active site. During acylation, the lid opens to allow substrate entry, resulting in the formation of an enzyme–SpaA acyl intermediate. Within this process, Trp83 in the lid stacks with Cys222 and His160, while Asp81 interacts with Arg231, suggesting a regulatory role in pilin polymerization (Chang et al., 2018). Additionally, mutations like H60A, V187D, and Y188G markedly impact enzyme's activity and pilus assembly (McConnell et al., 2021).

2.3.2. *Streptococcus pneumoniae*

S. pneumoniae, a prominent human pathogen frequently found in the upper respiratory tract, stands as a major cause of bacterial pneumonia, meningitis, septicemia, and otitis media (Mehr and Wood, 2012). Among the pneumococcal pili, Pilus-1 (P1) and Pilus-2 (P2) have been well studied (Ness and Hilleringmann, 2021).

Pilus island 1 (PI-1) is responsible for P1 formation, encoding three class C sortases consisting of SrtC1, SrtC2 and SrtC3, three P1 subunits RrgA, RrgB, and RrgC, and a transcriptional regulator (RirA). The P1 has a heterotrimeric structure, with RrgB as the major pilin and RrgA and RrgC as minor pilins (Barocchi et al., 2006; Izoré et al., 2010; Shaik et al., 2014). RrgB constitutes the core of the pilus, while RrgA situated at the pilus tip is a potential adhesin that can interact with extracellular matrix elements (El Mortaji et al., 2012; LeMieux et al., 2006; Shaik et al., 2014). In contrast, the functional role of RrgC within the pilus structure remains ambiguous. Unlike its dependence on pilus-specific sortases for its attachment to the cell wall, RrgC utilizes the catalytic activity of SrtA to bind the pre-assembled pilus in the cell wall, specifically anchoring the P1 structure (Shaik et al., 2014; Ness and Hilleringmann, 2021; Paterson and Mitchell, 2006). The structural elements at the ends of the pneumococcal pilus components display patterns similar to LPXTG, with a distinctive variation notably lacking Leu at the initial position. The sorting motifs consist of IPQTG for RrgB, YPRTG for RrgA and VPDTG for RrgC (Fälker et al., 2008; LeMieux et al., 2008; LeMieux et al., 2006). SrtA specifically anchors the P1 structure (Ness and Hilleringmann, 2021; Paterson and Mitchell, 2006). Additionally,

