



Staphylococcus aureus toxins – Their functions and genetics



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ABSTRACT

The outcome of encounters between *Staphylococcus* (*S.*) *aureus* and its human host ranges from life-threatening infection through allergic reactions to symptom-free colonization. The pan-genome of this bacterial species encodes numerous toxins, known or strongly suspected to cause specific diseases or symptoms. Three toxin families are in the focus of this review, namely (i) pore-forming toxins, (ii) exfoliative toxins and (iii) superantigens. The majority of toxin-encoding genes are located on mobile genetic elements (MGEs), resulting in a pronounced heterogeneity in the endowment with toxin genes of individual *S. aureus* strains. Recent population genomic analysis have provided a framework for an improved understanding of the temporal and spatial scales of the motility of MGEs and their associated toxin genes. The distribution of toxin genes among clonal lineages within the species *S. aureus* is not random, and phylogenetic (sub-)lineages within clonal complexes feature characteristic toxin signatures. When studying pathogenesis, this lineage association, which is caused by the clonal nature of *S. aureus* makes it difficult to discriminate effects of specific toxins from contributions of the genetic background and/or other associated genetic factors.

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1. Introduction

Staphylococcus (*S.*) *aureus* is notorious as the most common causative agent of hospital-acquired infections, and the spread of antibiotic resistant strains, particularly methicillin-resistant *S. aureus* (MRSA), in hospitals challenges health care systems worldwide. Moreover, *S. aureus* strains of increased virulence, known as community-acquired MRSA (CA-MRSA), can threaten even healthy individuals in the community (Chambers and DeLeo, 2009; David and Daum, 2010; DeLeo et al., 2010). In addition, *S. aureus* is currently being discussed as the trigger and/or enhancer of allergies of the respiratory system and the skin (Bachert and Zhang, 2012; Gould et al., 2007). Nevertheless up to now, no anti-*S. aureus* vaccine has been approved for medical practice (Schaffer and Lee, 2008; Spellberg and Daum, 2012). In spite of the above, the most frequent encounter of *S. aureus* with its human host is peaceful colonization, and around 20% of adults are persistent carriers of the microorganisms, while another 60% are intermittently colonized (van Belkum et al., 2009; Wertheim et al., 2005). What makes the species *S. aureus* so immensely successful?

1.1. Multiple virulence factors encoded in the pan-genome of *S. aureus*

A salient feature of *S. aureus* is its variability. By indexing nucleotide sequence diversity at seven universally present genetic loci,

multilocus-sequence typing (MLST) to date has revealed about 2,400 ‘sequence types’ (ST) for *S. aureus* (see <www.mlst.net>). The vast majority of these diverse STs, however, are clustered in a remarkably limited number of clonal complexes (CC) or lineages, each of which appears to be distributed worldwide (reviewed in (Nübel et al., 2011)). The predominant *S. aureus* lineages are CC1, 5, 8, 15, 22, 30, 45, 59, 80, 97 and 121 (Nübel et al., 2011).

About 75% of the *S. aureus* genes are shared by more than 95% of strains and hence may be considered the ‘core genome’ of the species. In addition, two kinds of variably present genes can be distinguished: (i) the core variable genes (~10% of genes), which are largely conserved within each of the *S. aureus* clonal complexes and constitute their respective “make up”, and (ii) mobile genetic elements (MGEs, ~15% of genes). The core variable genome includes most surface-associated genes (microbial surface components recognizing adhesive matrix molecules, MSCRAMMs) and regulator genes. Core variable genes are encoded on the bacterial chromosome and are, therefore, typically stable and transferred vertically (Lindsay et al., 2006). MGEs include bacteriophages, plasmids, *S. aureus* pathogenicity islands (SaPI), transposons, and staphylococcal chromosomal cassettes (SCC) (Feil et al., 2003; Lindsay, 2010; Lindsay and Holden, 2006; Lindsay et al., 2006). They mainly encode resistance (e.g. methicillin resistance genes) and virulence genes (e.g., Panton-Valentine leukocidin (PVL) genes, superantigen (Sag) genes). MGEs can be distributed either by vertical transmission to daughter cells or by horizontal transfer (Lindsay and Holden, 2006).

The full complement of all genes (also known as the pan-genome) of *S. aureus* encodes a wide array of secreted or cell-sur-

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Table 1
Toxin genes in the *S. aureus* pan-genome.

