0031-6997/08/6001-128–141$20.00
PHARMACOLOGICAL REVIEWS
Vol. 60, No. 1
7110/3331566
Pharmacol Rev 60:128–141, 2008
Printed in U.S.A

Sortase as a Target of Anti-Infective Therapy

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***Abstract*——The rise in antibiotic-resistant bacteria is a major concern, in particular because it includes many different species of pathogenic microbes. These "superbugs" are further characterized by high levels of virulence and disease-associated mortality. There seems to be few new antibiotics in the drug discovery pipeline; recent work has sought to define and validate new drug targets. The assembly of surface proteins and pili in the cell wall envelope of Gram-positive bacteria is catalyzed by sortase. Sortase cleaves a conserved C-terminal sequence of these polypeptides to generate an acyl-enzyme intermediate. The acyl-enzyme is next resolved by nucleophilic attack by the amino groups within cell wall cross-bridges or pilin proteins, thereby covalently attaching the polypeptides to the cell wall or the next pilin subunit. Sortase substrates function as adhesins, internalins, blood clotting and immune evasion factors, and transporters for nutrients across the microbial cell wall envelope; without them, most pathogens cannot sustain an infection. Here we review what is known about sortase catalysis and surface protein function, how surface protein anchoring can be inhibited, and what prospects such inhibition may have for anti-infective therapy.**

I. Targeting Virulence Factors as a Therapy for Bacterial Infections

Infectious microbes can be viewed as an endless tide, challenging individual human lives as well as entire populations and providing strong selective forces for evolution (Krause, 1981). There are many different outcomes to the endless number of encounters that occur between an individual and its pathogens, most of which

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This work was supported by National Institutes of Health Grants AI38897, AI057153, and U54-AI-057153 (to O.S.) and National Research Service Award Grant AI069697 (to A.M.).

This article is available online at http://pharmrev.aspetjournals.org. doi:10.1124/pr.107.07110.

shape innate and acquired immune responses and affect future encounters with the same pathogen. Until the first half of the twentieth century, infectious diseases were a major factor in determining the average life span in human populations (Krause, 1981). Therefore, it is not surprising that even the earliest experimental work on microbial infections was concerned with prevention or therapy of these diseases (Ehrlich, 1911; Koch, 1882). Several landmark discoveries over the course of the last century identified natural compounds derived from microbes (e.g., sulfonamides, penicillin, and streptomycin) that block replication of sensitive bacteria (Amyes, 2001). Such antibiotics not only transformed the diagnostic and therapeutic practices of the medical profession, they also expanded human life expectancy. Alongside a flurry of discoveries in the field of microbial antibiotics, the pharmacological industry achieved development of synthetic compounds that selectively block bacterial replication, thereby expanding the register of chemicals that can function as magic bullets. These widely celebrated research accomplishments instilled broad confidence into regulatory agencies as well as practicing physicians that all bacterial infections can be controlled by antibiotic treatment (Stewart, 1967). Such ambition is now broadly challenged by the emergence of microbial strains that are resistant to many, if not all, antibiotics currently in clinical use. Thus, the recent pressing challenge of microbial research is the identification of novel drug targets and the development of inhibitors that can be used for therapy of human infections.

The emergence of microbial strains resistant to antibiotics is a phenomenon of selection. Massive production and use of these compounds in industry and the clinic are not regulated and represent the basis for emergence of antibiotic-resistant strains. Considering the constellation of drug discovery, production, distribution, and use, the medical profession is engaged in an arms race because new antibiotics must be discovered to avoid therapeutic failure due to selection pressure of compounds currently in use (Bonomo, 2007). However, the generation of new antibiotics must be accompanied by an identification of novel bacterial targets whose inhibition would be of therapeutic value. The corollary to this is to develop anti-infectives that inhibit bacterial virulence strategies, such as attachment to host tissues, for example. Assuming that anti-infective therapy of this nature can be implemented for bacterial infections, such compounds may not be associated with the same resistance phenomena as antibiotics with bactericidal activity, a conjecture that remains speculative because such therapy has not been developed or approved for human use. At the outset, there are limitations to targeting virulence factors as possible therapies. Even if inhibited in their virulence strategies, pathogenic microbes require opsono-phagocytic killing by the immune system for host clearance, which would limit the use of such therapies to immune-competent individuals. Furthermore, this strategy requires knowledge of the invading pathogen, and any therapy would have to be delayed until a definitive identification of the pathogen has been made. In summary, the inhibition of virulence factors as a therapy for bacterial infections still represents a theory that requires experimental testing and proof-of-principle. Here, we discuss sortase, a prominent virulence factor in Gram-positive pathogens, as a target for the development of anti-infectives and examine recent research progress toward this goal.

II. *Staphylococcus aureus*

A. Clinical Disease and Epidemiology

Staphylococcus aureus is a Gram-positive, catalase-positive bacterium that colonizes human skin and mucosal surfaces. The microbe is isolated from at least 40% of the human population (Lowy, 1998, 2003; Peacock et al., 2001). Skin or soft tissue infections typically result from local breaches in epithelial barrier functions and may be resolved locally or lead to rapidly spreading infections with involvement of any internal organ system (Lowy, 1998). If sensitive to antibiotics, *S. aureus* infections can be effectively treated with β-lactam antibiotics, macrolides, and fluoroquinolones (Lowy, 2003). Staphylococcal resistance to β-lactam antibiotics is now widespread. Within one year of the introduction of methicillin, a nonhydrolyzable synthetic β-lactam, methicillin-resistant *S. aureus* (MRSA[1]) strain carrying the *mecA* gene and catalyzing peptidoglycan transpeptidation, even in the presence of antibiotic, was isolated from clinical infections (Jevons et al., 1963). The incidence of MRSA infection is increasing throughout the world, accounting for significant mortality and associated health care costs (Klevens et al., 2006, 2007; Noskin et al., 2007). Vancomycin and other glycopeptide inhibitors were once considered last resort therapies for MRSA; however, strains with intermediate or full resistance to vancomycin have been described previously (Chang et al., 2003; Weigel et al., 2003). Perhaps even more alarming is the rise of community-acquired MRSA strains (Klevens et al., 2007). Community-acquired -MRSA infections occur in children with no predisposing risk factors, young adults (including athletes, homosexual men, prisoners, and military recruits), and healthy newborns (Fridkin et al., 2005). Collectively, *S. aureus* precipitates up to 3 million clinical infections each year in the United States (Fridkin et al., 2005). For both community- and hospital-acquired *S. aureus* infections, antibiotic-resistant strains represent a formidable and increasing ther-

[1] Abbreviations: MRSA, methicillin-resistant *S. aureus*; SrtA, B, and C, sortases A, B, and C; SpaA, sortase-mediated pilin assembly A; AAEK, aryl β-amino(ethyl) ketone; HTS, high-throughput screen; MurNAc-GlcNAc, *N*-acetylmuramic acid-(β1–4)-*N*-acetylglucosamine; FRET, fluorescence resonance energy transfer; Isd, iron-regulated surface determinant.

apeutic challenge (King et al., 2006). It is estimated that up to 100,000 Americans will succumb to MRSA infections this year (Fridkin et al., 2005; King et al., 2006). Even with expert medical care, these highly destructive and debilitating infections can be fatal unless diagnosed early and treated aggressively (Gillet et al., 2002).

