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Review

Staphylococcus aureus as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity

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Although it is estimated that 20–30% of the general human population are carriers of *Staphylococcus aureus*, this bacterium is one of the most important etiological agents responsible for healthcare-associated infections. The appearance of methicillin resistant *S. aureus* (MRSA) strains has created serious therapeutical problems. Detailed understanding of the mechanisms of *S. aureus* infections seems necessary to develop new effective therapies against this pathogen. In this article, we present an overview of the biochemical and genetic mechanisms of pathogenicity of *S. aureus* strains. Virulence factors, organization of the genome and regulation of expression of genes involved in virulence, and mechanisms leading to methicilin resistance are presented and briefly discussed.

Keywords: *Staphylococcus aureus*, virulence, pathogenicity genes, toxins, methicillin resistance

BIOCHEMISTRY AND MOLECULAR GENETICS OF *STAPHYLOCOCCUS AUREUS*

Staphylococcus aureus is a Gram-positive spherical bacterium approximately 1 μm in diameter. Its cells form grape-like clusters, since cell division takes place in more than one plane. It is often found as a commensal associated with skin, skin glands, and mucous membranes, particularly in the nose of healthy individuals (Crossley & Archer, 1997). It has been estimated that approx. 20–30% of the general population are S. *aureus* carriers (Heyman, 2004). On a rich medium, *S. aureus* forms medium size "golden" colonies. On sheep blood agar plates, colonies of *S. aureus* often cause β-hemolysis (Ryan & Ray, 2004). The golden pigmentation of *S. aureus* colonies is caused by the presence of carotenoids and has been reported to be a virulence factor protecting the pathogen against oxidants produced by the immune system (Liu *et al.*, 2005).

Staphyloccoci are facultative anaerobes capable of generating energy by aerobic respiration, and by fermentation which yields mainly lactic acid. *Staphylococcus* sp. is catalase-positive, a feature differentiating them from *Streptococcus* sp., and they are oxidase-negative and require complex nutrients, e.g., many amino acids and vitamins B, for growth. *S. aureus* is very tolerant of high concentrations of sodium chloride, up to 1.7 molar.

Another feature of the *Staphylococcus* genus is the cell wall peptidoglycan structure that contains multiple glycine residues in the crossbridge, which causes susceptibility to lysostaphin (Crossley & Archer, 1997). *S. aureus* produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin.

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Abbreviations: AIP, auto-inducing peptide; IS, Insertion sequences; MRSA, methicillin resistant *Staphylococcus ureus*; MSSA, methicillin sensitive *S. aureus*; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; PBPs, penicillin-binding proteins; PNAG, poly-*N*-acetylglucosamine; PVL, Panton-Valentine leukocidin; SCVs, small-colony variants; SaPIs, superantigen toxins; SCC*mec*, staphylococcal cassette chromosome *mec*; TSST, toxic shock syndrome toxin.

Blood coagulation is used to distinguish *S. aureus* from others members of the genus, which are collectively designated as coagulase-negative staphylococci (Ryan & Ray, 2004). Since it is a frequent ethological agent of human diseases and exhibits resistance to a growing number of therapeutic agents, *S. aureus* is also one of the most intensively studied bacterial species.

S. AUREUS INFECTIONS

S. aureus is a commensal and a pathogen. The anterior nares are the major site of colonization in humans. About 20–30% of individuals are persistent carriers of *S. aureus*, which means they are always colonized by this bacterium, and 30% are intermittent carriers (colonized transiently) (Wertheim *et al.*, 2005). Colonization significantly increases the risk of infections since it provides a reservoir of the pathogen from which bacteria are introduced when host defense is compromised (Kluytmans *et al.*, 1997). Patients with *S. aureus* infections are usually infected with the same strain that they carry as a commensal (Williams *et al.*, 1959).

S. aureus is one of the main causes of hospital- and community-acquired infections which can result in serious consequences (Diekema *et al.*, 2001). Nosocomial *S. aureus* infections affect the bloodstream, skin, soft tissues and lower respiratory tracts. *S. aureus* can be a cause of central venous catheter-associated bacteremia and ventilator-assisted pneumonia. It also causes serious deep-seated infections, such as endocarditis and osteomyelitis (Schito, 2006). In addition to the infections listed above, *S. aureus* is often responsible for toxin-mediated diseases, such as toxic shock syndrome, scalded skin syndrome and staphylococcal foodborne diseases (SFD). Hospitalized patients are particularly exposed to *S. aureus* infections due to their compromised immune system and frequent catheter insertions and injections (Lindsay & Holden, 2004). The SENTRY Surveillance Program investigating worldwide *S. aureus* infections during a two-year period has revealed that this pathogen is the leading cause of bloodstream, lower respiratory tract and skin/soft tissues infections in all regions surveyed (Diekema *et al.*, 2001). The importance of this human pathogen, apart from its ability to cause life-threatening infections, is its remarkable potential to develop antimicrobial resistance.

VIRULENCE FACTORS

S. aureus is equipped with a great variety of virulence factors, which include both structural and secreted products participating in pathogenesis of infection.

Attachment-improving agents

S. aureus carries numerous surface proteins named "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) that mediate attachment to host tissues and initiate colonization leading to an infection (Gordon & Lowy, 2008). Fibronectin binding proteins A and B (FnbpA and FnbpB) participate in attachment of bacterial cells to an extra-cellular matrix component, fibronectin, and to plasma clot. Collagen binding protein, Cna, is necessary for adherence of *S. aureus* to collagenous tissues and cartilage (Switalski *et al.*, 1993) and it has been shown that antibodies against Cna block the bacteria attachment to those tissues (Patti *et al.*, 1994). Clumping factor A and B (ClfA and ClfB) mediate clumping and adherence of bacterial cells to fibrinogen in the presence of fibronectin. Clumping factors are thought to play a significant role in wound and foreign body infections and it has been shown that *clfA* mutant is less virulent than the wild type isogenic strain (Foster & Hook, 1998). Plasma-sensitive surface protein (Pls), once processed by plasmin, participates in binding to both fibrinogen and fibronectin (Hauck & Ohlsen, 2006). Protein A is a hallmark of *S. aureus* which is encoded by the *spa* gene and is a cell wall-associated protein that binds to the Fc domain of immunoglobin G (IgG). Protein A binds IgG in "wrong orientation" on the surface of *S. aureus* cells which is thought to disrupt opsonization and phagocytosis (Switalski *et al.*, 1993). Protein A also exhibits an ability to bind to von Willebrand factor, a protein present at sites of damage of endothelium, and as a result, it can play a role in adherence and induction of endovascular diseases by *S. aureus* (Hartleib *et al.*, 2000).

Implanted biomedical device-related *S. aureus* infections depend on the pathogen's ability to attach to the surface of the biomaterial and consequently to form a mucoid biofilm. Biofilms are complex bacterial populations which are surface-attached and enclosed in a polysaccharide matrix, composed of poly-*N*-acetylglucosamine (PNAG). PNAG production depends on proteins encoded by the *ica* (intracellular adhesion) operon (Fitzpatrick *et al.*, 2005). Biofilm-associated bacteria, unlike their planktonic counterparts, are resistant to the host immune responses and to antimicrobials, which often complicates treatment. It was reported that 60% of *S. aureus* strains were able to produce biofilm (Arciola *et al.*, 2001a; 2001b). However, contradictory results obtained by other investigators suggested that all *S. aureus* strains possess the *icaADBC* genes (Rohde

et al., 2001). In addition, there is a regulatory gene called *icaR* that, together with the *icaA* promoter, is subject to a multitude of regulatory effects linking *ica* gene expression to virulence regulators.

