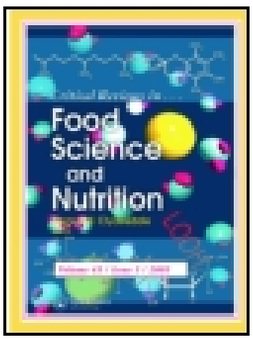

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Noreddine Benkerroum

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Staphylococcal Enterotoxins and Enterotoxin-like Toxins with Special Reference to Dairy Products: An overview

Noreddine Benkerroum

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Present address: Department of Food Science and Agricultural Chemistry, Macdonald-Stewart Building, McGill University, Macdonald Campus, 21,111 Lakeshore Road, Sainte-Anne-de-Bellevue QC H9×3V9, Canada

Corresponding author E-mail: n.benkerroum@gmail.com, Tel: +1 514-398-7761

ABSTRACT

Staphylococcal enterotoxins (SEs) have been raising health concerns for food safety due to their association with Staphylococcal Food Poisoning (SFP). As superantigens, they also cause the life threatening Toxic Shock Syndrome (TSS), the transmission of which via food cannot be ruled out despite the lack of epidemiological evidence. To date, at least 23 of these exotoxins are known and separated into SEs and staphylococcal enterotoxin-like (SEl) depending on whether or not they invoke emesis. This work presents an up-to-date overview on the presently known SEs/SEls from the perspective of their classification, pathogenesis, and genetic organisation. The incidence of these toxins in dairy products, the risk this poses to the public health, and possible control means are also reviewed.

Key words

Staphylococcal enterotoxins, Staphylococcal food poisoning, pathogenicity, Toxic shock syndrome, dairy products

INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a versatile pathogen that produces an array of exoproteins with toxicological effects on humans and animals, such as hyaluronidase, staphylokinase, nucleases, lipases, proteases, collagenases, hemolysins, exfoliative toxins, and superantigen proteins encompassing the toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin-like proteins (SEls) and staphylococcal enterotoxins (SEs). Among these exotoxins, SEs have been the most frequently associated with dairy-borne intoxications collectively referred to as Staphylococcal Foodborne Poisoning (SFP)(De Buyser et al., 2001; Asao et al., 2003). Although primarily produced by coagulase positive *S. aureus*, SEs were also reported to be secreted by other coagulase positive non *S. aureus*, coagulase negative *S. aureus* strains, and non-*S. aureus* staphylococci, such as *Staphylococcus cohnii*, *Staphylococcus epidermis*, *Staphylococcus xylosus*, *Staphylococcus haemolyticus*, *Staphylococcus hyicus* (coagulase-positive or -negative) and *Staphylococcus intermedius* (Adesiyun et al., 1984; Khambaty et al., 1994; Udo et al., 1999; Becker et al., 2001; Le Loir et al., 2003; Madhusoodanan et al., 2011).

Staphylococcal enterotoxins and SEls are low molecular weight, single-chain basic globular proteins, that specifically attack intestinal cells leading to gastroenteritis, typically evoking vomiting, diarrhoea and intestinal or gastric inflammation. They are stable to heat, digestive proteinases, irradiation, denaturing agents, and a wide pH range (Table 1). These toxins belong to the family of superantigens (SAgs), owing to their ability to induce excessive activation of T-cells with a subsequent massive release of cytokines invoking a life threatening toxic shock syndrome (TSS). Another superantigen produced by *S. aureus*, TSST-1, was

originally named Staphylococcal Enterotoxin F (SEF) for being considered as an SE; however, further characterisation revealed significant structural and functional divergence with SEs, and hence it was re-classified as a separate SAg (Bohach et al., 1990; Argudin et al., 2010). The family of SAgs also includes several streptococcal pyrogenic enterotoxins (SPEs) produced by *Streptococcus pyogenes*. *S. aureus* produces another family of toxic exoproteins sharing structural similarities with the staphylococcal superantigens, but are devoid of superantigenicity (Hermans et al., 2012). These exoproteins were first named Staphylococcal Exotoxin-like (SETs) proteins and subsequently renamed Staphylococcal Superantigen-Like (SSL) proteins to avoid confusion with the previously known Staphylococcal Enterotoxin T (SET) (Lina et al., 2004). A particular property of SEs among the other SAgs lies in the fact that they act primarily on the intestine to cause enteritis characterised by emesis among other symptoms. Conversely, diarrhoea, which is another common symptom of enteritis, is not always displayed in SE-mediated intoxications (Table 2). Emesis is therefore used as a benchmark criterion for the distinction between SEs and non-SEs in the classification of staphylococcal SAgs. A number of SAgs produced by *S. aureus* and sharing significant structural, biological and phylogenetic properties with the previously known SEs (i.e., SEA-SEE) have been identified recently. However, not all the newly identified staphylococcal SAgs induce emesis in humans or in non-human primate models; those that have already tested negative or have not been tested yet for this specific property are generically designated “Staphylococcal Enterotoxin-Like” (SEl) proteins. SEs and SEls now encompass over 23 members (Table 3), and the list is expected to continue growing in the coming years, due to the wider use of automated characterisation techniques at the molecular and genetic levels. Such sophisticated, rapid and sensible techniques

are expected to allow identification of new SEs/SEls or reconsideration of the classification of some formerly classified SEs/SEls. Consequently, the standard nomenclature of Sags is being continuously revised, as new variants, types or subtypes are discovered, or particular characteristics of known toxins are discerned (Lina et al., 2004).

This work presents a comprehensive overview on staphylococcal enterotoxins and enterotoxin-like proteins including their nomenclature, classification, pathogenicity mechanisms, and genetic expression with a special reference to their incidence in dairy products as the main SEs/SEls vehicles which have been frequently associated with SFP.

NOMENCLATURE OF SEs AND SEls

The current nomenclature of SEs uses the designation "SE" followed by an alphabetical letter in the order in which an SE is discovered, and the five first SEs discovered (SEA-E) are usually referred to as "the classical SEs." On the other hand, the designation "SEl" is assigned either permanently to SAgs that share significant structural, biological, and functional properties with the classical SEs but do not induce emesis in primate model (e.g., SElL, SElQ), or provisionally for SEls of which the emetic property awaits to be tested (Lina et al., 2004). In the latter case, the designation "SEl" will be maintained if the non-emetic character is confirmed or changed to "SE" if, on the contrary, emesis tests positive. Emesis induction is generally tested on primates by oral administration of the putative SE, although surrogate animal models such as rabbit, dog, and the small insectivore house musk shrew (*Suncus murinus*) have been occasionally used, but the results are not considered in the definite classification of the toxin as an SE (Schlievert et al., 2000; Ono et al., 2008; Pinchuk et al., 2010; Seo 2016). It was also suggested that newly

discovered SEs sharing ≥90% identity with previously known SEs be assigned the latter's SE designation followed by a number indicating that it is a subtype of an existing toxin (Lina et al., 2004). For example, SEs sharing more than 90% identity with SEC were considered to be subtypes of this SE (also referred to as SEC1) and designated SEC2, SEC3 and SEC4 (Argudin et al., 2010; Liu 2015). However, other designations for SE subtypes may refer to "variants" instead of being assigned numbers, as is the case for SEC variants produced by *S. aureus* strains isolated from mastitis cases of ovine, bovine or caprine origin, which were consequently named SEC-ovine/SECov, SEC-bovine/SECbov and SEC-caprine/SEC-cap, etc. (de Carvalho Uhl et al., 2004). Moreover, the International Nomenclature Committee for Staphylococcal Superantigens (INCSS) suggests switching to an alternative designation starting from SE26 if the number of SEs/SEls exceeds 25 letters of the alphabet (F is missing, as SEF is no longer assigned to any SE since it was renamed TSST-1) (Lina et al., 2004).

Considering the induction of emesis as the key criterion for these staphylococcal toxins to be considered as *bona fide* SEs, those that are proven not to be emetic or awaiting to be tested for this criterion are either definitely or provisionally designated SEls, respectively (Table 3). In the latter case, once the emesis feature is confirmed on primate models, on human volunteers or epidemiologically, the concerned SEl will be renamed as an SE. However, the non-emetic character of SEls may be controversial. For example, a recent study conducted by Omoe et al., (2013) showed that currently established SEls (SElL, SElM, SElN, SElO, SElP and SElQ) invoked emesis in monkeys (*Macaca fascicularis*) in 1.4 to 3.5 h after oral administration of 100 μg kg^{-1} body weight (Table 4). Consequently, the authors suggested switching the nomenclature of these staphylococcal toxins from SEls to SEs as stipulated by the INCSS (Lina et al., 2004).

However, these results remain to be confirmed by independent laboratories and researchers before they can be definitely validated and these SEls renamed SEs.

CLASSIFICATION OF SEs/SEls

To date, more than 23 different SEs/SEls are known and their amino acid sequence identities are highly variable, ranging between 21% to 83% (Table 5). However, an overall 11 to 15% residues are conserved to keep the common biological and functional properties, especially to mediate emesis and/or superantigenicity (Liu 2015). Therefore, many classification schemes have been suggested to range SEs/SEls in a limited number of coherent groups. Different criteria have been used for their classification on the basis of amino acid sequence identities, as separate superantigen proteins (Argudin et al., 2010) or as part of the broad family of pyrogenic superantigens produced by staphylococci and group A streptococci. Phylogenetic studies have also been used for SE/SEls classification based on their genetic relatedness and evolutionary pathways.

One of the most popular classification distributes SEs/SEls within the superfamily of pyrogenic superantigens produced by staphylococci and group A streptococci on four groups (I, II, III, and V) out of five defined on the basis of structural characteristics of SAgs (Spaulding et al., 2013). Table 6 presents these groups with their main distinctive structural features. Other authors have identified common ancestors of SAgs and classified SEs/SEls according to their phylogenetic relatedness (McCormick et al., 2001; Okumura et al., 2012; Xu and McCormick 2012). Although the phylogenetic and structure-based classifications share many similarities, some SEs/SEls are placed in different groups in each of these classifications. Conflicting data regarding the

accordance between structural features and the phylogenetic relatedness of some SEs/SEls have been reported (Wilson et al., 2011; Okumura et al., 2012; Xu and McCormick 2012; Spaulding et al., 2013; Ono et al., 2015).

STRUCTURE OF SEs/SEls

Staphylococcal enterotoxins and SEls are globular, single-chain proteins with molecular weights ranging between ~20 and 30 kDa (Table 3). Despite the variability in their primary amino acid sequence, their three-dimensional structure is well-conserved, as revealed by crystallographic analyses (Swaminathan et al., 1996; Rodstrom et al., 2015). Overall, these molecules consist of a mixture of α-helix and β-sheet components arranged in two unequal domains forming a compact ellipsoid shape. The larger domain (C-terminal domain) is a β-grasp motif comprised of β sheets (β6-β12) and α helices (α2, α4 and α5) of the C-terminal region. The smaller domain (N-terminal domain) is a β barrel-rich containing β sheets (β1-β5) and a short α3 helix; however, a section of 22 to 27 residues of the amino-terminus flips over the top of the C-terminal domain to be a part of it (Fig. 1). The disulphide loop, which characterizes SEs/SEls of the groups II and III, is located at the end of the N-terminal domain, while an α3-β8 loop inserted in the C-terminal domain characterizes the SAgs of group V. Figure 2 shows representative SEs/SEls of different groups illustrating the main structural characteristics that differentiate groups II, III and V.

LOCATION OF *se/sel* GENES

It is generally admitted that SEs/SEls are carried on various Mobile Genetic Elements (MGE), such as plasmids, prophages, transposons, *S. aureus* pathogenicity islands (SaPIs), and highly variable genomic regions vSa (Table 3). This is consistent with the frequent horizontal genetic

transfer and the widespread distribution of SEs/SEls among staphylococcal strains. However, *selw* and *selx* were reported to be located on the core chromosome as highly conserved ancestral *sel* genes (Wilson et al., 2011; Okumura et al., 2012).

Plasmids: Two main plasmid families, pIB485/pIB485-like and pF5/pF5-like, are known to host *se* and *sel* genes. The pIB485, 27.6 kb in size, typically encodes SED and SElJ, and is hence also called *sed*- and *sej*-carrying pIB485 plasmid (Bayles and Iandolo 1989; Zhang et al., 1998). However, *ser* gene was shown to be associated to *sed* and *selj* on pIB485-like plasmids isolated from diverse origins (Omoe et al., 2003; Fueyo et al., 2005; Suzuki et al., 2015). The *ser* gene was originally detected on a plasmid of *S. aureus* strain (F5) isolated from lunch boxes responsible of a foodborne intoxication outbreak in the Fukuoka city of Japan in 1997. This plasmid was thus termed pF5 and shown to harbours *selj*, *set* and *ses* genes in addition to *ser* gene (Table 3). Other plasmids in different *S. aureus* isolates from patients of the Fukukoa intoxication outbreak were found to carry *ser* and *selj* genes, and were subsequently designated pF5-like plasmids which, with pF5, form a family of closely related plasmids encoding *se/sel* genes (Omoe et al., 2003). In addition to the two plasmid families mentioned above, a 56.2-kb plasmid, referred to as pZA10, coding β lactamase and heavy metal resistance in *S. aureus* was reported to harbour *seb* gene (Johns and Khan 1988). The same authors have demonstrated that pZA10 is physically and segregationally unstable plasmid that can readily integrate the chromosome as a discrete genetic element.

Prophages: Prophages carrying *se*/*sel* genes are common among methicillin-resistant *S. aureus* and methicillin-susceptible *S. aureus*, community-acquired and hospital-acquired

staphylococcal strains, and they can be chromosomally integrated (Omoe et al., 2005a; Wu et al., 2011) or extrachromosomal (Utter et al., 2014). The chromosomally integrated prophages coding *se/sel* genes belong mainly to the family of *Siphoviridae*, integrase group Sa3, serogroups Fa and Fb, and holin groups 255a and 255b (Goerke et al., 2009). The *se/sel* genes confirmed to be associated with prophage are *sea*, *selk*, *selq* and *selp*, and a prophage can carry more than one *se/sel* gene. Indeed, SEA, SElK and SElQ are coded together by either of the two prophages ΦSa3ms (Sa3, Fb, 255b) and ΦSa3mw (Sa3, Fb, 255a) (Baba et al., 2002; Omoe et al., 2005a; Baba et al., 2008; Sato'o et al., 2014). However, the three *se/sel* genes carried together on the ΦSa3ms prophage have been reported to be *sea*, *seg* and *selk* rather than *sea*, *selk* and *selq* (Sumby and Waldor 2003), making of *seg* another prophage-borne *se* gene. On the other hand, SEA and SElP are separately coded by prophages; *sea* is coded by the prophages Φ252B (Sa3, Fa, 255b), ΦNM3 (Sa3, Fa, 255a) and ΦMu50a (Sa3, Fa, 255a), while *selp* is coded by ΦMu3A (Sa3, Fa, 255a) and ΦSa3n (Sa3, Fa, 255a), also designated ΦN315 according to its host strain, N315 (Kuroda et al., 2001; Baba et al., 2008; Goerke et al., 2009) (see also Table 3). In addition to the phage-coded SE/SEls mentioned above, SEE is reputed as being carried on a hypothetical prophage, based on the work of Couch et al., (1988) who showed that an *see* probe can hybridize to an *sea*-like prophage DNA fragment obtained from uv-induced *see*-producing *S. aureus* strains; nonetheless, the induced putative phage did not form plaques on a lawn of a reporter strain to provide convincing evidence, which has made authors to believe that *see* is a SaPI gene (Lindsay 2011). To our knowledge, no further studies have been conducted to settle this issue definitely.

Apart from the chromosomally integrated prophages, small, low-copy, linear/episomal or circular/plasmidial extra-chromosomal phages (ExPΦs) have been reported to be common in staphylococcal strains and may harbour *se/sel* genes (Utter et al., 2014). These authors have demonstrated the presence of *sei* and *selp* genes in ExPΦs elements isolated from two different strains of *S. aureus*; NRS26 and NRS19, respectively (Table 3).

