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1 *Staphylococcus aureus* toxins - their functions and genetics

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12

1 **Abstract**

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

17

18 **Keywords**

19 *S. aureus*, Pore-forming toxins, Exfoliative Toxins, Superantigens, Evolution,
20 Epidemiology

1 **1. Introduction**

2 *Staphylococcus (S.) aureus* is notorious as the most common causative agent of hospital-
3 acquired infections, and the spread of antibiotic resistant strains, particularly
4 methicillin-resistant *S. aureus* (MRSA), in hospitals challenges health care systems
5 worldwide. Moreover, *S. aureus* strains of increased virulence, known as community-
6 acquired MRSA (CA-MRSA), can threaten even healthy individuals in the community
7 (Chambers and Deleo 2009; David and Daum 2010; DeLeo, Otto et al. 2010). In addition,
8 *S. aureus* is currently being discussed as the trigger and/or enhancer of allergies of the
9 respiratory system and the skin (Gould, Takhar et al. 2007; Bachert and Zhang 2012).
10 Nevertheless up to now, no anti-*S. aureus* vaccine has been approved for medical
11 practice (Schaffer and Lee 2008; Spellberg and Daum 2012). In spite of the above, the
12 most frequent encounter of *S. aureus* with its human host is peaceful colonization, and
13 around 20% of adults are persistent carriers of the micro-organisms, while another 60%
14 are intermittently colonized (Wertheim, Melles et al. 2005; van Belkum, Verkaik et al.
15 2009). What makes the species *S. aureus* so immensely successful?

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

17 A salient feature of *S. aureus* is its variability. By indexing nucleotide sequence diversity
18 at seven universally present genetic loci, multilocus-sequence typing (MLST) to date has
19 revealed about 2,400 'sequence types' (ST) for *S. aureus* (see www.mlst.net). The vast
20 majority of these diverse STs, however, are clustered in a remarkably limited number of
21 clonal complexes (CC), each of which appears to be distributed worldwide (reviewed in
22 (Nubel, Strommenger et al. 2011)). The predominant *S. aureus* lineages are CC1, 5, 8, 15,
23 22, 30, 45, 59, 80, 97 and 121 (Nubel, Strommenger et al. 2011).

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

1 The full complement of all genes (also known as the pan-genome) of *S. aureus* encodes a
2 wide array of secreted or cell-surface-associated virulence factors (Foster 2005). These
3 include proteins that

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

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

25

26 **2. Pore-forming toxins**

27 *S. aureus* can produce several toxins that damage the membranes of host cells, which can
28 ultimately lead to cell lysis. At sublytic concentrations, these pore-forming toxins are
29 potent cell stressors. In synergy with other danger signals such as lipoproteins that
30 activate the toll-like receptor 2 the toxins trigger the NALP3-inflammasome response
31 resulting in release of cytokines IL1, IL18 and IL33 (Franchi, Munoz-Planillo et al. 2012).
32 Hla, hemolysin- γ (Hlg) and PVL have been shown to exert strong pro-inflammatory
33 effects in this manner (Munoz-Planillo, Franchi et al. 2009; Holzinger, Geldon et al.
34 2012; Kebaier, Chamberland et al. 2012; Perret, Badiou et al. 2012).

35

1 **2.1 Hemolysin- α (Hla, α -toxin)**

2 Hla is released by 95% of *S. aureus* strains as a water-soluble monomer of 33kDa with
3 pore-forming and pro-inflammatory properties. The *hla* gene is not mobile. Its
4 expression is regulated by at least three global regulatory systems including the
5 accessory gene regulator (*agr*) (Xiong, Willard et al. 2006); it is therefore not surprising
6 that Hla is produced in varying amounts by *S. aureus* strains. Upon binding to a
7 membrane receptor, Hla forms heptameric pores, thereby destroying a variety of host
8 cells, including epithelial cells, erythrocytes, fibroblasts, monocytes, macrophages, and
9 lymphocytes, but not neutrophils. The Hla receptor has long remained elusive and only
10 recently ADAM10 (A disintegrin and metalloproteinase 10) has been identified as a
11 binding partner of Hla (Wilke and Bubeck Wardenburg 2010; Inoshima, Inoshima et al.
12 2011). Binding of Hla and pore formation activates the enzyme, which degrades E-
13 cadherin in the epithelial adherens junctions (Inoshima, Inoshima et al. 2011).
14 Moreover, the ADAM10-Hla complex interferes with focal adhesion complexes (Wilke
15 and Bubeck Wardenburg 2010). Both mechanisms would be able to disrupt the integrity
16 of the epithelial and endothelial layers, thereby paving the way for *S. aureus* invasion.
17 The group of Julie Bubeck-Wardenburg has used murine infection models to
18 demonstrate that Hla strongly contributes to the pathogenesis of skin infections and
19 pneumonia induced by *S. aureus*-USA300, which produces the toxin in abundance
20 (Bubeck Wardenburg, Bae et al. 2007; Bubeck Wardenburg, Patel et al. 2007; Bubeck
21 Wardenburg and Schneewind 2008; Inoshima, Inoshima et al. 2011; Wardenburg 2011;
22 Inoshima, Wang et al. 2012; Powers, Kim et al. 2012).