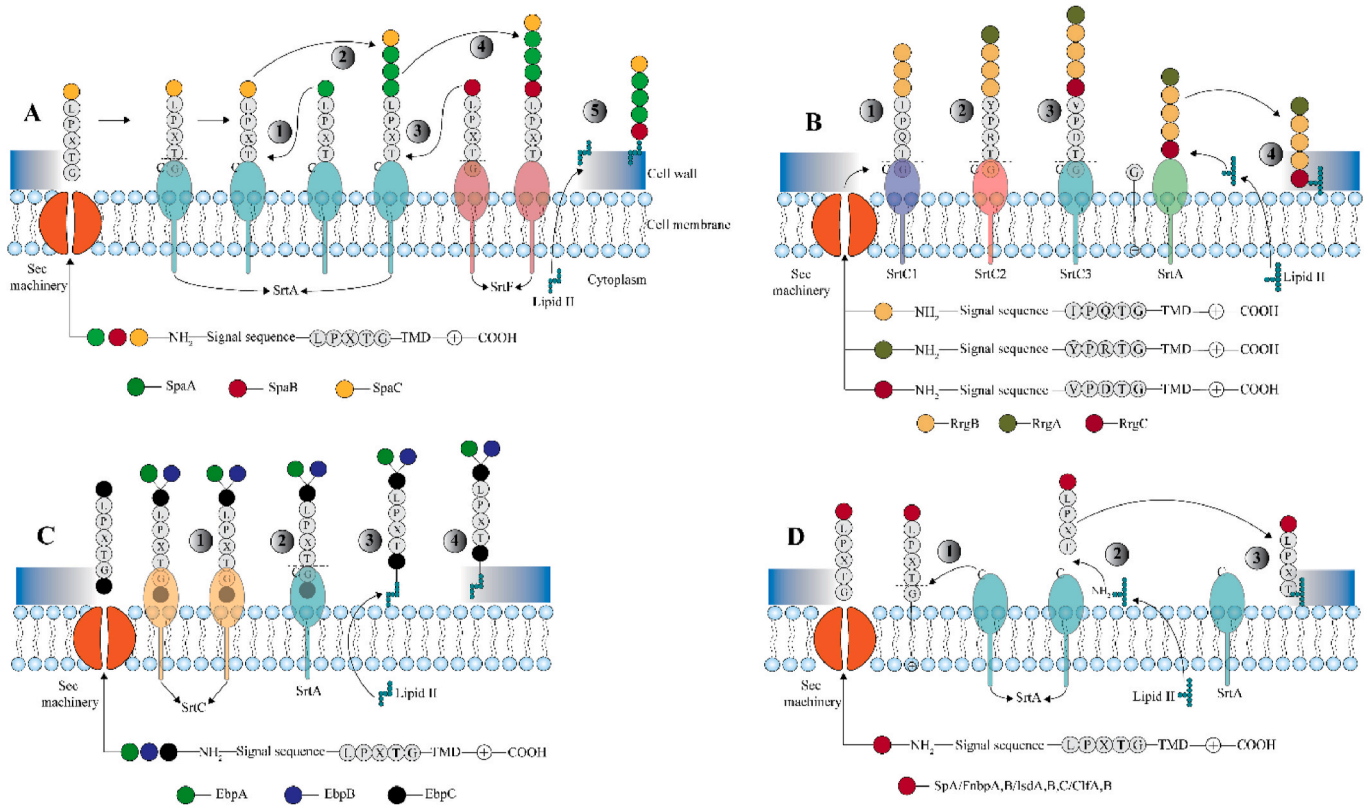

Fig. 5. Sortase assembled Pili of (A) *C. diphtheriae*- SrtA cleaves the substrates (SpaA, SpaB, and SpaC) at LPXTG motif facilitating the linkage of Thr to Lys within adjacent pilin motifs. (1) The first transpeptidation reaction occurs between SpaC and SpaA, linking the Thr of the SpaC LPXTG motif to the Lys of SpaA, (2) Subsequently, the pilus extends as SpaA pilins that forms pilus shaft, (3) SrtA recognizes SpaB and catalyzes the formation of SpaB and SpaA complex (4) The termination of pilus polymerization after integration of SpaB at the pilus base (5) SrtF anchors the Pili to cell wall through the lipid II intermediate complex. **(B) *S. pneumoniae*-** (1) SrtC-1 enzyme recognizes RrgB through the sorting signal and mediates the formation of an amide bond between Thr and the amino group of the cell wall, (2) SrtC-2 simultaneously polymerizes the minor pilin subunit RrgA, by recognizing the YPRTG motif, (3) SrtC-3 facilitates the attachment of RrgC to the pilus structure by recognizing VPDTG motif, (4) SrtA anchors the assembled pilus to the cell wall through lipid II intermediate complex. **(C) *E. faecalis*-**the pilin subunits, namely EbpA, EbpB (minor subunits), and EbpC (major subunits), are secreted across the cell membrane by the sec machinery. (1) SrtC facilitates the polymerization of the both major and minor pili subunits, (2) SrtA recognizes and cleaves the peptide bond between Thr and Gly in the LPXTG motif, leading to the formation of a thioester acyl-enzyme intermediate, (3) A nucleophilic attack occurs between the peptidoglycan precursor (Lipid II) and the intermediate, resulting in the anchoring of the assembled pilus structure to the cell wall by lipid II. **(D) *S. aureus,*** substrates including SpaA, FnbpA/B, ClfA/B, IsdA/B/C that have a conserved LPXTG motif and the substrates are translocated through sec machinery, (1) Cys residue present in active site of SrtA cleaves the LPXTG motif producing an intermediate, (2) The intermediate is then attacked by lipid II and the substrate is anchored to the cell wall, (3) The substrate protein is incorporated in the peptidoglycan layer during bacterial cell wall synthesis.

SrtC-1 catalyzes the covalent linkage of the major pilin subunit RrgB (El Mortaji et al., 2010). And, SrtC-2 is involved in the polymerization of the minor pilin subunit RrgA, while SrtC-3 catalyzes the attachment of RrgC to the pilus structure (Ness and Hilleringmann, 2021) (Fig. 5B). On the other hand, PI-2 encodes two pilins, PitA and PitB, along with sortases SrtG1 and SrtG2, as well as one signal peptidase-like protein (SipA). PitB functions as the core pilin, that forms P2. The sortase enzyme SrtG1 catalyzes the assembly of PitB units. In contrast, PitA and SrtG2 are both found to be less essential for P2 formation (Shaik et al., 2014; Bagnoli et al., 2008).

SrtC1 exhibits an architecture characterized by an eight stranded β-barrel, surrounded by three α-helices and three 3_{10} helices. The roof of the β-barrel is formed by α1, α2, and α3 helices, with the α3 region acting as a 'lid,' covering the active site. Within SrtC1, the active site comprises Cys193, His131, and Arg202. Specifically, Cys193 resides at the C-terminus of the β7 strand, His131 is located within the loop following the β4 strand, and Arg202 is situated at the N-terminus of the β8 strand (Manzano et al., 2008). These crucial residues play an integral role in the enzyme's transpeptidation reaction. The active sites are concealed by a lid, featuring the conserved DP(F/W/Y) anchor motif (Manzano et al., 2009; Persson, 2011; Spirig et al., 2011). Residues 50–72 form the lid, featuring interactions such as a salt bridge between Asp58 and the active site Arg202, along with a sulfur-aromatic interaction involving Trp60 and the active site Cys193. These interactions contribute to maintaining the lid in a closed and rigid state, which represents the prevalent form of the free enzyme in solution. Similarly, SrtC2 is expected to display a catalytic triad within a similar amino acid range. Conversely, the active site of SrtC3 is defined by Cys206, Arg215, and His144, positioned analogously to their counterparts in SrtC-1 (Fig. 2B). Additionally, a lid is present in the active site, anchored by Asp73 and Phe75 residues (Manzano et al., 2008).

2.3.3. *Enterococcus faecalis*

Enterococcal species is a prevalent commensal organism found in the gastrointestinal tract, and are recognized as causative agents in approximately 12% of healthcare-associated infections, contributing to conditions including endocarditis and urinary tract infections (UTIs) (Danne and Dramsi, 2012; Kreikemeyer et al., 2011). Ace adhesin (an adhesin to collagen) is a surface protein in *E. faecalis* that is responsible for the attachment to ECM proteins of the host tissue including collagen types I and IV, dentin and lamins (Nallapareddy et al., 2000).

The pilus loci, specifically *ebp* (endocarditis and biofilm-associated pili), is highly conserved among various strains of *E. faecalis* whereas *bee* (biofilm enhancer in enterococci) is identified in about 1% of strains

(Danne and Dramsi, 2012). The Ebp pili are heterotrimeric structures composed of three subunits: EbpA, EbpB, and EbpC (Sillanpää et al., 2013). EbpC is the major subunit, contributing to the formation of the pilus fiber backbone. The minor subunits, EbpA and EbpB, are localized at either the tip or the base of the pili structure and EbpA facilitates the initial attachment to host fibrinogen, collagen and platelets (Afonina et al., 2018). Translocation of sortase substrates across the cell membrane is mediated by the SecYEG translocation channel and the ATP-binding translocase, SecA (Kandaswamy et al., 2013). SrtA and SrtC are colocalized with SecA at the septum of *E. faecalis*. The polymerization of pili subunits is facilitated by SrtC, and subsequently, anchoring of these structures to the cell wall is facilitated by SrtA (Choo et al., 2023; Nielsen et al., 2013) (Fig. 5C). In the absence of SrtC, there is noticeable accumulation of pilin subunits in a single focal point (Choo et al., 2023; Kline et al., 2009).