Staphylococcal pathogenicity islands: SaPIs constitute a family of highly conserved mobile genetic elements of 14-17 kb in size, known to code a number of SE/SEl toxins. These genetic elements are widespread among *S. aureus* strains where they are stably inserted at specific sites in the chromosome owing to the integrases they express (Tallent et al., 2007). More than twenty SaPIs have been characterised and sequenced thus far, and some of them carry two or more *se*/*sel* genes (Table 3). Variable genomic islands: Two types of the highly variable genomic islands (vSaα and vSaβ) have been characterized in all of the *S. aureus* genomes sequenced thus far (Fitzgerald et al., 2003; Seo and Bohach 2010). The highly variable genomic islands vSaα and vSaβ differ from the other mobile genetic elements in that they harbour only non-functional remnants of their original integrase genes, and have thus lost their mobility. Nonetheless, unlike vSaα and vSaβ, other variable genomic islands, vSa3 and vSa4, can excise and replicate autonomously, and have been reported to harbour *se*/*sel* genes (Gill et al., 2005). However, vSaβ is the most known to be associated with *se* and *sel* genes where they are found as enterotoxin gene clusters (*egc)* organised in tandem orientation forming an operon (Fig. 3). Three types (I-III) of vSaβ can be distinguished, among which only the type I contains the four currently known *egc* operons (Fig. 3A). The first discovered *egc* (*egc1*) is the reference cluster (Jarraud et al., 2001), whose constitutive genes have undergone genetic rearrangements consisting of insertion,

deletion and/or recombination events leading to the emergence of novel genes and hence to the other *egc* operons. Figure 3B illustrates the genetic rearrangements that have occurred in *egc1* leading to the generating *egc2* and *egc4* operons. Furthermore, *egc* variants resulting from the integration of IS elements into different *es/esl* genes of *egc1* have been described and considered to be evolutionary intermediates. The location and orientation of these IS elements within *egc1* and resulting *egc* variants are shown in Fig. 3C.

Transposons: Few reports have descried the carriage of *se* genes by transposons. According to Noto and Archer (2006), *seh* flanked by truncated *seo* (Δ*seo*) gene is carried on a presumptive transposon, which is inserted in the chromosome, immediately downstream the staphylococcal cassette chromosome encoding methicillin resistance (SCC*mec*). In this location, the transposon with its *seh/*Δ*seo* genes appears to have stabilised SCC*mec* type IV which is, otherwise, thought to promote the rise of infections in community-associated methicillin-resistant staphylococcal because of its high mobility.

Core chromosome: Although it is well established that *se*/*sel* genes are generally located on MGEs, *selx* gene appears to be an exception, as it was reported to be located in the core genome of 95% of phylogenetically diverse *S. aureus* strains of different epidemiological and ecological origins (Wilson et al., 2011). According to these authors, *selx* is located 400 kb from the origin of replication in the oriC environ among a cluster of 4 genes specific for the *S. aureus* species, including 2 genes coding hypothetical proteins of unknown function and a predicted integrase pseudogene. The gene cluster is flanked by conserved genes coding ribosomal proteins and a DNA-binding protein involved in DNA replication. The genetic linkage of *selx* with an integrase

pseudogene, its wide distribution among *S. aureus*, and its absence in the genomes of other staphylococcal species indicate that this is an ancient gene that has been acquired by horizontal transfer likely to have occurred during *S. aureus* speciation. Similarly, Okumura et al., (2012) reported on *eslw* gene located in the core chromosome of different strains of *S. aureus* at 1.6 to 1.7 Mb proximal to a gene encoding 5'-methylthioadenosine nucleosidase/S-adenosylhomocysteine nucleosidase. This study also revealed that synteny of the *selw* locus, including the proximal 50 kb regions is well conserved in all the *S. aureus* genomes studied, and that the 50 kb regions have no factor related to mobile genetic elements. Such findings demonstrate that *selw* is a direct descendant of an ancestral staphylococcal SAg. Furthermore, these authors consider that staphylococcal SAgs, in addition to some streptococcal SAgs (Streptococcal Pyrogenic Exotoxin A; (SPEA), SPEI, and Streptococcal Superantigen A; SSA) belonging to the clade III, have derived from the *selw* gene which has undergone a positive selection during the evolutionary pathway of *S. aureus*. Meanwhile, ancestral genes of *selw* were transferred from staphylococci to streptococci via bacteriophages as indicated by the location of SPEA, SPEI, and SSA on prophages in streptococcal genomes (Dobrindt et al., 2015).

PATHOGENESIS OF SEs AND SEls

Staphylococcal enterotoxins have been classically associated with two main diseases; TSS and SFP. Symptoms of both of these health disorders are summarised in Table 2 showing that the first disease is systemic and far more severe than the second one consisting of a localised, usually self-limiting gastroenteritis. On the other hand, despite the high pathogenicity potential of SEls

due to structural and functional identities with SEs in addition to their ability to mediate TSS in laboratory animals, their actual implication in human diseases remains to be demonstrated.

TSS

This disease is the direct consequence of the superantigen activity of SEs/SEls due to their ability to stimulate T-cell proliferation driving an overproduction of cytokines with subsequent systemic inflammation and shock. This type of intoxication occurs because of the ability of SEs/SEls to by-pass the normal process of the cellular immune response whereby the antigen molecule is first processed by proteolysis inside of an antigen-presenting cell (APC). After the antigen digestion, a peptide (i.e., antigen peptide) derived thereof is displayed on the surface of the APC with the class II major histocompatibility complex (MHCII). The MHCII with the bound peptide will then attract a subset of T-cells bearing receptors known as T-cell receptors (TCRs) having variable alpha (Vα) and beta (Vβ) chains. Typically, the Vβ chain recognises and binds to the peptide presented by the MHCII of the APC to form the complex MHCII-peptide-TCR within an antigen-binding groove. The recognition of the peptide is highly specific and determines the specificity of the whole process of the conventional immune response, which normally results in the stimulation of approximately one out of 10^4-10^6 T-cells (Arcus et al., 2000; Stach et al., 2014). Conversely, SEs/SEls can bind directly (i.e., without prior processing in the APC) outside the antigen-binding groove to the α/β chain of MHCII, on one side, and to the Vβ chain C, on the other side. SEs may also engage the Vα TCR instead of Vβ while initiating T-cell activation in the same way (Li et al., 1998; Saline et al., 2010; Banke et al., 2014). As such, TCR and MHCII are cross-bridged together forming a ternary complex MHCII-SE-TCR (Fig. 4), which

activates about 5,000-fold more T-cells than does the conventional immune response (Fraser et al., 2000; Stach et al., 2014). Such an excessive T-cell activation drives a massive release of various pro-inflammatory cytokines from both APCs and TCR-bearing T-cells, among which interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumour necrosis factor α (TNF-α) are the main determinants of the TSS (Fraser et al., 2000; Krakauer and Stiles 2013; Liu 2015). The high levels of cytokines released evoke the so-called "cytokine storm" characterised by the symptoms listed in Table 2 (Dinges et al., 2000; Fraser and Proft 2008). Meanwhile, the massive production of IFN-γ suppresses, at least in part, the production of antibodies by B-cells, thereby down-regulating the humoral immunity of the host (Krakauer and Stiles 2013; Spaulding et al., 2013). The severity and outcome of the shock are a dose-dependent, and exposure to high doses (microgram levels) of one or more SEs results in a sudden onset (few minutes) of severe and often fatal symptoms (Fraser and Proft 2008; Krakauer and Stiles 2013).

Mechanism of Action

For better understanding of the mode of action, numerous structure-function studies have been conducted to characterize specific regions or amino acid residues involved in the massive activation of T-cells. For this purpose, different approaches have been used, but they have yielded inconsistent and contradictory results with regard to the exact localisation of the SE regions or specific amino acids involved in the interactions leading to T-cell proliferation (Table 7). An early study indicated that a 6.5-kDa N-terminal fragment obtained by tryptic hydrolysis of SEC plays crucial role in the superantigenicity of this SE (Spero and Morlock 1978). Additional evidence for the implication of the N-terminal region in the superantigenicity was provided by

subsequent studies (Pontzer et al., 1989; Pontzer et al., 1990; Pontzer et al., 1991; Griggs et al., 1992; Kappler et al., 1992; Mollick et al., 1993) (see also Table 7). On the contrary, other studies suggested that the domain A (C-terminal domain) rather than domain B (N-terminal domain) is responsible for T-cell activation (Hedlund et al., 1991; Binek et al., 1992; Irwin et al., 1992; Metzroth et al., 1993; Pontzer et al., 1993; Hoffman et al., 1996; Hakansson et al., 2000; Hu et al., 2009; Zhang and Rogers 2013). As matter of fact, both domains are necessary for SEs/SEls to elicit the superantigen activity, since the formation of the ternary complex (MHCII-SE-TCR) that triggers T-cell proliferation and cytokine release is the outcome of different interactions between SE and MHCII on one hand, and the resulting MHC-SE and TCR on the other hand (Griggs et al., 1992; Mollick et al., 1993; Komisar et al., 1994). These interactions involve different SE regions and amino acid residues not necessary located in the same domain. Indeed, Komisar et al., (1994) showed that fragments from the N- or C-terminal region play equally important roles in the interactions between SEB and MHCII. Also, the binding of a C-terminal fragment of SEA (107-233) to human MHCII products failed to activate T-cells, suggesting that T-cell activation is dependent on additional parts of the toxin (Hedlund et al., 1991).

It is now clear that SEs/SEls bind to one or two sites of MHCII molecule (Table 3); the first is a low affinity binding site (L) located in the α chain, whereas the second is a zinc-coordinated high affinity binding site (H) located in the β chain (Vallee and Auld 1990; Hudson et al., 1995; Rodstrom et al., 2015). The binding of an SE/SEl to the H-binding site of MHCII was demonstrated to involve cooperatively the L-binding site in a way that the SE/SEl binds on either side of the antigen-peptide groove providing enhanced stability to the ternary complex formed (Hudson et al., 1995). In these interactions, amino acid residues of the C-terminal domain

bind to the H-binding site via a zinc-dependant coordination, while residues of the N-terminal domain bind directly to the L-binding site (Johnson et al., 1991; Hudson et al., 1995; Hu and Nakane 2014). However, SEs/SEls of the zinc-binding family do not necessarily bind to both H- and L-binding sites of the same MCHII molecule. SEA and SEE were shown to bind two separate MHCII molecules each, one in the H-binding site and the other in L-binding site, which results in the formation of a quaternary complex [TCR-SE-$(MHCII)_2$] in the presence of TCR (Petersson et al., 2002; Rodstrom et al., 2015). The crystallographic structure solved by these authors shows that when the N-terminal domain of an SE binds to MCHII molecule via its L-binding site, the C-terminal domain is directed away from the bound MHCII molecule. Spatially, the latter domain cannot bind the same MHCII molecule and is, therefore, free to bind another MHCII molecule via its H-binding site without steric hindrance. As such, the MHCII molecule bound to the N-terminal domain of the SE/SEl can cross-bridges the TCR and initiate T-cell response. Conversely, the second MHCII molecule which is bound to the C-terminal domain is too distant from the TCR to cross-bridge it, but helps stabilise the quaternary complex for optimal T-cell response. Sequence alignment studies suggest that, in addition to SEA and SEE, SED and SElJ interact with MHCII molecules in the same way to form quaternary complexes with TCR (Petersson et al., 2002). Similarly, interactions of TCR with SEs/SEls involve amino acid residues of both SE domains as was demonstrated by mutational studies and confirmed by crystal structure determinations (Jardetzky et al., 1994; Swaminathan et al., 1996; Li et al., 1998; Sundberg et al., 2007; Fraser and Proft 2008; Banke et al., 2014; Rodstrom et al., 2015). Such studies have revealed that TCR binds to a shallow cavity at the surface of the SE molecule in the interface of the two domains (Fig. 1). The shallow cavity, also called the “TCR binding cleft’,

appears to be a common structural feature to all SE/SEls and comprises conserved residues in both domains involved in the interactions between TCR and SEs/SEls (Swaminathan et al., 1996; Saline et al., 2010). Despite the certainty that both domains A and B of SE/SEls are required for TSS mediation, the relative contributions of specific amino acid residues in each of these domains to the binding interactions are still unclear. Nonetheless, irrespective of the limitations and controversy surrounding the approaches used in such studies, the implication of specific amino acid residues in SE interactions with MHCII and TCR to invoke TSS has been repeatedly reported (Table 7). For example, there is a consensus that the phenylalanine at position 47 (F47) of the B domain is critical for SEA, SEE and SEB binding to the L-binding site of the MHCII α-chain and that two histidine residues (H187 and H225) in addition to an aspartic acid (D227) of the domain A constitute a zinc-binding motif that coordinates the zinc-mediated interaction of the SEs/SEls with the H-binding site of MHCII β-chain (Harris et al., 1993a; Hudson et al., 1995; Thibodeau et al., 1997; Petersson et al., 2002; Rodstrom et al., 2015). At least two residues of this motif (H225 and D227) were suggested to be conserved in members of zinc family of superantigens, including SEA, SED, SEE, SEH, SEI, SElJ and SEK (Petersson et al., 2001; Gunther et al., 2007; Rodstrom et al., 2015). Similarly, L48 and F44 were reported to play important roles in the interactions of SEA and SEB, respectively with MHCII (Harris et al., 1993a). In addition to F44, N23 also appears to be crucial for SEB to interact simultaneously with the α-chain of MHCII and the Vβ chain of TCR, thereby playing a dual role in T-cell activation (Kappler et al., 1992). Moreover, a recent study using crystallography techniques in conjunction with computational modelling has been conducted to elucidate interactions of SEE, as a representative of the group III SAgs, with human TCR (Rodstrom et al., 2015). According to

this study, SEE interacts with five regions of the human TCR Vβ chain (CDR1, CDR2, FR3, FR4 and HV4) through the establishment of hydrogen bonds and Van der Waals forces between 19 residues of SEE and 23 residues of the TCR. The SEE residues involved in these interactions are located in the TCR-binding cleft between the N- and C-terminal domains and are distributed on four main SE regions, including the α2-helix and the α4-β9 loop of domain B, and the hydrophobic patch consisting of β2-β3 and β4-β5a loops in addition to the upper side of the α5-helix located in the domain A (Fig. 5). The 19 SEE amino acid residues contacting TCR were identified to be N21, S24, N25, R27, Q28, Y32, N33, P62, W63, Y64, Y91, Y92, G93, Y94, S172, S174, F175, Y205, and P206, most of which, however, are not conserved in other SEs/SEls (Rodstrom et al., 2015). On the other hand, the interactions between group V SEs/SEls and TCR are characterised by the crucial role of the characteristic α3-β8 loop of these SAgs. In the case of SElK, the contact points with the human TCR consist of two amino acid residues, H142 and Y158, located in the α3-β8 loop; each of which establishes a hydrogen bond and a van der Waals interaction with residues of the TCR β-chain loops FR3 and FR4 (Gunther et al., 2007). The latter study showed that a 16-residues long N-terminal fragment (1-16) of SElK also contribute to these interactions by binding to the CDR2 loop of TCR through various hydrogen bonds and van Der Waals forces, with Q1 as the main contact point. The results of different studies reporting on regions and/or amino acid residues involved in biological functions of SEs are summarised in Table 7 illustrating the discrepancies and controversies in this regard starting from the earliest to the latest findings.