23

24 **2.2 Leukotoxins**

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

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

1 **2.2.1 Panton-Valentine leukocidin (PVL; lukF-PV + lukS-PV)**

2 Skin and soft tissue infections (SSTIs), such as furunculosis or abscesses, are the most
3 frequent diseases caused by *S. aureus* outside the hospital setting. Since Lina et al.
4 discovered a close epidemiological linkage between PVL and chronic or recurrent SSTIs
5 as well as necrotizing pneumonia (Gillet, Issartel et al. 2002), its role in these diseases
6 has remained under discussion. The genes of the F and S subunits are phage-encoded
7 (Table 2) and can thus be acquired by horizontal gene transfer (Kaneko, Kimura et al.
8 1998; Masiuk, Kopron et al. 2010). PVL genes are found in most CA-MRSA strains, which
9 exhibit increased virulence, e.g. in USA300 (Gillet, Issartel et al. 2002; Vandenesch,
10 Naimi et al. 2003; Diep, Gill et al. 2006; Badiou, Dumitrescu et al. 2008; Diep and Otto
11 2008; del Giudice, Blanc et al. 2009; Carpaij, Willems et al. 2011). CA-MRSA cause typical
12 diseases, namely skin and soft tissue infections (SSTIs), as well as necrotizing
13 pneumonia. However, PVL is not restricted to CA-MRSA, but the toxin is also found in the
14 majority of MSSA strains isolated from patients with community-acquired SSTIs
15 (Monecke, Slickers et al. 2007; Masiuk, Kopron et al. 2010). In contrast, the genes are
16 rare in other *S. aureus* strain collections such as nasal commensals or clinical isolates
17 from blood-stream infection (0-5%) (von Eiff, Friedrich et al. 2004; Holtfreter, Grumann
18 et al. 2007; Masiuk, Kopron et al. 2010). Thus, epidemiological evidence for a pathogenic
19 role of PVL is very strong, but the correlation is not absolute, and PVL-negative CA-
20 MRSA strains as well as MSSA strains associated with SSTI-patients have been isolated
21 (Diep and Otto 2008; Masiuk, Kopron et al. 2010; Otto 2010; Monecke, Coombs et al.
22 2011). Animal infection models have yielded conflicting results, which has been
23 attributed to differences between models, inoculum sizes, and, very importantly, the
24 host species (Labandeira-Rey, Couzon et al. 2007; Bubeck Wardenburg, Palazzolo-
25 Ballance et al. 2008; Brown, Dumitrescu et al. 2009; Montgomery and Daum 2009;
26 Tseng, Kyme et al. 2009; Villaruz, Bubeck Wardenburg et al. 2009; Olsen, Kobayashi et
27 al. 2010). In fact, the leukotoxic activity of PVL differs dramatically between species:
28 human and rabbit neutrophils are lysed by very low toxin concentrations (> 40 ng/ml),
29 whereas 1000-fold higher amounts are required for the lysis of mouse or java monkey
30 neutrophils (Loffler, Hussain et al. 2010).

31 **2.2.2 Other Leukotoxins (Hemolysin- γ , Hemolysin- γ 2, LukDE, LukF'M, LukGH)**

32 The *hlg* gene cluster giving rise to hemolysin- γ (Hlg) and hemolysin- γ 2 (Hlg2) is
33 encoded in the core genome; it is present in 99% of *S. aureus* strains. The toxins appear
34 to contribute to septic arthritis and weight loss in mice (Nilsson, Hartford et al. 1999)
35 and to endophthalmitis in rabbits (Supersac, Piemont et al. 1998). Recently, attention
36 has been focussed on LukDE and LukGH, which, similar to PVL, are expressed by the
37 majority of CA-MRSA strains. LukDE binds to CCR5 on immune cells, the chemokine
38 receptor which is also involved in immune cell targeting by the human
39 immunodeficiency virus (Alonzo, Kozhaya et al. 2013). Both leukotoxins contribute to
40 the virulence of *S. aureus* in murine sepsis and renal abscess models (Dumont, Nygaard