The crystal structure of *E. faecalis* sortase A (Ef-SrtA) was not available at the time of this article preparation. However, a computationally modeled 3D structure of Ef-SrtA was available. This was performed using Computed Atlas of Surface Topography of Proteins (CASTp) through homology modelling by taking the crystal structure of *S. mutans* sortase A (PDB:4TQX) as a template. Analyzing the modeled protein (Ef-SrtA) active sites exposes amino acid residues Leu20, Arg35, Arg62, Asn42, Thr64, Gly25, and Asn15 and potentially interacts with a well-studied phytochemical such as curcumin and its analogues (Sivaramakrishnan et al., 2019).

2.3.4. Streptococcus pyogenes

S. pyogenes, also known as Group A Streptococcus (GAS), is a prevalent bacterium responsible for infections in the nasopharynx and skin. The attachment of bacteria to mucosal tissues, along with the formation of cellular aggregates and microcolonies, relies significantly on GAS pili and SrtA (Manetti et al., 2007). The GAS pili comprises a major subunit (FctA), forming the shaft, and two ancillary proteins (Ap-1/Cpa and Ap-2/FctB) (Kreikemeyer et al., 2011; Young et al., 2014). Polymerization of GAS pili is facilitated by SrtC (Nakata and Kreikemeyer, 2021). Various substrates of *S. pyogenes* include Spy0125, Spy0128, FctB, SipA (Khare and VL Narayana, 2017).

In *S. pyogenes*, SrtA anchors several proteins, including protein F, M protein, and the protein GRAB, to the bacterial cell wall. Unlike typical sortase enzymes, SpSrtA has the ability to recognize not just the standard LPXTG motif, but also LPXTA and LPKLG motifs (Wójcik et al., 2020). Conversely, SrtB demonstrates specificity for the T antigen, a prominent component of the GAS pili, and recognizes the QVPTGV motif (Raz and Fischetti, 2008). SrtC1 plays a crucial role in pilus assembly and is essential for bacterial adhesion (Manetti et al., 2007). SrtC2 anchors surface proteins carrying the QVPTGV motif (Barnett et al., 2004).

The presence of an opened β7/β8 loop plays a crucial role in the broad substrate specificity. The eight-stranded β-barrel structure of SrtA encompasses an active site composed of Cys-208, His-142, and Arg-216 situated at the center of a long cleft. The structure features an extended groove, with the catalytic Cys positioned at its core, creating binding site for substrates. It also lacks Ca^{2+} binding pocket (Race et al., 2009) (Fig. 2C). The presence of Trp residue near the active site blocks access to the substrate groove (Wójcik et al., 2020).

Spy0129 is a class B sortase, featuring an eight stranded β-barrel core mainly composed of antiparallel β sheets. The active site region comprised of the residues Cys221, His126, His127, and Arg229. The connecting loops β2/β3 (residues 86–105), β4/β5 residues 126–150) and β6/β7 (residues 173–214) form the substrate binding region and also have some key insertions (Kang et al., 2011).

2.3.5. Bacillus anthracis

B. anthracis, a Gram-positive bacterium of high pathogenicity, is responsible for causing anthrax. This bacterium, capable of forming spores, exists in two distinct forms: as vegetative cells and as spores. The vegetative cells undergo multiplication within the host, and upon the host's death, these cells transition into spores. Remarkably resilient to environmental conditions, these spores have the capacity to re-enter hosts and revert to vegetative cells (Koehler, 2009; Marraffini and Schneewind, 2007). *B. anthracis* possess four sortase enzymes: SrtA, SrtB, SrtC, and SrtD and the substrate proteins include BasA to BasK (Fouet, 2009). SrtA is involved in anchoring surface proteins including BasA to G with a LPXTG motif, in vegetative cells (Gaspar et al., 2005; Marraffini and Schneewind, 2006).

During their infectious phase, *B. anthracis* requires iron for growth and relies on hemoproteins. The *srtB* locus encodes SrtB, IsdC, IsdJ/X1, and IsdK/X2 under iron-deprived conditions (Gat et al., 2008). SrtB facilitates the anchoring of IsdC to the cell wall by recognizing and cleaving the NPKTG motif (Maresso et al., 2006). SrtD is involved in cleaving the pilin precursor and contributes to pili assembly through the formation of intermolecular amide bonds. During sporulation, an asymmetric septum forms, dividing the cell into a forespore and a mother cell. BaSrtC (a class D sortase) anchors BasI to the cell wall of the pre-divisional cell by recognition of LPNTA motif (Marraffini and Schneewind, 2007). Additionally, BaSrtC plays a role in attaching BasH to the cell wall of the forespore (Aucher et al., 2011).

SrtA exhibits a structure comprising eight β-strands and four alpha helices. The active site, comprised of His126, Cys187, and Arg196 residues, is situated at the end of the sheet formed by the β4, β7, and β8 strands. Significantly, Cys187, located immediately after the β7/β8 loop, forms a critical surface for lipid II recognition (Weiner et al., 2010). The SrtB structure features an eight-stranded β-barrel and a structurally disordered β7/β8 loop, similar to SrtA. The active site is located at the end of the β-barrel and contains a catalytic triad composed of Cys194, His140, and Asp234. Binding sites include a major groove (formed by β3, β4, β7, and β8 strands, with highly conserved amino acid residues such as Asn63, Asp84, Asn87, Tyr99, Thr193, and Arg204) and a minor groove (formed by β2, β3 strands, C-terminal of α2, and the β2/β3 loop). Surface-located residues, including Asn39, Trp45, Gln59, Tyr66, and Ser80, contribute to these binding sites (Zhang et al., 2004). The active site of BaSrtC, consisting of His116, Cys173, and Arg185, is positioned at the end of a cleft formed by β4, β7, and β8 strands. Notably, BaSrtC lacks the N-terminal lid which is commonly found in most sortase C enzymes. SrtA and SrtD share similar structures, featuring a short 3_{10}-helix within the β6−β7 loop (Robson et al., 2012).

2.3.6. Staphylococcus aureus

S. aureus, a potent human pathogen, can induce severe and persistent conditions, including osteomyelitis, pneumonia, and profound soft tissue infections (Selvaraj et al., 2018). Most of these infections arise from the presence of a diverse array of surface proteins associated with virulence to the cell wall. Within its genetic repertoire, *S. aureus* possesses two separate sortase genes, each encoding for SrtA and SrtB (Baba et al., 2008; Kuroda et al., 2001).