Although the implication of SEs/SEls in TSS is well established, their ability to cause the disease as preformed toxins via food consumption remains uncertain. It is generally admitted that

SEs can cause TSS only through exposure from deep tissues (wound contamination with an SE-producing staphylococci), or as result of staphylococcal bacteraemia, or colonisation of intestinal or vaginal mucosa by SE-producing staphylococcal strains (McCormick et al., 2001). This was explained by the limited capacity of SEs/SEls to cause enterotoxaemia and, hence, their inability to be systemically disseminated via the enteric route to mediate the toxic shock (Chi et al., 2002). This observation is consistent with the results of a previous study conducted by Schlievert et al., (2000) who showed that oral administration of SEC in rabbit caused only emesis and diarrhoea without shock. The same authors reported that SEC has site-specific action, and is unable to cross the mucosal barrier and disseminate through the body via the bloodstream, unless too high doses (mg range) are ingested or administered intragastrically; they, therefore, concluded that SFP and TSS are two separate diseases. Conversely, several studies have demonstrated the ability of SEs (SEA, SEB, SEC, SEE) to transcytose across the epithelial layer of the intestine and reach other organs (Hamad et al., 1997; McKay and Singh 1997; Shupp et al., 2002; Brosnahan and Schlievert 2011). In addition, Shupp et al., (2002) showed that the transcytosis is a common feature of SEs, which is mediated by a conserved domain of 10 amino acid residues (KKKVTAQELD) that would be recognised by specific, but yet unidentified, receptors on the intestinal epithelial cells. Nonetheless, this *in vitro* study, conducted on monolayer models of human cell-lines (T-84 and Caco-2), did not specify whether or not the transcytosis was assisted by the superantigen activity of SEs. Other studies, however, demonstrated that for SEs to transcytose, they first have to injure the epithelium barrier through an inflammatory reaction. In this regard, evidence was provided by the prevention of SEB to disrupt the epithelium barrier function and transcytose across it when key mediators of enteric inflammation (IFN-ψ, TNF, IL-

8, and the monocyte chemoattractant protein-1; MCP-1) are neutralised with monoclonal antibodies (McKay and Singh 1997; Pinchuk et al., 2007). In addition, spread of orally ingested SEA and SEB from the Gastro Intestinal Tract (GIT) to other organs such as kidney, lymph nodes, liver and spleen subsequent to the intestinal inflammation onset has been demonstrated in monkeys (Sugiyama et al., 1963; Kent 1966). Furthermore, it has been reported that SEs can use non-damaging mechanisms to cross the intestinal epithelium barrier, suggesting that the inflammation is not a prerequisite for transcytosis. Oral administration of SEA was shown to cross intact rat gastrointestinal mucosal membrane and gain access to the kidney within 15 min without interfering with the epithelium integrity (Beery et al., 1984). Additionally, SEA and SEB were shown to move across an *in vitro* culture of human intestinal epithelial monolayer cells (Caco-2) at different rates by at least two different dose-dependent mechanisms; SEA passively translocated with water via paracellular diffusion, while SEB followed a typical facilitated transport pattern requiring specific receptors (Hamad et al., 1997). These results were subsequently confirmed *in vivo* in mice by peroral administration of SEA and SEB and their detection in the blood serum as function of time; both toxins reached peak doses in the blood after 2 h of ingestion, with SEB levels being 20-fold higher than those of SEA. Similarly, Shupp et al., (2002) showed that SEA and SEB can cross epithelial cell monolayers in a dose-dependent manner (increasing movement from 50 to 300 μg mL^{-1}) and at different rates, with SEB being more readily transcytosed than SEA. There is therefore a consensus regarding the ability of SEs to either cross intact small intestinal mucosa or after impairing its permeability function following inflammatory reaction induced by superantigen activity. Therefore, the limiting factor in systemic dissemination of SEs to cause TSS following intestinal mucosa exposure appears to

be the extent of toxin transcytosis, which in turn depends on the type and concentration of SEs. In fact, in some severe cases of SFP, symptoms such as prostration, low blood pressure, and shock, similar to those observed in TSS disease have been described (Hennekinne et al., 2012). An early report on SFP outbreak in the USA caused by the consumption of contaminated cream puffs, patients suffering from severe cases of the disease were described to be "*very definitely in a state of shock*" (Denison 1936). Therefore, it is probably the dose that makes the difference between SE leading to TSS or being limited to a benign SFP. In staphylococcal infections, the bacterium would produce *in situ* higher levels of SEs than could be found in food as preformed toxins to induce a shock (Cao et al., 2012). The well-established invasiveness feature of *S. aureus*, and its potential to cause bacteraemia, is an additional factor that would facilitate access of SEs to T-cells to ultimately evoke TSS, in the case of infection by an SE/SEl-producing *S. aureus* strain.

SFP

This is a disease caused by the ingestion of food containing preformed SEs exerting symptoms typically including vomiting, abdominal cramps and occasionally diarrhoea; however, other symptoms evoking a systemic effect may develop in susceptible individuals or when too high doses are ingested (Table 2). SFP is often associated with protein-rich food such as meat and dairy products providing favourable conditions for staphylococci to grow and produce enterotoxins. SEs are recognised as major causative agents of SFP outbreaks, most of which are associated with the classical SEs (SEA-SEE), with SEA being responsible for ~80% of the reported cases (Atanassova et al., 2001). Table 8 presents documented SE serotypes implicated

in dairy-borne SFP outbreaks in different countries. However, few reports on the implication of new SEs such as SEG, SEI (Cao et al., 2012) and SEH (Pereira et al., 1996; Ikeda et al., 2005) in SFP are available. A recent study strongly suggested that SER and SElJ are likely to have been involved in SFP outbreaks recorded in Japan during the period of 1992 and 2013 (Suzuki et al., 2015). On the other hand, the situation of SEls remains unclear, which has been attributed to the common use of commercial kits, available only for classical SEs, in the detection of staphylococcal toxins involved in SFP outbreaks (Table 9), which contributes to overlook the actual incidence of the newly described SEs and SEls. The frequent co-occurrence of genes encoding new SEs/SEls and those encoding classical SEs in staphylococcal strains isolated from SFP outbreaks (Kerouanton et al., 2007; Chiang et al., 2008; Sato'o et al., 2014) (Table 10), strongly suggests that new SEs and SEls are more involved in SFP than currently recorded. Such an assumption was illustrated in the case of the SFP caused in Japan by reconstituted skim milk in the year 2000 which was first ascribed to SEA alone (Ministry of Health and Welfare Osaka City 2001; Asao et al., 2003). Subsequent analysis (5 years later) using the more specific Western blotting technique revealed the presence of SEH at almost equal amounts as SEA in the same samples involved in the intoxication (Ikeda et al., 2005). Furthermore, there have been cases of foods confirmed to be responsible for SFP outbreaks on the basis of clinical symptoms, and yet none of the most common SEs (SEA-SEE, and SEG-SEI) was detected in the samples analysed (Wieneke et al., 1993; Kerouanton et al., 2007). Such a situation suggests the implication of one or more SEs/SEls that may have not been surveyed in the pertaining epidemiological studies. A recent study has demonstrated that SElJ and SER, in addition to a SED mutant, have been implicated in a number of SFP outbreaks in Japan without being detected

when these outbreaks were first characterised (Suzuki et al., 2015). Moreover, a staphylococcal food poisoning isolate was shown to produce SElY which is capable to induce emesis in home musk shrew and to cause superantigen activity in human Peripheral Blood Mononuclear Cells (PBMCs) (Ono et al., 2015). To improve the characterisation and detection of SEs/SEls involved in SFP, many approaches have been proposed including an integrated gene-to-protein approach (Hennekinne et al., 2010). However, the technical feasibility and cost effectiveness of such approaches on a routine basis should be scrutinized.

The toxic doses of SEs reported to cause SFP vary greatly depending on the source of information (e.g., outbreak surveys, reports or laboratory experiments on volunteers or animals), the group of exposed consumers (healthy adults or groups at risk), in addition to the type and number of the SEs responsible for the intoxication. However, it is generally accepted that the threshold toxic level varies between 20 and 100 ng per individual (Schelin et al., 2011), corresponding to 0.29 and 1.43 ng kg^{-1} of body weight (taking average adult body weight of 70 kg). The corresponding concentration in the contaminated food that causes SFP depends also on the food matrix, and the quantity ingested of the food in one or more servings. In the case of the SFP outbreak caused by reconstituted low fat skim milk in Japan, the SEA concentration causing the disease was estimated to range between 0.08 and 0.38 ng mL^{-1}. The corresponding intake per capita of SEA that caused this outbreak ranged between 20 and 259 ng for adults, depending on the age group, and a dose as low as 17 ng could induce SFP symptoms in children of less than 10 years old who are considered to be among the group at risk (Asao et al., 2003). Nonetheless, according to Ikeda et al., (2005), the latter doses are underestimated as SEA was not the only SE involved in this intoxication outbreak, and at least SEH was concomitantly present at

approximately the same concentration as SEA in the incriminated samples. Indeed, higher concentrations of SEA in chocolate milk (0.34 - 0.66 ng mL^{-1}) were reported to induce SFP symptoms in an outbreak in the USA, and the toxic doses varied between 94 and 184 ng for a serving of 280 mL (i.e. a half pint carton of chocolate milk); in some cases, three or more servings were necessary to cause symptoms (Evenson et al., 1988). In laboratory animals, orally administered SEs were shown to cause emesis at concentrations varying between 5 μg and 1 mg per animal depending on the animal species and the SE/SEl used in the test (Table 3). Nonetheless, it is well established that humans are significantly more sensitive to toxins than animals. Therefore, if these doses are to be considered to estimate a safe dose for humans, safety/uncertainty factors should be applied. In effect, early studies showed that 200 to 250 μg of pure SEB (i.e., 2.87--3.57 μg kg^{-1} of body weight) were necessary to cause SFP symptoms in human volunteers (Raj and Bergdoll 1969). In a more recent study where human volunteers ingested SEA, SEB or SEC, the toxic doses of these SEs ranged between 0.6 and 3.0 μg per individual (Bergdoll 1989).

GENETIC REGULATION OF SE/SEl PRODUCTION

Toxigenic staphylococci can produce SEs/SEls over a wide range of temperature, pH, $a_{w,}$ Eh and salt concentration (Table 11). However, a staphylococcal strain may harbour one or more *se*/*sel* genes and not be able to express some or all of them even under optimal conditions of growth (Omoe et al., 2002; Omoe et al., 2005a; Omoe et al., 2005b; Carfora et al., 2015). This may be due to the complexity of the genetic expression of *se/sel* genes involving an intricate network of regulatory systems acting independently or in coordination (Bronner et al., 2004). The best

known of such regulatory systems is the accessory gene regulator (*agr*) using the quorum-sensing signal in response to the cell density. This system has been known to upregulate the expression of *se* genes, such as *seb*, *sec* and *sed*, whose products are formed during the post-exponential phase of growth at a cell density of 10^6-10^8 cfu mL^{-1} or g (Morfeldt et al., 1996; Novick 2003; Derzelle et al., 2009). Nonetheless, the *agr* system was recently shown to downregulate the expression of *seh* gene (Sato'o et al., 2015) despite the production of SEH at the late phases of growth (Sakai et al., 2008; Derzelle et al., 2009; Schelin et al., 2011).

The *agr* system controls the expression of *se/sel* genes via an auto-inducing peptide (AIP); a ligand which is constitutively produced in an inactive form (pro-AIP) and exported outside the cell. During the export through a transmembrane protein (AgrB), the pro-AIP is processed by a proteolytic cleavage to reduce the number of amino acids to 7-9 residues followed by the formation of a cyclic thiolactone bond between a cysteine residue and the carboxyl terminus to yield the active AIP (Novick 2003). At certain concentration into the surrounding medium, the AIP activates the *agr* system in a typical two-component system manner resulting in increased production of RNAII and RNAIII transcripts. The latter transcript plays a major role in the regulation of *se* genes, owing to its antagonistic action against the Repressor Of Toxins (Rot). Meanwhile, the RNAII transcript is translated into four components (AgrB, AgrD, AgrC, and AgrA) to sustain the auto-activation of the *agr* system circuit. Figure 6A depicts the regulatory mechanisms of *seb*, *sec*, *sed*, and *seh* gene expression involving the *agr* system. In addition to the quorum sensing, various other genetic regulators were reported to be involved in the production of SEs/SEls as a response to environmental stimuli (Regassa and Betley 1993; Hennekinne et al., 2012; Sato'o et al., 2015). Such regulators include: (i) two-component systems, e.g., *S. aureus*

Exoproteins (*sae*), Staphylococcal Respiratory Response proteins (*srr*) and Autolysis-Related Locus (*arl*) (Schelin et al., 2011); and (ii) DNA-binding transcriptional regulators, e.g., Sar family proteins (SarA, SarR, SarS, SarT, SarU, SarV, SarZ) and sigma factor (Bronner et al., 2004). For example, SarA and sigma B were shown to control *seb*, *sec*, and *seh* genes upon exposure to catabolites (glucose, galactose, sucrose, glycerol, and maltose), environmental stress (high temperature or alkaline conditions), and high salt concentration (Regassa and Betley 1993; Yarwood and Schlievert 2003; Schmidt et al., 2004). These regulators were shown to upregulate or downregulate *se* gene expression, either independently (Chan and Foster 1998; Chien et al., 1999; Kusch et al., 2011) or in coordination with the *agr* system (Heinrichs et al., 1996; Bronner et al., 2004; Fujimoto et al., 2009). Also, anaerobic conditions and low redox potential were reported to activate the *ssr* system, which inhibits the RNAIII transcription from the *agr* operon, thereby downregulating *seb*, *sec*, and *sed* genes (Nouaille et al., 2014). Moreover, phage-inducing agents (e.g., low pH, oxidative stress, mitomycin C, and acetic acid) have been demonstrated to activate phage-borne *se/sel* genes in *agr*-negative staphylococcal strains concomitantly with the induction of the lytic cycle of the carrying phage (Sumby and Waldor 2003; Wallin-Carlquist et al., 2010a; Cao et al., 2012; Zeaki et al., 2015). Results of these studies suggest that the expression of prophage-borne *se/sel* genes is dependent on the life cycle of the carrier prophage rather than the *agr* system. The mechanism of activation of *se/sels* following the induction of the carrying prophage is likely to be similar to the classical genetic switch in the phage λ, as was demonstrated for *sea*, *seg2*, and *selk2* genes coded on phage ΦSa3ms (Sumby and Waldor 2003). Based on findings of the latter study, a proposed mechanism for the genetic regulation of phage-coded *se/sel* genes is suggested in Fig. 6.

HEALTH RISKS ASSOCIATED WITH SES IN DAIRY PRODUCTS

Predictive microbiology studies on the relationship between the growth of *S. aureus* and SEA production in milk and dairy products, suggest that staphylococcal enterotoxins (essentially SEA) are generally produced at detectable levels when the pathogen reaches a cell concentration of 5-8 log cfu mL^{-1} or g^{-1} (Lindqvist et al., 2002; Kim et al., 2009). Such a concentration would be reached in the mid-exponential phase of growth according to Combase™ predictive models (http://modelling.combase.cc/ComBase_Predictor.aspx). In an attempt to determine mathematical relationship between the growth of *S. aureus* and enterotoxin production in sterile milk, (Fujikawa and Morozumi 2006) demonstrated that SEA production (ng mL^{-1}) increases linearly with the time after reaching a cell concentration of 6.5 log cfu mL^{-1} at a constant temperature of 23°C or 32°C. On this basis, the authors established the following linear relationship between the rate of SEA production and temperature (within the interval of 15 and 32°C):

$$P = 0.0376 \times t - 0.559 \quad \text{Equation (1)}$$

Where: P is the production rate of SEA (ng mL^{-1} h^{-1}) and t is the temperature in°C. However, this function cannot predict the production level of SEA at temperatures higher than 32°C and lower than 15°C, while SEA may still be produced in food at these temperatures (Table 11).

Various risk assessment studies have been conducted using different approaches to determine the risk associated with SEs (mainly SEA) in dairy products (Fujikawa and Morozumi 2006; Soejima et al., 2007; Heidinger et al., 2009; Kim et al., 2009). Most of these studies attempted to

determine a relationship between the cfus g^{-1} or mL^{-1} of SE-producing *S. aureus* and the concentration of the SE produced by the bacterium in a food under different conditions of pH, temperature, food matrix, storage/holding time, etc. In this regard, Kim et al., (2009) suggested the following linear relationship based on data obtained by Soejima et al., (2007) on the dynamics of SEA production in skim milk concentrates:

$$Tox = 0.930075 \times C - 6.66209 \quad \text{Equation (2)}$$

Where Tox is the toxin production (log ng mL^{-1}) and C is the number of cells (log cfu mL^{-1}).

According to equation (2), SE is produced at detectable levels when the cell counts of the producer *S. aureus* exceeds 7.2 log cfu mL^{-1}. However, there are important gaps in the available knowledge (e.g., exact threshold dose unknown) in addition to the complexity and variability of the regulatory mechanisms that govern the expression of the *se* genes in different food products, and under different environmental conditions. Such imitations hinder seriously risk assessment studies aiming to determine the dose/response relationship, a key element to assess the risk associated with SEs in different food systems (Wallin-Carlquist et al., 2010b; Marta et al., 2011). Furthermore, it is well established that the level of SEs/SEls in food is not always correlated with the counts of the producer strains. SE-producing strains may be present in high counts in foods (e.g., $>10^8$ cfus g^{-1}), but SEs are either not produced or produced at undetectable levels (Scheusner et al., 1973). Conversely, SEs may be detected at toxic levels and the producer bacterium is either absent (Asao et al., 2003) or present at low counts ($<10^4$ cfu g^{-1}) (de Reu et al., 2002). In this regard, it has been demonstrated that milk and dairy products do not provide optimum conditions for SE production by staphylococcal strains compared with laboratory

media, although they do not restrict the growth of the pathogen. Conditions are less favourable in cheese and other fermented dairy products due to the weak competitiveness of staphylococci with the natural microflora of milk and the starter cultures used for fermentation (Smith et al., 1983). The implication of dairy products in SFP, in spite of the fact that they do not provide optimal conditions for the growth of toxigenic staphylococci, was explained by the accelerated growth of these strains immediately after milk drawing and/or during early stages of the production of cheese and fermented dairy products. At these stages, staphylococci may grow rapidly to reach high levels and produce significant amounts of enterotoxins. Then, the counts may decline sharply or be reduced to below detectable levels due to inhibition by the competing microbiota or other environmental factors. In dairy products that undergo microbicidal treatments such as heat treatment, use of antimicrobial additives and plant extracts, addition of flavouring and/or colouring agents, etc. (Kim et al., 2001; Galindo-Cuspinera et al., 2003; Pinto et al., 2011), SE-producing staphylococci can be removed, but the SEs, if already produced, will persist in the product through the manufacturing process and during storage (Tatini et al., 1971; Wieneke et al., 1993; Asao et al., 2003). The use of mastitis or subclinical mastitis milk as raw material in processed dairy products can be another explanation for the presence of SEs at toxic levels while enterotoxigenic staphylococci are either absent or present at low counts. In fact, during storage under abuse temperatures, SEs may recover most or all of their biological activities if they had been partially inactivated e.g., by heat treatment (Fung et al., 1973; Hennekinne et al., 2012). Given these conditions, the counts of enterotoxigenic staphylococci will be of little value as an indicator of the presence or absence of SEs in foods. Therefore, determination of the relationship between toxigenic staphylococci and SE

concentration in quantitative risk assessment (QRA) of SEs in dairy products provides only partial information to risk managers and stakeholders. Although this approach has proven to be useful to some extent from the food safety perspective, it appears worthwhile to consider such toxins as standalone chemical hazards in future QRA studies, as is now commonly done for mycotoxins. Such an approach is even more indicated as some authors are claiming that despite the well-established acute toxicity of bacterial toxins including SEs, they may have a sub-chronic or chronic effects upon repeated exposure to low or moderate doses (Pinchuk et al., 2010; Rajkovic 2014). Early studies showed that chronic administration of SEB to monkeys into different segments of the small intestine caused severe intestinal diseases including hypertrophy of mesenteric lymph node, an increase in lymphoid aggregates within the ileal submucosa and lymphoid hyperplasia in the mucosal lamina propria, submucosal fibrosis and thickening of the bowel wall (Van Prohaska 1963; Stiles and Denniston 1971). Furthermore, repeated exposure to SEs was suggested to affect immunological functions leading to the breakdown of oral tolerance (Principato and Qian 2014), thus allowing the development of severe immunopathologies including inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease and celiac disease (Garside et al., 1999). Chronic exposure to SEB produced *in situ* from *S. aureus* colonising the nose was reported to increase the level of IgE in the blood (Cui et al., 2015) and cause a multisystem autoimmune inflammatory disease presenting symptoms similar to those of the systemic lupus erythematosus (Chowdhary et al., 2012).