Recent studies indicated that among clinical isolates of *S. aureus*, only between 45% and 70% (depending on the type of infection) strains were able to form biofilm (Grinholc *et al.*, 2007). Those studies also suggested that no correlation exists between biofilm production and the type of staphylococcal infection.

Exotoxins

One of the important characteristics of *S. aureus* is its capability to secrete toxins that disrupt membranes of host cells. Cytolytic toxins form β-barrel pores in the cytoplasmic membranes and cause leakage of the cell's content and lysis (Foster, 2005). *S. aureus* secrets several cytolytic toxins, among them alpha-hemolysin, beta-hemolysin, gamma-hemolysin, leukocidin, and Panton-Valentine leukocidin (PVL) (Kaneko & Kamio, 2004). Alpha-hemolysin, encoded by the *hla* gene, inserts into eukaryotic membranes and oligomerizes into a β-barrel that forms a pore which causes osmotic cytolysis. Alpha-hemolysin is particularly cytolytic toward human platelets and monocytes (Menestrina *et al.*, 2001).

PVL is classified as a bicomponent cytolysin because it is dependent on two secreted proteins (LukF-PV and LukS-PV) that insert into the host's cytoplasmic membrane and hetero-oligomerize to form a pore (Kaneko & Kamio, 2004). PVL exhibits a high affinity toward leukocytes and is mostly associated with community-acquired methicillin resistant *S. aureus* (CA-MRSA) which causes severe necrotizing pneumonia and contagious skin infections (Foster, 2005). Other bicomponent toxins, gamma-hemolysin (Hlg) and leukocidin (Luk), are cytotoxic toward erythrocytes and leukocytes, respectively (Kaneko & Kamio, 2004).

Superantigen toxin

S. aureus generates a group of powerful immuno-stimulatory proteins implicated in gastroenteritis and toxic shock syndrome. They are resistant to heat denaturation and proteases. These toxins have the ability to cross-link MHC class II molecules located on antigen-presenting cells with T-cell receptors, forming a trimolecular complex. Formation of the complex induces intense T-cell proliferation in an antigen-independent manner resulting in massive cytokine production and release which causes capillary leak, epithelial damage and hypotension (Baker & Acharya, 2004). The primary function of superantigens is thought to weaken the host's immune system sufficiently to allow the pathogen to propagate and the disease to progress (Kotzin *et al.*, 1993). The staphylococcal enterotoxins A, B, C, D, E, G, Q are responsible for staphylococcal foodborne diseases and toxic shock syndrome, while TSST-1 is the cause of toxic shock syndrome (Baker & Acharya, 2004). The superantigen toxins are typically encoded by mobile genetic elements (Novick, 2003b) which will be described in later sections.

SMALL-COLONY VARIANTS

Small-colony variants (SCVs) represent a subpopulation of naturally occurring, slowly growing *S. aureus* with distinct phenotype and pathogenetic features. SCVs have been reported to cause recurrent, persistent infections many years after the initial infection had been cured (Proctor *et al.*, 1995). Very often they reside inside human cells avoiding host defenses and antimicrobial chemotherapeutics. SCVs are defective in their electron transport pathways and usually form non-pigmented, non-hemolytic tiny colonies on agar (Kaneko & Kamio, 2004). The small-colony variants display marked auxotrophisms for thymidine, menadione and/or hemin (Clements *et al.*, 1999; Bates *et al.*, 2003; Lannergard *et al.*, 2008; von Eiff, 2008). They exhibit reduced rate of metabolism and are less virulent, but due to their slow growth and reduced cell wall synthesis, they are more tolerant of β-lactam antibiotics than their wild-type parents. Their low membrane potential makes them also resistant to aminoglycoside antibiotics (Proctor *et al.*, 2006).

REGULATION OF GENES INVOLVED IN VIRULENCE

The genes coding for virulence factors are regulated in a tightly coordinated manner that is synchronized with the biological cycle of *S. aureus*. The production of factors involved in virulence is controlled by quorum sensing mechanism. In *S. aureus*, genes coding for surface proteins are down regulated during early stages of the growth whereas genes that encode secreted proteins are up regulated in late exponential phase. This pattern of gene expression in which surface proteins involved in adhesion and defense against host's immune system (protein A, coagulase, fibronectin binding proteins, among many others) are synthesized before production of secreted hemolysins, cytotoxins, proteases and other degradative enzymes seems to reflect a strategy of *S. aureus* in which the pathogen first establishes itself in the host and only then attacks it. This regulation is, in large part, due to the accessory gene regulator

(*agr*) two component system (Novick & Geisinger, 2008).

The *agr* locus consists of two divergent transcription units RNAII and RNAIII driven by two promoters, P2 and P3, respectively (Janzon & Arvidson, 1990). The P2 transcript, RNAII, contains four cistrons: *agrA*, *agrB*, *agrC* and *agrD*. The sensor, AgrC, and the response regulator, AgrA, comprise the two component system that responds to auto-inducing peptide (AIP). This peptide is present in the extracellular environment and drives transcription from both P2 and P3 promoters. *agrD* encodes the auto-inducing peptide, which is posttranslationally modified and secreted by AgrB (Novick, 2003a). The effector molecule of the *agr* system is a 514-nt transcript, derived from the P3 promoter, called RNAIII, which also carries the *hld* cistron that codes for delta-hemolysin. RNAIII stimulates the expression of post-exponentially synthesized extracellular toxins and enzymes and represses synthesis of exponential-phase surface proteins (Janzon & Arvidson, 1990). RNAIII acts primarily as an antisense RNA for translational activation of certain mRNAs or binds to the ribosome binding site in the case of repressed mRNAs, preventing ribosome binding and inducing fast mRNA degradation by endoribonuclease III (Morfeldt *et al.*, 1995; Boisset *et al.*, 2007).

ORGANIZATION OF THE *S. AUREUS* GENOME

The first genome sequences of *S. aureus* strains Mu50 and N315 were published in 2001 (Kuroda *et al.*, 2001). At present, complete genomic sequences of ten *S. aureus* strains are available, and the genomes of several others have been partially determined (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Gill *et al.*, 2005; Diep *et al.*, 2006). The genome of *S. aureus* is a circular chromosome that is 2.8–2.9 Mbp in size, with a G+C content of about 33% (Crossley & Archer, 1997). The chromosome encodes approximately 2700 CDSs (protein coding sequences) as well as structural and regulatory RNAs. It has been proposed that the *S. aureus* genome is composed of the core genome, accessory component and foreign genes.

The core genes are present in more than 95% of isolates, represent 75% of any *S. aureus* genome and determine the backbone of the genome. The organization of the core component is highly conserved and the identity of individual genes between isolates is 98–100%. The majority of core genes are associated with fundamental functional categories of housekeeping functions and central metabolism.