1 et al. 2011; Alonzo, Benson et al. 2012). LukGH exhibits potent cytolytic activity towards
2 neutrophils acting in synergy with PVL *in vitro* (Ventura, Malachowa et al. 2010; Rigby
3 and DeLeo 2012). The relative contribution of PVL, LukDE and LukGH to community-
4 acquired SSTI and necrotizing pneumonia remains to be determined. Finally, a PVL-
5 variant, the prophage-encoded LukF'M, has been identified in *S. aureus* strain P83 of
6 bovine origin (Zou, Kaneko et al. 2000). This toxin lyses bovine polymorphonuclear
7 leukocytes (PMNs) and has been implicated in bovine mastitis (Schlotter, Ehricht et al.
8 2012).

9 **2.2.3 Phenol-soluble modulins (PSMs)**

10 PSMs are a family of small, α -helical amphipathic peptides with cytolytic and pro-
11 inflammatory properties. Two subfamilies are described: the PSM α peptides, including
12 the long-known hemolysin- δ (Hld) (Yoshida 1963), are 20 to 26 amino acids long, and
13 the PSM β peptides are 43 to 44 amino acids in length (Wang, Braughton et al. 2007).
14 Most PSMs are core genome-encoded. All *S. aureus* strains contain the *hld* gene and the
15 two loci with the operons coding for the *psm α* and *psm β* genes (Wang, Braughton et al.
16 2007). In addition, a *psm-mec* gene is located on a chromosomal cassette, which is
17 restricted to hospital-acquiredMRSA (HA-MRSA) strains carrying SCC*mec* types II, III,
18 and VIII (Chatterjee, Chen et al. 2011). The *agr* is involved in the expression of PSMs
19 (Wang, Braughton et al. 2007; Queck, Jameson-Lee et al. 2008). The production of PSMs
20 is closely correlated with the capacity of staphylococcal species to cause invasive
21 infections (Rautenberg, Joo et al. 2011). CA-MRSA isolates show an increased production
22 of PSMs in comparison to HA-MRSA (Wang, Braughton et al. 2007). The group of Michael
23 Otto has described multiple functions of PSMs in pathogenesis. Firstly, PSM α peptides
24 effectively lyse white and red blood cells (Wang, Braughton et al. 2007; Cheung, Rigby et
25 al. 2010). PSM α -deletion in CA-MRSA strains resulted in lower mortality and decreased
26 ability to cause skin lesions in mice (Wang, Braughton et al. 2007). Secondly, PSMs have
27 proinflammatory properties, and they induce chemotaxis and activation of human
28 neutrophils, as well as cytokine expression (Wang, Braughton et al. 2007; Queck, Khan et
29 al. 2009; Cheung, Rigby et al. 2010). This response is mediated by activation of the
30 human formyl peptide receptor 2 (FPR2) (Kretschmer, Gleske et al. 2010). Thirdly,
31 proteolytically processed PSM α 1 and PSM α 2 peptides show antimicrobial activity and
32 interfere with competing colonizing pathogens (Joo, Cheung et al. 2011). Finally, they
33 appear to contribute to the structuring of biofilms during *S. aureus* infection (Periasamy,
34 Joo et al. 2012). Recently, Periasamy et al. have proposed that these functions in
35 virulence are indicative of a primary role for PSMs in the commensal colonization of
36 mammalian epithelia (Periasamy, Chatterjee et al. 2012).

37

38 **3. Exfoliative toxins (ETs)**

1 The three known *S. aureus* exfoliative toxins ETA, ETB and ETD are encoded on different
2 genetic elements (Table 1): *eta* is localized in the genome on a temperate phage,
3 whereas *etb* is found on plasmids and *etd* on a genomic island. The prevalence of *eta*
4 and/or *etb* ranges from 0.5 to 3% in MSSA (Becker, Friedrich et al. 2003; Sila, Sauer et al.
5 2009; Nhan, Leclercq et al. 2011), whereas around 10% of MRSA are *eta* positive (Sila,
6 Sauer et al. 2009). Holtfreter et al. observed a strong association of *etd* with invasive
7 CC25 isolates (*spa* type t078 and relatives) (Holtfreter, Grumann et al. 2007). The
8 expression of the ETs is *agr*-regulated (Sheehan, Foster et al. 1992). Functionally, ETs
9 are isoforms of enzymes with high species-specificity. They have glutamate-specific
10 serine protease activity and selectively cleave a single peptide bond in the extracellular
11 region of human and mouse desmoglein 1 (Dsg1; desmosomal intercellular adhesion
12 molecule), a keratinocyte cell-cell adhesion molecule. In this way, the ETs act as
13 “molecular scissors” facilitating bacterial skin invasion (Nishifuji, Sugai et al. 2008). By
14 loosening the keratinocyte junctions they cause blistering diseases known as bullous
15 impetigo and staphylococcal scalded-skin syndrome.