Mechanisms of Action of SEs to Cause SFP Symptoms

Emesis

Most studies on the mode of action of SEs in SFP aimed to elucidate the mechanism by which these toxins induce emesis as the hallmark of the disease. Nonetheless, contrary to the superantigen activity of SEs which is fairly well characterised, the mechanism of emesis still suffers from a shortage in scientific knowledge to be elucidated, essentially due to difficulties accessing adequate laboratory animals to conduct relevant studies. In particular, the access to monkeys, considered to be the best surrogate for humans, is severely restricted by the high cost, availability of the animals and ethical considerations.

The main uncertainty in the mechanism of SE-mediated emesis relates to the early steps of the disease onset, especially the identification of hypothetical receptor sites for SEs in the GIT. Although there is a general agreement that the small intestine is the primary site of action (Beery et al., 1984), the exact location and molecular characterisation of the putative receptors of SEs remain unclear and controversial. Early studies have demonstrated that the emesis stimulus in monkey (*Macaca mulatta*) initiates from the abdominal viscera and reaches the vomiting centre in the brain by means of vagal and sympathetic nerves. These observations were substantiated by the lack of responsiveness to very high doses of SEA administered orally in monkeys that had been subjected to complete denervation of afferent and sympathetic nerves of the abdominal viscera (Sugiyama and Hayama 1965). More recently, Hu et al., (2007) confirmed the implication of the vagal afferent nerve in SE-mediated emesis by demonstrating the loss of the emesis reflex in vagotomised house musk shrew (*Suncus murinus*) challenged with SEA. These

authors further demonstrated that the small intestine is the crucial site of SEA-induced emesis, and that serotonin (5-hydroxytryptamine; 5-HT) is the main neurotransmitter involved in emesis. The same study, showed that the induction of emesis by SEA originates at the mucosa of the small intestine where the SE attaches to unknown receptors on enterochromaffin cells causing them to release 5-HT, which in turn binds to 5-HT3-receptors on vagal afferent neurones. The binding of 5-HT to the vagal afferent neurones depolarises/activates them resulting in the stimulation of the emetic centre of the medulla oblongata in the brain, which eventually triggers the emetic reflex (Fig. 7). This signalling pathway was evidenced by the inhibition of SEA-mediated emesis in the presence of a 5-HT synthesis-inhibitor and by a 5-HT3 receptor antagonist, indicating that both 5-HT and the receptor 5-HT3 are required for emesis induction. Nonetheless, the characterisation of the putative receptors of SEs on the epithelial enterochromaffin cells remains the major limitation to delineate a valid and comprehensive mechanism for SE-mediated emesis. Meanwhile, other studies have suggested that SFP symptoms, including emesis, are in fact the result of SE-induced inflammation involving degranulation of mast cells (Dinges et al., 2000). Mast cells in the GIT were indeed shown to play an important role in SE-mediated emesis as demonstrated by the implication of various inflammatory mediators normally released by mast cells upon degranulation. These mediators include histamine, serotonin (5-HT), prostaglandine E_2, cysteinyl leukotriene and 5-hydroxyeicosatetraenoic acid (Scheuber et al., 1987; Jett et al., 1990; Dinges et al., 2000; Ono et al., 2012). The emetic activity was indeed completely abolished or drastically reduced when antagonistic substances to these mediators were co-administered to animals along with SEs, thereby confirming the role of mast cells in the development SFP symptoms. For example, the

emetic response to SEB was inhibited by H_2 (a histamine receptor antagonist) and calcium channel-blockers which also block the release of histamine (Scheuber et al., 1985). The implication of mast cells in the emetic response to SE stimuli was also substantiated by immediate-type skin reaction through degranulation of cutaneous mast cells upon intradermal injection of SEB into monkeys (Scheuber et al., 1985). Komisar et al., (1992) further demonstrated that the degranulation of murine mast cells *in vitro* by SEB releases 5-HT (serotonin) in a dose-dependent manner with observable effects, starting from 50 $\mu g\ mL^{-1}$ of SEB. However, there is a controversy as to whether SEs act directly or indirectly on mast cells to drive their degranulation with a consequent generation of inflammatory mediators. Alber et al., (1989) found no evidence on a direct link between SEs and the degranulation mast cells in monkey with subsequent release of pro-inflammatory mediators. On the contrary, an increased body of evidence confirms that SEs cause the degranulation of mast cells in the GIT by direct binding. For example, Ono et al., (2012) used different immunostaining techniques and demonstrated that SEA binds directly to intestinal mast cells causing their degranulation with subsequent release of 5-HT.

At present, there is a consensus to consider 5-HT as the main neurotransmitter involved in SE-mediated emesis; yet, conflicting data are available regarding the exact type and location of the cells responsible for its release in response to an SE stimulus. Enterochromaffin (EC) cells, neuronal cells, and mucosal and sub-mucosal mast cells in the GIT contain 5-HT, and they all are possible targets for SE-induced activation. Hu et al., (2007) showed that SE causes the release of 5-HT from enterochromaffin cells in the mucosa and from the myenteric plexus neurons. However, for Ono et al., (2012), 5-HT is released from mast cells of the submucosal layer of the

small intestine rather than from the epithelial enterochromaffin cells. The latter study conducted on home musk shrew, showed that SEA transcytose from the mucosal epithelium in the GIT lumen to the submucosa where it accumulates and binds to mast cells. The binding of SEA to submucosal mast cells induces their degranulation with subsequent release of 5-HT which, in turn, binds to 5-HT3 receptors of the vagal afferent nerve causing its activation to eventually stimulate the emetic centre of the brain (Fig. 7). Although the SEA receptor on the surface of submucosal mast cells remains unknown, the latter study indicated that (i) this receptor is not MHCII, and (ii) SEA preferably binds to the putative SEA-receptor of mast cells rather than to MHCII of the APC.

These findings suggest that orally administrated or ingested SEA is more likely to cause SFP than TSS. Nevertheless, given the present state of knowledge and the controversial results available on this issue, further studies are needed before a mechanism is widely accepted and validated, and a relationship between the emetic and superantigen activities is definitely established.

As for the functional characterisation of SEs regions with regard to emesis induction, intensive studies have been done to relate specific fragments or amino acids to this hallmark SFP symptom. Although the relationship between structural components of SEs and their biological function as causative agents of SFP remains unclear, it is generally admitted that the emetic and the superantigen activities reside in separate domains of SEs (Spero et al., 1975; Spero and Morlock 1978; Harris et al., 1993b; Hoffman et al., 1996). The physical separation of these two biological functions was first demonstrated in SEB which retained it mitogenic function despite

the abrogation of the emetic activity by formaldehyde treatment (Spero et al., 1975). Subsequently, mutation studies showed that modified SEA and SEB with suppressed mitogenic activity could still elicit emesis in monkeys (Harris et al., 1993a). Conversely, other studies have demonstrated that the emetic and superantigen activities are correlated, as SEA with suppressed superantigenicity failed to evoke emesis in monkeys (Hoffman et al., 1996; Hu et al., 2009). This may be explained by the fact that among the amino acid residues involved in the functional activities of SEs, some have a dual functionality for being involved in the emetic and superantigen activities (Hoffman et al., 1996). The substitution of such specific residues results in the loss of both biological activities, whereas the substitution of residues involved in either emesis or superantigenicity can only supress the activity in which they play crucial role.

The emetic activity of SEs has been thought to be primarily related to the cystine loop located at the N-terminal region of SE molecules, as indicated by the lack of this loop in the SElL and SElQ, generally considered to be the prototypes of non-emetic SEls. However, it is now evident that other newly discovered SEs such as SER, SES, SET, and SEI do not possess the loop and can still elicit emesis in primates (Table 3). In addition, the recent study of Omoe et al., (2013) is challenging the prevailing evidence that SElK-Q, all devoid of cystine loop, are non-emetic. Conversely, streptococcal pyrogenic enterotoxin A (SPEA) belonging to the same structural group of superantigens as SEB-D possess a cystine loop but dot not possess emetic activity (Hovde et al., 1994; Schlievert et al., 2000; Spaulding et al., 2013). Studies on the role of the cystine loop in emesis have established that despite its usefulness to stabilise the conformation of SE molecules for a maximum activity, it is neither necessary nor sufficient for an SE to mediate emesis (Spero and Morlock 1978; Grossman et al., 1990; Hovde et al., 1994;

Spaulding et al., 2013). The substitution of one or both cysteine residues for serine in SEC to disrupt the cysteine loop, did not abrogate emesis, whereas the substitution of cysteine residues of the same SEC molecule for alanine produced mutants deficient in emesis. This was explained by the ability of serine hydrogen bonds to stabilize the critical loop structure as in the case of the disulphide bond (Hovde et al., 1994). Therefore, it is the tertiary structure rather than the disulphide bond itself that would be responsible for emesis induction.

In an effort to define the role of specific amino acids in emesis, early studies revealed that histidine residues are crucial for this biological function of SEs, as demonstrated by the complete loss of the emetic activity of SEA and SEB upon inactivation of all their histidine residues by carboxymethylation (Stelma and Bergdoll 1982; Alber et al., 1990). Subsequently, series of site-directed mutagenesis studies attempted to either confirm the role of histidine residues or demonstrate the implication of other residues in emesis. Hoffman et al., (1996) confirmed the role of histidine in emesis and, also, demonstrate its importance in superantigen activity depending on its position within the molecule; at position 61 (H61), this residue plays a role in emesis only, while at position 225 (H225) it is critical for both emetic and superantigen activities. This was demonstrated by the replacement of histidine residue at positions 61 (H61) by either alanine or aspartic acid and the modified SEA molecules (SEA-H61A or SEA-H61D) were defective in emesis but still able to illicit mitogenic activity towards T-cells (Hoffman et al., 1996). On the other hand, a modified SEA where histidine at position 225 was replaced by alanine (SEA-H225A) had impaired emetic and superantigen activities.

Other amino acid residues than histidine were shown to mediate emesis by SEs. According to Harris et al., (1993a), either phenylalanine at position 47 (F47) or cysteine at position 106 (C106) is essential for SEA to cause emesis. These authors demonstrated that modified SEA molecules (SEA-F47G and SEA-C106A) obtained by the substitution of phenylalanine and cysteine for glycine or alanine, respectively failed to induce emesis in monkeys challenged intragastrically with as a high dose as 375 μg per animal. The same study showed that aspartic acid at positions 9 (D9) and asparagine at position 23 (N23) are crucial for the emetic activity of SEB. Furthermore, the substitution of D227 for alanine in SEA has supressed both emesis and superantigenicity indicating that this residue plays a dual role in the toxicity of this SE (Hu et al., 2009). These findings suggest that even though the emetic and superantigen activities can be dissociated from one another in the SE molecule, as is widely admitted, overlapping residues may have a dual functionality and be essential for the superantigenicity as well as emesis regardless of their position within the SE molecule (Table 7). It appears, therefore, that the precise amino acid residue(s) responsible for the function and its/their exact role in mediating emesis is not fully elucidated and may vary among SEs. This is another limitation that needs to be clarified for practical applications such as the design of therapeutic (Dutta et al., 2015) or prevention means (e.g., vaccine development) (de Carvalho Uhl et al., 2004), or even be used in cancer treatment as was suggested for SEA/SEE chimera (Borghaei et al., 2009; Forsberg et al., 2010; Hedlund et al., 2013).

SE-Mediated Diarrhoea

Few works have been done to elucidate the molecular mechanism by which SEs cause diarrhoea, since this symptom has not been systematically associated with SFP, although frequently reported (Asao et al., 2003). Nevertheless, it is generally admitted that SEs would act in a similar way as the cholera toxin (CT) to induce diarrhoea, as they also were shown to reduce sodium flux out of the intestinal lumen of dogs (Elias and Shields 1976). Such an effect results from the stimulation of adenylate cyclase enzyme which becomes continually active upon bonding of the toxin to GM1 gangliosides on the enterocytes of the intestinal mucosa cells making them overproduce cyclic AMP (cAMP). In turn, cAMP activates the protein kinase A that causes enterocytes to excrete abundantly chloride ions (Cl^-) into the intestine (De Haan and Hirst 2004). Electrolytes, such as Na^+, and water are pumped inside the lumen because of the osmotic and electric gradients created by the excretion of Cl^- thereby inducing perfuse diarrhoea and ultimately dehydration; the typical symptoms of cholera disease (Asakura and Yoshioka 1994) which can also occur in some cases of SFP (Denison 1936a; Mead et al., 1999). Although diarrhoea is not a typical symptom of SFP, it has been reported to be the main cause of death in susceptible individuals (infants, elderly and persons affected by an underlying illness) suffering from SFP (Hennekinne et al., 2012). As matter of fact, diarrhoea was more frequently recorded in the SFP outbreaks in Japan than emesis (Asao et al., 2003). However, the exact mechanism of diarrhoea induction by SEs is not yet fully investigated to provide a direct evidence for this mode of action. Therefore, any comparison between these toxins and SEs in this regard remains a mere speculation, and alternative mechanisms such as a direct action on mucosal transport as well as

an alteration of the blood flow and gastrointestinal motility have been suggested (Elias and Shields 1976) and this warrants due attention.

CONCLUSION

Foodborne intoxications caused by toxins of bacterial origin continue to raise concern to the public health worldwide. Among these, SEs are the most frequent aetiologies of dairy-borne intoxications causing substantial losses in national and international economies. However, their actual incidence and impact on human health remain overlooked due to the lack of appropriate surveillance programs and accurate assessment methodologies to estimate the risk associated with them as standalone hazards. The great diversity of SEs/SEls in terms of amino acid sequences, mode of action, genetic, immunological activities, and interactions with the food matrix and environmental parameters add to difficulties in assessing accurately their risks and hamper the implementation of efficient control measures. Despite the undeniable scientific progress and the significant advances in many aspects related to some SEs/SEls, much remains to be done before achieving enough knowledge to design efficient preventive and/or therapeutic means to overcome these intoxications. The shortage in scientific information is particularly evident for the new SEs and SEls whose epidemiology and incidence in dairy products is poorly documented. Yet, the coding genes of many new SEs/SEls have frequently been detected in staphylococci implicated in dairy-borne SFP. In addition, the causal relationship between some of these SEs/SEls and SFP episodes has been reported. The design of detection methods for as many SEs/SEls as possible appears to be crucial for an accurate estimate of the overall incidence of these toxins in dairy products. This will allow official food safety authorities establish

adequate surveillance programs and perform meaningful assessment studies that would ultimately help taking appropriate control actions. It is also of paramount importance to conduct pertinent epidemiological studies in order to settle the question of whether or not the SEs/SEls can cause TSS via foods. Since TSS is generally far more severe and devastating than SFP, this will certainly impact the perception by consumers and stakeholders of food contamination with SEs/SEls.

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Table 1: Resistance of SEs/SEls to physico-chemical treatments.