	Toxin genes	References
Core genome		
	<i>hla</i>	Bhakdi and Tranum-Jensen (1991)
	<i>hld</i>	Janzon et al. (1989)
	<i>hlg</i> gene cluster	Kaneko and Kamio (2004)
	<i>psm</i> α , <i>psm</i> β	Wang et al. (2007)
	<i>selx</i>	Wilson et al. (2011)
Genomic islands		
vSa β	enterotoxin gene cluster (<i>egc</i>): <i>seg</i> , <i>sei</i> , <i>selm</i> , <i>seln</i> , <i>selo</i> , (<i>selu</i> , <i>selu2</i> , <i>selv</i>)	Baba et al. (2002), Letertre et al. (2003), Thomas et al. (2006)
	<i>lukDE</i>	Baba et al. (2002), Barrio et al. (2006), Lindsay and Holden (2006)
vSa γ	<i>etd</i>	Highlander et al. (2007), Yamaguchi et al. (2002)
Plasmids		
pIB485	<i>sed</i> , <i>sej</i> , <i>ser</i>	Bayles and landolo (1989), Omoe et al. (2003), Zhang et al. (1998)
pF5	<i>sej</i> , <i>ser</i> , <i>ses</i> , <i>set</i>	Omoe et al. (2003), Ono et al. (2008)
pGSA ₁₈ rep32 (pETB)	<i>etb</i>	McCarthy and Lindsay (2012), Yamaguchi et al. (2001)
pGSA ₁₁ rep22 (SAP057A)	<i>etb</i>	McCarthy and Lindsay (2012)
Staphylococcal cassette chromosomes		
SSCmec types II, III, VIII	<i>psm-mec</i>	Chatterjee et al. (2011), Queck et al. (2009)
Pathogenicity islands		
SaPI _{N1} (N315)/SaPI _{m1} (Mu50) (vSa4 type I)	<i>sell</i> , <i>sec</i> , <i>tst</i>	Novick and Subedi (2007)
SaPI ₃ (COL vSa1)	<i>seb</i> , <i>selk</i> , <i>selq</i>	Novick and Subedi (2007)
SaPI _{mw2}	<i>sell</i> , <i>sec</i>	Baba et al. (2002), Lindsay and Holden (2006)
Bacteriophages		
ϕ Sa1	<i>lukFM</i> (ϕ PV83)	Choorit et al. (1995), Kaneko and Kamio (2004), McCarthy et al. (2012), Zou et al. (2000)
	<i>eta</i> (ϕ ETA)	Kuroda et al. (2001), McCarthy et al. (2012), Yamaguchi et al. (2000)
ϕ Sa2	<i>lukFS-PV</i>	Baba et al. (2002), Kaneko et al. (1998), Narita et al. (2001)
ϕ Sa3	<i>sea</i> ; <i>seip</i> ; <i>sea</i> , <i>selq</i> , <i>selk</i>	Baba et al. (2002), McCarthy et al. (2012)

face-associated virulence factors (Foster, 2005). These include proteins that

- (1) mediate adherence to damaged tissue, extra-cellular matrix and the surface of host cells (Foster and Hook, 1998),
- (2) facilitate tissue destruction and spreading,
- (3) promote iron uptake (Skaar and Schneewind, 2004),
- (4) bind to proteins in the bodily fluids to help evade antibody- and complement-mediated immune responses, including the action of phagocytes,
- (5) lyse host cells and
- (6) manipulate the innate and adaptive immune responses.

However, a clear association between virulence genes and disease symptoms has been established or is strongly suspected only for some potent *S. aureus* toxins causing, for example, toxic shock syndrome (TSS), staphylococcal scalded skin syndrome (SSSS), necrotizing pneumonia, or deep-seated skin infections (Dinges et al., 2000; Holtfreter and Bröcker, 2005; Jarraud et al., 1999, 2002; Ladhani, 2003). This review focuses on such toxins, including pore-forming toxins, like PVL and hemolysin- α (Hla, α -toxin), exfoliative toxins (ET) and the SAGs. They damage the membranes of host cells, degrade inter-cellular junctions, or modulate the immune response by aberrant activation of immune cells. Only a few *S. aureus* toxins, such as Hla and the phenol-soluble modulins (PSMs), are core genome-encoded, while most of the other toxin genes are localized on MGEs (Table 1). Hence, the species *S. aureus* is characterized by extraordinary heterogeneity regarding the toxin gene equipment of individual clinical isolates.

2. Pore-forming toxins

S. aureus can produce several toxins that damage the membranes of host cells, which can ultimately lead to cell lysis. At sublytic concentrations, these pore-forming toxins are potent cell

stressors. In synergy with other danger signals such as lipoproteins that activate the toll-like receptor 2 the toxins trigger the NALP3-inflammasome response resulting in release of cytokines IL1, IL18 and IL33 (Franchi et al., 2012). Hla, hemolysin- γ (Hlg) and PVL have been shown to exert strong pro-inflammatory effects in this manner (Holzinger et al., 2012; Kebaier et al., 2012; Munoz-Planillo et al., 2009; Perret et al., 2012).