B. Staphylococcal Virulence Genes

The development of animal models for infection together with molecular biology techniques permitted studies on the genetic basis for staphylococcal diseases. We now know that *S. aureus* requires a plethora of virulence factors to establish an infection. *S. aureus* strains secrete more than fifty polypeptides that enable bacterial escape from innate immune responses, promote tissue and cellular damage, or degrade connective tissues (Sibbald et al., 2006). The aggregate of all exoprotein secretion events provides for staphylococcal spread in infected tissues (Novick, 2003b). Expression of many exoprotein genes (and therefore the secretion of encoded polypeptides) is controlled by *agr*, the staphylococcal quorum sensor and regulator of virulence genes (Recsei et al., 1986; Ji et al., 1995). Other important regulatory and sensory molecules include two-component systems (SaeRS) and transcription factors (Sar) (Cheung and Zhang, 2002; Novick, 2003a; Steinhuber et al., 2003). It is noteworthy that deletion of a single exoprotein gene typically does not cripple the resulting mutant strain or diminish its virulence properties. An important exception to this notion is the secretion of α-hemolysin, a pore-forming toxin that is essential for the pathogenesis of experimental lung infections in mice (Wardenburg et al., 2007). However, mutations that abrogate the function of regulatory factors involved in the coordinated expression of exoproteins (*agr*, *saeRS* mgrA, or *sar*) cause virulence defects in almost every animal model system examined, indicating that many exoproteins fulfill redundant yet essential functions (Cheung et al., 1994). In agreement with this hypothesis, pioneering studies by Richard Novick, Tom Muir, and colleagues developed inhibitors of Agr function that display therapeutic effects when added to skin infections (Lyon et al., 2000). These compounds do not affect staphylococcal growth in laboratory media but demonstrate therapeutic efficacy and can therefore be considered as examples of compounds that target virulence strategies.

The most extensive knowledge for animal models that develop invasive staphylococcal diseases has been accumulated in mice. Intraperitoneal or blood stream infection of mice with many different human clinical *S. aureus* isolates, typically at a dose of 1 to 10×10^6 colony-forming units, is followed by opsono-phagocytic killing of up to 99% of invading staphylococci within 1 to 2 h (Albus et al., 1991; Mazmanian et al., 2000). Approximately 10^3 to 10^4 staphylococci escape phagocytic killing, exit the bloodstream, and adhere eventually to various organ tissues. Within 3 to 4 days, staphylococci replicate to large numbers and are sequestered by immune cell-mediated liquefaction necrosis (Bubeck Wardenburg et al., 2006). Staphylococcal abscesses harbor central lesions filled with staphylococci and cellular debris that are surrounded by large numbers of polymorphonuclear cells and macrophages (Bubeck Wardenburg et al., 2006). Peripheral fibrin deposits confine abscesses from other tissues and restrict fluid circulation into the lesion (Bubeck Wardenburg et al., 2006). The elucidation of abscesses in infected animals is often used as a marker for the severity and progression of staphylococcal disease (Albus et al., 1991; McKenney et al., 1999; Mazmanian et al., 2000).

C. The Staphylococcal Cell Wall Envelope

The cell wall of staphylococci and other Gram-positive bacteria represents a surface organelle composed of a primary polymer, peptidoglycan, and secondary polymers (protein, carbohydrates, and teichoic acids) that are immobilized in the peptidoglycan scaffold. In *S. aureus*, the 30 to 100-nm thick cell wall is composed of the repeating disaccharide *N*-acetylmuramic acid-(β1–4)-*N*-acetylglucosamine (MurNAc-GlcNAc) (Ghuysen and Strominger, 1963; Ghuysen et al., 1965; Dmitriev et al., 2004). MurNAc is amide-linked to alanine of the cell wall tetrapeptide [L-Ala-D-isoGln-L-Lys(NH_2-Gly_5)-D-Ala], which is linked to adjacent strands of tetrapeptide through a pentaglycine cross-bridge (Fig. 1) (Ghuysen et al., 1965; Tipper and Strominger, 1965; Tipper et al., 1965; Tipper, 1968; Archibald, 1972). This cross-linking gives rise to the murein sacculus, a single large macromolecule that completely envelopes the cell (Beveridge, 1981). In addition to imparting shape and architecture, the cell wall contains covalently and noncovalently associated proteins involved in adhesion, nutrient sensing/uptake, immunomodulation, and the transfer of DNA (Moks et al., 1986; Flock et al., 1987; Chen and Cleary, 1990; Galli et al., 1990, 1992; Gaillard et al., 1991; Clewell, 1993; Burne and Penders, 1994; Patti et al., 1994; Dramsi et al., 1995; Herwald et al., 2004). Furthermore, it can be decorated with (lipo)teichoic acids, teichuronic acids, and polysaccharide (Robbins and

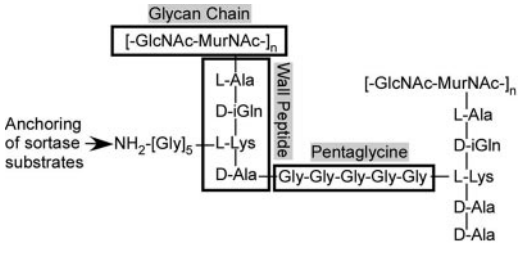

FIG. 1. The cell wall of *S. aureus*. The repeating disaccharide *N*-acetylmuramic acid-(β1–4)-*N*-acetylglucosamine (GlcNAc-MurNAc) is amide-linked to the alanine of the pentapeptide [L-Ala-D-iGln-L-Lys-(Gly_5)-D-Ala]. Its pentaglycine cross-bridge is linked to carboxyl group of D-Ala at position 4 of a neighboring cell wall tetrapeptide. The amino group of pentaglycine cross-bridges is also the site of sortase-mediated anchoring of surface proteins (arrow).

Schneerson, 1990; Fischer, 1994; Neuhaus and Baddiley, 2003). During infection, the sum of all cell wall polymers enables many of the pathogenic strategies of *S. aureus*.

D. Surface Protein

Sequence analysis of some known surface proteins in Gram-positive bacteria identified a conserved LPXTG motif (Fischetti et al., 1990). The LPXTG motif is one element of cell wall-sorting signals positioned at the C-terminal end of precursor surface proteins, which contain also a hydrophobic domain and positively charged tail (Schneewind et al., 1993). Disruption of two or more of the positively charged residues leads to secretion into the media, suggesting that the polybasic region of surface proteins acts as a retention signal for cell wall anchoring (Schneewind et al., 1993). Moreover, fusion of the sorting signal to the C-terminal end of precursor proteins with N-terminal signal peptides directs their hybrid product to the cell wall envelope (Schneewind et al., 1992; Navarre and Schneewind, 1994, 1996). Chemical analysis of anchored polypeptides liberated from the cell wall envelope by enzymatic cleavage of peptidoglycan fragments revealed that the C-terminal end of surface protein, the threonine of the LPXTG motif, is amide-linked via its carboxyl group to the amino group of the pentaglycine cross-bridge in the cell wall (Schneewind et al., 1995; Ton-That et al., 1997; Navarre et al., 1998, 1999). A screen of mutant populations of *S. aureus* led to the identification of the sortase gene, which is responsible for the anchoring of surface proteins to the cell wall envelope (Fig. 2). Since this discovery, hundreds of homologs of the sortase gene have been identified, encompassing ~900 potential substrates in over 50 different species of Gram-positive bacteria. Sortase-like genes have even been identified in Gram-negative organisms (Pallen et al., 2001, 2003; Comfort and Clubb, 2004). In most cases, multiple sortases are found in the same genome and can be grouped based on their homology into four or five classes (Comfort and Clubb, 2004; Dramsi et al., 2005). Sortase A is the prototypical enzyme for members of the class A family, the so-called "house-keeping" sortase. One of the shared features of sortase A members is that they are responsible for the anchoring of proteins that mediate bacterial adhesion. Members of this class of sortase seem to be ubiquitously expressed. Class B sortases are regulated by the availability of iron and anchor proteins that are involved in heme-iron acquisition. Class C sortases are responsible for the elaboration of pili on the bacterial surface. Finally, class D sortases are expressed during bacterial sporulation, and their anchored products contribute to the developmental programs that define the life cycle of bacilli or streptomyces (Fig. 3). Throughout this review, unless a specific sortase (i.e., sortases A–D) is mentioned, we use the term sortase to refer to the general properties of this class of transpeptidases using sortase A as a prototype.