Toxin	Stability of toxins			
	Heat[1]	pH	Irradiation (kGy)	Proteinases and denaturing agents
SEs/SEls	SEA:	2.0 - 10.0[2]	> 30	- Resistant to pepsine, chymotrypsine, trypsin, rennin, papain (SEB is destroyed by pepsin at pH 2 but not at higher pH values)
	– 121°C for 28 min			
	–130°C for 4 sec[3]			- Denatured by urea and guanidine hydrochloride
	SEB:			
	–100°C for 3 h			
	–121°C for 30 min			
	–100°C for 5 min[4]			
	SEC			
	–100°C for 3 h			
	–121°C for 30 min			

[1] These values vary widely according to the nature and purity of the SE as well as the composition and pH of the food or laboratory media used for testing thermal resistance. Protein-rich foods such as dairy products were reported to be more protective to SEs. However, it is generally admitted that the thermal processes commonly used in the food industry do not destroy completely SEs, and that the toxins may retrieve up to 100% of their initial activity depending on the temperature conditions after the heat treatment (Fung et al., 1973; Hennekinne et al., 2012)

[2] Stable for several days to more than a week at room temperature (22 -- 25°C); this stability increases with decreasing temperature and *vice-versa* (Schantz et al., 1965). In cheddar and Colby cheeses, SEA persisted for more than 3 years at pH values varying from 5.2 to 6.2 when stored at 4.4°C (Tatini et al., 1971)

[3] Biological activity retained after this treatment (Asao et al., 2003)

[4] 50% loss of the initial activity (Schantz et al., 1965)

Data compiled from (Schantz et al., 1965; Fung et al., 1973; Tatini 1976; Anderson et al., 1996; Asao et al., 2003; Le Loir et al., 2003; Hennekinne et al., 2012; Rajkovic 2014; Liu 2015)

Table 2: Health disorders and symptoms caused by preformed bacterial toxins in humans with relevance to dairy products

Toxin	Threshold toxic dose/person	Disease	Time to onset	Duration for recovery (h)	Typical symptoms	Site of action	References
SE	94-200 ng[1] See text for more details	Staphylococcal food poisoning syndrome (SFP)	30 min - 8 h	24 - 48 (self-limiting) Often does not require hospitalisation, and rarely leads to death in susceptible persons, mainly elderly.	Typical: Nausea, vomiting, abdominal cramps, occasionally fever and diarrhoea (generally as the last established symptom), and intestinal inflammation Severe cases: In addition to the typical symptoms; dizziness, shivering and general weakness and moderate fever.	Small intestine	(Denison 1936b; Wieneke et al., 1993; Ostyn et al., 2010; Hennekinne et al., 2012)

					Extremely severe cases: In addition to the typical symptoms; headaches, prostration, low blood pressure, collapse, and fainting or shock		
		Toxic shock syndrome (TSS)[2]	Within minutes or hours depending on the dose	May lead to death within hours or require hospitalisation	High fever, hypotension, oedema, diffuse erythematous rash, convalescent desquamation (palms and soles), nausea, vomiting, diarrhoea, and dysfunction of three or more organ systems, frequently including kidneys and/or lungs. Possibly death	Immune system (systemic)	(Pinchuk et al., 2010)
				High fatality rate in severe cases (high doses or susceptible individuals such as children and elderly)			

[1]Other doses are available in the literature and a dose as low as 17 ng was reported to cause food poisoning in children under 10 years old of age (Asao et al., 2003), see text for further details

[2]No evidence for its association with foodborne intoxication

Table 3: Properties of staphylococcal superantigen proteins having enterotoxin activities. For molecular weights (MW), number of residues (NR) and cystein residues involved in S-S bonds, only mature molecules of SEs/SEls were considered, unless otherwise indicated in the footnotes.

	Emesis induction (μg per animal)[1]	MW (kDa)	NR	S-S bond (Residues involved)	Health significance	Genetic determinant[2]	MHCII binding site[3]
Confirmed SE							
SEA	25	27.1	233	96-106	Major role in SFP	ΦSa3ms, ΦSa3mw, Φ252B, ΦNM3, ΦMu50A, Φ Mu3A	L and H
SEB	100	28.4	239	93-113	SFP-TSS[4]	pZA10, SaPI3	L
SEC/SEC1	5	27.5	239	93-110	SFP-TSS[4]	SaPIn1, SaPIm1, SaPImw2, SaPIbov1	L
SEC2	1000[5]	27.5	239	93-110	SFP-TSS[4]	SaPI3, SaPIm1, SaPIn1	L
SEC3	<50	27.6	239	93-110	SFP-TSS[4]	SaPI3, SaPIm1, SaPIn1	L
SEC-bovine	NA	27.6	239	93-110	Specific for bovine	SaPIbov	L
SEC-ovine	NA	27.0	239	93-110	Specific for ovine	SaPIov	L

SED	40[5]	26.9	233	96-106	SFP	pIB485	L and H
SEE	10[5]	26.4	230	93-103	SFP	ΦSa (hypothetical)	L and H
SEG	160--320	27.0	233	91-107	Minor role in SFP	*egc1*; *egc2*; *egc3*; egc4, ΦSa3ms[6]	L
SEH	30	25.1	217	82-92	SFP	Putative transposon next to scc (*she/Δseo*)	L and H
SEI	300-600	25.1	216	None	Minor role in SFP	*egc1*, *egc2*, *egc3*, ExPΦ (from NRS26 strain)	L and H
SER	<100	27.0	233	None	NA	pIB485-like; pF5	L and H
SES	<100	26.2	227	None	NA	pF5	L and H
SET	<100	22.6	189	None	NA	pF5	L and H
Confirmed SEl							
SElL	100[7]	24.7	216	None	NA	pF5, SaPIm1, SapIn1, SaPImw2, SapIbov1	L and H
SElQ	100[7]	25.1	216	None	NA	SaPI1, SaPI3, SaPI5, ΦSa3mw, Φ	L and H

						Sa3ms	
Provisional SEl					NA		
SElJ	NT	28.2	242	94-104	NA	pIB485-like; pF5	L and H
SElK	100[7]	25.4	219	None	NA	SaPI1, SaPI3, SaPI5, Φ Sa3ms[6], Φ Sa3mw[6]	L and H
SElM	100[7]	24.8	217	None	NA	*egc1*, *egc2*, *egc3*	L and H
SElN	100[7]	26.1	227	92-102	NA	*egc1*, *egc2*, *egc3*, *egc4*	L and H
SElO	100[7]	26.8	232	98-108	NA	*egc1*, *egc2*, *egc3*, *egc4*, transposon (*seh/Δseo*) adjacent to *ccm*	L and H
SElP	100[7,8]	26.7	230	93-103	NA	ΦN315[9], ΦMu3A, ExPΦ (ΦBU01 from NRS19 strain)	L and H
SElU	NT	28.6	244	94-113	NA	*egc2*, egc3	L
SElU2	NT	23.7	202	None	NA	*egc4*	L
SElV	NT	25	217	None	NA	*egc4*	L and H
SELW[10]	NT	23.2[11]	203[11]	None	NA	Core chromosome	NA

SElY	500[12]	22.5	192	None	SFP	?	?
SElX[13]	NT	19.4 (23.3)[11]	168 (203)[11]	None	NA	Core chromosome	L

NA: not available (no clinical records available on their implication in SFP or TSS, although all SEs/SEls were experimentally proven to act at least as superantigens in laboratory animals)

NT: Not tested yet

N: Negative for emesis in primate models

SaPI: *Staphylococcus aureus* pathogenicity island

egc: Enterotoxin gene cluster

scc: Staphylococcal cassette chromosome; gene coding methicillin resistance

ExPΦ: Extra-chromosomal phage

[?]Unknown

[1]The test animal is a primate unless stated otherwise

[2]For the exact location of the genetic element, see (Argudin et al., 2010)

[3]L: Low-affinity □-chain binding-site; H: High-affinity □-chain binding-site (Spaulding et al., 2013)

[4]Reported to be implicated in TSS incidents by non-oral routes (Bohach et al., 1990; Wang et al., 2009)

[5]Intraperitoneal administration in house musk shrew *Suncus murinus* (Ono et al., 2008)

[6]Prophage bearing SE variant

[7]Pending change nomenclature to SEs instead of SEl as suggested by Omoe et al., (2013) following demonstration of their ability to induce emesis in monkeys (*Macaca fascicularis*) at 100 □g per animal (see Table 4 for detailed results)

[8]Emetic in in the house musk shrew starting from a dose of 50 □g/animal (Omoe et al., 2005b)

[9]Also designated ΦSa3n

[10]Previously proposed as an alternative designation for SElU2 (Collery and Smyth 2007)

[11]estimated length and MW of the precursor

[12]Oral and Intraperitoneal administration in house musk shrew (Ono et al., 2015)

[13]The smaller size of this SEl compared with the other SEs/SELs is probably due to its truncated B domain (Wilson et al., 2011).

Data compiled from (Hudson et al., 1995; Munson et al., 1998; Kuroda et al., 2001; Omoe et al., 2003; Sumby and Waldor 2003; Omoe et al., 2005a; Fraser and Proft 2008; Argudin et al., 2010; Lindsay 2011; Schelin et al., 2011; Hennekinne et al., 2012; Okumura et al., 2012; Spaulding et al., 2013; Hu and Nakane 2014), or generated from UniProt knowledgebase (UniProtKB) website (http://www.uniprot.org).

Table 4: Emetic activity testing of SEls in monkeys (*Macaca fascicularis*) using SEA and SEB as controls. Adapted from Omoe et al., (2013)

SEl	Dose (µg/kg)	Positive responses/Number of monkeys tested (% positive)
SEA	10	5/10 (50)
	100	6/7 (86)
SEB	100	4/4 (100)
SElK	100	2/6 (33)
SElL	100	1/6 (17)
SElM	100	1/7 (14)
SElN	100	2/6 (33)
SElO	100	1/8 (13)
SElP	100	2/6 (33)
SElQ	100	2/6 (33)

Table 5 : Pairwise identities (%) between SEs/SEls (mature molecules) were calculated by using the clustal omega alignment program (Sievers et al., 2011) available at the Uniprot knowledgebase website (http://www.uniprot.org). The percent identity with superscript was taken from the orginal publication. NB: Precent identities may differ from those of previous publications [(Balaban and Rasooly (2000); Le Loir et al., (2003); Cunha and Calsolari (2008)] due to differences in the algorithms used to determine SE identities and to the specific SE sequence of the strains deposited in the databank. Nonetheless, these variations generally remain within a small range and do not affect SE classifications on the basis of sequence identities.

SE/SEl	SEA	SEB	SEC	SED	SEE	SEG	SEH	SEI	SEJ	SElK	SElL	SElM
SEA	100	35	32	52	82	31	37	41	66	35	34	36
SEB		100	67	36	34	45	35	31	33	31	30	32
SEC			100	33	33	43	30	34	30	28	28	30
SED				100	55	28	35	38	55	38	41	42
SEE					100	32	38	38	65	32	35	37
SEG						100	34	29	31	28	28	28
SEH							100	32	35	31	31	32
SEI								100	37	59	59	64
SEJ									100	36	33	37
SElK										100	61	59
SElL											100	56
SEM												100

Table 5 : (Continued …)

SE/SEl	SElN	SElO	SElP	SElQ	SER	SES	SET	SElU	SElV	SElX	SElY

SEA	41	40	78[1]	40	33	41	22	34	37	32	27
SEB	34	37	35	32	47	34	27	56	33	31	30
SEC	30	35	32	35	43	32	21	57	27	44	23
SED	41	41	52	37	30	34	32	30	38	31	30
SEE	41	40	76[1]	38	30	41	29	32	37	26	24
SEG	31	32	31	30	61	35	26	39	28	56	28
SEH	47	36	37	30	33	38	26	31	33	31	26
SEI	31	37	41	97	31	38	31	31	62	28	25
SEJ	43	36	37	31	33	38	26	31	33	31	23
SElK	64	35	35	59	32	31	25	32	63	39	22
SElL	30	36	34	59	30	38	30	31	58	35	25
SElM	33	32	37	63	31	31	21	32	87	39	24
SElN	100	45	41	31	32	52	28	31	31	47	21
SElO		100	40	33	40	47	37	35	34	50	26
SElP			100	41	33	41	22	34	37	32	27
SElQ				100	32	39	31	32	62	28	29
SER					100	37	26	44	31	50	30
SES						100	24	32	32	42	23
SET							100	26	23	26	38
SElU								100	32	28	33
SElV									100	31	25
SElX										100	31
SElY											100

[1] After Omoe et al., (2005b)

Table 6: Grouping of staphylococcal and streptococcal superantigens[1] based on structural features (Adapted from Spaulding et al., 2013).

Group	Superantigen	Distinctive features
I	TSST-1, TSST-ovine, SElX, SElY[2],	- Unique amino acid sequence - Absence of cystine loop
II	SEB, SEC1-3, SEG, SElU, SElU2, SPEA, SSA	- Presence of cystine loop with a variable length (10 to 19 amino acid span between the two cystein residues)
III	SEA, SED, SEE, SEH, SElJ, SElN, SElO, SElP	- Presence of cystine loop of 9 amino acid span between the two cystein residues
		- Presence of a zinc-binding domain (H-site) in addition
		to the L-site
		- Restricted to staphylococcal SAgs
		- The main causative agents of SFP
IV	SPEC, SPEG, SPEJ, SMEZ	- Absence of a cystine loop
		- Restricted to streptococcal SAgs
V	SEI, SElK, SElL, SElM, SElQ, SER, SES, SET, SElV, SPEH	Mostly new SE/SEls with a characteristic α3-β8 loop insertion[3]

SPE: Streptococcal pyrogenic exotoxin (streptococcal superantigen)

SMEZ: Streptococcal mitogenic exotoxin Z

SSA: Streptococcal superantigen A

L: Low-affinity binding site to the α-chain of MHCII

H: High-affinity binding site to the β-chain of MCHII

[1]Non-group A streptococcal enterotoxins are not included

[2]Recently added to this group (Ono et al., 2015)

[3]An extra 15-amino acid insert between the third α-helix and the β-strand 8, which appears to be critical for the specificity of the superantigens interaction with a set of T-cells, leading to TSS (Brouillard et al., 2007; Gunther et al., 2007; Kang et al., 2015)

Table 7: Regions and amino acid (AA) residues involved in biological functions of staphylococcal enterotoxins (SEs) as reported in different studies.

Peptide or AA involved in SE toxicity	SE /domain	Binding/interacting with:				Biological function		Technique	Reference
		MHC-II		TCR					
		α Chain	β Chain	αV	βV	Super-antigenicity	Emesis		
Peptide fragments[1]									
9-20[2]	SEB/A	NS	NS	NT	NT	+	NT	Competitive inhibition of MHCII binding with synthetic peptides	(Komisar et al., 1994)
30-38	SEB/B	NS	NS	NT	NT	+	NT		
61-70	SEB/B	NS	NS	NT	NT	+	NT		
90-114[3]	SEB/B	NS	NS	NT	NT	+	NT		
169-181	SEB/A	NS	NS	NT	NT	+	NT		
9-23	SEB/A	NS	NS	NT	+	+	NT	Site-directed mutagenesis	(Kappler et al., 1992)
41-53	SEB/B	NS	NS	-	-	+	NT		
60-61	SEB/B	-	-	NT	+	+	NT		
200-207	SEA/A	-	-	NT	+	+	NT	Chimeric constructs of truncated SEs	(Mollick et al., 1993)
200-207	SEE/A	-	-	NT	+	+	NT		
1-70	SEA/A and B	NS	NS	-	-	+	NT		