2.1. Hemolysin- α (Hla, α -toxin)

Hla is released by 95% of *S. aureus* strains as a water-soluble monomer of 33 kDa with pore-forming and pro-inflammatory properties. The *hla* gene is not mobile. Its expression is regulated by at least three global regulatory systems including the accessory gene regulator (*agr*) (Xiong et al., 2006), it is therefore not surprising that Hla is produced in varying amounts by *S. aureus* strains. Upon binding to a membrane receptor, Hla forms heptameric pores, thereby destroying a variety of host cells, including epithelial cells, erythrocytes, fibroblasts, monocytes, macrophages, and lymphocytes, but not neutrophils. The Hla receptor has long remained elusive and only recently ADAM10 (A disintegrin and metalloproteinase 10) has been identified as a binding partner of Hla (Inoshima et al., 2011; Wilke and Bubeck Wardenburg, 2010). Binding of Hla and pore formation activates the enzyme, which degrades E-cadherin in the epithelial adherens junctions (Inoshima et al., 2011). Moreover, the ADAM10-Hla complex interferes with focal adhesion complexes (Wilke and Bubeck Wardenburg, 2010). Both mechanisms would be able to disrupt the integrity of the epithelial and endothelial layers, thereby paving the way for *S. aureus* invasion. The group of Julie Bubeck Wardenburg has used murine infection models to demonstrate that Hla strongly contributes to the pathogenesis of skin infections and pneumonia induced by *S. aureus*-USA300, which produces the toxin in abundance (Bubeck Wardenburg et al., 2007a,b; Bubeck Wardenburg and

Schneewind, 2008; Inoshima et al., 2011, 2012; Powers et al., 2012; Wardenburg, 2011).

2.2. Leukotoxins

The bi-component (hetero-oligomeric) pore-forming leukotoxins can lyse cells of the myeloid lineage, namely monocytes, macrophages, and neutrophils, which is considered important for *S. aureus* immune evasion (Dumont et al., 2011; Ventura et al., 2010). In different animal infection models, they contribute to disease progression (not yet demonstrated for LukM/LukF'-PV) (Choorit et al., 1995; Kaneko et al., 1997; Morfeldt et al., 1995; von Eiff et al., 2004).

Leukotoxins consist of one class S and one class F protein of 32–35 kDa, whose genes are either core genome- or phage-encoded. The class S and F proteins are non-toxic on their own, but upon oligomerization, they form a β -barrelled pore-structure (Kaneko and Kamio, 2004). Five class F subunits (HlgB, LukF'-PV, LukD, LukF'-PV, and LukG) and six class S subunits (HlgA, HlgC, LukS-PV, LukE, LukM, and LukH) have been described (Dumont et al., 2011; Kaneko and Kamio, 2004; Morinaga et al., 2003; Prevost et al., 2001; Ventura et al., 2010). Table 2 shows that most S and F subunits exhibit monogamous pairing, whereas HlgB, the F subunit of the hemolysin- γ (*hlg*) gene cluster, can pair with either of two S subunits, HlgA or HlgC Table 2.

2.2.1. Panton-Valentine leukocidin (PVL; lukF-PV + lukS-PV)

Skin and soft tissue infections (SSTIs), such as furunculosis or abscesses, are the most frequent diseases caused by *S. aureus* outside the hospital setting. Since the discovery of a close epidemiological linkage between PVL and chronic or recurrent SSTIs as well as necrotizing pneumonia (Gillet et al., 2002), its role in these diseases has remained under discussion. The genes of the F and S subunits are phage-encoded (Table 2) and can thus be acquired by horizontal gene transfer (Kaneko et al., 1998; Masiuk et al., 2010). PVL genes are found in most CA-MRSA strains, which exhibit increased virulence, e.g. in USA300 (Badiou et al., 2008; Carpaiz et al., 2011; del Giudice et al., 2009; Diep et al., 2006; Diep and Otto, 2008; Gillet et al., 2002; Vandenesch et al., 2003). CA-MRSA cause typical diseases, namely skin and soft tissue infections (SSTIs), as well as necrotizing pneumonia. However, PVL is not restricted to CA-MRSA, but the toxin is also found in the majority of MSSA strains isolated from patients with community-acquired SSTIs (Masiuk et al., 2010; Monecke et al., 2007b). In contrast, the genes are rare in other *S. aureus* strain collections such as nasal commensals or clinical isolates from blood-stream infection (0–5%) (Holtfreter et al., 2007; Masiuk et al., 2010; von Eiff et al., 2004). Thus, epidemiological evidence for a pathogenic role of PVL is very

strong, but the correlation is not absolute, and PVL-negative CA-MRSA strains as well as MSSA strains associated with SSTI-patients have been isolated (Diep and Otto, 2008; Masiuk et al., 2010; Monecke et al., 2011; Otto, 2010). Animal infection models have yielded conflicting results, which has been attributed to differences between models, inoculum sizes, and, very importantly, the host species (Brown et al., 2009; Bubeck Wardenburg et al., 2008; Labandeira-Rey et al., 2007; Montgomery and Daum, 2009; Olsen et al., 2010; Tseng et al., 2009; Villaruz et al., 2009). In fact, the leukotoxic activity of PVL differs dramatically between species: human and rabbit neutrophils are lysed by very low toxin concentrations (>40 ng/ml), whereas 1000-fold higher amounts are required for the lysis of mouse or java monkey neutrophils (Löffler et al., 2010).