The catalytic process commences with the Cys residue of SrtA, leading to the cleavage of the peptide bond between Thr and Gly within the sorting signal. This initiates the formation of a tetrahedral intermediate, ultimately resulting in the establishment of a stable thioacyl intermediate that forms a covalent bond between sortase and its protein substrates (Novick, 2000). SrtA then identifies its second substrate, lipid II, which serves as the cell wall precursor (Ruzin et al., 2002). It facilitates a reaction where the amino group within the cross-bridge peptide of lipid II attacks the carbonyl carbon within the thioacyl bond. This gives rise to a transient tetrahedral intermediate, leading to the formation of the protein-lipid II product. Subsequently, this complex is incorporated into the peptidoglycan through transpeptidation and glycosylation reactions, contributing to the synthesis of the cell wall (Jacobitz et al., 2017; Perry et al., 2002) (Fig. 5D). On the other hand, the second sortase type, referred to as "pilin polymerases," constructs bacterial pili through the polymerization of pilin protein subunits (Hendrickx et al., 2011; Kline et al., 2010). These enzymes responsible for pilin assembly utilize a transpeptidation reaction similar to that of

SrtA of *S. aureus*. However, in contrast to using lipid II as a nucleophile for anchoring proteins to the cell wall, these enzymes employ a lysine amino group present in a protein pilin subunit as a secondary substrate. This lysine amino group attacks the thioacyl intermediate formed between the sortase and the protein, facilitating the assembly process (Cozzi et al., 2011; Z. Wu and Guo, 2012). The lysine amino group initiates an attack on the thioacyl intermediate formed between the pilin subunit and sortase, creating lysine-isopeptide bonds that connect protein subunits and ultimately construct pili. Both processes, involving the anchoring of proteins to the cell wall and pili construction, are catalyzed by sortase enzymes and occur in the extracellular membrane (Suree et al., 2007).

The structure of SrtA *in S. aureus* is formed by eight β strands, an α helix and two 3-turn helices where the β4, β7 and β8 strands form a substrate binding pocket. Active site is formed by Cys184 (located at the end of β7 strand), His120 (located at end of β6 strand), Arg197 (located on β8 strand) (Marraffini et al., 2004) (Fig. 2D). SrtA also has a Ca^{2+} binding site near the active site, which stimulates catalysis by altering the conformation of a surface loop that recognizes the substrate. The Ca^{2+} binding site is formed by Glu105, Glu108, Asp112, Asn114, and Glu171. In the β6/β7 loop, situated outside the β3/β4 loop, the Glu171 coordinating residue plays a key role in substrate recognition and binding, and in presence of Ca^{2+} the stability of the loop is enhanced (Naik et al., 2006).

3. Antivirulence compounds and their mode of action

3.1. Sortase specific inhibitors and their mode of action

Sortase inhibitors in most cases do not kill the bacteria (Kudryavtsev et al., 2021), however, it does disarm the pathogenic properties of bacterial strains thereby providing a significant advantage over conventional antibiotics as the inhibitors do not harm the microbiota and provides low selectivity pressure thereby preventing the emergence of drug-resistant strains (Cascioferro et al., 2015; Maresso et al., 2007). Furthermore, recent studies describe sortase inhibitors as compounds with great potential to be used as antivirulence drugs (Dickey et al., 2017; Jaudzems et al., 2019). Therefore, this section of the article highlights the roles of various protein (antimicrobial peptides) and non-protein (Diarylacrylonitriles, Rhodanines, Morpholinobenzoate, and Dihydro-β-carboline derivatives) molecules in inhibiting the sorting machinery of pathogenic bacteria. In addition, this section also critically evaluates the limitations of various SrtA inhibitors.

Diarylacrylonitriles class of compounds is one among the well-studied SrtA inhibitors, as those compounds bind to the active site of SrtA and prevent it from interacting with its substrate proteins (e.g., pilin proteins) resulting in an inaccessible active site (Ha et al., 2020). In addition, the inhibitors also form covalent bonds with the amino acid Cys present in the catalytic site of SrtA (Fig. 6), resulting in an irreversible modification of the active site (Maresso et al., 2007; Oh et al., 2004). For instance, (Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile (DMMA) is recently demonstrated to inhibit SrtA by forming hydrophobic binding pockets with active site of SrtA