16

17 **4. Superantigens (SAGs)**

18 The staphylococcal SAGs belong to the most potent T-cell mitogens known. Some of
19 these toxins stimulate human T-cells at femtomolar concentrations. Originally, the SAGs
20 of *S. aureus* were termed staphylococcal enterotoxins (SEs) because they elicit vomiting
21 and diarrhea after oral uptake, the hallmarks of *S. aureus* food poisoning. This feature is
22 different from their superantigenicity, however, because some of the recently identified
23 SAGs apparently lack emetic properties. Therefore, the International Nomenclature
24 Committee for Staphylococcal Superantigens introduced a new nomenclature in 2004 to
25 distinguish SAGs with proven emetic activity in primates (staphylococcal enterotoxins;
26 SEs) from those whose emetic properties remain unconfirmed (staphylococcal
27 enterotoxin-like toxins; SEIs) and toxic shock-syndrome toxin 1 (TSST-1) (Lina, Bohach
28 et al. 2004). So far, 23 different staphylococcal SAGs have been described: the
29 staphylococcal enterotoxins A-E, G-J, and R-T (SEA-SEE, SEG-SEJ, SER-SET), the
30 staphylococcal enterotoxin-like toxins K-Q and U-X (SEI/K-SEI/Q, SEI/U-SEI/X) and TSST-1
31 (Proft and Fraser 2003; Lina, Bohach et al. 2004; Holtfreter and Broker 2005; Thomas,
32 Chou et al. 2007; Ono, Omoe et al. 2008; Wilson, Seo et al. 2011).

33 Genetic analysis of *S. aureus* clinical isolates, including whole genome sequencing, has
34 shown that ~80% of *S. aureus* clinical isolates harbor an average of five to six SAG genes.
35 There is extensive heterogeneity of SAG gene patterns between *S. aureus* strains
36 (Jarraud, Peyrat et al. 2001; Baba, Takeuchi et al. 2002; Becker, Friedrich et al. 2003;
37 Holtfreter, Bauer et al. 2004). In addition, the *S. aureus* strains that harbor SAG genes
38 produce varying levels of the toxins. This can be attributed to the involvement of at
39 least four global regulators, *agr*, *sarA*, σ^B and *saeRS* (Tseng, Zhang et al. 2004; Andrey,
40 Renzoni et al. 2010; Kusch, Hanke et al. 2011). Most SAGs are encoded on MGEs, such as

1 bacteriophages, plasmids, SaPIs, and genomic islands (Johns and Khan 1988; Fitzgerald,
2 Monday et al. 2001; Lindsay and Holden 2006; Ono, Omoe et al. 2008), whereas the
3 recently described SE IX is core genome-encoded (Wilson, Seo et al. 2011) (Table 1).