2.2.2. Other Leukotoxins (Hemolysin- γ , Hemolysin- γ 2, LukDE, LukF'M, LukGH)

The *hlg* gene cluster giving rise to hemolysin- γ (Hlg) and hemolysin- γ 2 (Hlg2) is encoded in the core genome; it is present in 99% of *S. aureus* strains. The toxins appear to contribute to septic arthritis and weight loss in mice (Nilsson et al., 1999) and to endophthalmitis in rabbits (Supersac et al., 1998). Recently, attention has been focussed on LukDE and LukGH, which, similar to PVL, are expressed by the majority of CA-MRSA strains. LukDE binds to CCR5 on immune cells, the chemokine receptor which is also involved in immune cell targeting by the human immunodeficiency virus (Alonzo et al., 2013). Both leukotoxins contribute to the virulence of *S. aureus* in murine sepsis and renal abscess models (Alonzo et al., 2012; Dumont et al., 2011). LukGH exhibits potent cytolytic activity towards neutrophils acting in synergy with PVL *in vitro* (Rigby and DeLeo, 2012; Ventura et al., 2010). The relative contribution of PVL, LukDE and LukGH to community-acquired SSTI and necrotizing pneumonia remains to be determined. Finally, a PVL-variant, the prophage-encoded LukF'M, has been identified in *S. aureus* strain P83 of bovine origin (Zou et al., 2000). This toxin lyses bovine polymorphonuclear leukocytes (PMNs) and has been implicated in bovine mastitis (Schlotter et al., 2012).

2.3. Phenol-soluble modulins (PSMs)

PSMs are a family of small, α -helical amphipathic peptides with cytolytic and pro-inflammatory properties. Two subfamilies are described: the PSM α peptides, including the long-known hemolysin- δ (Hld) (Yoshida, 1963), are 20–26 amino acids long, and the PSM β peptides are 43–44 amino acids in length (Wang et al., 2007). Most PSMs are core genome-encoded. All *S. aureus* strains contain the *hld* gene and the two loci with the operons coding for the *psm* α and *psm* β genes (Wang et al., 2007). In addition, a *psm-mec* gene is located on a chromosomal cassette, which is re-

Table 2
Leukotoxins.

Leukotoxins	Components ^a		Localisation	Prevalence ^b	Cell specificity
	Class F subunit	Class S subunit			
Hemolysin- γ (Hlg)	HlgB (Hlg1, LukF)	HlgA (Hlg2)	<i>hlg</i> gene cluster; genome	~99%	erythrocytes from humans and other mammalian species
Hemolysing- γ 2 (Hlg2)/Leukocidin (Luk)	HlgB (Hlg1, LukF)	HlgC (LukS)			human and rabbit PMN and rabbit erythrocytes
Panton-Valentine Leukocidin (PVL)	LukF-PV	LukS-PV	<i>pvl</i> locus; phage	0–5%	human and rabbit PMN
LukDE	LukD	LukE	pathogenicity island	30–87%	murine and rabbit PMN
LukF'M	LukF'-PV	LukM	phage	0%	bovine PMN
LukGH (LukAB)	LukG (LukB)	LukH (LukA)	n.d.	n.d.	human PMN

References: Alonzo et al. (2012), Choorit et al. (1995), Dumont et al. (2011), Gravet et al. (2001), Kaneko and Kamio (2004), Rigby and DeLeo (2012), Vandenesch et al. (2012), Ventura et al. (2010), von Eiff et al. (2004).

^a Synonyms are indicated in brackets.

^b Prevalence in clinical *S. aureus* isolates from humans.

stricted to hospital-acquired MRSA (HA-MRSA) strains carrying SCCmec types II, III, and VIII (Chatterjee et al., 2011). The *agr* is involved in the expression of PSMs (Queck et al., 2008; Wang et al., 2007). The production of PSMs is closely correlated with the capacity of staphylococcal species to cause invasive infections (Rautenberg et al., 2011). CA-MRSA isolates show an increased production of PSMs in comparison to HA-MRSA (Wang et al., 2007). The group of Michael Otto has described multiple functions of PSMs in pathogenesis. Firstly, PSM α peptides effectively lyse white and red blood cells (Cheung et al., 2010; Wang et al., 2007). PSM α -deletion in CA-MRSA strains resulted in lower mortality and decreased ability to cause skin lesions in mice (Wang et al., 2007). Secondly, PSMs have proinflammatory properties, and they induce chemotaxis and activation of human neutrophils, as well as cytokine expression (Cheung et al., 2010; Queck et al., 2009; Wang et al., 2007). This response is mediated by activation of the human formyl peptide receptor 2 (FPR2) (Kretschmer et al., 2010). Thirdly, proteolytically processed PSM α 1 and PSM α 2 peptides show antimicrobial activity and interfere with competing colonizing pathogens (Joo et al., 2011). Finally, they appear to contribute to the structuring of biofilms during *S. aureus* infection (Periasamy et al., 2012b). Recently, Periasamy et al. have proposed that these functions in virulence are indicative of a primary role for PSMs in the commensal colonization of mammalian epithelia (Periasamy et al., 2012a).