III. Sortase

A. Sortase-Catalyzed Transpeptidation

S. aureus sortase A is a 206 amino acid protein with an N-terminal signal peptide/membrane anchor. Replacement of its N-terminal 25 residues with an affinity tag provides for a single step purification strategy of a soluble hexahistidyl-tagged enzyme on nickel-nitrilotriacetic acid-Sepharose from lysates of recombinant *Escherichia coli* that express *srtA* under control of the T7 promoter. The substrate *abz*-LPETG-*dnp* harbors an N-terminal fluorophore (2-amino-benzyl) and a C-terminal quencher (2,4-dinitrophenyl); fluorescence resonance energy transfer (FRET) occurs in the substrate due to the close proximity of fluorophore and quencher.

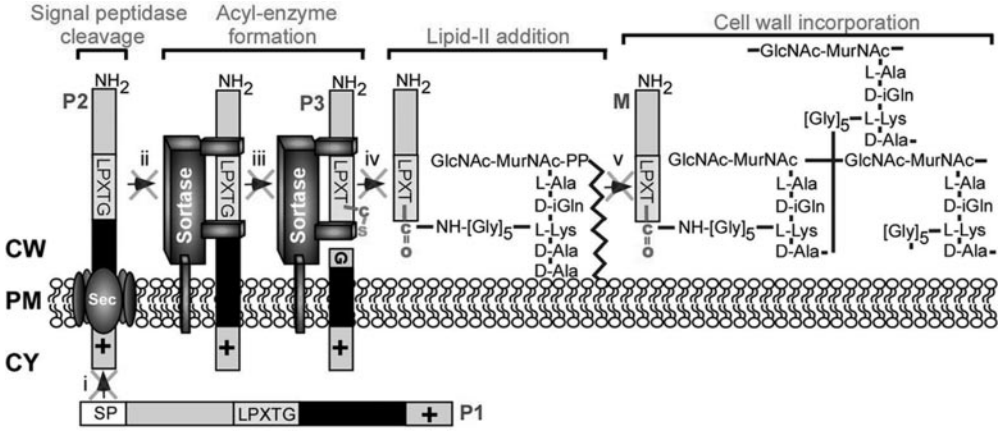

FIG. 2. Sortase-mediated anchoring of surface proteins. i, proteins destined for cell wall anchoring are first initiated into the Sec pathway by an N-terminal signal peptide (SP). ii and iii, following cleavage of the LPXTG motif, a thioester-linked acyl-enzyme intermediate is formed between sortase and C-terminal threonine of surface protein. iv and v, resolution of the acyl-enzyme occurs through nucleophilic attack of the amino group of the pentaglycine of lipid II to generate lipid II-linked surface protein, which is incorporated into the cell wall. The black bar and + represent the hydrophobic domain and positively charged tail of cell wall sorting signals, respectively. X indicates potential points of inhibition in the "sorting pathway."

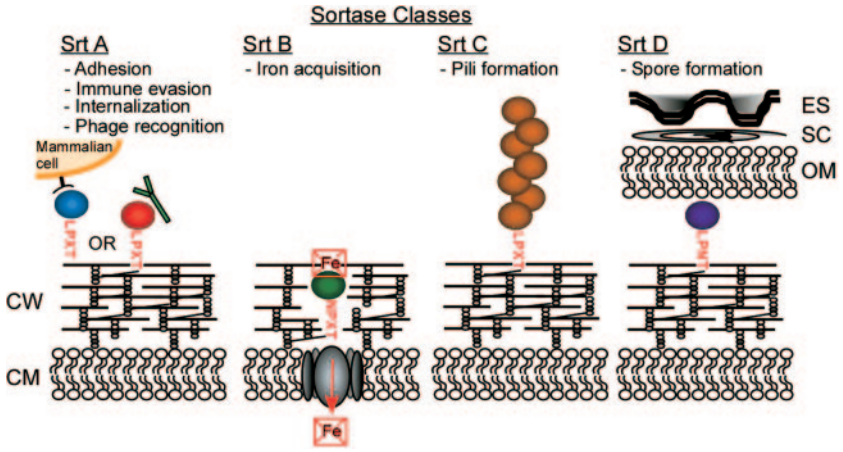

FIG. 3. Diversity of sortase biology. Four different classes of sortase enzymes can be delineated based on sequence homology and distinct function in Gram-positive organisms. Class A enzymes, SrtA or housekeeping sortase is responsible for the cell wall anchoring of proteins that are involved in bacterial adhesion, immune evasion, internalization, or function as receptors for phage binding. Class B enzymes, SrtB, anchor proteins to the cell wall envelope that are specifically involved in iron acquisition. Class C enzymes, SrtC, assemble pili on the surface of Gram-positive bacteria, whereas class D sortases anchor proteins to cell wall peptidoglycan as bacilli or streptomyces engage in sporulation, a developmental program that generates dissimilarly sized daughter and mother cells. Recognition motifs for each class of sortase are highlighted. CM, cell membrane; CW, cell wall; OM, outer membrane; SC, spore coat; ES, exosporium.

Sortase A-mediated cleavage precipitates separation of reaction products, *abz*-LPET and G-*dnp*, and fluorescence increase serves as a measure for sortase cleavage of surface protein substrates (Ton-That et al., 1999, 2000). Addition of hydroxylamine (NH_2OH), a strong nucleophile, to this reaction increases the velocity of sortase cleavage both in vitro and in vivo. Sortase forms surface protein LPET-hydroxamate in vivo, indicating that NH_2OH attacks the acyl-enzyme intermediate (Mazmanian et al., 1999; Ton-That et al., 2000). Similar to hydroxylamine, peptidoglycan substrates and their surrogates, for example, Gly, Gly_2, Gly_3, Gly_4, or Gly_5 (i.e., the pentaglycine cross-bridge), also stimulate sortase cleavage of *abz*-LPETG-*dnp*. The products of this reaction, *abz*-LPET-Gly, *abz*-LPET-Gly_2, and so forth, document that the amino group of the cell wall cross-bridge performs the nucleophilic attack at the acyl-enzyme intermediate of sortase A both in vivo and in vitro (Mazmanian et al., 1999; Ton-That et al., 1999, 2000). When offered a peptidoglycan substrate with amino group nucleophile, sortase A invariably functions as a transpeptidase but not as a peptide hydrolase. The physiological nucleophile for the sortase A-catalyzed anchoring reaction is lipid II, the peptidoglycan biosynthesis precursor [C_{55}-PP-MurNAc(-L-Ala-D-isoGln-L-Lys(NH_2-Gly_5)-D-Ala-D-Ala)-GlcNAc], because this compound can be linked to surface proteins both in vivo and in vitro (Perry et al., 2002; Ruzin et al., 2002). Vancomycin, which binds to lipid II and prevents its incorporation into the cell wall, and moenomycin, a lipid II analog, block cell wall synthesis and the sorting pathway of surface proteins (Ton-That and Schneewind, 1999).