4 The mechanism of action of SAGs was discovered by Bernhard Fleischer and Hubert
5 Schrezenmeier and first described in 1988 (Fleischer and Schrezenmeier 1988). In
6 contrast to conventional peptide antigens, SAGs activate a large fraction of T
7 lymphocytes simultaneously. Conventional antigens are taken up by antigen-presenting
8 cells (APCs) and processed by protease digestion. The resulting antigenic peptides are
9 bound to major histocompatibility complex (MHC) molecules and displayed on the APC
10 surface as MHC/peptide complexes. These are recognized by T-cells via the
11 hypervariable loops of their T-cell receptor (TCR) α and β chains. SAGs can bypass this
12 highly specific antigen-driven interaction between T-cells and APCs. They directly cross-
13 link certain TCR V β domains with conserved structures on MHC class II (MHC II)
14 molecules expressed on professional APCs. They interact with MHC II by binding to the
15 α -chain (antigen peptide-dependent or independent) or to a conserved histidine in the
16 β -domain via a zinc complex (peptide-dependent) (Fraser and Proft 2008)..
17 Furthermore, each SAG interacts with a defined TCR repertoire determined by the TCR
18 V β sequences. As the human genome encodes approximately 50 TCR V β elements, which
19 are unevenly represented in the T-cell pool of an individual, up to 20% of T cells can be
20 activated by a given SAG (Proft and Fraser 2003). In contrast, conventional peptide
21 antigens stimulate only 1 out of 10^5 to 10^6 naïve T-cells (Fraser, Arcus et al. 2000). The
22 V β -restricted T-cell expansion is thus the hallmark of SAGs (Kappler, Kotzin et al. 1989;
23 White, Herman et al. 1989; Choi, Lafferty et al. 1990) with two exceptions: (i) the SAG
24 SEH also contacts TCR V α chains (Petersson, Pettersson et al. 2003; Thomas, Dauwalder
25 et al. 2009) and (ii) the staphylococcal protein A, which is universally expressed by
26 *S. aureus*, acts as a B cell SAG targeting B-cell receptors (membrane-anchored
27 antibodies) which use the immunoglobulin-V $\text{H}3$ gene element (Silverman and Goodyear
28 2006). In addition, many T-cell SAGs also trigger cytokine release by the APCs, which are
29 activated via MHC-II engagement (Proft and Fraser 2003).

30

31 **4.1 Role of SAGs in staphylococcal virulence**

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

38 Staphylococcal toxic shock syndrome (TSS) is characterized by high fever, rash,
39 desquamation, vomiting, diarrhea and hypotension, frequently resulting in multiple

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

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

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

28 Finally, a prominent role for SAGs is being discussed in skin and airway allergies. For
29 atopic dermatitis, a correlation between clinical severity and colonization with SEA- and
30 SEB-producing *S. aureus* as well as with IgE specific for SEA and SEB was documented in
31 one study, but not confirmed in others (Bunikowski, Mielke et al. 1999; Zollner,
32 Wichelhaus et al. 2000). Bronchial asthma afflicts around 300 million people worldwide,
33 thus belonging to the most common diseases. In allergic asthma, the triggers are known
34 inhalative allergens (= allergy-driving antigens), while the causative agents of non-
35 allergic or intrinsic asthma, around 10% of cases, are not known. Intrinsic asthma is
36 often of late onset (3rd-4th decade of life) and takes a severe disease course, which is
37 refractory to established treatment strategies. Chronic rhinosinusitis, a pronounced
38 inflammation of the mucosal tissue of the nose and sinuses, with or without the
39 development of polyps, is also very frequent and often accompanied by intrinsic asthma.
40 Since many patients possess high titres of SAG-specific IgE in their serum or locally in

1 the polyps, several research groups promote the opinion that allergic reactions to
2 *S. aureus* SAGs drive or at least amplify chronic airway inflammation (Gevaert,
3 Holtappels et al. 2005; Bachert, Zhang et al. 2008; Barnes 2009; Bachert, Zhang et al.
4 2010; Zhang, Holtappels et al. 2011).

5

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

7 Toxin gene clustering and sequence homologies imply evolution from ancestral genes by
8 gene duplication and variation. These features are prominent in the families of
9 leukotoxins, ETs, and SAGs. The enterotoxin gene cluster (*egc*), discovered on a
10 staphylococcal genomic island (vSa β) by the group of Jarraud, is given here as an
11 example (Jarraud, Peyrat et al. 2001; Letertre, Perelle et al. 2003). *Egc* SAGs are the most
12 prevalent SAG genes in commensal and invasive *S. aureus* isolates, with frequencies
13 ranging between 46% and 66% in different strain collections (Becker, Friedrich et al.
14 2004; Fueyo, Mendoza et al. 2005; Holtfreter, Grumann et al. 2007; Monecke, Luedicke
15 et al. 2009). Most *egc*-positive *S. aureus* strains harbor five SAG genes (*seg*, *sei*, *selm*, *seln*,
16 and *selo*) and the pseudogenes ψ *ent1* and ψ *ent2* (Jarraud, Peyrat et al. 2001). The *egc* is
17 unusually variable. The *S. aureus* clonal cluster CC30, for example, harbors an additional
18 SAG gene, designated *selu*, a fusion product of ψ *ent1* and ψ *ent2* (Letertre, Perelle et al.
19 2003; Thomas, Jarraud et al. 2006; Holtfreter, Grumann et al. 2007). Given that the
20 members of the *egc* display considerable sequence differences and each of the *egc* SAGs
21 shows closest similarity to SAGs encoded outside the *egc*, Jarraud et al. proposed that the
22 *egc* functions as an “enterotoxin gene nursery” (Jarraud, Peyrat et al. 2001).