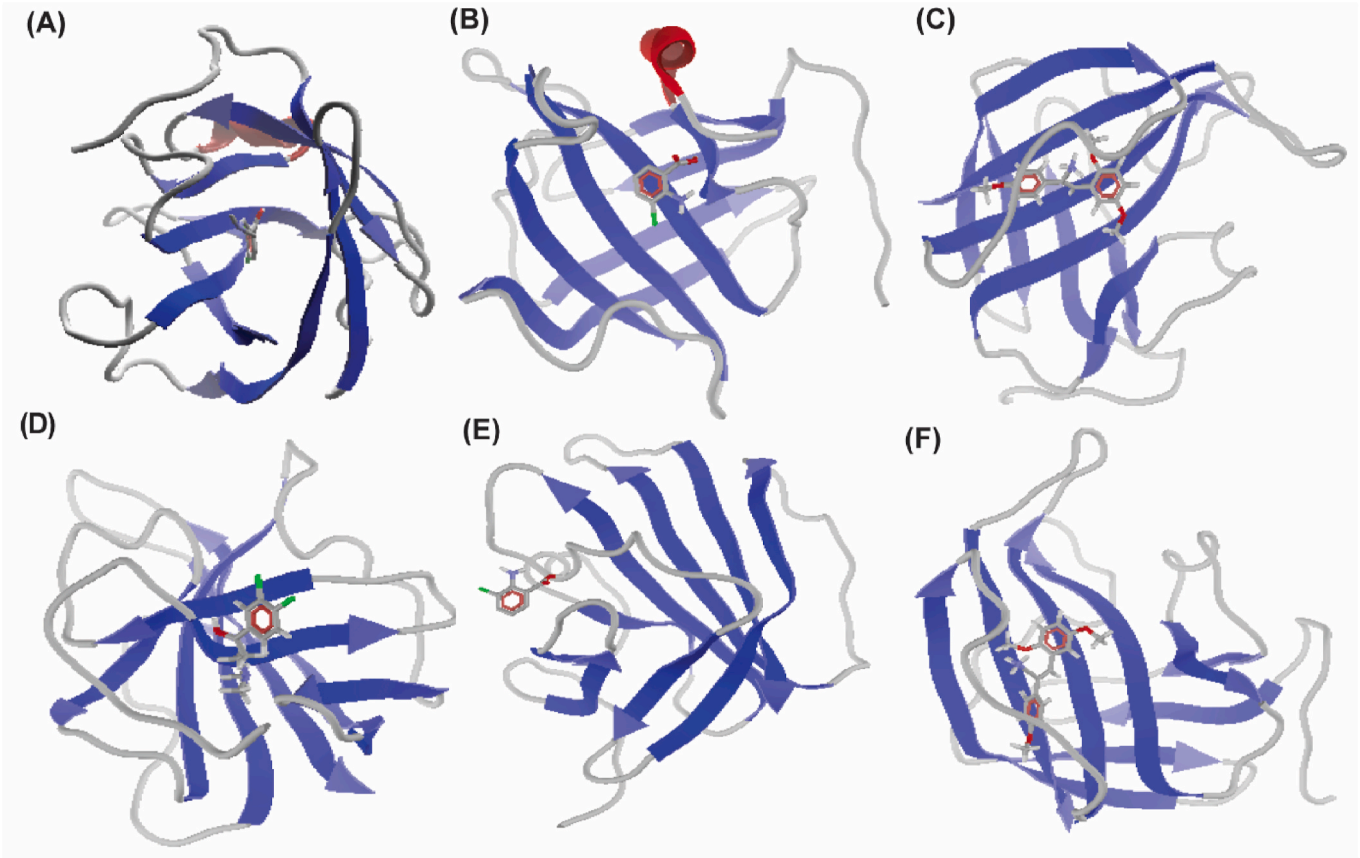

Fig. 6. Docked complexes of sortase inhibitors and sortase proteins of various bacterial species- (A) Interaction between 1-(3,4-dichlorophenyl)-3-(dimethylamino) propan-1-one and *S. aureus* SrtA (PDB: 1IJA), **(B)** Interaction between 2-(2-amino-3-chlorobenzoylamino)-benzoic acid and *S. aureus* SrtA (PDB: 1IJA), **(C)** Interaction between (Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile (DMMA) and *S. aureus* SrtA (PDB: 1IJA), **(D)** Interaction between 1-(3,4-dichlorophenyl)-3-(dimethylamino)propan-1-one and *S. aureus* SrtA (PDB: 2MLM), **(E)** Interaction between 2-(2-amino-3-chlorobenzoylamino)- benzoic acid and SrtA from *S. aureus* (PDB: 2MLM), and **(F)** Interaction between (Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile (DMMA) and *S. aureus* SrtA (PDB: 2MLM).