1-70	SEE/A and B	NS	NS	-	-	+	NT		
1-45	SEA/A and B	NS	NS	NT	NT	+	NT	Competitive inhibition with synthetic peptides	(Pontzer et al., 1990)
1-27	SEA/A	NS	NS	NT	NT	+	NT		
121-149	SEA/A	NS	NS	NT	NT	+	NT	Competitive inhibition with monoclonal antibodies, and induction of TNFα and IL-1 production by monocytes	(Pontzer et al., 1993)
28-45	SEA/B	NS	NS	NT	NT	NT	NT	Competitive inhibition with synthetic peptides and antiserum	(Griggs et al., 1992)
39-66	SEA/B	NS	NS	NT	NT	NT	NT		
62-86[4]	SEA/B	NS	NS	NT	NT	NT	NT		
121-149	SEA/A	NS	NS	NT	NT	NT	NT		
18-58	SEC/B	NT	NT	NT	NT	+	-	Serological studies and in vivo tests for emesis	(Spero and Morlock 1978)
104-239	SEC/A	NS	NS	NS	NS	-	+		
20-34	SEB/B	NS	NS	-	-	+	+	Competition and mitogenesis	(Zhang and Rogers 2013)
206-220	SEB/A	_	-	NT	+	+	NT		

								studies using chimeric constructs of truncated SEs	
Amino acid residues									
F44	SEB/B	NS	NS	-	-	+	NT	Site-directed mutagenesis and *in vivo* tests on mice	(Kappler et al., 1992)
N23	SEB/A	NS	NS	NT	+	+	+		
N60 and Y61	SEB/B	-	-	NT	+	+	NT		
P203 and D204	SEE/A	-	-	NT	+	+	NT	Chimeric constructs of truncated SEs	(Irwin et al., 1992)
S206 and N207	SEA/A				+	+	NT		
C106	SEA/B	NS	NS	NS	NS	+/-	+	Site-directed mutagenesis	(Harris et al., 1993a)
F47	SEA/B	NS	NS	NS	NS	+	+		
L48	SEA/B	NS	NS	NS	NS	+	-		
N25	SEA/A	NS	NS	NS	NS	+/-	-		
D9/N23	SEB/A	NS	NS	NS	NS	+/-	+		
F44	SEB/B	NS	NS	NS	NS	+	-		
H61	SEA/B	-	-	-	-	-	+	Site-directed mutagenesis	(Hoffman et al., 1996)
H225	SEA/A	NS	NS	NS	NS	+	+		
F47	SEA/A	+	-	NT	NT	+	NT	Site-directed	(Thibodeau et

								mutagenesis	al., 1997)
F47	SEA/B	+	-	-	-	+	NT	Site-directed mutagenesis	(Hudson et al., 1995)
H187, H225 and D227	SEA/A	-	+	-	-	+	NT		
F47, L48 and H50[5]	SEA/B	+	-	-	-	+	NT	Crystallography	(Petersson et al., 2002)
H187, H225, and D227	SEA/A	-	+	-	-	+	NT		
19 residues (see text)	SEE/A and B	NT	NT	-	+	+	NT	Crystallography	(Rodstrom et al., 2015)
H225 and D227	SEE/A	-	+	-	-	+	NT	*In silico* (Computational modelling)	
H142 and Y158	SElK/A	-	-	-	+	+	NT	Crystallography and mutational studies	(Gunther et al., 2007)
H169, H207 and D209	SElK/A	-	+	-	-	+	NT		
D227	SEA/A	NT	NT	NT	NT	+	+	Site-directed	(Hu et al.,

								mutagenesis, and serological and in vivo tests	2009)
H206 and D208	SEA/A	-	+	-	-	+	NT	Crystallography	(Hakansson et al., 2000)
H206 and D208	SEH/A	-	+	-	-	-	NT	Crystallography	(Petersson et al., 2001)

P: Probable implication
NT: Not tested

NS: Not specified (The specific chain MHCII or TCR involved in the interactions with the SE was not determined)

+: Has a crucial role in the activity

-: Has no role in the activity

+/-: Reduction of the activity, but not complete loss

[1] Numbers correspond to the first and last amino acid residues of the peptide in the sequence of the mature SE
[2] Although part of the N-terminal primary sequence, this fragment is folded in the A domain (C-terminal domain) in the tertiary structure
[3] Fragment containing the disulphide loop in SEB
[4] The most effective fragment in binding MHCII
[5] These residues from a ridge as the main contact region of SEA with MHCII; other residues in the vicinity (L39, G46, H50, D70 and G95) contribute to the interactions

Table 8: Documented implication of SEs in dairy-borne SFP outbreaks in selected countries

Pays	Dairy product[1]	Number of outbreaks/Number of cases	SE type	Period or year	References
France	• Unpasteurised milk soft cheese	6/23	E	2009	(Ostyn et al., 2010)
	• Raw milk semi-hard cheese, raw milk soft cheese, soft cheese, sheep's milk cheese, raw milk cheese, raw milk sheep's cheese, chocolate milk, cream, sliced soft cheese	15/228[2]	A and/or D, or B[3]	1981--2002	(Kerouanton et al. 2007)
	Raw milk ewe cheese	1/20	A and D	1983	(De Buyser et al., 1985)
Canada	•Cheese curd	1/62	A and C	1980	(Todd et al., 1981)
UK and Wales	Milk and desserts containing milk or cream, cheeses	28/NA	A[4]	1969-1990	(Wieneke et al., 1993)
Scotland	Ewe cheese (raw milk	1/27	A	1984	(Bone et al., 1989)
Austria	Milk, cacao milk or vanilla milk	1/40	A and D	2007	(Schmid et al., 2009)
Japan	Low-fat	1/ 13 420	A and H	2000	(Asao et al.,

	reconstituted milk				2003; Ikeda et al., 2005)
Brazil	• Cheese	7	H	1994	(Pereira et al., 1996)
	• Minas cheese	1/50	A, B and C	1999	(Carmo et al., 2002)
	• Raw milk	1/328	C and D	1999	

[1]When the origin of milk is not specified, it is either bovine milk or the origin was not provided by the authors

[2]This figure in underestimated, as the number of cases was not known in some of the 15 outbreaks

[3]SEA largely dominating (73% of cases); other SEs (e.g., SEG, SEH and SEI) may have been involved as suggested by the characterisation of the corresponding genes in the strains isolated from the incriminated products

[4]Detected only in Halloumi cheese and raw ewe's milk cheese; no SE could be detected in the other milk products implicated in outbreaks exerting symptoms, and incubation and recovery periods typical of SFP.

Table 9: Most used commercial kits to detect classical SEs in dairy products involved in Staphylococcal food poisoning (SFP) or in bacterial culture of putative staphylococcal enterotoxin (SE)-producing strains of *Staphylococcus* sp.

Test trade name	Target SEs	Technique used	Sensitivity ng g^{-1} or mL^{-1}	Producer/Supplier
Ridascreen® set	A, B, C, D and E	Enzyme-linked immonosorbent assay (ELISA)	0.25/0.38[1]	R-Biopharm, Germany
3M® TECRA® Staph Enterotoxins VIA SETVIA48 (SET A-E)	A, B, C1[2], C2, C3, D and E	ELISA	> 1.0	3M®, USA
3M® TECRA® Staph enterotoxins, Identification Test[3]	A, B, C, D and E	ELISA	1.0	3M®, USA
SET-RPLA Staphylococcal enterotoxins kit	A, B, C and D	Reversed passive latex agglutination (RPLA)	1.0	Oxoid microbiology, UK
Transia® tube staphylococcal enterotoxins	A, B, C1, C2, C3, D and E	ELISA using tubes as solid phase	0.5	Raisio diagnostics Spain
Transia® plate staphylococcal enterotoxins Plus[4]	A, B, C1, C2, C3, D and E	ELISA in microtitre plates	0.25	Raisio diagnostics Spain
TRANSIA® Id	A, B, C, D and E	ELISA in	0.02	Raisio diagnostics

Staphylococcal Enterotoxins		microtitre plates		Spain
TRANSIA® IAC Staphylococcal Enterotoxins	A, B, C, D and E	Immunoaffinity chromatography	0.1	Raisio diagnostics Spain
VIDAS® SET 2	A, B, C, D and E	Enzyme Linked Fluorescent Assay (ELFA)	1.0	Biomerieux France

[1] 0.38 ng g^{-1} is the detection limit when solid food is used (e.g., cheese samples)

[2] SEC1 is the same as SEC

[3] SEs can be specifically identified

[4] Sample preparation adapted to milk and dairy products

Table 10: Detected genes coding for the production of staphylococcal enterotoxins or enterotoxin-like toxins in staphylococcal strains isolated from milk and dairy products, some of which were implicated in staphylococcal food poisoning (SFP) episodes.

Dairy products	Origin of milk	Enterotoxin genes detected	References
Cream cheese	Caprine	*sea*, *sec*, *seg*, *sei*	(Carmo et al., 2002)
Fresh cheese	Bovine (raw milk)	*sea, sec, seg,*	(Akineden et al., 2008)
	Caprine	*sei, seh*	
Monte Veronese (Italy)	Bovine	*sea, seb, sec, sed, seg, seh, sei, sej, sek, sel, sem, seo, sep, ser*	(Rosengren et al., 2010)
Sheep milk cheeses	Ovine	*sea, seb, sed*	(Poli et al., 2007)
Soft cheese (Italy)	NA	*sea*, *sed*, *sej*, *sec*, *sel*, *seg*, *sea*	(Ertas et al., 2010)
Raw-milk fresh unpripened cheese	Bovine	*sed, seg, seh, sei*	(Bernini et al., 2010)
Canastra cheese (Brazil)	Bovine	*sea, seb, sec, sed*	(Little et al., 2008)
Various dairy	NA	*sea, seb*	(Borelli et al., 2006)

products (Brazil)			
Raw milk and raw-milk dairy products (Turkey)	NA	*sea, sec, seh, seo, sep, sem, sen, seu, seg, sei*	(Veras et al., 2008)
Lben and Jben (Morocco)	NA	*sea, seb, sec, sed, seh*	(Aydin et al., 2011)
Minas Frescal (Brazil)	Bovine	*sea, seb, sec, sed, seg, seh, sei, selj, sell*	(Bendahou et al., 2009)
Soft, semi-hard and Hard (Norway)	Bovine	*sec, seb. seg, sei*	(Arcuri et al., 2010)

NA: Not available

Table 11: Main factors affecting the growth of *Staphylococcus aureus* and production of staphylococcal enterotoxin (SE) (Adapted from Loncarevic et al., 2005)

Environmental parameter	Growth		SE production	
	Optimum	Limits	Optimum	Limits
Temperature (°C)	35 -- 41	6--48	34--40	10--46
pH	6--7	A: 4.0--9.8 AN: 4.6-9.8	7--8	A: 4.0--9.6 AN: 5.3-9.6
Water activity	A: 0.83- >0.99 AN: 0.90→0.99	0.83-0.99	0.87- >0.99	A: 0.86-0.99 AN: 0.92→0.99
NaCl (%)	0	0--20	0	20-10
Red/ox potential (Eh, mV)	> +200	-200 - > +200	> +200	-200 - > +200
Atmosphere	Aerobic	Aero-anaerobic	Aerobic (5--	Aero-

			20% dissolved O_2)	anaerobic

A: Under aerobic conditions

AN: Under anaerobic conditions

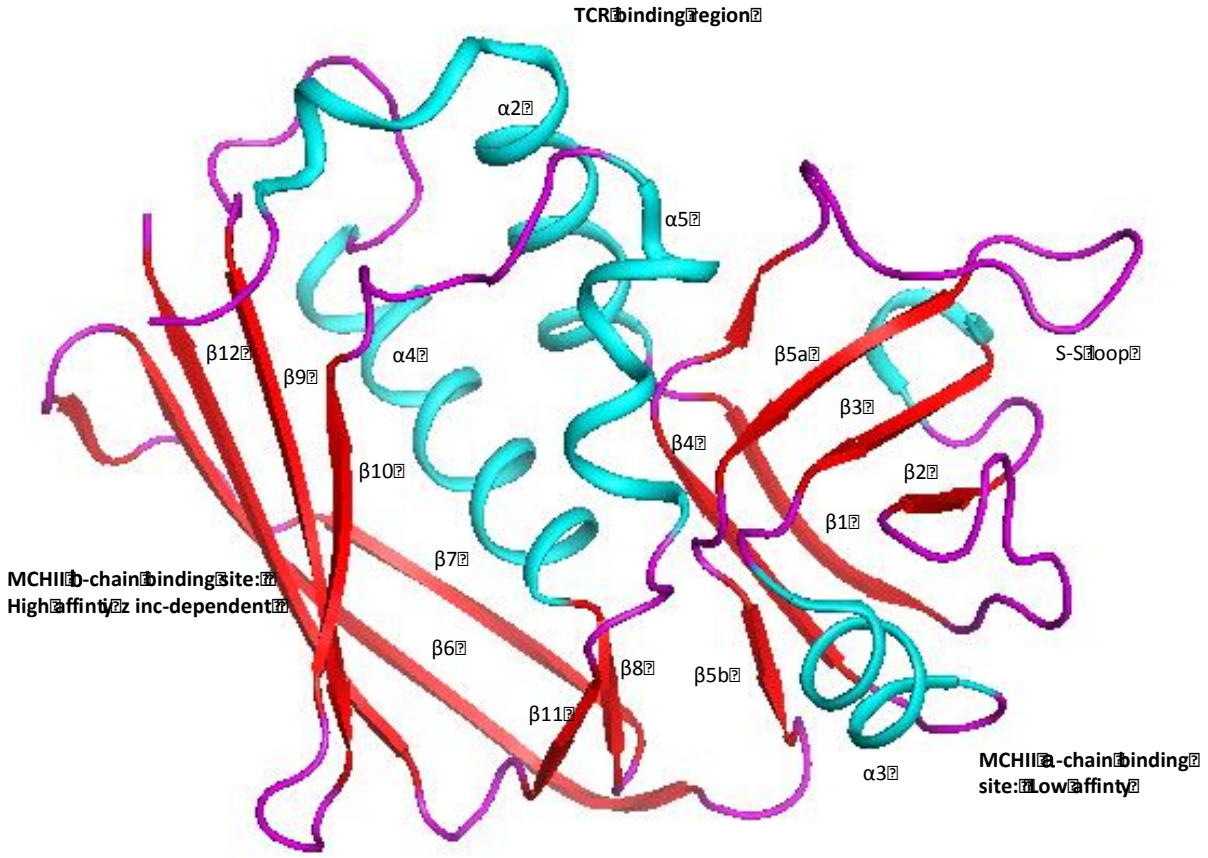
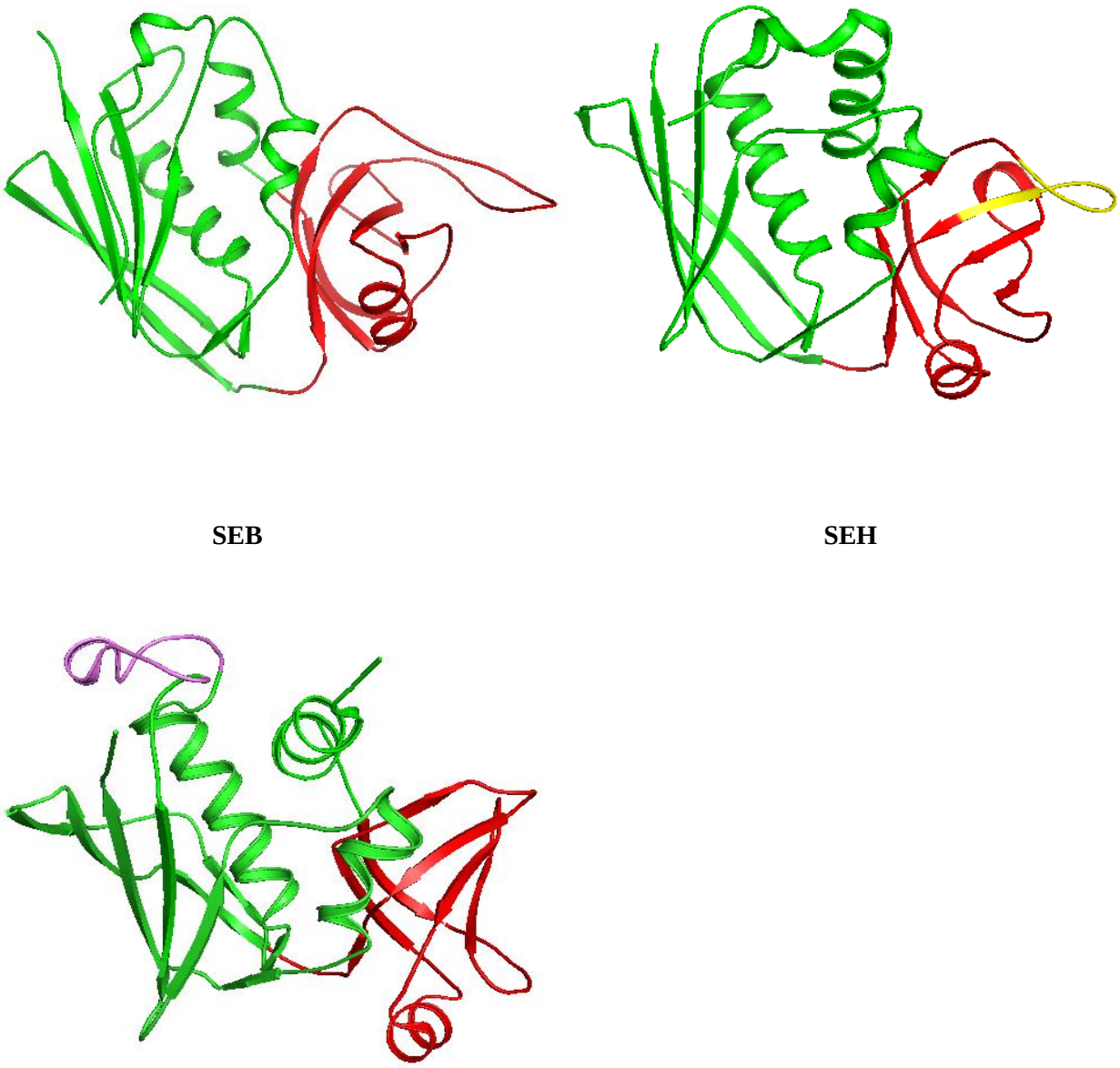