23

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

25 MGEs and toxin genes they carry can be disseminated either by vertical transmission to
26 daughter cells upon cell replication or by horizontal gene transfer. Transduction through
27 bacteriophages commonly is considered the predominant mechanism for acquisition of
28 genetic material in *S. aureus*, whereas DNA transformation (Morikawa, Takemura et al.
29 2012) and conjugation are assumed to be less relevant (Lindsay and Holden 2006).

30 Over the past decade, studies based on sequencing a limited number of representative
31 *S. aureus* genomes (Lindsay and Holden 2006), comparative genomic hybridization to
32 microarrays (Monecke, Slickers et al. 2008; McCarthy and Lindsay 2012; McCarthy,
33 Witney et al. 2012; Shore, Brennan et al. 2012), or dedicated PCR assays (Holtfreter,
34 Grumann et al. 2007; Masiuk, Kopron et al. 2010) have provided an overview of the
35 distribution of MGEs and their associated toxin genes in the *S. aureus* population. An
36 overwhelming feature of the resulting data is that there exists remarkable variation in
37 the endowment with MGEs, both within and among clonal complexes (Holtfreter,
38 Grumann et al. 2007; Monecke, Luedicke et al. 2009; Masiuk, Kopron et al. 2010). It

1 further appeared that MGEs and hence the toxin genes they carried were not randomly
2 distributed between lineages, suggesting restrictions to horizontal transfer (Holtfreter,
3 Grumann et al. 2007; Monecke, Slickers et al. 2008; Monecke, Luedicke et al. 2009;
4 Masiuk, Kopron et al. 2010; Shore, Brennan et al. 2012).

5 Due to their location on different genetic elements (Table 1), individual *S. aureus* toxin
6 genes differ greatly in their distribution and horizontal genetic mobility. Some by
7 definition belong to the core genome because they are present in almost all strains: *hla*,
8 the *hlg* gene cluster, and the *psm* loci. Most are much more variably distributed, but,
9 importantly, all are more or less closely linked to the genetic background of *S. aureus*.
10 One extreme example is the *egc*-encoding genomic island vSA β . Among the dominant
11 clonal clusters, *egc* is found in all strains of CC5, CC22, and CC45 as well as in CC25. CC30
12 and CC121 isolates are characterized by a variant of vSA β harboring an *egc* with a
13 functional *selu* gene. On the other hand, CC1, CC8, CC15 and CC395 appear to lack *egc*
14 completely (Holtfreter, Grumann et al. 2007; Masiuk, Kopron et al. 2010; Grumann,
15 Ruotsalainen et al. 2011). Other SAGs with strong clonal complex linkage are the SaPI-
16 encoded *tst* and *sec-sell* (CC45), or the plasmid-encoded *sed*, *sej* and *ser* (CC8). SAG genes
17 with a broader distribution are the phage-encoded *sea*, and the SaPI-encoded *seb*
18 (Peacock, Moore et al. 2002; Fueyo, Mendoza et al. 2005; Diep, Gill et al. 2006; Lindsay
19 and Holden 2006; Lindsay, Moore et al. 2006; Holtfreter, Grumann et al. 2007; Monecke,
20 Berger-Bachi et al. 2007; Monecke, Slickers et al. 2007; Masiuk, Kopron et al. 2010;
21 Monecke, Coombs et al. 2011). The pore-forming toxin lukFS-PV genes are broadly
22 distributed among *S. aureus* clonal lineages with the notable exception of CC45
23 (Monecke, Slickers et al. 2007; Goerke, Pantucek et al. 2009; Masiuk, Kopron et al. 2010)
24 and a subclade of CC121, which in turn is associated with *eta* and/or *etb* (Masiuk,
25 Kopron et al. 2010) (our unpublished data). In contrast, *etd* is rare and characterizes
26 CC80 MRSA isolates as well as some CC25 strains (Holtfreter, Grumann et al. 2007;
27 Masiuk, Kopron et al. 2010; Monecke, Coombs et al. 2011). All the studies cited above
28 were limited, however, by the typing techniques applied (i. e., MLST or *spa* typing), and
29 their associated discriminatory power. Because substantial variation of MGE
30 endowment was observed within the phylogenetic groupings resolved (clonal
31 complexes, mostly), the dynamics of MGE motility could not be investigated. In contrast,
32 more recent work has provided novel, quantitative insights into the frequency of MGE
33 acquisition and loss in *S. aureus*, based on extended MLST (analysing variation at >100
34 genetic loci; (Nubel, Roumagnac et al. 2008; Lowder, Guinane et al. 2009; Nubel, Dordel
35 et al. 2010)) or whole-genome sequencing, respectively (Lindsay and Holden 2006;
36 Harris, Feil et al. 2010; McAdam, Templeton et al. 2012; Holden, Hsu et al. 2013), applied
37 to globally representative population samples. In these studies, abundant
38 polymorphisms were ascertained in the *S. aureus* core genome, which provided robust,
39 high-resolution phylogenetic frameworks for each of the populations investigated. One
40 important discovery borne from these investigations was that *S. aureus* constitutes
41 'measurably evolving populations', accumulating detectable genetic variation over