3. Exfoliative toxins (ETs)

The three known *S. aureus* exfoliative toxins ETA, ETB and ETD are encoded on different genetic elements (Table 1): *eta* is localized in the genome on a temperate phage, whereas *etb* is found on plasmids and *etd* on a genomic island. The prevalence of *eta* and/or *etb* ranges from 0.5 to 3% in MSSA (Becker et al., 2003; Nhan et al., 2011; Sila et al., 2009), whereas around 10% of MRSA are *eta* positive (Sila et al., 2009). Holtfreter et al. observed a strong association of *etd* with invasive CC25 isolates (*spa* type t078 and relatives) (Holtfreter et al., 2007). The expression of the ETs is *agr*-regulated (Sheehan et al., 1992). Functionally, ETs are isoforms of enzymes with high species-specificity. They have glutamate-specific serine protease activity and selectively cleave a single peptide bond in the extracellular region of human and mouse desmoglein 1 (Dsg1; desmosomal intercellular adhesion molecule), a keratinocyte cell–cell adhesion molecule. In this way, the ETs act as “molecular scissors” facilitating bacterial skin invasion (Nishifuji et al., 2008). By loosening the keratinocyte junctions they cause blistering diseases known as bullous impetigo and staphylococcal scalded skin syndrome.

4. Superantigens (SAGs)

The staphylococcal SAGs belong to the most potent T-cell mitogens known. Some of these toxins stimulate human T-cells at femtomolar concentrations. Originally, the SAGs of *S. aureus* were termed staphylococcal enterotoxins (SEs) because they elicit vomiting and diarrhea after oral uptake, the hallmarks of *S. aureus* food poisoning. This feature is different from their superantigenicity, however, because some of the recently identified SAGs apparently lack emetic properties. Therefore, the International Nomenclature Committee for Staphylococcal Superantigens introduced a new nomenclature in 2004 to distinguish SAGs with proven emetic activity in primates (staphylococcal enterotoxins; SEs) from those whose emetic properties remain unconfirmed (staphylococcal enterotoxin-like toxins; SELs) and toxic shock-syndrome toxin 1 (TSST-1) (Lina et al., 2004). So far, 23 different staphylococcal SAGs have been described: the staphylococcal enterotoxins A–E,

G–J, and R–T (SEA–SEE, SEG–SEJ, SER–SET), the staphylococcal enterotoxin-like toxins K–Q and U–X (SEK–SEIQ, SEIU–SEIX) and TSST-1 (Holtfreter and Bröker, 2005; Lina et al., 2004; Ono et al., 2008; Proft and Fraser, 2003; Thomas et al., 2007; Wilson et al., 2011).

Genetic analysis of *S. aureus* clinical isolates, including whole genome sequencing, has shown that ~80% of *S. aureus* clinical isolates harbor an average of five to six SAG genes. There is extensive heterogeneity of SAG gene patterns between *S. aureus* strains (Baba et al., 2002; Becker et al., 2003; Holtfreter et al., 2004, 2007; Jarraud et al., 2001). In addition, the *S. aureus* strains that harbor SAG genes produce varying levels of the toxins. This can be attributed to the involvement of at least four global regulators, *agr*, *sarA*, σ^B and *saeRS* (Andrey et al., 2010; Kusch et al., 2011; Tseng et al., 2004). Most SAGs are encoded on MGEs, such as bacteriophages, plasmids, SaPIs, and genomic islands (Fitzgerald et al., 2001; Johns and Khan, 1988; Lindsay and Holden, 2006; Ono et al., 2008), whereas the recently described SEIX is core genome-encoded (Wilson et al., 2011) (Table 1).

The mechanism of action of SAGs was discovered by Bernhard Fleischer and Hubert Schrezenmeier and first described in 1988 (Fleischer and Schrezenmeier, 1988). In contrast to conventional peptide antigens, SAGs activate a large fraction of T lymphocytes simultaneously. Conventional antigens are taken up by antigen-presenting cells (APCs) and processed by protease digestion. The resulting antigenic peptides are bound to major histocompatibility complex (MHC) molecules and displayed on the APC surface as MHC/peptide complexes. These are recognized by T-cells via the hypervariable loops of their T-cell receptor (TCR) α and β chains. SAGs can bypass this highly specific antigen-driven interaction between T-cells and APCs. They directly cross-link certain TCR V β domains with conserved structures on MHC class II (MHC II) molecules expressed on professional APCs. They interact with MHC II by binding to the α -chain (antigen peptide-dependent or independent) or to a conserved histidine in the β -domain via a zinc complex (peptide-dependent) (Fraser and Proft, 2008). Furthermore, each SAG interacts with a defined TCR repertoire determined by the TCR V β sequences. As the human genome encodes approximately 50 TCR V β elements, which are unevenly represented in the T-cell pool of an individual, up to 20% of T cells can be activated by a given SAG (Proft and Fraser, 2003). In contrast, conventional peptide antigens stimulate only 1 out of 10^5 – 10^6 naïve T-cells (Fraser et al., 2000). The V β -restricted T-cell expansion is thus the hallmark of SAGs (Choi et al., 1990; Kappler et al., 1989; White et al., 1989) with two exceptions: (i) the SAG SEH also contacts TCR V α chains (Petersson et al., 2003; Thomas et al., 2009) and (ii) the staphylococcal protein A, which is universally expressed by *S. aureus*, acts as a B cell SAG targeting B-cell receptors (membrane-anchored antibodies) which use the immunoglobulin-V H 3 gene element (Silverman and Goodyear, 2006). In addition, many T-cell SAGs also trigger cytokine release by the APCs, which are activated via MHC-II engagement (Proft and Fraser, 2003).