The accessory component includes genetic regions present in 1–95% of isolates and accounts for about 25% of any *S. aureus* genome. It typically consists of mobile genetic elements that have or previously had the ability of horizontal transfer between strains. These genetic elements include pathogenicity islands, genomic islands, prophages, chromosomal cassettes and transposons (Lindsay & Holden, 2004).

Pathogenicity islands

The family of staphylococcal pathogenicity islands that carry genes for superantigen toxins (SaPIs) are 15–20 kb elements located at constant positions in the chromosome. SaPIs possess certain bacteriophage-related attributes: genes coding for integrases, helicases and terminases, and flanking direct repeats (Novick, 2003b). The archetype of this family, SaPI1, codes for toxic shock syndrome toxin TSST (*tst*) and is excised and induced to replicate as well as transduced at high frequency by phage 80α. DNA of SaPI1 is encapsulated into 80α phage-like particles for transfer (Lindsay *et al.*, 1998; Ruzin *et al.*, 2001). Another member of SapI family, SapI3, encodes enterotoxin B and is thought to be mobilized and encapsulated by phage 29 (Novick, 2003b). Certain bovine isolates of *S. aureus* carry SaPIbov1 which encodes toxin shock syndrome toxin (*tst*) and can be induced by three phages: 80α, ϕ11 and ϕ147 (Ubeda *et al.*, 2005). Members of the SaPI family have been found in almost all strains of *S. aureus* sequenced so far (Kuroda *et al.*, 2001; Baba *et al.*, 2002; Diep *et al.*, 2006).

In addition to SaPIs, *S. aureus* strains contain genomic islands from the *v*Sa family. These islands carry genes coding for about half of the *S. aureus* toxins and virulence factors, and greatly contribute to the pathogenicity of this species (Gill *et al.*, 2005). They are found in all sequenced strains in the same locations and some of the genes carried by them are highly conserved (Lindsay & Holden, 2004). They encode their own integrase and usually are spontaneously excised from the host chromosome (Baba *et al.*, 2002). Members of this family of genomic islands include, but are not limited to, *v*Sa1 (carrying enterotoxin genes *seb*, *tsst*, *ear*), *v*Sa2 (containing genes encoding enterotoxine (*sec*) and toxic shock syndrome toxine — (*tsst*)) (Gill *et al.*, 2005). Other examples include *v*Saα and *v*Saβ, which are found in all sequenced isolates and have a defective transposase gene, and therefore are not excised from the chromosome. Additionally they carry a cassette encoding a restriction-modification system and genes encoding leukocidin (*lukDE*) (Baba *et al.*, 2002).

Prophages

Prophages of *S. aureus* can be classified into three groups based on the size of their genome. Class I includes phages with genomes of less than 20 kb, class II has a genetic material of approximately 40 kb and class III of more than 125 kb (Kwan

et al., 2005). Prophages are thought to play an important role in evolution and pathogenicity of *S. aureus* and very often offer means for the horizontal transfer of genetic information. Each of the *S. aureus* strains sequenced so far contains between one and three prophages, most of them carry virulence determinants exemplified by enterotoxins A, G, K, exfoliative toxin, staphylokinase and Panton-Valentine Leukocidin (Kuroda *et al.*, 2001; Lindsay & Holden, 2004; Diep *et al.*, 2006).

Insertion sequences and transposons

Insertion sequences (IS) carry at least one gene coding for a transposase which participates in the recombination required for transposition. Most IS elements also contain short inverted terminal repeats acting as transposase binding sites (Baba *et al.*, 2004). Insertion elements are randomly scattered throughout the genome of *S. aureus*, both in coding and non-coding regions. In MRSA, *S. aureus* N315 and Mu50 strains, eight copies of IS1181 have been found (Kuroda *et al.*, 2001; Gill *et al.*, 2005).

Transposons are larger transposable genetic elements that, in addition to a transposase gene, carry other genes which very often are antibiotic resistance determinants. *S. aureus* is the host to more than ten transposons, the majority of which carry antibiotic resistance genes (Baba *et al.*, 2004).

Plasmids

Plasmids, defined as extrachromosomal genetic elements bearing only non-essential genes which, however, may provide a benefit to the host under special environmental conditions, often encode factors determining resistance to antibiotics or heavy metals, virulence factors and proteins facilitating survival in the presence of unusual nutrients (Wegrzyn, 2005). Plasmids of *S. aureus* have been categorized into three classes. Class I plasmids are of the size of 1–5 kb and occur in high copy number (15–50 per cell). They usually carry a single antibiotic resistance determinant. The class II plasmids are of intermediate size and occur in intermediate copy number, and they usually code for β-lactamase and confer resistance to inorganic ions. The last group of staphylococcal plasmids, class III, consists of large conjugative plasmids (40–60 kb). Class III plasmids carry multiple resistance determinants, exemplified by resistance to trimethoprim, gentamycin and ethidium bromide (Novick, 1989). The plasmids often can serve as means by which antibiotics resistance is transmitted. Moreover, the conjugative plasmids encode their own conjugative horizontal transfer mechanism by *tra* genes that offer an advantage by which transfer of extrachromosal genetic information to other bacteria occurs (Hartleib *et al.*, 2000; Kuroda *et al.*, 2001; Holden *et al.*, 2004 ; Gill *et al.*, 2005; Diep *et al.*, 2006).

METHICILLIN RESISTANT *S. AUREUS* (MRSA)

Methicillin resistant *Staphylococcus aureus* (MRSA) is defined by the presence of a large mobile genetic element called staphylococcal cassette chromosome, *mec* (SCC*mec*). It carries the *mecA* gene that codes for an alternative penicillin binding protein, PBP2a, with low binding affinity to all β-lactams (described in next sections) (Ito *et al.*, 1999). MRSA strains were first described in hospital settings, after the introduction of β-lactamase-insensitive penicillins into medical practice, and they continue to be a serious problem in health care due to their ability to acquire multidrug resistance determinants. Although outbreaks of diseases in a hospital may also be caused by methicillin sensitive *S. aureus* (MSSA) (Kurlenda *et al.*, 2009), MRSA infections are especially easily spread throughout a hospital and, without implementation of a special surveillance program with control procedures, a risk of an epidemy in such a hospital is high (Kurlenda *et al.*, 2007).

Staphylococcal chromosome cassette (SCC*mec*)

SCC*mec* is a 21–67-kb genetic element that is found in the chromosome of methicillin resistant *S. aureus* at a unique site designated *att*B*scc*, located near the origin of replication. *att*B*scc* is found in an open reading frame of unknown function, identified as *orfX*, that is well conserved among *S. aureus* strains. The integration site of SCC*mec*, *att*B*scc*, contains a 15-bp sequence that, when SCC*mec* is inserted in the chromosome, is found at both chromosome-SCC*mec* junctions (*att*L and *att*R). However, unlike the direct repeats found in transposons, which are created by target duplication, one of the two repeat sequences is located within SCC*mec* at its right end. Degenerate inverted repeats are also present at both ends of SCC*mec* (Ito *et al.*, 2001; Noto *et al.*, 2008). These incomplete inverted repeats are thought to be recognized by SCC*mec*-specific recombinase during excision and integration of this element from and to chromosome (Fig. 1) (Hiramatsu *et al.*, 2001; Noto *et al.*, 2008).