Separation of sortase reaction products by reversed phase-high-performance liquid chromatography and mass spectrometry of eluate fractions demonstrated that LPETG is cleaved between the threonine and the glycine. These experiments also revealed the formation of the acyl-enzyme intermediate, i.e., the thioester linkage between LPET and the active site cysteine thiol of sortase A in vitro (Huang et al., 2003). The addition of [2-(trimethylammonium)ethyl]methanethiosulfonate and *p*-hydroxymercuribenzoic acid, two sulfhydryl-modifying agents, to staphylococcal cultures or to the in vitro sortase reaction each inhibits substrate cleavage by modifying the active site cysteine (Ton-That and Schneewind, 1999; Ton-That et al., 1999). Iodoacetamide, a compound that reacts rather slowly with thiol, cannot inactivate sortase. One explanation for this observation may be that the cysteine of sortase is ionized (Ton-That and Schneewind, 1999). In agreement with the proposed role of the sole cysteine residue in catalysis, substitution of Cys^{184} with alanine abolishes sortase A activity both in vivo and in vitro (Ton-That et al., 1999, 2000). Bioinformatic analysis revealed that the active site cysteine is a conserved feature of sortases, in agreement with the general hypothesis that mechanisms of surface protein anchoring to the cell wall envelope are conserved in Gram-positive bacteria (Ton-That et al., 1999).

B. Sortase Structure and Catalysis

NMR and X-ray crystallography of *S. aureus* sortase A revealed that the enzyme adopts a unique eight-stranded β-barrel, which contains several short helices and loops (Ilangovan et al., 2001a,b; Zong et al., 2004a). The active site is located in a hydrophobic depression formed by two β-stands, features that are preserved in sortase B from *S. aureus* and *Bacillus anthracis*. Two conserved residues, His^{120} and Arg^{197}, are positioned in close proximity to the active site sulfhydryl of Cys^{184} (Ilangovan et al., 2001b; Zong et al., 2004a). Cocrystals of sortase and LPETG revealed that Cys^{184} and Arg^{197}

reside between the side chains of the scissile peptide bond, suitably positioned to act as a catalytic cysteine-arginine dyad, where Arg^{197} may function as a base for thiol ionization (Zong et al., 2004a). Both His^{120} and Arg^{197} are involved in catalysis; however, their mechanistic contributions are still under investigation (Ton-That et al., 2002; Conolly et al., 2003; Dessen, 2004; Marraffini et al., 2004; Zhang et al., 2004; Zong et al., 2004b; Frankel et al., 2005). It seems unlikely that the catalytic contributions of His^{120} and Arg^{197} can be resolved until detailed structural appreciation for binding of peptidoglycan and polypeptide substrates to sortase is achieved. Using a cyanoalkene analog of LPETG and NMR analysis, three residues within the hydrophobic compression adjacent to the active site (Thr^{180}, Ile^{182}, and Ala^{118}) were demonstrated to undergo rearrangement as the enzyme engages its substrate. In agreement with the hypothesis that Thr^{180}, Ile^{182}, and Ala^{118} are involved in recognition and stabilization of bound substrate, amino acid substitution of each of these residues can have profound effects on sortase activity (Liew et al., 2004). Calcium ions stimulate *S. aureus* sortase A activity 8-fold but have little or no impact on the activity of *S. aureus* sortase B or *B. anthracis* sortase A, sortase B, or sortase C (see below). Calcium concentrations required for stimulation of sortase A activity are found under physiological conditions in host tissues, and these ions are involved in structural rearrangements of a disordered loop covering the active site that enable substrate binding (Ilangovan et al., 2001b; Zong et al., 2004a; Naik et al., 2006). Interestingly, sortase also forms dimers in vitro, a function that may stimulate catalysis (Lu et al., 2007).

C. The Pathway of Surface Protein Anchoring

A model illustrated in Fig. 2 assimilates many of the observations summarized here for *S. aureus* sortase A. Surface proteins are synthesized in the bacterial cytoplasm as precursors harboring an N-terminal signal peptide and a C-terminal sorting signal (P1 precursor). After initiation of surface proteins into the Sec secretion pathway, signal peptidase cleaves the N-terminal signal peptide to generate the P2 precursor. The hydrophobic domain of the sorting signal is thought to retain the polypeptide in the membrane, thereby allowing the LPXTG motif to be recognized by sortase A. The sulfhydryl of Cys^{184}, presumably activated for nucleophilic attack via deprotonation by Arg^{197}, forms a covalent bond with the carbonyl carbon of threonine at the C-terminal end of the cleaved polypeptide, releasing the amino group of the cleaved C-terminal fragment. The product of the first part of the sortase-catalyzed transpeptidation reaction, the thioester linked acyl-enzyme intermediate, is subject to nucleophilic attack by the amino group of the pentaglycine cross-bridge within the peptidoglycan precursor lipid II and thereby forms the P3 intermediate [C_{55}-PP-MurNAc(-L-Ala-D-isoGln-L-Lys(surface protein-Gly_5)-D-Ala-D-Ala)-GlcNAc]. P3 intermediates are subsequently incorporated into the envelope via the transpeptidation and transglycosylation reactions of cell wall biosynthesis, ultimately generating mature product (M), i.e., surface proteins that are covalently linked at the C-terminal end to the cell wall envelope.

D. Sortases, Surface Proteins, and the Pathogenesis of Microbial Infections

Interest in sortase as a target for the development of anti-infectives primarily stems from findings that loss of the sortase gene leads to defects in the pathogenesis of many different infections caused by Gram-positive bacteria. For example, *S. aureus* virulence can be assessed in a mouse model of infection (Lee et al., 1987; Albus et al., 1991; McKenney et al., 1999). When injected at a sublethal dose into the blood stream of mice, most staphylococci survive innate immune response, disseminate to peripheral tissues and establish abscesses in multiple organ systems over 72 to 96 h. The products of the infection can be quantified as bacterial load in homogenized tissue (colony-forming units) or via histopathology for their anatomical substrate. In these models, *srtA* mutants display a 2-log reduction in bacterial growth in multiple organ systems as well as a 1.5-log increase in the lethal dose (LD_{50}) compared to wild-type strains (Mazmanian et al., 2000). These findings prompted further investigations into the contribution of *S. aureus* sortase A to other types of infection, including septic arthritis and endocarditis (Bremell et al., 1991, 1992). In the septic arthritis model, staphylococcal blood stream inoculations lead to joint infections with cartilage and bone destruction, which can be assessed by histopathology. In experimental endocarditis, lesions on the heart valves serve as a site of deposition of staphylococci, causing destructive damage of endocardial tissue. In both model systems, *srtA* mutants displayed large reductions in pathogenesis (Jonsson et al., 2002, 2003; Weiss et al., 2004). Recently, an experimental model of *S. aureus* lung infection was developed, which monitors the development of acute pneumonia and disease-associated mortality following intranasal inoculation of mice with staphylococci. Animals infected with *srtA* mutants show less disease than their wild-type counterparts (Wardenburg et al., 2007). Finally, the ability of surface proteins to illicit protective immunity following vaccination with purified components was assessed for Group B streptococcal, pneumococcal, and staphylococcal infections. By combining sortase substrates as vaccine antigens that generated the highest protective immunity, animals could be protected against lethal challenge with the pathogen. For example, the combination of IsdA, IsdB, SdrD, and SdrE as vaccine antigens protected mice against lethal challenge with *S. aureus* strains that had been isolated from human clinical infections, including MRSA strains USA100, USA300, and USA400, the

most common cause of infections in the United States (Stranger-Jones et al., 2006). Virulence attributes of sortase and surface protein extend well beyond staphylococci, as defects in the pathogenesis of sortase mutants have been reported for animal infections with many different species, including actinomyces, enterococci, streptococci, bacilli, or listeria (Bierne et al., 2002; Garandeau et al., 2002; Kharat and Tomasz, 2003; Lee and Boran, 2003; Chen et al., 2005; Lalioui et al., 2005; Levesque et al., 2005; Paterson and Mitchell, 2005; Sabet et al., 2005; Yamaguchi et al., 2006). Collectively, these studies illustrate the many important and diverse roles that surface proteins play during the pathogenesis of infections caused by Gram-positive pathogens.