4.1. Role of SAGs in staphylococcal virulence

SAGs have been implicated in a broad range of diseases. SEs are the causative agents of staphylococcal food poisoning resulting from ingestion of contaminated food. Due to their extraordinary stability in denaturing conditions, such as heat and low pH, SEs are not completely destroyed by mild cooking or digestion of food in the stomach. Nausea, emesis, abdominal pain or cramping and diarrhea ensue after a short incubation time. The disease is usually self-limiting (Thomas et al., 2007).

Staphylococcal toxic shock syndrome (TSS) is characterized by high fever, rash, desquamation, vomiting, diarrhea and hypotension, frequently resulting in multiple organ failure. In TSS, *S. aureus*

is usually localized, either at mucosal sites (vagina or nasopharynx) or in abscesses (Fraser and Proft, 2008), but the released SAGs act systemically, triggering large numbers of T-cells to produce massive amounts of pro-inflammatory cytokines, such as IL-2, IFN- γ and TNF. This cytokine storm causes the symptoms (Bergdoll et al., 1981; McCormick et al., 2001). This is followed by a state of profound T-cell unresponsiveness or anergy, where the T-cells fail to proliferate or secrete IL-2 (Rellahan et al., 1990), or they even undergo cell death (Alderson et al., 1995). It has, therefore, been proposed that SAGs might confer an evolutionary advantage to *S. aureus* by deleting T-cells that help B-cells to mount an effective antibody response against the bacteria (Fraser et al., 2000). This view has been challenged by the observation that SAGs themselves are potent immunogens eliciting an effective and highly specific neutralizing antibody response (Grumann et al., 2011; Holtfreter et al., 2006).

The role of SAGs in other forms of sepsis is less well defined. In animal models, SAGs and LPS, a major component of the outer membrane of Gram-negative bacteria and highly potent stimulator of the innate immune system, most effectively synergize in the induction of lethal shock (Blank et al., 1997; Schlievert, 1982). This observation prompted the development of the two-hit model of septic shock (Bannan et al., 1999), which was later generalized by Holtfreter and Bröker: A first hit by SAGs or other potent T cell stimuli is potentiated by a second hit by pathogen-associated molecular patterns (PAMPs), which activate the innate immune system. This sequence of events culminates in a dramatic, often lethal activation of the whole immune system (Holtfreter and Bröker, 2005). The sequence of events varies in the different accounts.

Kawasaki disease is an acute febrile disease in children that resembles TSS. A role for SAGs has been suggested (Yarwood et al., 2000). Intravenous immunoglobulin therapy is highly effective when given early, suggesting that the agent is a toxin that is neutralized by anti-toxin antibodies contained in pooled human serum.

Finally, a prominent role for SAGs is being discussed in skin and airway allergies. For atopic dermatitis, a correlation between clinical severity and colonization with SEA- and SEB-producing *S. aureus* as well as with IgE specific for SEA and SEB was documented in one study, but not confirmed in others (Bunikowski et al., 1999; Zollner et al., 2000). Bronchial asthma afflicts around 300 million people worldwide, thus belonging to the most common diseases. In allergic asthma, the triggers are known inhalative allergens (=allergy-driving antigens), while the causative agents of non-allergic or intrinsic asthma, around 10% of cases, are not known. Intrinsic asthma is often of late onset (3rd–4th decade of life) and takes a severe disease course, which is refractory to established treatment strategies. Chronic rhinosinusitis, a pronounced inflammation of the mucosal tissue of the nose and sinuses, with or without the development of polyps, is also very frequent and often accompanied by intrinsic asthma. Since many patients possess high titres of SAG-specific IgE in their serum or locally in the polyps, several research groups promote the opinion that allergic reactions to *S. aureus* SAGs drive or at least amplify chronic airway inflammation (Bachert et al., 2010, 2008; Barnes, 2009; Gevaert et al., 2005; Zhang et al., 2011).

5. The evolution of the *S. aureus* toxin gene families

Toxin gene clustering and sequence homologies imply evolution from ancestral genes by gene duplication and variation. These features are prominent in the families of leukotoxins, ETs, and SAGs. The enterotoxin gene cluster (*egc*), discovered on a staphylococcal genomic island (*vSA β*) by the group of Jarraud, is given here as an example (Jarraud et al., 2001; Letertre et al., 2003). *Egc* SAGs are the most prevalent SAG genes in commensal and invasive *S.*

aureus isolates, with frequencies ranging between 46% and 66% in different strain collections (Becker et al., 2004; Fueyo et al., 2005; Holtfreter et al., 2007; Monecke et al., 2009). Most *egc*-positive *S. aureus* strains harbor five SAG genes (*seg*, *sei*, *selm*, *seln*, and *selo*) and the pseudogenes *ψ ent1* and *ψ ent2* (Jarraud et al., 2001). The *egc* is unusually variable. The *S. aureus* clonal cluster CC30, for example, harbors an additional SAG gene, designated *selu*, a fusion product of *ψ ent1* and *ψ ent2* (Holtfreter et al., 2007; Letertre et al., 2003; Thomas et al., 2006). Given that the members of the *egc* display considerable sequence differences and each of the *egc* SAGs shows closest similarity to SAGs encoded outside the *egc*, Jarraud et al. proposed that the *egc* functions as an “enterotoxin gene nursery” (Jarraud et al., 2001).