SCC*mec* is a variable genetic element with certain conserved features. Among the conserved elements, SCC*mec* contains the *mec* operon composed of *mecA* and its regulatory genes, as well as the cassette chromosome recombinase complex *ccr* (Ito *et al.*, 2001; 2004; Baba *et al.*, 2002; Holden *et al.*, 2004 ; Gill *et al.*, 2005; Diep *et al.*, 2006). The *ccr* locus is composed of the cassette chromosome recombinase genes,

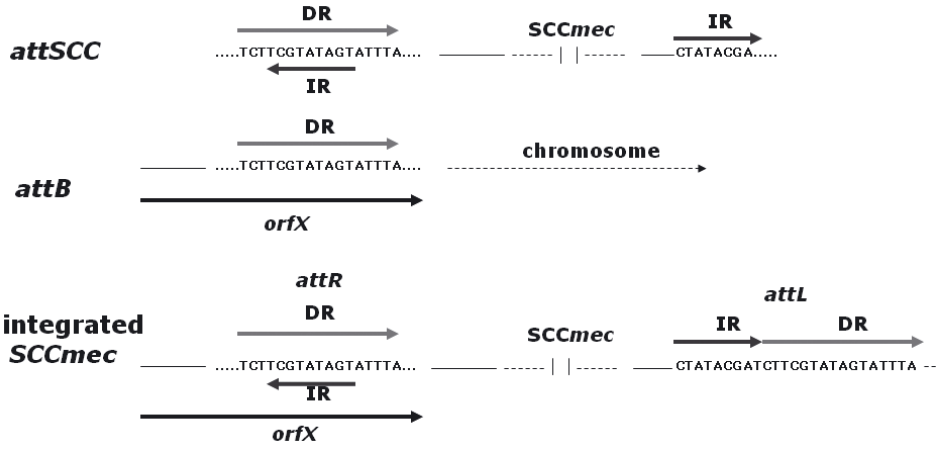

Figure 1. Schematic representation of attachment sites for SCC*mec* integration.
The SCC*mec* attachment site (*att*SCC) as it is found on the circular extra-chromosomal SCC*mec* elements (top panel). Chromosomal attachment site (*attB*) located within *orfX* near origin of replication (middle panel). Integrated into chromosome, SCC*mec* with hybrid attachment sites at both ends (*attL* and *attR*) (bottom panel). Light-grey arrows indicate direct repeat sequences, dark-grey arrows — indirect repeat sequences, and black arrows — coding region of *orfX* (Noto *et al.*, 2008).

ccrA, *ccrB* or *ccrC*, that are involved in the integration into the chromosome and in precise excision form the chromosome of the *SCCmec* element (Katayama *et al.*, 2000; Ito *et al.*, 2001; 2004). The variable regions of SCC*mec*, called J-region, contain integrated genetic elements such as plasmids (pT181, pUB110 and p1258), transposons (Tn554) and insertion sequences (IS431, IS1272 and IS256) (Hanssen & Ericson Sollid, 2006).

The hallmark of SCC*mec* is the *mec* operon that consists of *mecA* and its regulatory genes *mecI* and *mecR1* (Fig. 2). The operon is found in several variants as a component of different SCC*mec* elements. The variants of the *mec* operon are divided into two main categories: those with both *mecI* and *mecR1* genes intact and those with portions of one or both of these regulatory genes deleted. The first group of the *mec* complex is known as class A *mec* operon while the latter have been categorized as classes B, C, D, E.

All classes of the *mec* operon include a copy of IS431 associated with the *mecA* gene and therefore designated IS431*mec*. Classes B–E contain deletions of *mecI* that may be extended to part of the *mecR1* gene. Usually these deletions coincide with insertion of IS elements. Moreover, the *ccr* locus of SCC*mec* elements also exists in several variants. As mentioned above, the

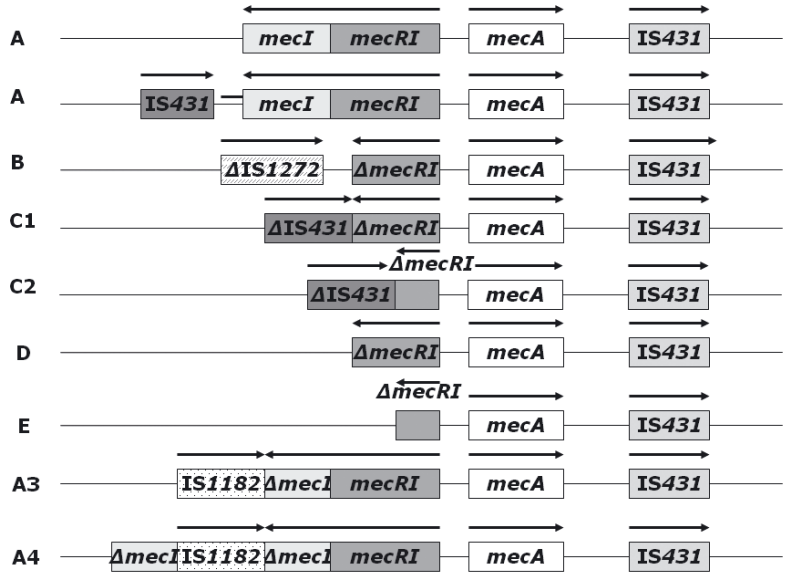

Figure 2. Structural classes of *mec* operon.
Direction of transcription is indicated by arrows above each element. Designation of each variant is shown on the left. After Hanssen and Ericson-Sollid (2006).

Table 1. Characteristics of eight types of SCC*mec* elements.

(Based on: Hiramatsu *et al.*, 2001; Deurenberg & Stobberingh, 2008; Zhang *et al.*, 2009)

SCC*mec* type	*mec* complex	*ccr* genes	Size	Other resistance determinants
I	class B–E	*ccrA1B1*	34 kb	None
II	class A	*ccrA2B2*	52–58 kb	Erythromycin, spectinomycin, bleomycin, tetracycline
III	class A	*ccrA3B3*	67 kb	Erythromycin, spectinomycin, tetracycline, mercury, cadmium
IV	class B–E	*ccrA2B2 or ccrA4B4*	20–25 kb	None
V	class B–E	*ccrC*	28 kb	None
VI	class B	*ccrB4*	20–25 kb	None
VII	class C	*ccrC2, ccrC8*	28–30 kb	None
VIII	class A	*ccrA4, ccrB4*	32 kb	Erythromycin, spectinomycin

ccr locus contains either *ccrA* and *ccrB* or *ccrC*. Based on differences in nucleotide sequences, the combination *ccrA* and *ccrB* allotypes are divided into eight complexes of the *ccr* gene (types I through VIII, Fig. 3) (Komatsuzawa *et al.*, 1994; Katayama *et al.*, 2001).

To date, at least five types of SCC*mec* elements have been defined based on combination of different *mec* (Fig. 2) and *ccr* (Table 1) complexes they contain. Table 1 describes the characteristics of the eight main SCC*mec* types.