1 epidemiological timescales that can be directly observed. Importantly, this feature
2 enabled the application of coalescence-based methods for dating evolutionary events
3 and tracking staphylococcal spatial spread on the basis of temporally structured
4 population samples. Furthermore, the motility of variably present genomic components
5 could be evaluated in relation to the evolutionary history of the *S. aureus* core
6 genome. By mapping the presence of motile genomic components onto core-genome
7 based phylogenetic trees, multiple events of gene acquisition or loss were identified.
8 Using the accumulation of SNPs in the core genome for calibration, these genetic events
9 could be dated and pinpointed to geographic areas in which they likely had
10 occurred. Even though genomic plasticity was not the main focus of any of these recent
11 studies, warranting further in-depth analyses of the available data, a deepened
12 understanding of the temporal and spatial scales of MGE motility in *S. aureus* currently
13 emerges.

14 Each of the clonal complexes forms a radiation of multiple phylogenetic sub-lineages
15 that had not been resolved by MLST, *spa* typing, or DNA macrorestriction (Nubel,
16 Roumagnac et al. 2008; Harris, Feil et al. 2010; McAdam, Templeton et al. 2012; Holden,
17 Hsu et al. 2013). These sub-lineages evolve mostly clonally, as homologous
18 recombination is extremely rare (Nubel, Roumagnac et al. 2008; Holden, Hsu et al. 2013)
19 [Castillo-Ramirez 2012; PMID: 23270620]. The most recent common ancestors of
20 individual sub-lineages date back only few decades (Lowder, Guinane et al. 2009; Harris,
21 Feil et al. 2010; Nubel, Dordel et al. 2010; McAdam, Templeton et al. 2012; Holden, Hsu
22 et al. 2013). Notably, sub-lineages are equipped with specific MGEs (and associated
23 toxin genes). Within a given clonal complex, sub-lineages commonly share several of
24 their MGEs (e. g., SaPIs and prophages, which may have been acquired prior to the sub-
25 lineage split), but they also show multiple differences (typically, additional prophages,
26 *SCCmec* elements, transposons, plasmids) (Nubel, Roumagnac et al. 2008; Holden, Hsu et
27 al. 2013) (our unpublished work).

28 A recently published study compared comprehensive accessory genomes derived from
29 *de-novo* assemblies of Illumina sequencing reads from 193 *S. aureus* isolates from CC22
30 and mapped their components onto a high-resolution phylogeny (Holden, Hsu et al.
31 2013). This analysis demonstrated the accumulation over time of genetic determinants
32 for antimicrobial resistance in a hospital-associated sub-lineage of CC22, in response to
33 selective pressure experienced in a clinical environment. There was also evidence of
34 occasional loss of resistance genes, prophages, and even entire SaPIs, and one example
35 of a replacement of an *SCCmec* element through another, structurally different *SCCmec*
36 element. Such events were rare, however, each affecting a small minority of isolates in
37 the sample, whereas the majority of MGE appeared fully conserved within the sub-
38 lineage (Holden, Hsu et al. 2013). Thus, once acquired, MGE presence in the genome
39 commonly remained stably maintained throughout sub-lineage evolution (i. e., over
40 decades), even though some minor structural variation was acquired through
41 recombination.