E. Iron Acquisition

Pathogenic bacteria have an absolute requirement for iron as a nutrient during the infectious process (Crosa et al., 2004). *S. aureus* sortase B (SrtB) recognizes a surface protein with an NPQTN sorting signal, which plays a role in iron acquisition (Mazmanian et al., 2002). In *S. aureus*, *srtB* is located in the Isd locus; name was given because it contains genes whose products are all involved in heme-iron scavenging and transport. Specifically, the locus encodes cell wall-anchored heme-binding proteins (IsdA, IsdB, and IsdC), a membrane-based heme transport system (IsdD, IsdE, and IsdF), a heme degrading monooxygenase (IsdG), and SrtB. The locus is regulated by the ferric uptake regulator protein, a repressor of DNA transcription at the promoter of target genes during iron-replete conditions (Mazmanian et al., 2003; Skaar et al., 2004). IsdC appears to be the only surface protein substrate in *S. aureus* that is anchored by SrtB, which cleaves the substrate at its NPQTN motif-sorting signal and immobilizes it at cell wall crossbridges. IsdA and IsdB, however, are anchored to the cell wall by sortase A (Mazmanian et al., 2002). In contrast to IsdA and IsdB, IsdC remains buried within the staphylococcal cell wall envelope, presumably because it is linked to mature assembled peptidoglycan but not to lipid II. As a consequence, IsdC is not incorporated into linear peptidoglycan strands that eventually reach the envelope surface, unlike all sortase A-anchored products (Mazmanian et al., 2002; Marraffini and Schneewind, 2005). The aforementioned results were also the first indication that sortase genes, which are positioned in the same transcriptional unit as putative surface proteins, generally anchor these substrates to the envelope. Loss of *srtB* reduces both staphylococcal growth on heme as the sole source of iron and transport of heme across the cell membrane, implicating sortase B function in the pathway whereby staphylococci acquire iron during infection. In the renal abscess model, *srtB* mutant staphylococci show defects in the persistence of bacterial infections and an overall decrease in virulence in a murine infectious arthritis model, corroborating the hypothesis that heme uptake is important during the infectious process (Mazmanian et al., 2002; Jonsson et al., 2003; Weiss et al., 2004). SrtB homologs are also found in other Gram-positive pathogens, including *Listeria monocytogenes* and *B. anthracis*. In listeria, SrtB surface anchors two proteins, SpvA and an IsdC homolog. *B. anthracis* IsdC, which is also a substrate of SrtB, binds heme and is required for growth on heme as the sole source of iron (Bierne et al., 2004; Pucciarelli et al., 2005; Maresso and Schneewind, 2006; Maresso et al., 2006; Skaar et al., 2006). As iron acquisition is essential for bacterial replication during infection, inhibition of sortase B offers an opportunity to disrupt infectious processes.

F. Sortase and Pili

Pili represent some of the most important virulence determinants for bacterial infection of a mammalian host. These macro-molecular structures often consist of repeating protein subunits, which extend from the bacterial surface into the surrounding medium. The tip or cap of these fimbriae typically display adhesive properties that promote bacterial binding to extracellular matrices or host cell receptors in the context of an infection. The first indication that sortase may anchor components of this structure was revealed in actinomyces, pathogens responsible for tooth decay (Yeung and Ragsdale, 1997; Yeung et al., 1998). Reconstitution of immunoreactivity in a heterologous host (*E. coli*) using anti-pilin antisera allowed for the identification of an operon, which contained a pilin-like gene and a gene later characterized as sortase. Mutagenesis of the putative sortase abolished actinomyces adherence and led to the loss of pili from the bacterial surface (Yeung and Ragsdale, 1997; Yeung et al., 1998).

Definitive evidence that some sortases polymerize pili in the cell wall envelope occurred with the discovery of six sortase-like genes in *Corynebacterium diphtheriae*, a human respiratory pathogen that causes diphtheria (Hadfield et al., 2000). Specific surface proteins or pilin subunits encoded by the same operon as sortases identify specific pilus types, of which *C. diphtheriae* expresses at least three. For example, antibodies against the SpaA protein (sortase-mediated pilin assembly A), a component of one of these loci, stained fimbrae-like structures in a uniform fashion, whereas those against SpaC, a second pilin component of the locus, stained the tip of pilus fibers. A third component, SpaB, is also distributed along the fiber shaft. Deletion of both the *spaA* and *srtA* genes abolished formation of SpaA pili, whereas deletion of *spaB* and *spaC* abrogated only binding of the corresponding pilin-specific antibodies without affecting assembly of SpaA pilin into pili by their cognate sortase. Furthermore, as assessed by immunoblotting, only *srtA* and *spaA* mutants failed to form high molecular weight species consistent with pilus assembly (Ton-That and Schneewind, 2003). Assembly was also dependent on a conserved lysine residue of SpaA, the

critical residue in the "pilin motif", which in conjunction with the sorting signal is sufficient for the formation of pili in a SrtA-dependent manner. The requirement of lysine is explained by the presumed role of its ε-amino group in relieving pilin acyl-enzyme intermediates with sortase. If this is the case, class C sortases may polymerize pili by forming covalent bonds between individual pilin subunits, and the bonds involve the cleavage site of pilin precursors as well as the pilin motif lysine residue. Class C sortases and their corresponding pili have been discovered in *Actinomyces viscosis*, *Actinomyces naeslundii*, *Bacillus cereus*, *Clostridium perfringens*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (Dramsi et al., 2005). Following the assembly of the pilus by SrtC, it is hypothesized that SrtA catalyzes the covalent attachment of the full-length structure onto the peptidoglycan (Swaminathan et al., 2007)

G. Sortase and Sporulation

B. anthracis harbors three sortase genes, *srtA* and *srtB*, with properties similar to those described for *S. aureus*, and a third gene, *srtC* (originally called *srtC* but is actually a member of the *srtD* class), in a locus that also encodes a two-component regulatory system (*sctR* and *sctS*) as well as *basI* (an LPNTA motif-containing protein) (Fig. 3) (Dramsi et al., 2005; Marraffini and Schneewind, 2006). Another LPNTA-motif containing protein, *basH*, is located elsewhere on the *B. anthracis* genome. Purified SrtC cleaves LPNTA between its threonine and alanine residue but does not cut the recognition motifs of sortase A (LPXTG) and sortase B (NPKTG), suggesting that both BasI and BasH may be in vivo substrates for sortase C. Using transcriptional fusions of *srtC* to *gfp*, *srtC* expression was observed only during sporulation and was dependent on the response regulator *sctR*. BasH-GFP hybrid expression was observed only in the developing forespore and was dependent on the presence of the transcription factor σ^F RNA polymerase. Analysis of cell wall fragments demonstrated that BasH and BasI are anchored to the forespore and mother cell peptidoglycan, respectively, and that both are dependent on a functional *srtC* gene, whose product is localized to the mother cell and the forespore envelopes. Deletion of the *srtC* gene caused no defect in virulence in *B. anthracis* strain Ames and carcasses of animals infected with the mutant teamed with vegetative forms, similar to carcasses derived from infection with the wild-type parent strain. However, vegetative forms of the *srtC* mutant were unable to form spores in carcass tissue, even 3 weeks after animal death, whereas wild-type bacilli formed spores within 24 h. A similar phenotype was observed in sheep's blood, as *srtC* mutants were unable to sporulate unless blood was rotated and exposed to oxygen. A general model was derived from these observations, whereby the sortase C-mediated anchoring of BasI and BasH to the cell wall envelope was critical for the generation of spores in host tissues (Marraffini and Schneewind, 2006). Although it is unlikely that this class of sortase would be specifically targeted for anti-infective development, given that spore formation is not usually observed during human infection, we should not discount the possibility for infection control after addition of sortase inhibitors to foodstuff contaminated with spores.