Two PCR-based methods of SCC*mec* typing have been developed allowing characterization of this genetic element. Ito and coworkers (2001) established a method in which portions of the *mec* complex as well as specific parts of *ccr* genes are amplified by means of PCR. Using this method SCC*mec* elements are typed according to the combination of the *mec* operon and *ccr* genes present. Oliveira and de Lancastre developed a multiplex PCR strategy that detects other regions of SCC*mec* in addition to

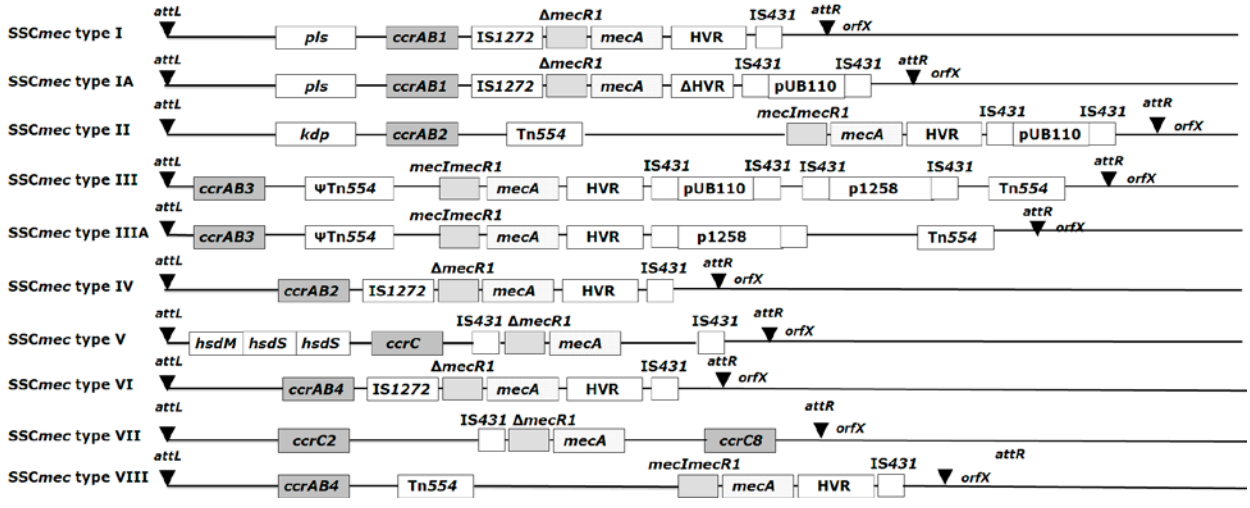

Figure 3. Schematic representation of SCC*mec* types I–VII and their variants.
Labels indicate names of genes, insertion sequences, transposons and plasmids, *pls,* plasmin sensitive surface protein; *kdp,* operon involved in ATP-dependent potassium transport; HVR, hypervariable region; *ccrAB1*, *ccrAB2*, *ccrAB3*, *ccrC,* allelic variants of SSC*mec*-specific recombinases; *hsd,* type I restriction-modification system; pUB*110*, pT*181*, *pI*258, integrated plasmids; Tn*554*, Ψ Tn*554,* transposon and pseudo-transposon, respectively; IS*431*, IS*1272,* insertion sequences; *mecI*, *mecRI,* repressor and sensor, respectively, of the *mec* two-component system; *mecA,* low affinity penicillin binding protein. After Ito *et al.* (2001; 2004), Oliveira *et al.* (2001), Okuma *et al.* (2002), Hanssen and Ericson-Sollid (2006).

the *mec* and *ccr* loci. This method allows detecting structural variants of SCC*mec* elements (Au *et al*., 2005; Milheirico *et al*., 2007).

The first clinical isolates of MRSA were reported just one year after introduction of methicillin, and they were resistant to multiple antibiotics in addition to β-lactams. It was proposed that these strains had probably been selected by the common use of a variety of antibiotics in healthcare settings (Rice, 2006). The isolates were identified as MRSA SCC*mec* types I, II and III. The phenomenon of multi-resistance among these strains was largely due to the presence of particular SCC*mec* types I and II which contain integrated transposons and plasmids that carry additional resistance determinants. The copy of IS431 adjacent to the *mecA* gene and designated IS431*mec* is thought to facilitate the acquisition of resistance determinants through homologous recombination (e.g., plasmids pUB110 and pT181) (Katayama *et al*., 2000; Ito *et al*., 2001). Since 1990, several MRSA have been isolated as the pathogenic agents of severe infections in the community. These isolates caused disease among individuals without the typical factors for MRSA infections, such as recent hospitalization, admission in long term care facility or preexisting health problems. These community-acquired MRSA, designated CA-MRSA, were susceptible to many antibiotics other than β-lactams and contained the SCC*mec* type IV (Fey *et al*., 2003). Some CA-MRSA (USA300) contain a newly discovered mobile genetic element, arginine catabolic mobile element (ACME). The physical linkage between SCC*mec* and ACME suggests that selection for antibiotic resistance and for pathogenicity may be interconnected (Diep *et al*., 2008). Another variant of pathogenic CA-MRSA strain was isolated which contains type V SCC*mec* (Ito *et al*., 2004). Recently, nevely discovered types of SCC*mec*, designated types VI, VII and VIII, have been described (Deurenberg & Stobberingh, 2008; 2009; Zhang *et al*., 2009).

SCC*mec* contains many open reading frames exhibiting atypical codon usage and skewed G+C content at the third codon positions indicating that this genetic element was acquired by *S. aureus* relatively recently from another species (Kuroda *et al*., 2001). The origin of SCC*mec* and the mechanism of transfer are unknown. A possible evolutionary precursor of the *mecA* gene of MRSA strains, exhibiting 88% identity, was found in *S. sciuri* (Wu *et al*., 2001). It has been proposed that the *ccr* and the *mec* genes were brought together in coagulase-negative staphylococci where deletions in the *mec* regulatory genes occurred before the transfer to *S. aureus*. There is also evidence supporting the hypothesis of SCC*mec* transfer from coagulase-negative staphylococci to *S. aureus.* The IS1272 element is found in intact form and in multiple copies in the genome of *S. haemolyticus*, whereas it typically contains deletions in *S. aureus* and *S. epidermidis*. This suggests that *S. haemolyticus* was the primary host of this element and it was secondarily acquired by *S. aureus* and *S. epidermidis* by means of horizontal transfer (Kobayashi *et al*., 1999). There is also report of *in vivo* MRSA formation by horizontal transfer of *mecA* between *S. epidermidis* and *S. aureus* during antibiotic therapy (Wielders *et al*., 2001). Methicillin resistance is common in *S. epidermidis* (more than 70% hospital isolates) and is less common among *S. aureus* strains. The presence of SCC*mec* type IV (Fig. 3) was common among *S. epidermidis* isolates from the 1970s and was not reported in methicillin resistant *S. aureus* strains during that time. The rapid emergence and dissemination of community-acquired MRSA containing SCC*mec* type IV lead to the hypothesis that *S. epidermidis* may act as a reservoir of SCC*mec* type IV in the community and the recurring transfer of this element into methicillin sensitive *S. aureus* (MSSA) is partly responsible for the increasing occurrence of CA-MRSA (Wisplinghoff *et al*., 2003). It has been proposed that recombination between SCC*mec* type I and other sequences occurred in coagulase-negative staphylococci leading to the generation of SCC*mec* type IV which was subsequently transferred to *S. aureus* (Wisplinghoff *et al*., 2003). This data supports the hypothesis that SCC*mec* are mobile genetic elements in the environment and their transfer into methicillin sensitive *S. aureus* contributes to the spread of MRSA. It has been estimated that *S. aureus* has acquired SCC*mec* element at least in 20 independent occasions (Robinson & Enright, 2003; Deurenberg *et al*., 2007). Hospital-acquired MRSA (HA-MRSA) infections historically have been caused by internationally dispersed clones, including five major ones (the Iberian, Brazilian, Hungarian, New York/Japan and Pediatric). These multi-drug resistant clones are disseminated globally and account for the majority of HA-MRSA infections. However, it remains unclear why particular clones are more successful in becoming the established "HA-MRSA" in certain regions (Robinson & Enright, 2003).