Fig. 1: A cartoon representation of staphylococcal enterotoxin H (SEH) showing the typical structure of SE/SEl with two constitutive domains A and B that characterise superantigens. The larger domain A (left) contains a beta grasp motif and the smaller domain B (right) is a beta barrel-rich structure known as "oligomer-binding fold; OB-fold" as it can bind carbohydrate and nucleic acid oligomers. The beta sheets and alpha helices are shown in red and blue, respectively. The interface between A and B domains consists of a set of α-helices forming a long groove in the backside of the molecule and a shallow cavity at the top. Drawing were made by using PyMol software (Schrodinger 2015)



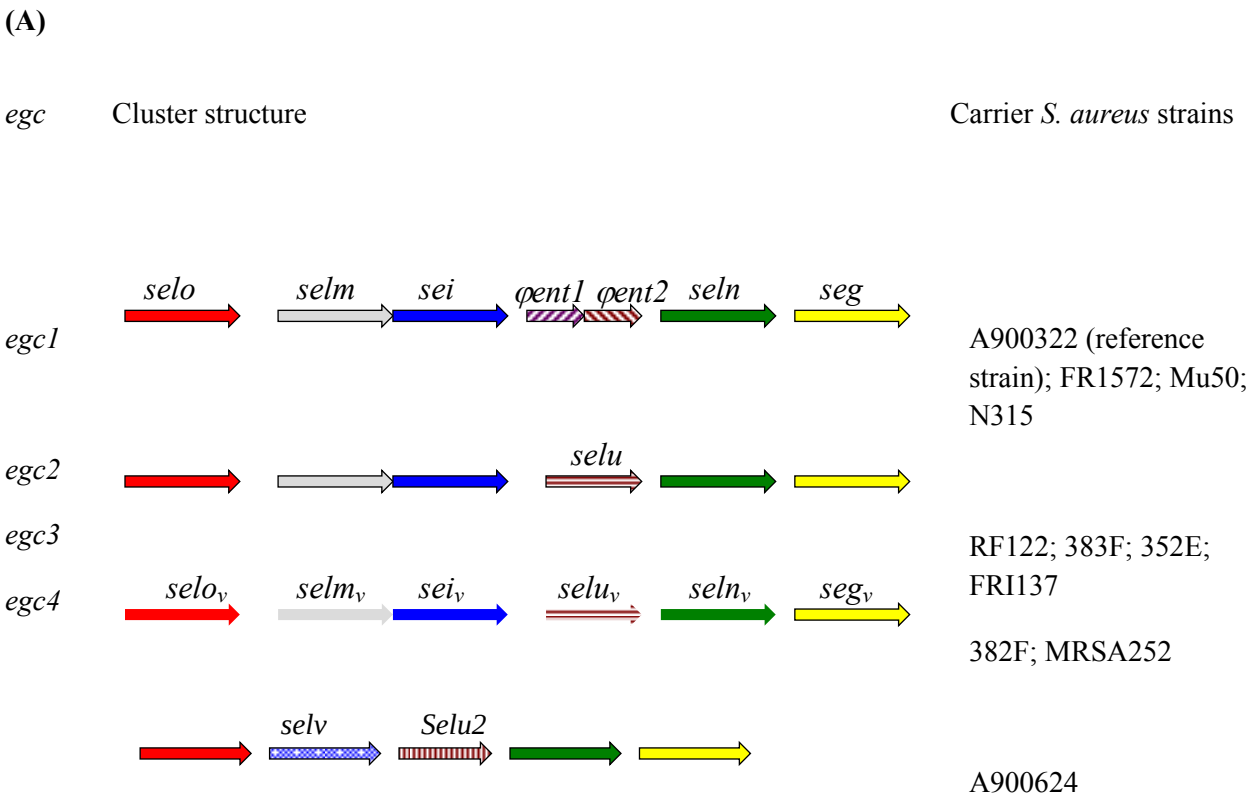
SEB **SEH**

SEIK

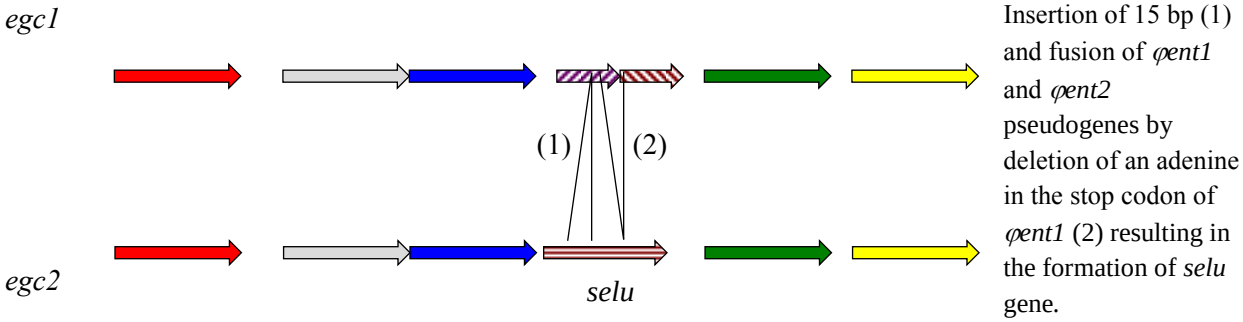
Figure 2: Cartoon structures of SEB, SEH and SElK, each representing an evolutionay group of SEs/SEls showing the main structural distinctive features. The presence of the disulfide loop (yellow) in SEH (representative of group III) and its absence in SEB (reprsenttive of group II)

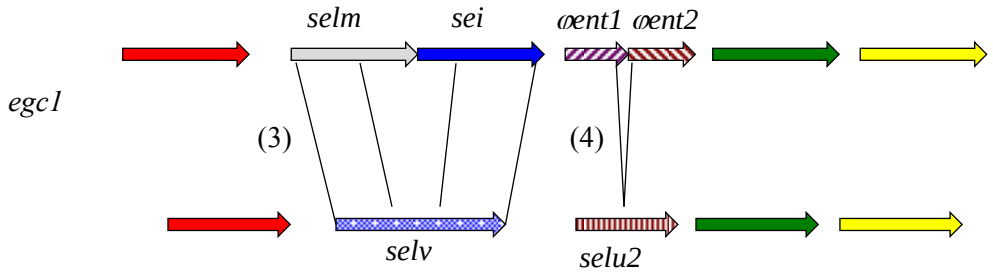
and SElK (representative of group V); the presene of the α3-β8 insert (purple) in SElK and its absence in SEB and SEH. Domains A (C-terminal) and B (N-terminal) are in green and red, respectively. Drawing were made by using PyMol software (Schrodinger 2015)

(A)



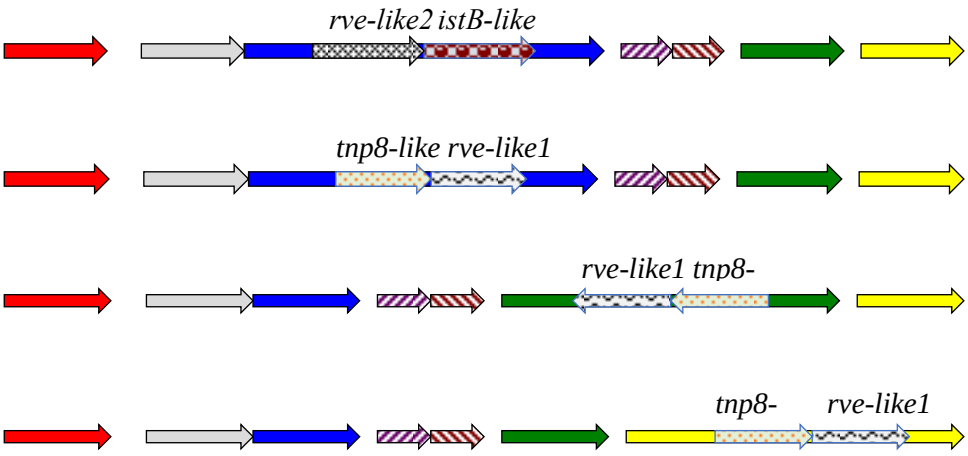
(B)





Recombination between *selm* and *sei* to generate *selv* (3), and deletion of an adenine between the 5' and 3' ends of the *φent1* and *φent2* (4) leading to the fusion of both pseudogenes in one gene, *selu2*.

(C)



Carrier strain of *S. aureus*

A980341

A940440

LY1991287

LY1991222

Fig. 3: Enterotoxin gene cluster organisation and generation of new variant clusters: Inventory of the presently known *egc* operons with the *se*/*sel* genes they carry and the producer strains of *S. aureus*. Elements ***ϕent1 ϕent2*** located in the intergenic region between *sei* and *seln* in *egc1* are not-protein coding pseudogenes. Genes of *egc3* bearing « *v* » indice are allelic variants of the corresponding *se /sel* genes found in *egc2* (Letertre et al., 2003; Collery et al., 2009).(A) Genetic rearrangements of *es/esl* genes within the reference *egc1* leading to the emergence of *egc2* and *egc4* operons.(B) Insertion of transposons in *se/sels* of *egc1* generating evolutionary intermediates upon insertion of each of two IS elements; one inserts in either one of *sei*, *seln* or *seg* genes, and the second inserts in *sei* gene. The first IS, a 757 to 1222-bp DNA fragment consists of two genes (*tpn8-like* and *rve-like1*) coding a transposase 8-like (Tnase 8-like) and integrase-like 1 (Rve-like 1) proteins, respectively and the second IS, a 2122-bp DNA fragment, bearing two genes (*istB* and *rve-like 2*) coding an ATP-binding and integrase-like 2 proteins, respectively (Adapted from Thomas et al., 2006; Collery et al., 2009).

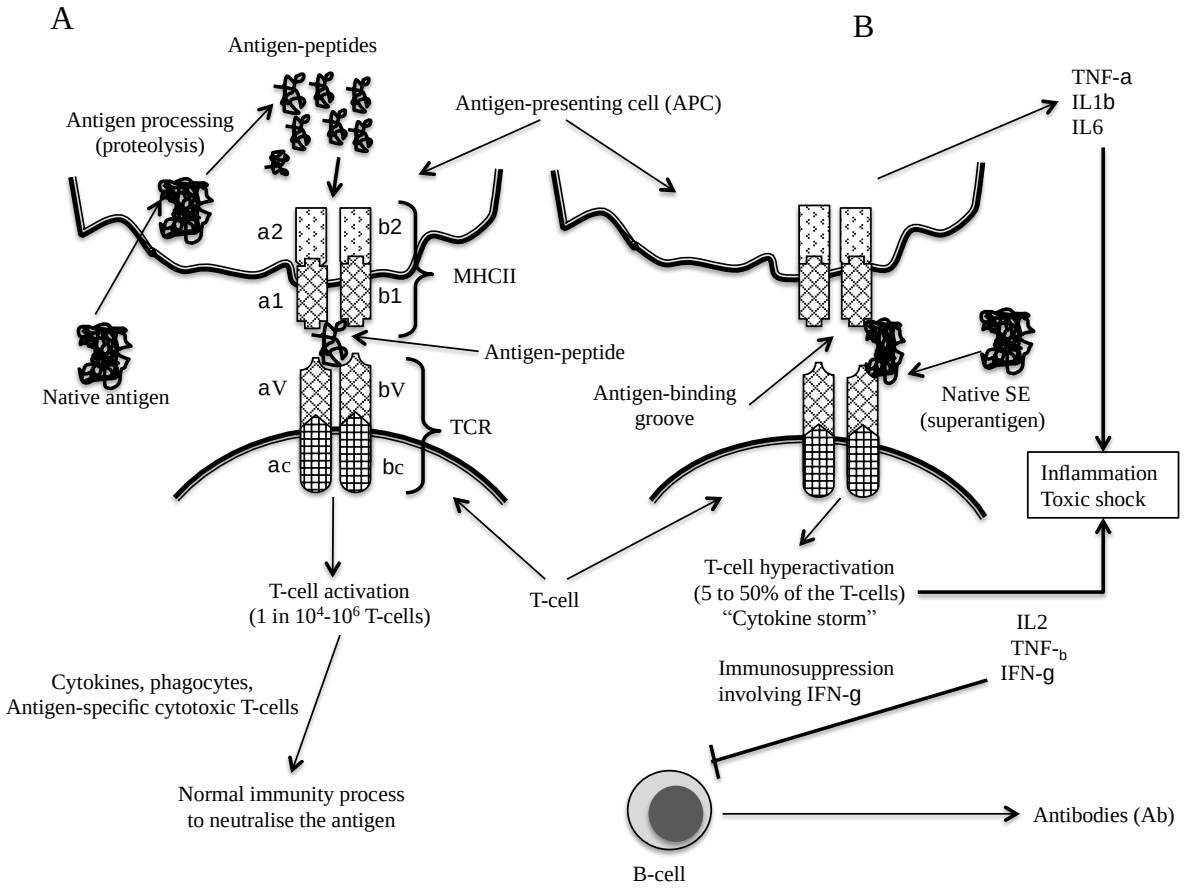
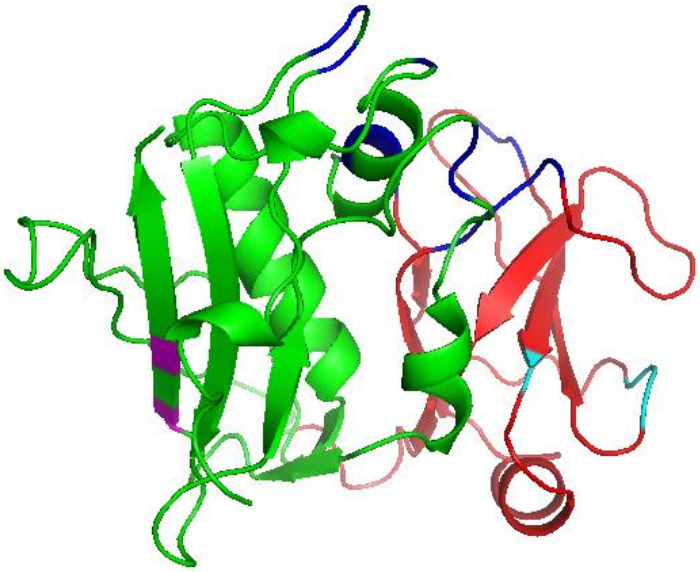
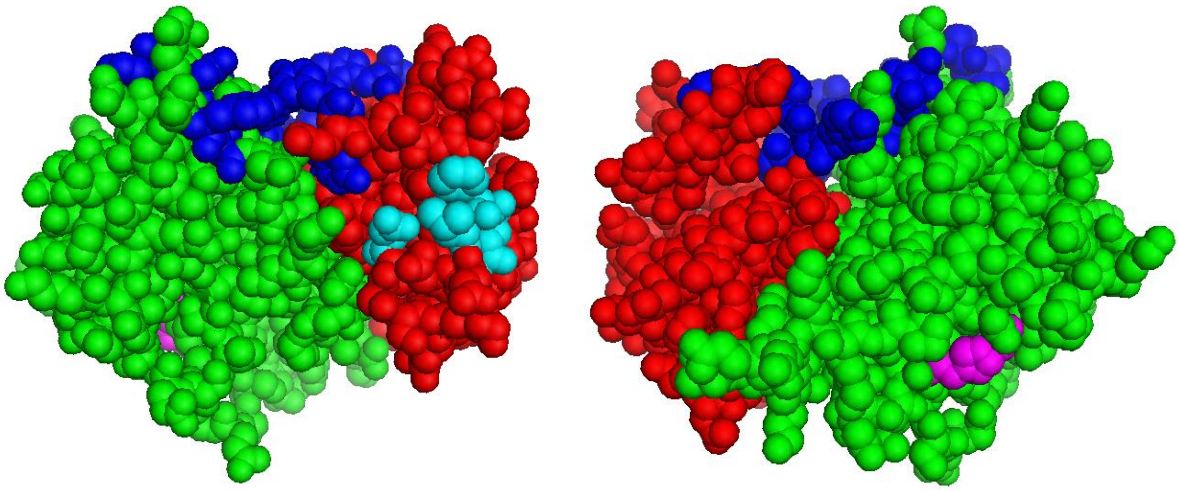


Fig. 4: Model for the mechanism of immunological reaction showing specific (A) and non-specific (B) activation of T-cells in response to the presence of a conventional antigen or superantigen, respectively (For explanation of the mechanisms, see the text). Abbreviations: SE, Staphylococcal Enterotoxin; TCR, T-Cell Receptor; MHCII, Major Histocompatibility Complex II; IL, Interleukin; TNF, Tumor Necrosis Factor; INF, Interferon