IV. The Inhibition of Sortase

A. Early Observations

The first inhibitors of the cell wall sorting reaction were discovered even before sortase was identified (Ton-That and Schneewind, 1999). At that time, it was known for *S. aureus* that surface proteins are linked through an amide bond to the amino group of pentaglycine cross-bridges (Ton-That et al., 1997, 1998; Navarre et al., 1998). The similarity of this mechanism to the transpeptidation reaction of cell wall biosynthesis led to the investigation of antibiotics as inhibitors of the sorting pathway. Penicillin, an inhibitor of the transpeptidation reaction of cell wall synthesis, had no effect on the generation of mature anchored surface proteins; however, vancomycin and moenomycin reduced surface protein anchoring. As vancomycin and moenomycin both target steps in the lipid II biosynthesis cycle, it was presumed that lipid II may be a substrate for the sorting reaction. Interestingly, compounds that are reactive with sulfhydryl groups, such as methanethiosulfonates, i.e., [2-(trimethylammonium)ethyl]methanethiosulfonate, were also inhibitors (>2 log units) of surface protein anchoring. *p*-Hydroxymercuribenzoic acid, which inhibits cysteine proteases, also inhibited anchoring, whereas iodoacetamide and DTT did not (Ton-That and Schneewind, 1999). These findings not only implicated sulfhydryl (cysteine) as a catalyst for surface protein anchoring but also demonstrated that inhibition of sortase in vivo could be measured.

B. Natural Products

After this discovery and the cloning of sortase, there have been several investigations for sortase inhibitors in compound libraries of natural and synthetic products (Table 1). Taking the approach that plants may harbor antimicrobial compounds, extracts of 80 medicinal plants from Korea were examined for inhibition of recombinant sortase (Kim et al., 2002). *Cocculus trilobus*, *Fritillaria verticillata*, *Liriope platyphylla*, and *Rhus verniciflua* extracts had the best inhibitory activity with IC_{50} values ranging from 1.5 to 8.4 μg/ml. Further purification of *F. verticillata* extracts led to the isolation of β-sitosterol-3-*O*-glucopyranoside as the active sortase inhibitor with an IC_{50} of 18.3 μg/ml, two times lower then *p*-hydroxymercuribenzoic acid (Kim et al., 2003). It is not known why crude extracts display a lower IC_{50} than the purified inhibitor. Elimination of the glucopy-

TABLE 1
Summary of sortase inhibition

Name[a]	Source/Type	IC_{50} in Vitro[b]	In Vivo Inhibition	Mechanism	References
Methanethiosulfonate	Synthetic	N.D.	Seb anchoring	Most likely covalent	Ton-That and Schneewind (1999)
p-Hydroxymercuribenzoic acid	Synthetic	N.D.	Seb anchoring	Most likely covalent	Ton-That and Schneewind (1999)
β-Sitosterol-3-*O*-glucopyranoside	*F. verticillata* (plant)	18 μg/ml	Binding to fibronectin	N.D.	Kim et al. (2003)
Berberine chloride	*C. chinensis* (plant)	SrtA: 8.7 μg/ml SrtB: 6.3 μg/ml	Binding to fibronectin	N.D.	Kim et al. (2004) Oh et al. (2006)
Psammaplin A1	*A. rhax* (sponge)	SrtA: 39 μg/ml SrtB: 23 μg/ml	Binding to fibronectin	N.D.	Oh et al. (2006)
Bromodeoxytopsentin	*T. genitrix* (sponge)	19.4 μg/ml	Binding to fibronectin	N.D.	Oh et al. (2005)
Curcumin	*C. longa* (plant)	13 μg/ml	Binding to fibronectin	N.D.	Park et al. (2005)
Flavonoid phenols	*R. verniciflua* (bark) and natural products	SrtA: 37–52 μM; SrtB: 8–36 μM	Clumping	N.D.	Kang et al. (2006)
Diazo/chloromethyl ketone	Synthetic, substrate mimic	N.D.	N.D.	Most likely covalent	Scott et al. (2002)
3,3,3-Trifluoro-1-(phenylsulfonyl)-1-propene	Synthetic	190 μM	Binding to fibronectin	Covalent (C184)	Frankel et al. (2004)
Phosphinic- peptidomimetic	Synthetic, transition state mimic	10 mM	N.D.	N.D.	Kruger et al. (2004a)
Diarylacrylonitrile	Small-molecule library	SrtA: 2.7 μg/ml; SrtB: 10 μg/ml	Binding to fibronectin	Possibly noncovalent	Oh et al. (2004, 2006)
Aryl β-amino(ethyl) ketones	Small-molecule library	SrtA: 4.8 μM; SrtB: 14 μM; SrtC: 15 μM	N.D.	Covalent, mechanism-based (C184)	Maresso et al. (2007)

N.D., not determined; Seb, staphylococcal enterotoxin B; C184, SrtA cysteine 184.
[a] In the absence of a specific inhibitor, the general name for the class is indicated.
[b] The lowest IC_{50} for each specific class is presented.

ranoside side chain moiety led to a ~1.5 log reduction in inhibitory activity, indicating that this moiety may be the active component of the compound. This compound also inhibited *Bacillus subtilis* and *S. aureus* growth with minimal inhibitor concentrations at 50 and 200 μg/ml, respectively. Bactericidal activity in a sortase inhibitor would indeed be a surprising feature, and it seems more likely that these compounds block other reactions that are essential for bacterial survival and growth on laboratory media. A simple test for the possibility that sortase inhibitors display antibiotic properties may be to determine the plating efficiency of wild-type and sortase mutants in the presence of compound. Assuming that sortase inhibitors perturb envelope functions in a manner that affects bacterial growth, such phenomena could not occur in strains lacking sortases. Until proven experimentally, it seems more likely that β-sitosterol-3-*O*-glucopyranoside exhibits cytotoxic effects on bacterial cells that are distinct from the inhibition of sortase.

Isoquinoline alkaloids (berberine chloride) from rhizomes of *Coptis chinensis* were also found to be inhibitors of sortase (IC_{50} of 8.7 μg/ml) and exert antibiotic activity (Kim et al., 2004). Further examination of marine invertebrate extracts (*Topsentia genitrix*), which are known to contain antimicrobial activity, lead to the identification of bis(indole) alkaloids of the topsentin class as sortase inhibitors with an IC_{50} of ~15 μg/ml. One of these, bromodeoxytopsentin, was evaluated for its ability to inhibit *S. aureus* adherence to fibronectin, a proposed method for evaluating sortase inhibition in vivo. At approximately the IC_{50} in vitro, near complete inhibition of adherence to fibronectin was achieved, with 50% inhibition of adherence occurring at 2.5 μg/ml (Oh et al., 2005). Considering the IC_{50} in vivo is approximately six times better than the IC_{50} in vitro, it seems likely that there are nonspecific effects on adherence that are not related to the inhibition of sortase. Measurements of bacterial adherence to fibronectin are not a direct measurement of sortase activity, and these assays suffer from pitfalls associated with inhibition of other pathways that also effect adherence (e.g., inhibition of protein synthesis or secretion of polypeptide through the Sec secretion pathway). Curcumin and derivatives from *Curcuma longa* also inhibited sortase with appreciable activity (IC_{50} 13–32 μg/ml) and potentially prevented adherence to fibronectin (Park et al., 2005). In similar fashion, inhibitors of SrtB from *S. aureus* were isolated from the bark of the plant *R. verniciflua*. These inhibitors, identified as flavonoid phenols, showed appreciable in vitro inhibition of sortase in the low micromolar range (Kang et al., 2006). It is noteworthy that three flavonoids, morin, kaempferol, and galangin, along with the already identified β-sitosterol-3-*O*-glucopyranoside, displayed at least a 3-fold difference in inhibition between SrtA and SrtB. These results suggest that it may be possible to identify inhibitors that discriminate between specific members of the sortase family, highlighting discrete differences in active site architecture between these enzymes. Preliminary structure-activity relationships suggest that the co-occurrence of a hydroxyl group at C-3 of ring C and *meta*-hydroxy groups at C-2′ and C-4′ of the B ring is required for appreciable sortase inhibition. Furthermore, many of these com-