Cell wall structure and biosynthesis

The staphylococcal cell wall is a dynamic, semi-rigid structure. It is composed of three components: peptidoglycan, teichoic acids and surface proteins. Of these three constituents, peptidoglycan is the major component which builds the murein sacculus. Structurally, peptidoglycan forms a macromolecular net in which glycan strands are cross-linked by short peptides. The glycan strands are composed of repeating disaccharide units of β-1-4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid (Labischinski, 1992). The majority of glycan chains have a length of 3–10 disaccharide units. Extending from the carboxyl residue of the β-1-4 linked acetylglucos-

amine moiety is the stem peptide with the sequence: L-alanyl-D-isoglutaminyl-L-lysyl-D-alanyl-D-alanine (Scheffers & Pinho, 2005). A series of five L-glycine residues are attached to the L-lysine of the stem peptide which is a characteristic feature of the *S. aureus* cell wall. The pentaglycine cross-bridge is synthesized in a sequential manner by a family of FemABX non-ribosomal peptide transferases (Berger-Bachi & Tschierske, 1998; Rohrer *et al.*, 2003).

The first step of peptidoglycan synthesis takes place in the cytoplasm and leads to the synthesis of nucleotide sugar-linked precursors: UDP-*N*-acetylmuramyl-pentapetide (UDP-murNAc-pentapeptide) and UDP-*N*-acetylglucosamine (UDP-GlcNAc). In the second stage, which occurs at the cytoplasmic membrane, UDP-murNAc-pentapeptide is transferred to the membrane-bound acceptor (bactoprenol) yielding lipid I and is followed by addition of UDP-GlcNAc resulting in lipid II formation. Bactoprenol (undecaprenol phosphate) is a lipophilic molecule that enables the cell to translocate hydrophilic precursors from the aqueous cytoplasm across the hydrophobic membrane to the externally localized sites of polymerization of peptidoglycan (Scheffers & Pinho, 2005; Bouhss *et al.*, 2008).

The final step of biosynthesis takes place outside the cytoplasmic membrane and involves incorporation of the recently synthesized disaccharide-peptide units into the peptidoglycan. The last step of peptidoglycan synthesis is carried out by penicillin-binding proteins (PBPs) which catalyze the transglycosylation and transpeptidation reactions, i.e., formation of the glycosidic and peptide bonds, respectively.

The transglycosylation is catalyzed by multimodular penicillin-binding proteins, particularly PBP2 and by the monofunctional glycosyltransferase, Mtg, and results in incorporation of the lipid-linked precursors into the glycan polymer (Wang *et al.*, 2001; Barrett *et al.*, 2005). In the transpeptidation (cross-linking) reaction, which is also catalyzed by PBPs, the terminal L-glycine of the pentaglycine interpeptide bound to L-lysine of one stem peptide is attached to the D-alanine of another stem peptide. The terminal D-alanine of this stem peptide is cleaved off during this reaction. Thus, a flexible pentaglycine cross-bridge is formed between two peptidoglycan moieties, resulting in a strong and flexible cell wall structure (Gally & Archibald, 1993). *S. aureus* typically possesses four PBPs which are able to catalyze the transpeptidation reaction. PBPs 1, 2 and 3 are high molecular mass proteins (87, 80 and 75 kDa, respectively) and PBP4 is a low molecular mass protein (41 kDa). Figure 4 depicts the cell wall structure as well as the transglycosylation and transpeptidation reactions (Scheffers & Pinho, 2005).

Another component of the *S. aureus* cell wall are teichoic acids, which are polymers of ribitol residues or polymers of glycerol phosphate. Teichoic acids contribute to the negative charge present on the cell surface that plays a role in acquisition of ions, also and have been reported to be a component of *S. aureus* phage receptor (Chatterjee, 1969).

The last component of the *S. aureus* cell wall are surface proteins, including microbial surface components recognizing adhesive molecules (MSCRAMMs). These proteins contain a signal sequence directing their secretion and the LPXTG motif. The LPXTG motif is cleaved by sortase and than the proteins are covalently attached to the peptidoglycan. Protein A, fibronectin binding protein, collagen binding protein and clumping factor A, among many others, are components of the cell wall and are attached in this manner (Foster & Hook, 1998).

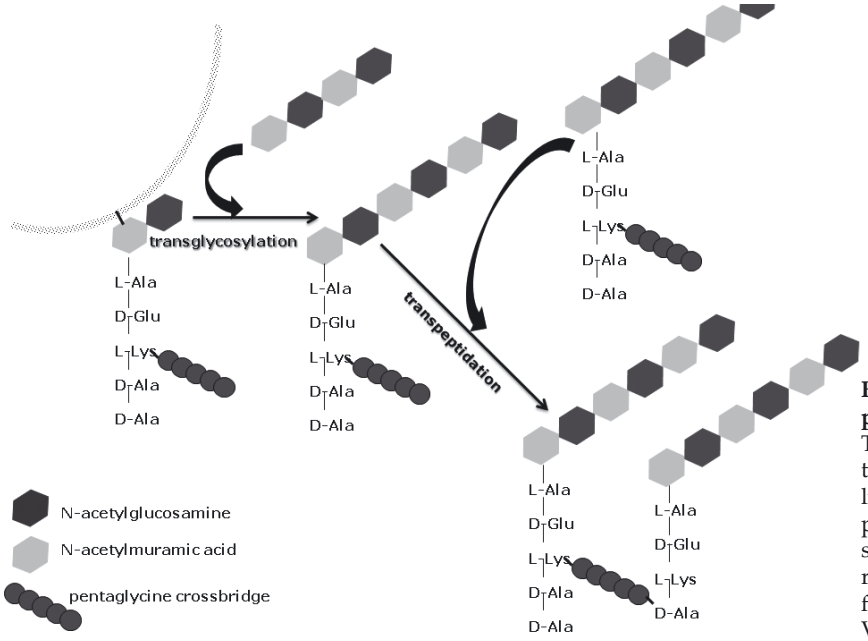

Figure 4. Scheme of staphylococcal peptidoglycan biosynthesis. Transglycosylation and transpeptidation reactions catalyzed by penicillin binding proteins are shown. Stem peptides and cross-bridges are not shown on all *N*-acetylmuramic acid residues of the glycan chains. Modified after (Scheffers & Pinho, 2005; Vollmer *et al.*, 2008).

Mechanism of β-lactams antibiotic action

β-Lactam antibiotics include penicillins, cephalosporins, and penicillinase-insensitive β-Lactams such as methicillin and oxacillin. β-Lactams are bactericidal, cell wall-active agents that target the transpeptidation step of the peptidoglycan synthesis. This is achieved by binding and inactivating the transpeptidase domain of PBPs in the cell wall (Chambers, 2004). β-Lactams are structural analogs of the natural substrate of PBPs, D-alanyl-D-alanine of the peptidoglycan stem peptide (Fig. 5).