6. The phylogenetic distribution and motility of *S. aureus* toxin genes

MGEs and toxin genes they carry can be disseminated either by vertical transmission to daughter cells upon cell replication or by horizontal gene transfer. Transduction through bacteriophages commonly is considered the predominant mechanism for acquisition of genetic material in *S. aureus*, whereas DNA transformation (Morikawa et al., 2012) and conjugation are assumed to be less relevant (Lindsay and Holden, 2006).

Over the past decade, studies based on sequencing a limited number of representative *S. aureus* genomes (Lindsay and Holden, 2006), comparative genomic hybridization to microarrays (McCarthy and Lindsay, 2012; McCarthy et al., 2012; Monecke et al., 2008; Shore et al., 2012), or dedicated PCR assays (Holtfreter et al., 2007; Masiuk et al., 2010) have provided an overview of the distribution of MGEs and their associated toxin genes in the *S. aureus* population. An overwhelming feature of the resulting data is that there exists remarkable variation in the endowment with MGEs, both within and among clonal complexes (Holtfreter et al., 2007; Masiuk et al., 2010; Monecke et al., 2009). It further appeared that MGEs and hence the toxin genes they carried were not randomly distributed between lineages, suggesting restrictions to horizontal transfer (Holtfreter et al., 2007; Masiuk et al., 2010; Monecke et al., 2008, 2009; Shore et al., 2012).

Due to their location on different genetic elements (Table 1), individual *S. aureus* toxin genes differ greatly in their distribution and horizontal genetic mobility. Some by definition belong to the core genome because they are present in almost all strains: *hla*, the *hlg* gene cluster, and the *psm* loci. Most are much more variably distributed, but, importantly, all are more or less closely linked to the genetic background of *S. aureus*. One extreme example is the *egc*-encoding genomic island *vSA β* . Among the dominant clonal clusters, *egc* is found in all strains of CC5, CC22, and CC45 as well as in CC25. CC30 and CC121 isolates are characterized by a variant of *vSA β* harboring an *egc* with a functional *selu* gene. On the other hand, CC1, CC8, CC15 and CC395 appear to lack *egc* completely (Grumann et al., 2011; Holtfreter et al., 2007; Masiuk et al., 2010). Other SAGs with strong clonal complex linkage are the SaPI-encoded *tst* and *sec-sell* (CC45), or the plasmid-encoded *sed*, *sej* and *ser* (CC8). SAG genes with a broader distribution are the phage-encoded *sea*, and the SaPI-encoded *seb* (Diep et al., 2006; Fueyo et al., 2005; Holtfreter et al., 2007; Lindsay and Holden, 2006; Lindsay et al., 2006; Masiuk et al., 2010; Monecke et al., 2007a,b; Monecke et al., 2011; Peacock et al., 2002). The pore-forming toxin *lukFS-PV* genes are broadly distributed among *S. aureus* clonal lineages with the notable exception of CC45 (Goerke et al., 2009; Masiuk et al., 2010; Monecke et al., 2007b) and a subclade of CC121, which in turn is associated with *eta* and/or *etb* (Masiuk et al., 2010) (our unpublished data). In contrast, *etd* is rare and characterizes CC80 MRSA isolates as well as some CC25 strains (Holtfreter et al., 2007; Masiuk et al., 2010; Monecke et al., 2011). All the studies cited above were limited, however, by the

typing techniques applied (i.e., MLST or *spa* typing), and their associated discriminatory power. Because substantial variation of MGE endowment was observed within the phylogenetic groupings resolved (clonal complexes, mostly), the dynamics of MGE motility could not be investigated. In contrast, more recent work has provided novel, quantitative insights into the frequency of MGE acquisition and loss in *S. aureus*, based on extended MLST (analysing variation at >100 genetic loci; (Lowder et al., 2009; Nübel et al., 2008, 2010)) or whole-genome sequencing, respectively (Harris et al., 2010; Holden et al., 2013; Lindsay and Holden, 2006; McAdam et al., 2012), applied to globally representative population samples. In these studies, abundant polymorphisms were ascertained in the *S. aureus* core genome, which provided robust, high-resolution phylogenetic frameworks for each of the populations investigated. One important discovery borne from these investigations was that *S. aureus* constitutes ‘measurably evolving populations’, accumulating detectable genetic variation over epidemiological timescales that can be directly observed. Importantly, this feature enabled the application of coalescence-based methods for dating evolutionary events and tracking staphylococcal spatial spread on the basis of temporally structured population samples. Furthermore, the motility of variably present genomic components could be evaluated in relation to the evolutionary history of the *S. aureus* core genome. By mapping the presence of motile genomic components onto core-genome based phylogenetic trees, multiple events of gene acquisition or loss were identified. Using the accumulation of SNPs in the core genome for calibration, these genetic events could be dated and pinpointed to geographic areas in which they likely occurred. Even though genomic plasticity was not the main focus of any of these recent studies, warranting further in-depth analyses of the available data, a deepened understanding of the temporal and spatial scales of MGE motility in *S. aureus* currently emerges.