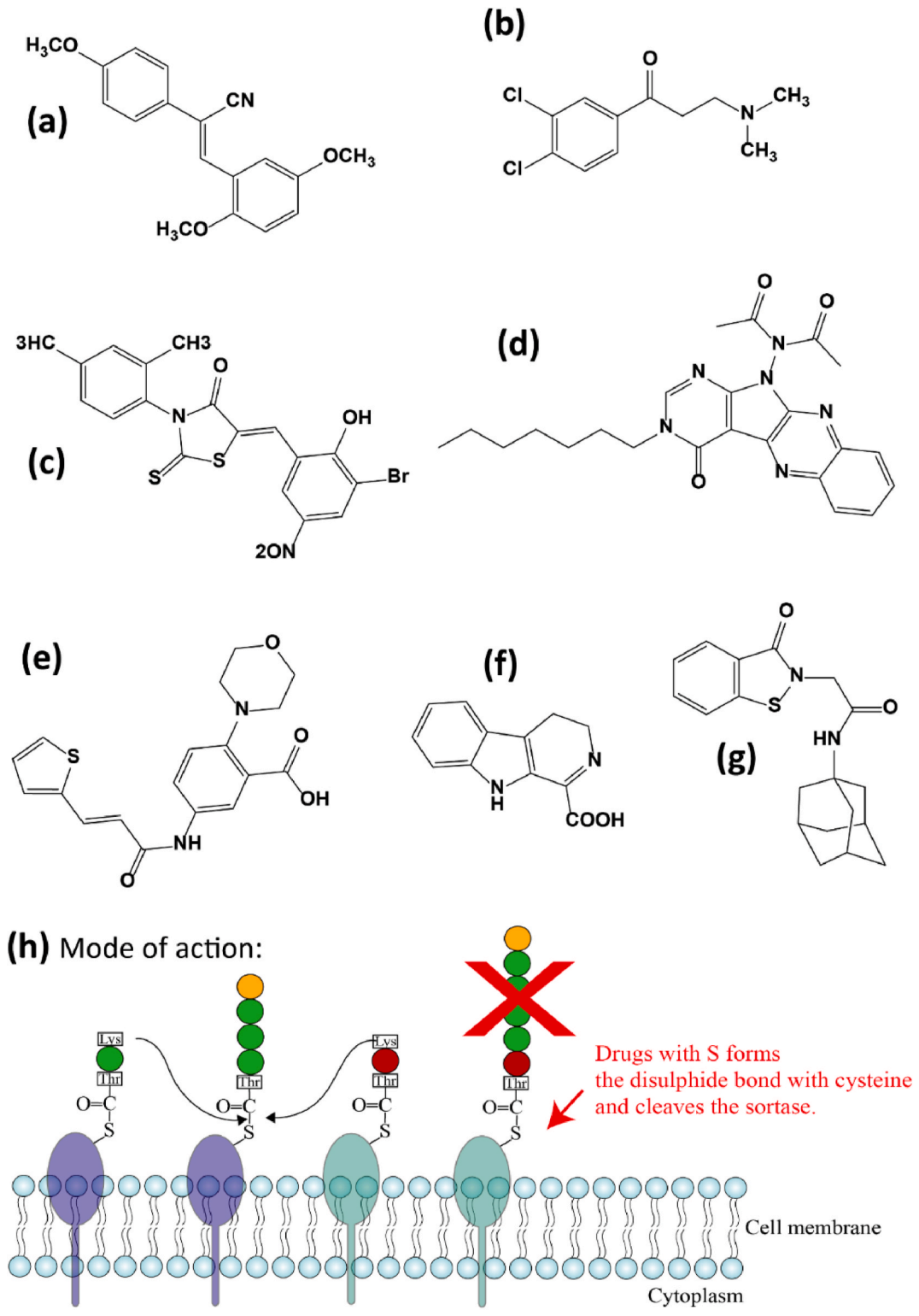

Fig. 7. Structure of sortase inhibitors (a) DMMA, (b) 1-(3,4-dichlorophenyl)-3-(dimethylamino) propan-1-one, (c) 2-(2-amino-3-chlorobenzoylamino)-benzoic acid, (d) Rhodanines series of compounds containing 2,4-dimethyl and 2-OH functional groups, (e) methyl 2-(N-morpholino)-5-nitrobenzoate, (f) 6-hydroxydihydro-β-carboline, (g) N-(adamantan-1-yl)-2-(3-oxo-2,3-dihydro-1,2-benzothiazol-2-yl)-acetamide, (h) the sortase inhibitors interacts with the cysteine residues and form a disulphide bond to cleave the sortase from the cell membrane.

(Cascioferro et al., 2015), Specifically with amino acids such as Val193, Trp194, Ala92, Ala104, Leu169, Val168, and Ile182 found in active site (Table 3, Fig. 6A). In addition, studies involving mouse models have also shown that intravenous administration of DMMA reduced the mortality of mice and decreased the *S. aureus* infection in kidneys and joints. Furthermore, the fibronectin-binding assay reveals the DMMA inhibits the binding of *S. aureus* to fibronectin (an adhesive glycoprotein that has affinity towards to SrtA) found on host tissue (Oh et al., 2006, 2010). However, the inhibitory effect of DMMA on other classes of sortases such as SrtC remains unclear and requires thorough investigation.

Table 3
Sortase inhibitors and their mode of action.

Inhibitor Name	Sortase Class and bacterial species	Type of Compound	Effective inhibitory concentration	Mode of inhibition	References
(Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl) acrylonitrile (DMMA) (Fig. 7a)	SrtA of *S. aureus*	Diarylacrylonitriles	IC_{50} value of 9.2 μM	Forms hydrophobic binding pockets with active site of SrtA. Specifically, with amino acids (Val193, Trp194, Ala92, Ala104, Leu169, Val168, and Ile182) found in active site (Fig. 6C and F)	(Cascioferro et al., 2015; Oh et al., 2004).
1-(3,4-dichlorophenyl)-3-(dimethylamino) propan-1-one (Fig. 7b)	SrtA of *S. aureus* and *B. anthracis*	Aryl(β-amino)-ethyl Ketones (AAEK)	IC_{50} value of 15 μM in *S. aureus* and IC_{50} value of 5.6 μM in *B. anthracis*	The thiol group of Cys184 (present in active site SrtA) was inhibited by AAEK type of compound through non-competitive and irreversible covalent modification of amino acids present in active site. (Fig. 6A and D)	(Cascioferro et al., 2015; Maresso et al., 2007)
2-(2-amino-3-chlorobenzoylamino)-benzoic acid (Fig. 7c)	SrtA of *S. aureus*	Unknown or Novel Class of compound	IC_{50} value of 59.7 μM	Inhibition of amino acids found in the active site of SrtA. In addition, reduces biofilm thickness and alters biofilm structure. (Fig. 6B and E)	(Volynets et al. 2022)
Rhodanines series of compounds containing 2,4-dimethyl and 2-OH functional groups (Fig. 7d)	SrtA of *S. aureus* and SrtA of *B. anthracis*	Rhodanines	IC_{50} value of 3.7 μM	The exchange of thiol-disulfide groups between inhibitor and Cys 184 (of SrtA) occurs in reversible manner and blocks the SrtA active site.	(Cascioferro et al., 2015; Nuttee Suree, Yi et al., 2009; Uddin et al., 2012)
Modified version of commercially available methyl 2-(N-morpholino)-5-nitrobenzoate (2) (Fig. 7e)	Recombinant form of $SrtA_{\Delta 59}$ in *S. aureus*	Morpholino benzoate derivatives	IC_{50} value of 75 μM	The presence of an electron withdrawing group (e.g., NO_2, Cl, F) and hydrophobic groups in the thiophene increases SrtA inhibitory activity	(Cascioferro et al., 2015; Chenna et al., 2010; Zong et al., 2004)
6-hydroxydihydro-β-carboline (Fig. 7f)	SrtA of *S. aureus*	Dihydro-β-carboline	IC_{50} value of 290 μM	Disarm the virulence mechanism without altering the growth of the bacteria. Molecular level of interaction between inhibitor and SrtA remains unclear.	(Cascioferro et al., 2015; Lee et al., 2010)
N-(adamantan-1-yl)-2-(3-oxo-2,3-dihydro-1,2-benzothiazol-2-yl)-acetamide (Fig. 7g)	SrtA of *S. aureus*	Benzo[d]isothiazol-3 (2H)-one-adamantane amine derivatives	IC_{50} of 6.11 μM	Formation of covalent bond between sulfur atom of active site cysteine (Cys 184 of SrtA) and benzothiazole group of inhibitor leads to high velocity irreversible binding.	(Cascioferro et al., 2015; Zhulenkovs et al., 2014)

In addition to Diarylacrylonitriles, Rhodanines are natural compounds of flavonoid glucosides that have been reported to effectively inhibit the activity of SrtA without affecting the growth and survival of bacteria (Wang et al., 2023). In addition, Rhodanin attenuates the virulence-related phenotype of Methicillin-resistant *S. aureus* (MRSA) by reducing the adhesive properties of the surface protein (SpA- Staphylococcal protein A) and decreasing the binding to fibrinogen, and reducing biofilm formation (Nuttee Suree, Yi et al., 2009; Wang et al., 2023). In particular, one of the Rhodanines series of compounds containing 2,4-dimethyl and 2-OH as functional groups (Table 3) block the active site of SrtA by exchanging thiol-disulfide groups between the inhibitor and Cys 184 (of SrtA) and this occurs in a reversible manner. Similarly, the Pyridazinones, and Pyrazolethiones class of compounds inhibit SrtA by blocking the active site but without causing any detrimental effect to the bacteria (Cascioferro et al., 2015; Dubey and Bhosle, 2015; Nitulescu et al., 2021). In particular, the pharmacophore feature of Pyridazinones class of compounds reveal the presence of two hydrophobic region and three H-bond acceptors as functional groups (Table 3), studies have confirmed a strong interaction between those functional groups and cysteine residue (Cys 184) of SrtA (Cascioferro et al., 2015; Dubey and Bhosle, 2015; Kudryavtsev et al., 2021). Such interactions of SrtA with inhibitors severely compromises the ability of sortase enzyme in performing crucial tasks such as substrate recognition, thio-esterification, transpeptidation that takes place during pilus assembly of *S. aureus* (Ha et al., 2020).