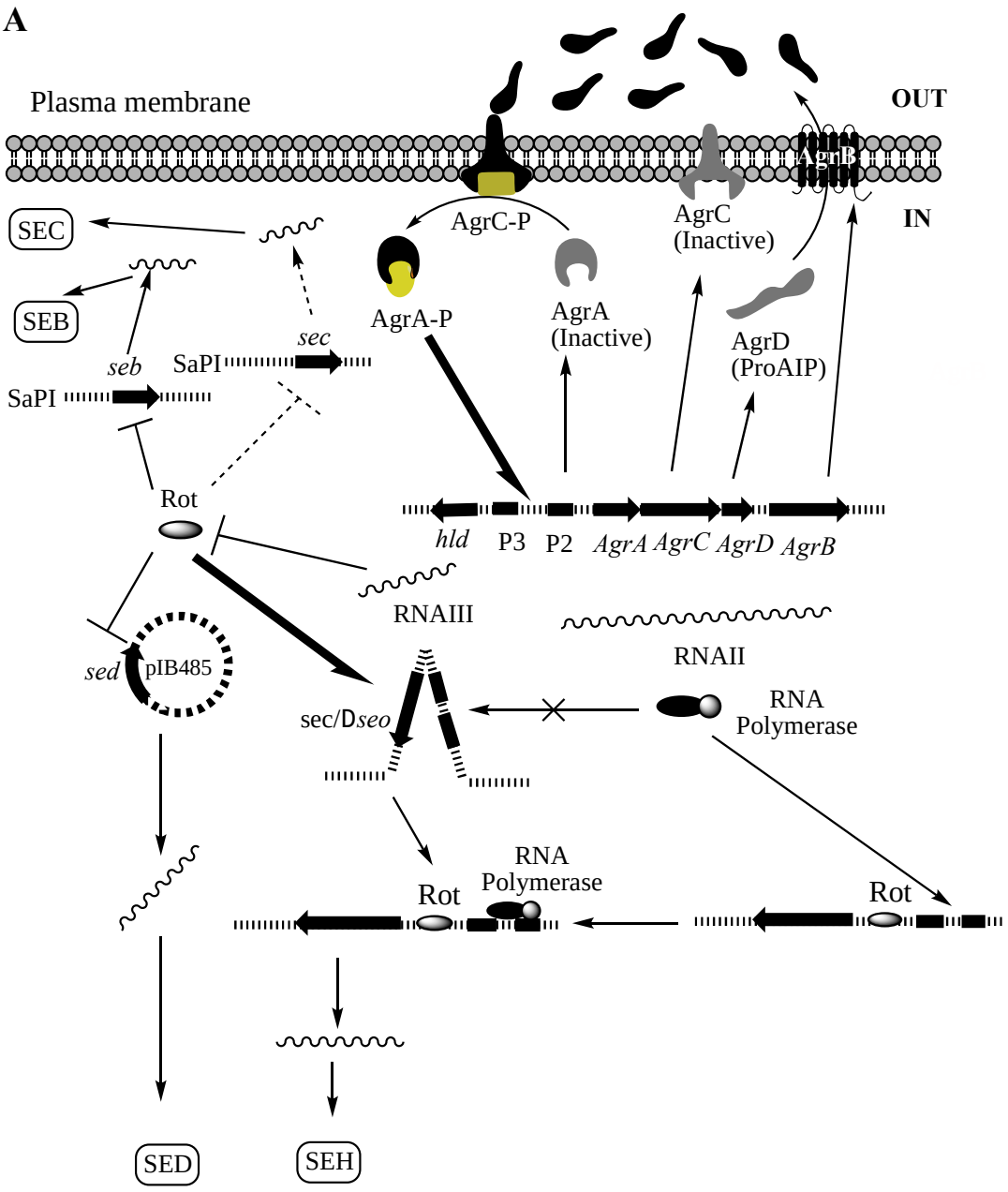
A

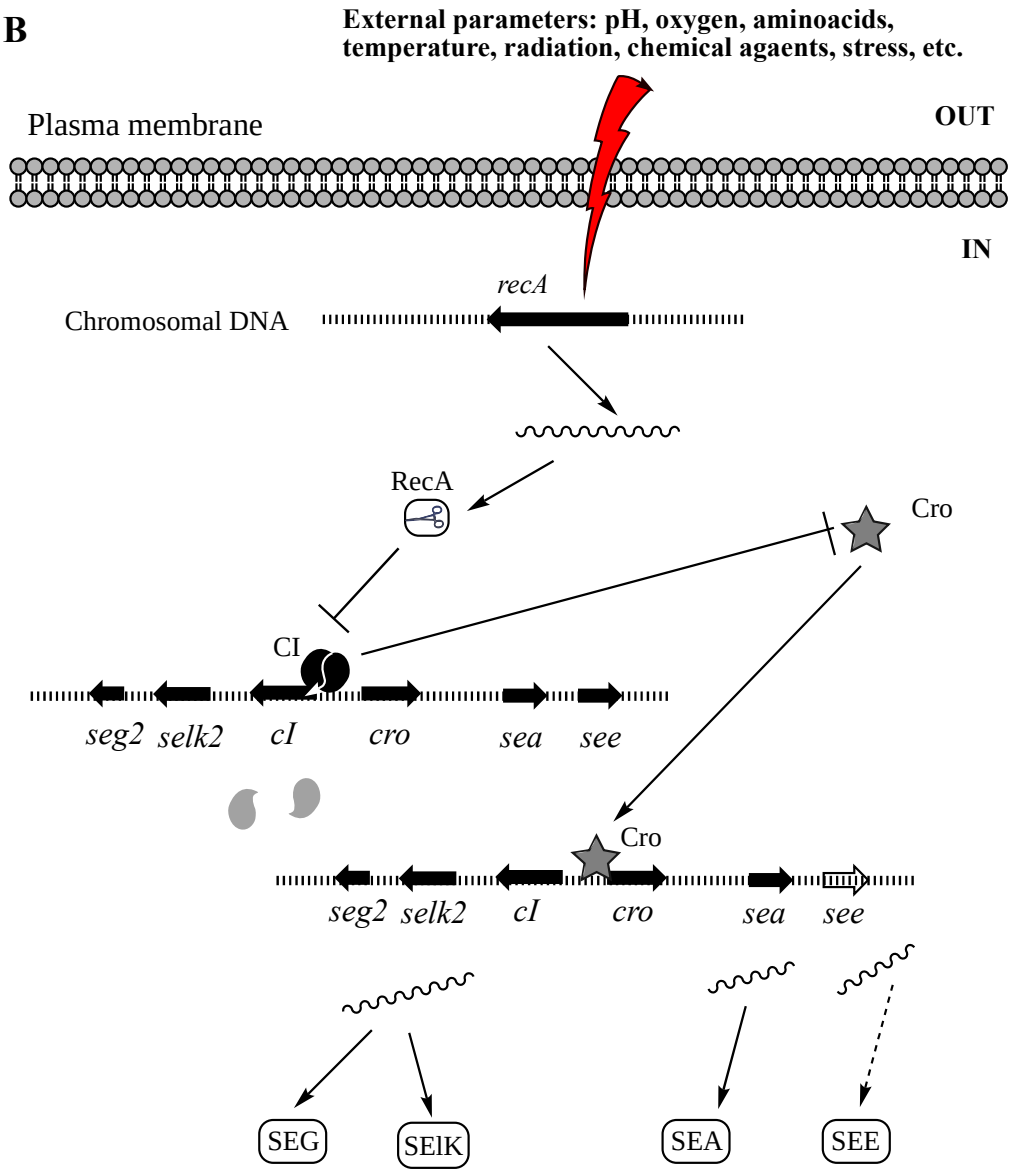


B **C**

Figure 5: Figure 5: Regions of SEE involved in the interactions with the MHCII high-affinity binding site (magenta) and the low -affinity binding site (cyan) as well as with TCR to invoke

TSS, as determined by Rodstrom et al., (2015). Representations of SEE show the N-terminal (green) and the C-terminal (red), as a cartoon (A) and as spheres for the front side (B) and the back side (C) of the molecule. Molecules were drawn by using PyMol software (Schrodinger 2015)





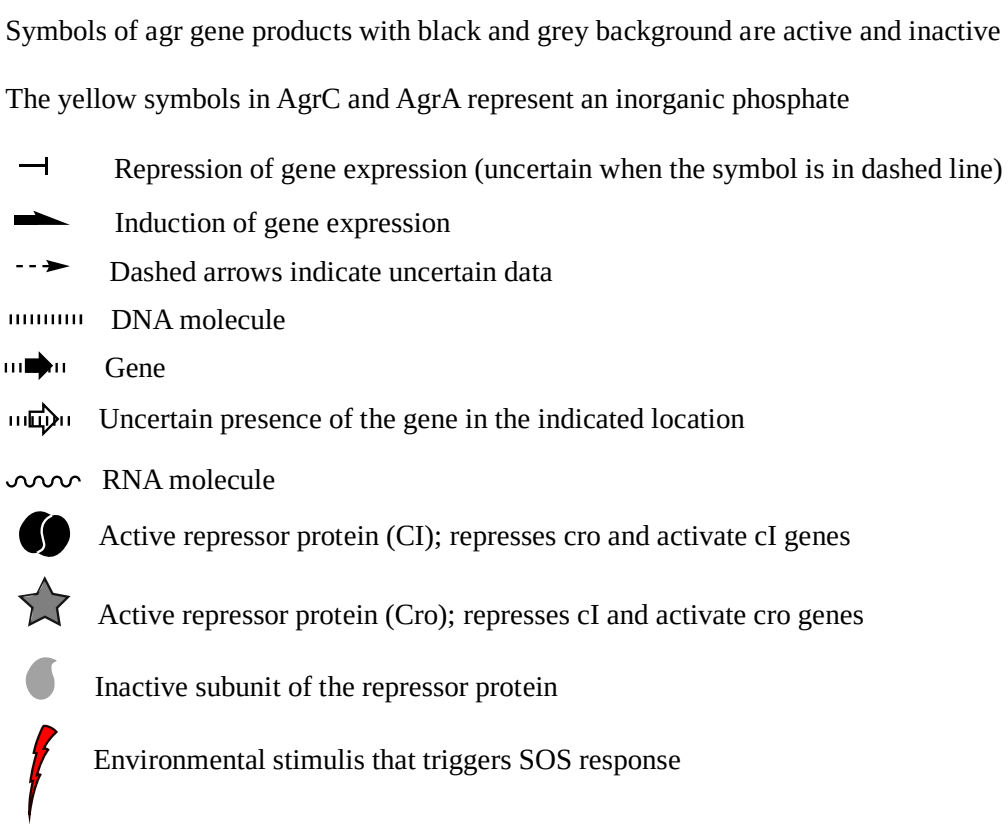


Fig. 6: Mechanisms for the regulation of the expression of *se*/*sel* genes under the control of *agr* system (A) and as a response to environmental stimuli (B). The *agr* systems consists of two operons, *agrACDB* and *hld*, which are activated by the auto-inducing peptide (AIP). The activation of the *agrACDB* operon induces the production of four RNAII transcript proteins (AgrB, AgrD, AgrC and AgrA), each playing a specific role in the *agr* activation. AgrB is a transmembrane protein that processes the AgrD (pro-AIP) and excretes it out of the cell in the form of active AIP. As a ligand, the AIP binds to and induces the auto-phosphorylation of AgrC (the sensor histidine kinase), which then transfers the phosphate to AgrA (the response regulator). The phosphorylated AgrA, in turn, binds to a DNA sequence between P2 and P3 promoters and activates both of them, with a consequent increase in the production of RNAII and

RNAIII transcripts. The latter transcript, inactivates Rot thereby de-repressing *seb*, *sed* and *sec* genes, as they are normally repressed in the presence of Rot. On the other hand, *seh* gene is originally folded forming a loop that prevents the binding of RNA polymerase to the promoter region, which can still bind Rot. The binding of Rot to the *seh* promoter unfolds the loop providing the access of RNA polymerase to *seh* promoter to which it binds and initiates the transcription.(A) (B) Environmental stimuli trigger a typical SOS response as is known for the lambda phage. The exposure to external stimuli (pH, oxygen, UV-irradiation, chemical agents, etc.), activates the chromosomal *recA* gene of an *S. aureus* lysogenic strain carrying phage. The resulting product (RecA) induces the auto-proteolysis of the CI repressor and its dissociation from the binding site which overlaps with the *cro* promoter. The *cro* gene is then de-repressed and its product (Cro) can bind to its specific site being free after CI dissociation from *cI* promoter. This terminates the lysogenic state and activates the latent prophage genes to drive irreversibly the phage into the lytic cycle. The induction of the phage ΦSa3ms also activates the expression of *sea*, *seg*, and *selk* carried by the phage. The drawing of *see* gene as a part of the ΦSa3ms genome is hypothetical; however, this gene, which is believed to be borne on Sa phage, would be activated in a similar way (Adapted from Sumby and Waldor 2003)

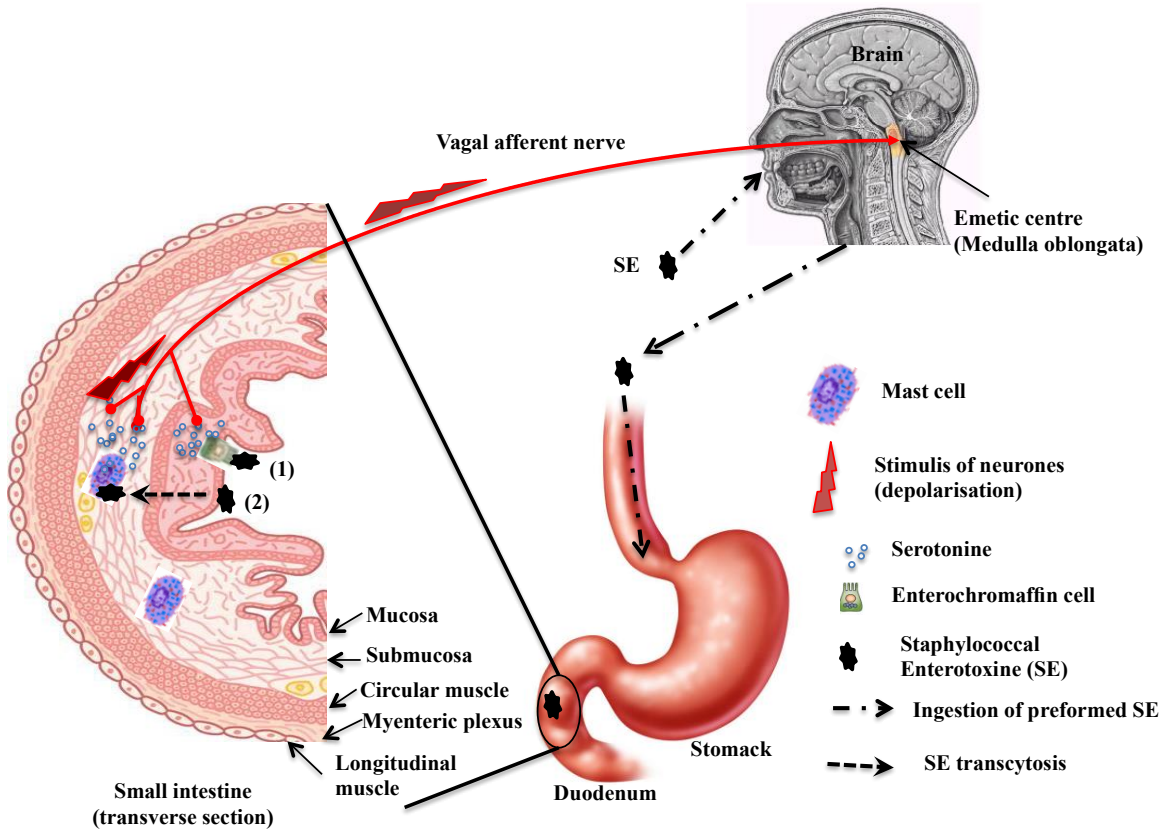


Fig. 7: Hypothetical mechanisms of SE-mediated emesis as demonstrated in house musk shrew. The ingested SE reaches the small intestine, and either attaches to the Enterochromaffin Cells (ECC) in the mucosa (1) or translocates the intestinal mucosa into the submucosal layer where it binds to Mast Cells (MC) (2). SE-binding to ECC or MC causes the release of serotonin (5-HT) that attaches to 5-HT3 receptors on the vagal afferent nerve causing its depolarisation thereby stimulating the emetic centre in the brainstem (medulla oblongata). Left figure is an enlargement of the site of action for SE in the upper part of the small intestine (duodenum). Signalling pathways (1) and (2) are as described by Hu et al., (2007) and Ono et al., (2012), respectively. NB: Drawings are not in scale