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The formation of *Staphylococcus aureus* enterotoxin in food environments and advances in risk assessment

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Key words: *Staphylococcus aureus*, staphylococcal enterotoxins, staphylococcal food poisoning, foodborne illness, virulence regulation, risk assessment

Abbreviations: SFP, staphylococcal food poisoning; SE, staphylococcal enterotoxin; SaPI, *Staphylococcus aureus* pathogenicity island

The recent finding that the formation of staphylococcal enterotoxins in food is very different from that in cultures of pure *Staphylococcus aureus* sheds new light on, and brings into question, traditional microbial risk assessment methods based on planktonic liquid cultures. In fact, most bacteria in food appear to be associated with surfaces or tissues in various ways, and interaction with other bacteria through molecular signaling is prevalent. Nowadays it is well established that there are significant differences in the behavior of bacteria in the planktonic state and immobilized bacteria found in multicellular communities. Thus, in order to improve the production of high-quality, microbiologically safe food for human consumption, in situ data on enterotoxin formation in food environments are required to complement existing knowledge on the growth and survivability of *S. aureus*. This review focuses on enterotoxigenic *S. aureus* and describes recent findings related to enterotoxin formation in food environments, and ways in which risk assessment can take into account virulence behavior. An improved understanding of how environmental factors affect the expression of enterotoxins in foods will enable us to formulate new strategies for improved food safety.

Introduction

The battle against bacterial foodborne diseases is facing new challenges due to rapidly changing patterns of human consumption, the globalization of the food market and climate change. Today, consumers want more natural food products that are less processed, without preservatives, with low salt, sugar or fat contents, but with an extended shelf-life and high quality.[1] The demand for convenient, ready-to-eat food has also increased, and the food industry has developed new food processing techniques such as semi-prepared, minimally processed, chilled food in response to these demands.[2,3] Convenience food offers a suitable growth environment for toxin-producing bacteria such as *Staphylococcus aureus*, which is able to grow and express virulence in a wide variety of foods such as milk products, mixed foods, meat and meat products, egg and egg products, cakes and ice cream.[4] The European Food Safety Authority (EFSA) reported in 2009 that cheese followed by mixed or buffet meals were the two main food vehicles in verified outbreaks of food poisoning caused by staphylococcal toxins.[5] As in all industries, there is a desire to minimize production costs, leading to the search for low-cost raw materials globally, resulting in ingredients from many countries being combined into one dish. Consequently, food is prepared, produced and stored differently, and the behavior of foodborne pathogens under these different conditions is not yet fully understood, potentially increasing the risk of foodborne illness.

To improve the production of microbiologically safe food for the consumer, data related to the physiology of foodborne pathogens in authentic food situations is required to complement existing knowledge on the growth and survival of planktonic bacteria in liquid cultures. In fact, recently reported data have shown that there are significant differences in the behavior of bacteria in the planktonic state and in actual food matrices.[6-8] Knowledge about the effects of critical food-related factors on microbial responses such as virulence gene expression, lag phase duration, growth rate and extracellular virulence formation, will not only aid in the prevention of foodborne diseases, but also enable the advancement of quantitative microbial risk assessment (QMRA). A risk assessment consists of four steps: (1) hazard identification, (2) hazard characterization, (3) exposure assessment and (4) risk characterization. Steps 1 to 3 are combined to assess the health risk in relation to the specific risk questions addressed. The dose-response relationship is crucial in the hazard characterization step, while the ability to estimate the likelihood and amount of the hazardous material ingested is important in exposure assessment. The latter usually involves the use and development of predictive microbiology models for quantification of growth, inactivation and toxin production. The application of risk analysis frameworks and

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preventive approaches (e.g., hazard analysis and critical control points, HACCP) and the responsibility of food producers, are key principles in the new paradigm to address foodborne illnesses.[9] A scientific assessment of the risk to health is the basis for the management of foodborne hazards in terms of the measures chosen to control risk to an appropriate level of protection (ALOP). The ALOP must be translated into a metric, a safety level, useful for setting limits that producers can relate to.[10] Proposed metrics include Food Safety Objectives (FSO), Performance Objectives (PO), Performance Criteria (PC) and Microbial Criteria (MC).[11] Regardless of the metric chosen, the key in this process is the development of a risk assessment procedure.[12]

This review focuses on *S. aureus* and describes recent findings related to enterotoxin expression, formation and regulation in food environments, and ways in which risk assessment can be improved by in situ virulence data. In general, the enterotoxin(s) are formed during *S. aureus* multiplication in food, but new findings show that bacterial growth and enterotoxin production may be decoupled in food products. Different metabolic regulatory systems involved in enterotoxin expression will be discussed, as well as recent risk assessment approaches.