pounds seemed to effect the clumping of *S. aureus* cells to one another. Collectively, these studies represent the first steps toward the identification of inhibitors of sortase from natural sources and have generated compounds that warrant further development.

However, care must be taken in considering in vitro inhibition solely on the basis of IC_{50} values (Copeland, 2005). A determination of the mechanism of inhibition of sortase by these classes is required to completely understand their relative effectiveness and specificity; i.e., is inhibition reversible/irreversible or mechanism-based? What are the affinities and kinetics of inhibition? Does inhibition occur through interaction with active site residues, or is this elsewhere in the polypeptide? Is the inhibition competitive in the presence of substrate? In light of the fact that many compounds can inhibit enzyme activity based on redox action, promiscuous aggregation, denaturization, pH changes, and so forth, not to mention possible quenching of fluorescence or spurious reaction with peptide substrate when considering the sortase activity assay, it seems prudent to carefully investigate the nature of the inhibition (Seidler et al., 2003; Kruger et al., 2004b).

C. Rational Design

Several investigators have taken advantage of the active site thiol features of sortase or the conserved structure of its peptide substrate and designed inhibitors in a rational manner. The first such report investigated the use of synthetic substrate-derived inhibitor sequences with reactive electrophiles aimed at irreversibly modifying the active site thiol of sortase (Scott et al., 2002). Using the substrate recognition motif of staphylococcal SrtA (LPXTG), the authors replaced the scissile peptide bond between the Thr and Gly with diazoketone (-$COCHN_2$) or chloromethyl ketone (-$COCH_2Cl$). Both compounds displayed micromolar inhibitor constants (K_i $\sim 2.0 \times 10^{-7}$), with the chloromethyl ketone demonstrating faster kinetics of inactivation. Along these lines, vinyl sulfones, electrophilic inhibitors of cysteine proteases, were examined for inhibition of sortase (Hanzlik and Thompson, 1984; Frankel et al., 2004). Collectively, these compounds displayed time-dependent irreversible inhibition of sortase. One compound, 3,3,3-trifluoro-1-(phenylsulfonyl)-1-propene, was most effective with an IC_{50} of 190 μM and a single-step inhibition mechanism, most likely due to its highly electrophilic character relative to the phenyl vinyl sulfone-related compound. Mass spectrometry confirmed covalent bond formation with the active site (Cys^{184}) of SrtA for one member of this class of inhibitors, thereby confirming their reactivity to nucleophilic cysteines. In addition to substrate-derived inactivators, inhibitors aimed at imitating the transition state of the sorting reaction were evaluated for activity (Kruger et al., 2004a). A nonhydrolyzable phosphinic peptidomimetic (NH_2-YALPE-Ala[PO_2H-CH_2]Gly-EE-NH_2) showed competitive inhibition with an IC_{50} of 10 mM. Although poor in inhibition, the usefulness of this reagent was realized when a kinetic analysis of the inhibitory pattern suggested Ping-Pong Bi Bi hydrolytic shunt kinetic model for sortase catalysis (Kruger et al., 2004a). For a detailed description of the proposed kinetic mechanisms of sortase catalysis, the reader is referred to several thorough studies on this topic (Kruger et al., 2004b; Frankel et al., 2005, 2007). Substrate analogs can also be used to map peptide-binding regions on sortase. The partial amino acid sequence of the SrtA substrate LPAT fused to cyanoalkene and sulfhydryl moieties allowed for the identification of the hydrophobic surface between the β4 and β7 strands as substrate-anchoring regions (Liew et al., 2004). Others have used threonine analogs to covalently modify the active site of sortase (Jung et al., 2005).

D. High-Throughput Screen

With the increasing availability of small molecules, drug-like libraries, and robotic automation, the search for sortase inhibitors has now entered the era of high-throughput screening. A screen of 1000 diverse compounds for inhibition of sortase yielded a diarylacrylnitrile with an IC_{50} of 231 μM (Oh et al., 2004). Examination of the structure-activity relationships of this compound indicated placing the two benzene rings in the *trans*-orientation as a (*Z*)-diarylacrylonitrile lowered the IC_{50} to 28 μM. Further structure-activity relationship indicated that a 2,5-dimethoxy configuration was the most potent with a competitive inhibition profile. Dialysis and activity recovery experiments suggested that inhibition was reversible. Modeling studies suggested further that the phenyl rings of the inhibitor may interact with lipophilic residues of the sortase substrate binding pocket. Future work on this class of inhibitors will be needed to achieve a structural appreciation of sortase inhibition.

Another HTS for sortase inhibition used 135,000 compounds from a diverse small molecule library. Upon monitoring cleavage of the FRET substrate LPETG via an increase in fluorescence, approximately 6000 compounds displayed inhibitory activity compared to positive controls (no compounds added) (Maresso et al., 2007). Due to the high hit rate (~4%) in this screen, a "drug-like" filter was applied to eliminate all hits that encompasses reactive, genotoxic, promiscuous (frequent hits in unrelated assays) compounds, or molecules that lacked drug-like properties. The remaining molecules were clustered via structural similarities and sampled on the basis of activity, properties, and potential to provide SAR. A final set of 407 compounds were subjected to secondary screens aimed at i) determining the inhibitory activity toward a structurally related thiol protease (papain) and ii) determining the broad-based inhibitory activity toward different sortase family members (B and C) and species (SrtA from *B. anthracis*). All compounds that showed appreciable inhibition of different sortases

but not papain were considered to demonstrate specificity and were examined further. This selection process, i.e., imposing cheminformatic filters on HTS data sets, followed by confirmation assays and specificity constraints, allowed for the identification of some promising lead compounds (Fig. 4a).

Using this methodology, several unique classes of reversible and nonreversible sortase inhibitors were uncovered. One class, termed the aryl β-amino(ethyl) ketone (AAEK), was selected for further study due to its simple drug-like structure, marked selectivity, and inhibitor activity toward sortase (Fig. 4b). Inhibition with this class of compounds was time/dose-dependent, irreversible, and showed low micromolar IC_{50} and K_i values. Mass spectrometry confirmed that the inhibition was covalent; the inhibitor adduct differed from the parent compound in that the amino moiety had been lost during the reaction with sortase (Fig. 4b). This result suggested the AAEK class of inhibitors were mechanism-based inactivators, a type of covalent modifier that has clear precedence in on-market drugs (Copeland, 2005). A model was proposed and tested whereby an electrophilic olefin intermediate was generated following interaction with sortase, which reacted with the active site thiol. Cocrystallization of several AAEK family members with *B. anthracis* sortase B suggested that binding of inhibitors to the active site of sortase may first be mediated by an interaction of a charged ammonium species with an anionic pocket followed later by stabilization of the inhibitor in the pocket via stacking interactions between the aryl ring and an active site tyrosine (Fig. 4, c and d) (Maresso et al., 2007). Preliminary SAR-hinted anionic substituents in the *para*-position on the aryl ring are required for inhibition and lie in a cationic pocket above the site of reaction with the active site cysteine (unpublished observations).