Each of the clonal complexes forms a radiation of multiple phylogenetic sub-lineages that had not been resolved by MLST, *spa* typing, or DNA macrorestriction (Harris et al., 2010; Holden et al., 2013; McAdam et al., 2012; Nübel et al., 2008). These sub-lineages evolve mostly clonally, as homologous recombination is extremely rare (Castillo-Ramirez et al., 2012; Holden et al., 2013; Nübel et al., 2008). The most recent common ancestors of individual sub-lineages date back only few decades (Harris et al., 2010; Holden et al., 2013; Lowder et al., 2009; McAdam et al., 2012; Nübel et al., 2010). Notably, sub-lineages are equipped with specific MGEs (and associated toxin genes). Within a given clonal complex, sub-lineages commonly share several of their MGEs (e. g., SaPIs and prophages, which may have been acquired prior to the sub-lineage split), but they also show multiple differences (typically, additional prophages, SCCmec elements, transposons, plasmids) (Holden et al., 2013; Nübel et al., 2008) (our unpublished work).

A recently published study compared comprehensive accessory genomes derived from *de-novo* assemblies of Illumina sequencing reads from 193 *S. aureus* isolates from CC22 and mapped their components onto a high-resolution phylogeny (Holden et al., 2013). This analysis demonstrated the accumulation over time of genetic determinants for antimicrobial resistance in a hospital-associated sub-lineage of CC22, in response to selective pressure experienced in a clinical environment. There was also evidence of occasional loss of resistance genes, prophages, and even entire SaPIs, and one example of a replacement of an SCCmec element through another, structurally different SCCmec element. Such events were rare, however, each affecting a small minority of isolates in the sample, whereas the majority of MGE appeared fully conserved within the sub-lineage (Holden et al., 2013). Thus, once acquired, MGE presence in the genome commonly remained stably maintained throughout sub-lineage evolution (i.e., over decades),

even though some minor structural variation was acquired through recombination.

In summary, acquisition and loss of MGE appears to be rare events, and horizontal gene transfer is not as rampant as perhaps may have been anticipated. Differences between phylogenetic sub-lineages sufficiently explain the gene content variation within clonal complexes that had been observed in previous studies. After all, the association of genetic traits with phylogenetic (sub-)lineages is to be expected from a highly clonal population, provided the genotyping procedure provides sufficient discriminatory power to resolve such lineages (Kurt et al., 2013; Turner and Feil, 2007).

The linkage of toxin genes with the genomic background has practical implications for the epidemiological investigation of *S. aureus* virulence. In most cases, the contribution of individual toxin genes to a phenotype of interest cannot be clearly separated from that of the genomic background at the species level. In order to avoid the erroneous attribution of pathophysiological effects to *S. aureus* toxins, these should be studied by comparing toxin-positive and toxin-negative isolates within a given clonal cluster or sub-clade and/or by using multi-factorial approaches. This will optimize the power of genetic investigations into the mechanisms of *S. aureus* pathogenesis.

Extensive antigenic variation such as it is observed in *S. aureus* toxins may, in principle, be driven by immune selection. However, the impact this may have on toxin gene evolution and diversity in *S. aureus* has not been assessed systematically, considering the very recently accumulated, abundant genome sequence data.

7. Outlook: toxins as vaccination targets

Toxins are interesting vaccine candidates because (i) they are dangerous and significantly contribute to pathogenesis and (ii) their toxic functions can be neutralized by specific antibodies. In fact, antibody-mediated protection from the effects of *S. aureus* toxins has been convincingly demonstrated in humans and in animal models (Cheung and Otto, 2012; Daum and Spellberg, 2012; Holtfreter et al., 2010; Spellberg and Daum, 2012). However, the extra-ordinary variability of toxins in the pan-genome of *S. aureus* constitutes an enormous challenge for the development of broad-spectrum anti-toxin vaccines. It is highly unlikely that a “one fits all”-strategy can solve the problem; an effective anti-*S. aureus* vaccine will contain multiple toxins or toxoids. Selection criteria should include their role in pathogenesis, their distribution among *S. aureus* strains and the degree of natural immunization in the human host population. In view of the pronounced heterogeneity, even within toxin families, it appears mandatory to establish in each case where the natural (or induced) human antibody response is located on a scale ranging from exquisite antigen specificity to broad cross-reactivity with a whole toxin family.

Finally, it must be borne in mind that besides antibodies, T lymphocytes are also necessary for clinical protection against *S. aureus*. Hence, any anti-toxin vaccine approach should be complemented with a strategy to elicit a powerful *S. aureus*-specific T cell response (Spellberg and Daum, 2012).

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