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

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

17

18 **3. Outlook: Toxins as vaccination targets**

19 Toxins are interesting vaccine candidates because (i) they are dangerous and
20 significantly contribute to pathogenesis and (ii) their toxic functions can be neutralized
21 by specific antibodies. In fact, antibody-mediated protection from the effects of *S. aureus*
22 toxins has been convincingly demonstrated in humans and in animal models (Holtfreter,
23 Kolata et al. 2010; Cheung and Otto 2012; Daum and Spellberg 2012; Spellberg and
24 Daum 2012). However, the extra-ordinary variability of toxins in the pan-genome of
25 *S. aureus* constitutes an enormous challenge for the development of broad-spectrum
26 anti-toxin vaccines. It is highly unlikely that a “one fits all”-strategy can solve the
27 problem; an effective anti-*S. aureus* vaccine will contain multiple toxins or toxoids.
28 Selection criteria should include their role in pathogenesis, their distribution among
29 *S. aureus* strains and the degree of natural immunization in the human host population.
30 In view of the pronounced heterogeneity, even within toxin families, it appears
31 mandatory to establish in each case where the natural (or induced) human antibody
32 response is located on a scale ranging from exquisite antigen specificity to broad cross-
33 reactivity with a whole toxin family. Generally, immune selection may drive antigenic
34 variation, but the impact this could have on toxin gene evolution and diversity in *S.*
35 *aureus* has not been assessed systematically, considering recently accumulated,
36 abundant genome sequence data.

37

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

5

6

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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, Lofdahl et al. 1989)
	<i>hlg</i> gene cluster	(Kaneko and Kamio 2004)
	<i>psmα</i> , <i>psmβ</i>	(Wang, Braughton et al. 2007)
	<i>selx</i>	(Wilson, Seo 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>) <i>lukDE</i>	(Baba, Takeuchi et al. 2002; Letertre, Perelle et al. 2003; Thomas, Jarraud et al. 2006) (Baba, Takeuchi et al. 2002; Barrio, Rainard et al. 2006; Lindsay and Holden 2006)
vSa γ	<i>etd</i>	(Yamaguchi, Nishifuji et al. 2002; Highlander, Hulten et al. 2007)
Plasmids		
pIB485	<i>sed</i> , <i>sej</i> , <i>ser</i>	(Bayles and Iandolo 1989; Zhang, Iandolo et al. 1998; Omoe, Hu et al. 2003)
pF5	<i>sej</i> , <i>ser</i> , <i>ses</i> , <i>set</i>	(Omoe, Hu et al. 2003; Ono, Omoe et al. 2008)
pGSA ₁₈ <i>rep32</i> (pETB)	<i>etb</i>	(Yamaguchi, Hayashi et al. 2001; McCarthy and Lindsay 2012)
pGSA ₁₁ <i>rep22</i> (SAP057A)	<i>etb</i>	(McCarthy and Lindsay 2012)
Staphylococcal Cassette Chromosomes		
SSC <i>mec</i> types II, III, VIII	<i>psm-mec</i>	(Queck, Khan et al. 2009; Chatterjee, Chen et al. 2011)
Pathogenicity Islands		
SaPI _n 1 (N315)/ SaPI _m 1 (Mu50) (vSa4 type I)	<i>sell</i> , <i>sec</i> , <i>tst</i>	(Novick and Subedi 2007)
SaPI3 (COL vSa1)	<i>seb</i> , <i>selk</i> , <i>selq</i>	(Novick and Subedi 2007)
SaPI _m w2	<i>sell</i> , <i>sec</i>	(Baba, Takeuchi et al. 2002; Lindsay and Holden 2006)
Bacteriophages		
ϕ Sa1	<i>lukFM</i> (ϕ PV83)	(Choorit, Kaneko et al. 1995; Zou, Kaneko et al. 2000; Kaneko and Kamio 2004; McCarthy, Witney et al. 2012)
	<i>eta</i> (ϕ ETA)	(Yamaguchi, Hayashi et al. 2000; Kuroda, Ohta et al. 2001; McCarthy, Witney et al. 2012)
ϕ Sa2	<i>lukFS-PV</i>	(Kaneko, Kimura et al. 1998; Narita, Kaneko et al. 2001; Baba, Takeuchi et al.

φSa3

sea; selp; sea, selq, selk

2002)

(Baba, Takeuchi et al. 2002; McCarthy, Witney et al. 2012)

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-γ2 (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

^aSynonyms are indicated in brackets.

^bPrevalence in clinical *S. aureus* isolates from humans.

References: (Choorit, Kaneko et al. 1995; Gravet, Couppie et al. 2001; Kaneko and Kamio 2004; von Eiff, Friedrich et al. 2004; Ventura, Malachowa et al. 2010; Dumont, Nygaard et al. 2011; Alonzo, Benson et al. 2012; Rigby and DeLeo 2012; Vandenesch, Lina et al. 2012)

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