Structure Activity Relationship (SAR) studies demonstrate an active inhibition of $SrtA_{\Delta 59}$ (Table 3) by modified version of Morpholino benzoate class of compounds. $SrtA_{\Delta 59}$ is a fully active variant of SrtA with truncated 59 amino acids at N-Terminal region of active SrtA (Chenna et al., 2010). The morpholine ring, amide carbonyl, and NH groups of the inhibitor was found to be crucial in inhibiting SrtA activity of *S. aureus* without affecting cell viability (Chung et al., 2019). In addition, inhibitory effects were also observed on compounds derived from marine derived *Streptomyces* sp. MBTH32, compounds such as lumichrome exhibit strong inhibitory activity against SrtA of *S. aureus*. Furthermore, cell clumping assay revealed a reduction in immobilization with fibrinogen-binding protein thus limiting the anchoring ability suggesting lumichrome as potent antivirulence compound to treat *S. aureus* infections (Chung et al., 2019).

In addition to aforementioned non-protein inhibitors, Antimicrobial Peptides (AMPs) were proven to disrupt the virulence machinery (Govindarajan and Kandaswamy, 2023). In particular, cationic AMPs such as human β-defensin 2(hBD2) was proven to alter the localization patterns of both virulence protein (SrtA) and a conserved protein conducting channel (SecA), and inhibits the growth of *E. faecalis* (Järvå et al., 2018). Furthermore, hBD2 was also proven to kill *Candida albicans* by forming pores on cell membrane (Järvå et al., 2018). In another study, it was shown that the oxidized form of Human α-defensin 5 (HD5) causes bleb formation (on cell division site), cellular elongation, and clumping of *E. coli* cells (Chileveru et al., 2015). Given the inhibitory effects of AMPs against bacteria and fungi they hold a promising potential to treat a wide range of microbial infections and some of them are under clinical trials (Mohaideen et al., 2023). Nonetheless, AMPs are unlikely to be considered as mainstream drugs as they are limited by various factors such as toxicity, protease susceptibility and high manufacturing cost (Huan et al., 2020).

3.2. Synergistic effects of sortase inhibitors and antibiotics

Recent studies have demonstrated that sortase inhibitors when

combined with traditional antibiotics inhibits the virulence but does not kill the bacterial cells. For instance, Punicalagin (a natural compound found in pomegranate) in combination with traditional antibiotics such as Cefotaxime effectively inhibits the virulence of MRSA without causing bacterial cell death. Molecular docking studies have identified key residues (Lys190, Tyr187, Ala104, and Glu106) involved in the binding mechanism between punicalagin and SrtA, with a calculated binding free energy of −8.4 kcal/mol(Song et al., 2022). Similarly, molecular dynamics simulations, Quercitrin (QEN), a bioflavonoid from *Sabina pingii* var. *wilsonii* was found to bind to SrtA via hydrogen bonding and hydrophobic interactions, localizing to the active region crucial for SrtA function. The interaction involves strong bonds with Ala104 and Ser106, along with van der Waals interactions with Val193, Val166, Gly167, and Val168, leading to reduced flexibility of SrtA residues in the binding site. QEN demonstrates notable inhibitory activity against SrtA, displaying an IC_{50} of 32.18 ± 5.36 μg/mL. Despite this effect, QEN shows minimal impact on bacterial growth, even at concentrations as high as 256 μg/mL. These findings highlight the specific inhibitory action of QEN against SrtA without significantly affecting *S. aureus* proliferation (Liu et al., 2015).

Phytochemicals such as Taxifolin (Tax), a flavonoid compound isolated from the larch tree (a Chinese herb), inhibits SrtA activity with an IC_{50} of 24.53 ± 0.42 μM. Importantly, it exhibits no toxicity to mammalian cells at concentrations up to 200 μM. Tax effectively reduces *S. aureus* virulence by diminishing bacterial adherence and biofilm formation. Molecular modelling and site-directed mutagenesis studies elucidate its mechanism of action, revealing its predominant binding to SrtA's pocket through hydrogen bonding, electrostatic interactions, and van der Waals forces, with Asp170 and Gln172 playing crucial roles (Wang et al., 2021). Another flavonoid by the name Dryocrassin ABBA (ABBA), has been identified as a potent inhibitor *of S. aureus* SrtA activity, demonstrated by Fluorescence Resonance Energy Transfer (FRET) assays. Inhibitory effect of ABBA was validated through decreased fluorescence signal in the presence of ABBA, with an IC_{50} of 24.17 μM. Molecular modelling revealed that ABBA interacts with SrtA via intermolecular forces, binding to the catalytic pocket. Specifically, ABBA forms strong interactions with Glu105, Val166, Gly167, Val168, Trp194, and Val193. The binding of ABBA decreases the flexibility of residues (100–110, 120–140) in the binding site, indicating stable complex formation. These residues are crucial for stabilizing ABBA binding with SrtA and significantly reducing the catalytic activity (Zhang et al., 2014).