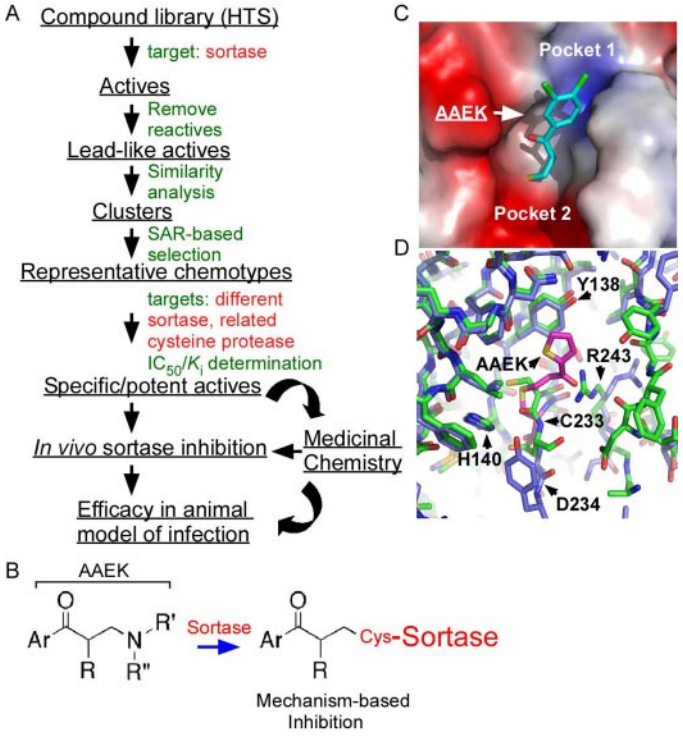

FIG. 4. HTS identifies mechanism-based inhibitors of sortase. A, proposed workflow for the identification and validation of sortase inhibitors. B, following sortase-induced β-elimination of the amine, the AAEK is converted to an electrophilic olefin, which undergoes a Michael-type addition by the thiol of the active site cysteine of sortase, thereby inactivating the enzyme. C, the crystal structure of sortase with the AAEK inhibitors identified keystone interactions and ionic pockets that can be exploited for inhibitor improvement. Electrostatic potential (red for negative and blue for positive) of the active site was generated using GRASP. Ligand atoms are color-coded as follows: yellow, sulfur; red, oxygen; green, chloride; and light blue, carbon. D, the catalytic triad of sortase B (His^{140}, Asp^{234}, and Cys^{233}) and Arg^{243} is in close proximity to the inhibitor adduct and undergoes substantial structural shifts. The SrtB (green) and SrtB-AAEK adduct (blue) are superimposed. The figure was generated using PyMOL. Figure 4D was reproduced from Maresso et al. (2007) with permission from the American Society for Biochemistry and Molecular Biology.

V. On the Development of Sortase Inhibitors

To date, the search for inhibitors of sortases has involved natural, synthetic, and high-throughput screening methodologies. As the field moves toward the development of compounds that can be interrogated for therapeutic efficacy, several issues outlined below must be considered. Although still preliminary in nature, the chemical diversity of the already uncovered inhibitors of sortases provides important assets for future improvements of specificity and potency.

A. Assay Design

When offered a choice, drug developers prefer noncovalent inhibitors over those that modify enzymes covalently, with mechanism-based inactivation perhaps representing the exception to this rule (Copeland, 2005). Most of the inhibition studies described thus far utilize the FRET-based cleavage of LPETG as a measure of sortase activity. Although this assay is well suited for inhibitor identification via HTS (Z' of 0.94), there are several drawbacks. For instance, the concentration of enzyme and substrate required to attain appropriate Z' are high. Low catalytic turnover, combined with long incubation times, probably introduces bias toward selection of covalent inhibitors. This phenomenon has indeed been observed in our own studies (unpublished data) and may be circumvented by the addition of physiologic nucleophiles (i.e., pentaglycine of lipid II) to the reaction for greater activity at lower enzyme concentration. Of concern is also the bias toward thiol reactive compounds, because sortase utilizes a catalytic cysteine residue. A possible solution to such systematic error would be to perform sortase assays in the presence of reducing agent (e.g., dithiothreitol). Finally, the potential for fluorescence quenching or spurious reactions of compounds with peptide substrate and/or fluorophore must be examined with secondary assays that use different outputs for sortase activity (Kruger et al., 2004b).

B. In Vivo Evaluation of Sortase Inhibitors

It is clear that lead compounds identified by in vitro screens will have to be evaluated for in vivo inhibition of surface protein anchoring to the cell wall. One should be cautious in the evaluation of readouts that are not direct measurements of this process, such as adhesion of *S. aureus* to extracellular matrix proteins, for example. A plethora of mechanisms in protein secretion, folding, and anchoring to the cell wall envelope (including synthesis of peptidoglycan and other envelope components) affect the abundance of functional surface proteins in the staphylococcal envelope. A direct assessment of sortase activity is achieved by pulse-labeling polypeptides with [^{35}S]methionine. Immunoprecipitation and separation of surface proteins on SDS-polyacrylamide gel electrophoresis followed by autoradiography can monitor the processing of pulse-labeled P1 precursor to P2 and mature species, thereby providing an in vivo measure for the sortase-catalyzed anchoring reaction. This time-consuming protocol is not useful for HTS; however, it can be used to characterize lead compounds. For HTS, we advocate the use of fluorescence-activated cell sorting (FACS) to monitor the abundance of anchored sortase substrates in the cell wall envelope. Finally, validation should include efficacy testing in animal models of infection that involve Gram-positive pathogens with sortases and surface proteins. In this respect, the commonly used renal abscess model appears suitable due to its large dynamic range (>3 log units) of virulence assessment, albeit that many other models may also be useful. However, it should not be forgotten that effective in vivo sortase inhibitors must also display favorable pharmacologic properties, including low toxicity and reactivity with mammalian proteases.

VI. Summary

In an era of global spread of antibiotic resistance in many human pathogens, concerted efforts must be applied toward identification and validation of new targets for drug development. These include essential factors necessary for bacterial survival, even under nondisease conditions as well as virulence determinants whose actions are mostly manifested during the infectious process. Whereas antibiotics have a long-standing history of success in the treatment of bacterial infections, drugs that target virulence factors still suffer from a lack of experimental proof-of-principle. However, unless agents with novel inhibition mechanisms against essential factors are uncovered, the problem of resistance will continue. Sortase, a major virulence factor responsible for the covalent attachment of surface proteins to the cell wall, is a target candidate for the treatment of Gram-positive infections. Progress toward this goal has been made and several distinct sortase inhibitor classes have been identified. Compounds with reasonable inhibition, specificity, and mechanisms of inactivation have been uncovered. For these inhibitors to be realized as effective drugs, direct assessment of the inactivation of sortase and attenuation of virulence in animal models must be demonstrated. In a continuous struggle to stay ahead of the ever-changing drug resistance traits of pathogenic bacteria, the selective inhibition of virulence factors may prove to be an effective therapeutic strategy that may either augment or perhaps even supplant the more traditional approaches.

Acknowledgments. We apologize for the omission of any work that was not mentioned in this review due to space constraints. We acknowledge membership within and support from the Region V "Great Lakes" Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Consortium (1-U54-AI-057153).

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