The reaction between PBP and a β-lactam antibiotic begins with a non-covalent association between these two molecules. This intermediate can either dissociate or undergo an irreversible reaction of acylation, when the PBP covalently binds the antibiotic at its active site, cleaving the cyclic amide bond in the β-lactam ring. The natural substrate for PBP, D-alanyl-D-alanine, undergoes quick deacylation by hydrolysis that liberates the PBP for a next round of transpeptidation. However, when the substrate is a β-lactam antibiotic, the deacetylation process is very slow and the PBP is effectively inactivated. Without functional PBPs, the cell wall synthesis is inhibited and cell death occurs (Chambers, 2003). The features of β-lactams which make them attractive antimicrobial agents stem from the fact that their targets are easily accessible (localized outside of the cytoplasm membrane) and that they are specific to bacteria (lacking functional and structural equivalents in the human organism) (Wilke *et al.*, 2005).

Mechanism of resistance: β-lactamases

β-Lactamases are proteins with enzymatic activities that contribute to β-lactam resistance by inactivation of many of these antibiotics in a reaction similar to the one β-lactams use to inhibit PBPs. β-Lactamases bind β-lactams, which results in formation of an acylated intermediate. Unlike the reaction catalyzed by PBPs during peptidoglycan biosynthesis, resolution of the acylated intermediate results in the cleavage of the amide bond of the β-lactam ring. The inactivated β-lactam antibiotic and active β-lactamase are released (Frere, 1995). Based on sequence similarity four different types of β-lactamases have been described so far in *S. aureus* which differ in their substrate specificity (Zygmunt *et al.*, 1992).

The gene coding for β-lactamase (*blaZ*) is usually carried on a plasmid or located on a transposon. Expression of β-lactamase is induced by the presence of β-lactam antibiotics through a regulatory system composed of a repressor, BlaI, and a signal transducer, BlaRI. Genes *blaI* and *blaRI* are located a in two-gene operon that is divergently transcribed from *blaZ*. BlaRI is a membrane protein composed

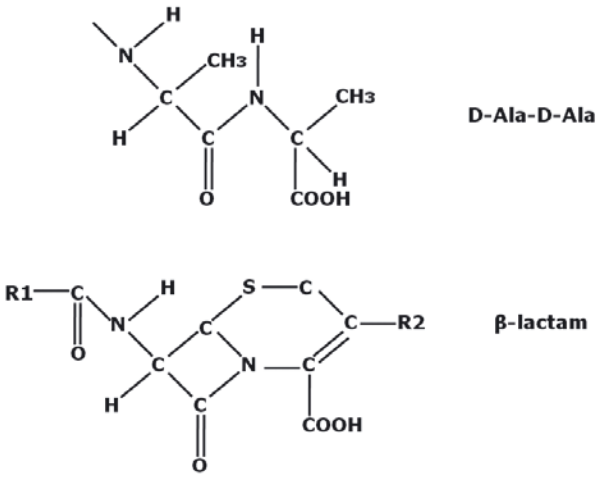

Figure 5. Structures of D-alanyl-D-alanine and a β-lactam antibiotic.
β-Lactams and D-Ala-D-Ala have similar structures that allow them to be bound by PBPs. R1 and R2 indicate groups that differ among various β-lactam antibiotics.

of an extracellular sensor domain that is acylated by β-lactam antibiotics, a membrane-spanning domain that transduces the signal across the membrane, and an intracellular zinc metalloprotease domain which is proteolytically activated upon acylation of the sensor domain. BlaI repressor forms a homodimer which binds to the operator region of the operon encoding BlaI and BlaRI and containing the β-lactamase structural gene (*blaZ*). Upon binding of a β-lactam antibiotic to the sensor domain of BlaRI, its metalloprotease domain undergoes autocleavage and subsequent cleavage of BlaI, which prevents its dimerization, operator binding and leads to derepression of *blaZ* transcription (Zhang *et al.*, 2001).

Mechanism of resistance: methicillin resistance

Methicillin, oxacillin and nafcillin are semisynthetic β-lactamase-insensitive β-lactams. *S. aureus* has developed resistance to this class of β-lactams by acquiring the *mecA* gene carried on the SCC*mec* element described above. Strains containing *mecA* are known as methicillin resistant *S. aureus* (MRSA), although they are, in fact, resistant to all β-lactam antibiotics (Berger-Bachi, 1994; Chambers, 1997; 2003). *mecA* encodes an alternative penicillin binding protein PBP2a, that has low affinity for β-lactams. PBP2a belongs to the group of high molecular mass (78 kDa) family of PBPs and consists of a transpeptidase domain and a non-penicillin binding domain of unknown function (Goffin & Ghuysen, 1998). PBP2a is known to posses low affinity for β-lactams that allows MRSA strains to grow in antibiotic concentrations that inactivate all native PBPs (Gaisford & Reynolds, 1989). PBP2a appears to be a rather poorly active enzyme, comparing to other native PBPs

that synthesize highly cross-linked peptidoglycan (de Jonge & Tomasz, 1993). Even when the transpeptidase activity of all native PBPs is inhibited by the presence of methicillin, PBP2a has been shown to rely on the transglycosylase, β-lactam-insensitive, domain of the native PBP2 to confer resistance (Pinho *et al.*, 2001).

The serine in the active site of the transpeptidase domain in PBP2a is responsible for nucleophilic attack on both the β-lactam ring and the D-alanyl-D-alanine substrate and is located in an extended narrow groove. The groove mediates noncovalent interactions with the β-lactam that place the β-lactam in an unfavorable position for interaction with the serine in the active site. As a result, the acylation between the β-lactam and the active site does not occur. PBP2a successfully balances the crucial transpeptidase activity with a decreased affinity toward β-lactam antibiotics. Therefore PBP2a is able to synthesize the cell wall at otherwise lethal concentrations of β-lactams (Pinho *et al.*, 2001; Lim & Strynadka, 2002; Chambers, 2003).

Regulation of *mecA* expression

The expression of the *mecA* gene is regulated in analogous manner to that of the β-lactamase gene, *blaZ*. Similarly to *blaZ*, *mecA* is divergently transcribed from its two regulatory genes organized in an operon, *mecI* and *mecR1*. Homodimeric methicillin repressor, MecI, constitutively represses expression of *mecA* as well as transcription of the *mecI-mecRI* operon by binding to two palindromes contained within the promoter-operator region (Safo *et al.*, 2006). Derepression occurs through cleavage of MecI upon activation of the metalloprotease domain of the mecRI sensor-transducer by a β-lactam antibiotic.