Hibifolin, a flavonoid, exhibits potential as an SrtA inhibitor in *S. aureus*, showing significant synergy with cefotaxime against MRSA in vitro, as evidenced by a fractional inhibitory concentration index (FICI) of 0.312, indicating enhanced antibacterial effects. This combination not only attenuates the pathogenicity of *S. aureus* but also enhances the antibacterial activity of cefotaxime. Hibifolin inhibits SrtA activity with an IC_{50} of 31.20 mg/mL and interacts with SrtA's active pocket through hydrogen bonding and electrostatic interactions, involving crucial residues such as Thr180, Asn114, Ala104, Arg197, and Trp194. Among these, Arg197 plays a pivotal role in the binding interaction. Mutated SrtA, particularly the Arg-197 mutant, shows significantly reduced activity compared to the wild-type group, highlighting the critical involvement of specific amino acids in the direct binding interaction between SrtA and hibifolin (Zhang et al., 2014). Scutellarin, a flavonoid, exhibited significant inhibition of SrtA activity with an IC_{50} of 53.64 μg/mL. Its direct binding to the SrtA molecule was confirmed through fluorescence quenching assay and molecular docking, with a KA value of 7.58×10^4 L/mol. Furthermore, when combined with vancomycin, scutellarin demonstrated enhanced inhibition of MRSA strain USA300, reducing the MIC of vancomycin from 3 μg/mL to 0.5 μg/mL, indicative of a synergistic effect (Wang et al., 2022).

Echinacoside (ECH), a natural polyphenol, has been identified via FRET as a potential SrtA inhibitor, demonstrating an IC_{50} of 38.42 μM. It effectively inhibits SrtA-mediated *S. aureus* fibrinogen binding, protein A anchoring, and biofilm formation. Direct interaction with SrtA is confirmed by fluorescence quenching assays, revealing a KA-binding constant of 3.09×10^5 L/mol. Molecular dynamics simulations further elucidate ECH-SrtA interactions at specific residues, including A92G, A104G, V168A, G192A, and R197A. Additionally, when combined with vancomycin, ECH protects murine models from MRSA-induced pneumonia, underscoring its potential as an antivirulence agent against *S. aureus* infections (Jiang et al., 2023). Similarly, Isovitexin demonstrates notable inhibition of SrtA activity in *S. aureus*, with an IC_{50} of 28.98 μg/mL. This inhibitory effect is confirmed through a FRET assay and indirectly supported by reduced fibrinogen binding, decreased biofilm formation, and diminished display of SpA on bacterial surfaces. Remarkably, Isovitexin does not impact *S. aureus* growth, indicating its potential as an antivirulence agent specifically targeting SrtA-mediated pathogenesis (Jiang et al., 2023).

In addition to phytochemicals, a chitosan-based oligolysine antimicrobial peptide named CSM5-K5 (is a synthetic antimicrobial peptide composed of chitosan monomer and lysine repeat units) was specifically designed to target multidrug-resistant bacteria. It exhibits rapid bactericidal activity against MRSA, MDR *E. coli*, and vancomycin-resistant *E. faecalis* (VRE). When used in combination with conventional antibiotics, CSM5-K5 effectively restores antibiotic sensitivity and enhances their efficacy against resistant strains. CSM5-K5 alone significantly reduces preformed biofilms by 2–4 orders of magnitude, and when combined with antibiotics at subinhibitory concentrations, the reduction exceeds 2–3 orders of magnitude. In the case of *S. aureus*, CSM5-K5 demonstrates partial synergy with oxacillin and meropenem, as indicated by a fractional inhibitory concentration index (FICI) of >0.5 to ≤ 0.1. When combined with CSM5-K5 at 0.5 × MIC (8 μg/mL), the MIC of oxacillin decreases from 32 to 0.5 μg/mL and the MIC of meropenem decreases from 8 to 1.0 μg/mL. Similarly, for *E. faecalis*, which exhibits resistance to vancomycin and oxacillin (MIC 32 μg/mL), CSM5-K5 at 0.25 × MIC (16 μg/mL) reduces the vancomycin MIC to 4 μg/mL and the oxacillin MIC to 2 μg/mL. Additionally, for *E. coli*, CSM5-K5 demonstrates synergy with streptomycin and tetracycline (FICI <0.5), reducing the MIC of streptomycin from 32 to 8 μg/mL and the MIC of tetracycline from 32 to 4 μg/mL when used at 0.25 × MIC (Thappeta et al., 2020).

4. Conclusion

Sortases are crucial for covalently anchoring of secreted virulence factors (e.g., Pili and adhesins) to the bacterial cell wall. Drug molecules that target sortases not only disarm the virulence machinery but also prevent the emergence of drug resistant strains. Therefore, extensive research has been conducted over the past decade to identify a sortase inhibitor that not only targets the active sites of sortase protein but also disrupts their localization pattern and eventually leads to defective pilus assembly and attenuated virulence.

Studies have shown that Non-Protein Inhibitors (NPI) such as Diarylacrylonitriles and Rhodanines possess very high inhibitory effect on sortase pathway. Although those studies gave promising results, it is important to note that the majority of those NPIs are still in the early stages of development and some are in the clinical trial phase. Taken together, more research is needed to determine the efficacy and safety of NPIs before it can be considered as a mainstream antimicrobial drug. Furthermore, studies related to chemical modifications of NPI such as Morpholinobenzoate derivatives gave promising results, a similar approach could potentially help improve the efficacy of protein-based inhibitors such as AMPs.

Besides NPIs, the combination of peptides (e.g., synthetic CSM5-K5) with traditional antibiotics (e.g., Oxacillin) not only inhibits the growth of multi-drug resistant bacterial strains but also offers collateral sensitivity. Recent trends in screening for phytochemicals (e.g., Punicalagin) that affects sortase activity are very intriguing, as they are both cost effective and could be easily combined with antibiotics (e.g., Cefotaxime) to enhance bactericidal activity. In summary, antivirulence

compounds such as NPI, peptides, and phytochemicals hold promising drug-like characteristics, and their usage could soon help us manage disease outcome and combat against emergence of drug resistant pathogenic strains.

CRediT authorship contribution statement

Sowmiya Sri Sivaramalingam: Conceptualization, Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Deepsikha Jothivel:** Conceptualization, Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Deenadayalan Karaiyagowder Govindarajan:** Conceptualization, Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Lohita Kadirvelu:** Conceptualization, Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Muthusaravanan Sivaramakrishnan:** Software, Validation, Visualization, Writing – review & editing. **Dhivia Dharshika Chithiraiselvan:** Conceptualization, Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Kumaravel Kandaswamy:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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