MecRI is a membrane protein that has an extracellular penicillin-binding domain that, when bound by β-lactams, undergoes a conformational change inducing autocleavage of the intracellular protease domain. The active MecRI cleaves MecI which leads to derepression of *mecA* as well as the *mecI-mecRI* operon (Mallorqui-Fernandez *et al.*, 2004). Additionally to regulation of *mecA* expression by its cognate MecI and MecRI regulators, it can also be regulated by structurally and functionally similar β-lactamase regulators, BlaI and BlaRI. Because of the structural and functional similarity, MecI as well as BlaI are able to bind as homodimers to the promoter-operator region of *mecA* (Gregory *et al.*, 1997). The MecI or BlaI-mediated repression is only relieved by induction through homologous, and not heterologous sensor-transducers, demonstrating the repressor specificity of induction. Induction of *mecA* expression by the MecRI-MecI system is slower than

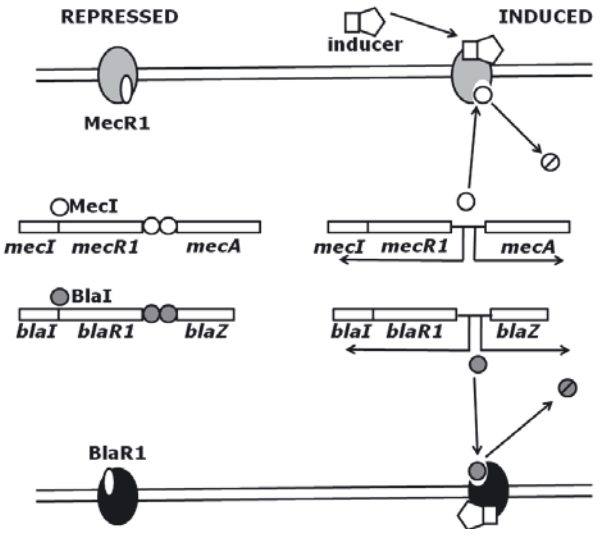

Figure 6. Two pathways regulating PBP2a and β-lactamase production.
MecI represses transcription of *mecA* and *mecRI-mecI* operon. Upon binding of β-lactam to sensor domain of MecRI, intracellular peptidase domain cleaves MecI repressor, which triggers *mecA* and *mecI* transcription. Analogous system controls *blaZ* and *blaRI-blaI* expression. Despite BlaI and MecI repressors are being interchangeable and both recognizing *mec* and *bla* regulatory sequences, cleavage of the expression regulators by BlaRI and MecRI is specific. Adapted form Berger-Bachi and Rohrer (2002).

the induction by BlaRI-BlaI and never leads to maximal *mecA* transcription (Potempa *et al.*, 1991). Figure 6 shows the regulation of *mecA* and *blaZ* expression.

Repression of *mecA* by MecI and BlaI is even stronger if these two repressors are present together (Rosato *et al.*, 2003a). The majority of clinical MRSA strains contain mutations in *mecI*, however, in the vast majority of these cases, *mecA* expression is commonly regulated by BlaI. This highlights the need of controlled transcription of *mecA* by at least one of the two regulators. Given the more rapid rate of induction through BlaRI-BlaI, it has been proposed that it might be the preferred system of *mecA* regulation (Rosato *et al.*, 2003b). In so called pre-MRSA strains, carrying both functional regulatory systems, *mecA* expression is controlled by both MecI and BlaI. This strict repression can prevent *mecA* transcription, and thus cause inhibition of β-lactam resistance, leading to the misinterpretation of these isolates as methicillin sensitive *S. aureus* (MSSA) (Niemeyer *et al.*, 1996; Weller, 1999). It has also been shown that certain genetic backgrounds of MSSA strains carrying plasmid-borne, unregulated *mecA* are restrictive and select against *mecA* expression because *mecA* is often mutated or deleted to avoid production of PBP2a in such strains. However, when the *mec* or *bla* regulatory genes are introduced together with *mecA*,

the system is tolerated. These findings emphasize not only the importance of tight regulation of *mecA* expression but also the influence of particular genetic backgrounds on *mecA* stability and the resistance phenotype (Katayama *et al.*, 2005).

At present, MRSA infections have a higher frequency than methicillin-susceptible *S. aureus* (MSSA) infections in some settings. MRSA was accountable for 59% of skin and soft tissue infections diagnosed in eleven emergency departments in the United States (Okuma *et al.*, 2002). Moreover, MRSA accounted for 59.5% of all *S. aureus* infections in intensive care units patients in 2004 (Rice, 2006). Also, the occurrence of MRSA infections outside healthcare facilities, in the community, more than doubled between 2002 and 2004. The increasing rate of MRSA infections has shifted chemotherapy away from β-lactam antibiotics toward antibiotics more effective against MRSA, such as vancomycin and daptomycin (Berger-Bachi *et al.*, 1992).

Heterogeneous and homogeneous methicillin resistance

The level of methicillin resistance of *S. aureus* varies extremely from one strain to another, spanning the range from several micrograms per milliliter (a value very close to the resistance level of MSSA) to several milligrams pre milliliter. Certain MRSA strains are composed of cells expressing varied levels of resistance to β-lactams. They are apparently made up of several bacterial sub-populations that significantly differ in their degree of antibiotic resistance. This peculiar non-consistency in the phenotypic expression of antibiotic resistance is called heteroresistance (Tomasz *et al.*, 1991).

Heterogeneous expression of methicillin resistance is characterized by a majority of cells expressing low level resistance from which, upon challenge with methicillin, a small proportion of highly, uniformly resistant clones segregate. The frequency of segregation of the highly resistant sub-clones designated homoresistant is a reproducible and strain-dependent property. The homoresistant phenotype is stable and the highly resistant clones generally maintain their resistance level even in the absence of selective pressure (Berger-Bachi & Rohrer, 2002). The ability of MRSA strains to produce PBP2a is essential for their methicillin resistance but there is no correlation between homo- and heteroresistance and the cellular concentration of PBP2a. PBP2a production does not seem to explain the observed variety of resistance levels. Therefore it is thought that additional chromosomal genes are involved in optimal methicillin resistance (Hackbarth *et al.*, 1994; Murakami & Tomasz, 1989). It has been proposed that selection of a homoresistant derivative from a heteroresistant population is due to unspecified mutations or genetic rearrangements occurring outside the SCC*mec* element. However, the size of the homoresistant subpopulation selected upon challenge with oxacillin is well above the frequency of spontaneous mutations (Finan *et al.*, 2002).

The same mechanism of selection of a highly homoresistant subpopulation from a heteroresistant population is thought to operate in clinical environments and it could be blamed for the failure of β-lactam treatment against MRSA (Berger-Bachi, 1999). Moreover, very low-level resistant MRSA strains are dangerous since they can evade standard phenotypic detection while they appear phenotypically susceptible. These strains still carry the *mecA* determinant and express resistance heterogeneously and upon β-lactam exposure they are able to segregate highly resistant subpopulations at a frequency well above spontaneous mutation rate (Ender *et al.*, 2008).

CONCLUDING REMARKS

Although it is estimated that approximately 20–30% of the general human population are "healthy" *S. aureus* carriers, this bacterium can also be a serious infectious agent responsible for relatively frequent and severe diseases. Particularly MRSA strains are dangerous due to their resistance to most antibiotics currently used in clinical practice. A better understanding of the biochemical and genetic mechanisms of *S. aureus* pathogenicity will lead to improved prevention and treatment strategies. Nevertheless, although various approaches are being considered, including special procedures of antibiotic treatment, photodynamic therapy or bacteriophage therapy (see, for example: Dzwonkowska *et al.*, 2007; Grinholc *et al.*, 2008; Jurczak *et al.*, 2008; Mann, 2008; Grinholc *et al.* 2008), it appears to be a formidable task.

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