The Organism

S. aureus is a Gram-positive coccus occurring singly or in irregular clusters. The bacteria produce a carotenoid pigment resulting in golden-colored colonies, giving rise to the species epithet aureus (meaning golden). They are nonmotile and nonsporing chemoorganotrophics with both respiratory and fermentative metabolism.[13,14] *S. aureus* is found in the nostrils and on the skin of warm-blooded animals, and the primary source of food contamination is the hands of food handlers.[15,16] The organism can also be endemic in the processing environment.[17] *S. aureus* has the ability to grow, and produce staphylococcal enterotoxins (SE), the causative agent of staphylococcal food poisoning (SFP), over an extensive range of temperature, pH, sodium chloride concentration and water activity (**Table 1**).[4] The robustness of the organism permits its growth in many types of food, producing enterotoxins subsequently causing food poisoning. The bacteria can be killed through heat treatment of the food, but the enterotoxins are very heat resistant. Thus, although the bacteria are eliminated, the toxins will remain and can cause SFP.[15]

Staphylococcal food poisoning and enterotoxins. Food safety is an important issue throughout the world, and is one of the WHO's 13 strategic objectives for 2008–2013. A study on the impact of food-related illness has recently been published in the USA and, due to a number of serious incidents in recent years, the US Food and Drug Administration (FDA) has stepped up efforts to improve the traceability of contaminated products.[18] In Europe, the EFSA reported a total of 5,550 outbreaks of foodborne illness in 2009, affecting almost 49,000 people and causing 46 deaths. Among these, 293 outbreaks were caused by *Staphylococcus* spp and bacterial toxins (produced by Bacillus, Clostridium and Staphylococcus) were the fourth most common causative agent in foodborne outbreaks.[5]

SFP is a foodborne intoxication that develops in people who ingest food that has been improperly prepared or stored. The severity of the illness depends on the amount of food ingested, the amount of toxin in the ingested food and the general health of the victim.[16] SFP can be caused by as little as 20–100 ng of enterotoxin.[19] After ingestion, symptoms appear rapidly and abruptly, consistent with diseases caused by preformed toxins. The symptoms include copious vomiting, diarrhea, abdominal pain or nausea.[13,14] Ingested bacteria do not produce toxin, and the symptoms therefore normally wear off within 24 h.

To date, 21 SEs or enterotoxin-like proteins (SEls) have been identified and designated SEA to SElV (**Table 2**).[20,21] While SEs are the toxins that induce emesis, the related SEls either lack emetic activity or have not yet been tested for this.[22] The genes encoding the different enterotoxins are carried and disseminated by different mobile genetic elements, i.e., prophages, plasmids, pathogenicity islands (SaPIs), enterotoxin gene cluster (*egc*) and the staphylococcal cassette chromosome (SCC).[23-40] Enterotoxins are short, extracellular proteins that are water-soluble. They are most commonly described as very stable, and are resistant to heat as well as degrading enzymes.[15,41,42] However, some cases have been reported where the toxins disappeared. Recently, SEA and SED were found to decrease in boiled ham after a period of accumulation,[7,8] and a number of earlier studies have reported the disappearance of SEA in broth, minced food and raw and pasteurized milk.[43,44] The apparent decrease in enterotoxin levels could simply be an analytical artifact, such as loss of serological recognition using immuno-based methods such as ELISA, which is a technique commonly used to detect enterotoxins. However, it has also been proposed that proteases produced by lactic acid bacteria (LAB) cause the decrease in SEA levels, or that SEA becomes cell-associated and is, therefore, not detected.[45,46] Furthermore, it has been reported that the expression of genes encoding potential proteases were increased upon acid shock of *S. aureus*.[47] These findings suggest that *S. aureus,* or other organisms present in the surrounding environment, e.g., LAB, could cause the decrease in enterotoxin level observed under certain conditions, possibly through extracellular protease activities. The enterotoxins, which are classified as superantigens, display the common characteristics of this group, i.e., pyrogenicity, immune suppression and a mitogenic effect on T cells.[15] Superantigens can also cause toxic shock syndrome, a serious condition characterized by rashes, hypovolemic shock and respiratory distress syndrome.[48] The majority of reported SFP outbreaks are associated with the classical enterotoxins, SEA-SEE; staphylococcal enterotoxin A (SEA) being considered the most common cause of SFP.[49-51]

Regulation of Enterotoxin Formation

The classical enterotoxins (SEA-SEE). *Prophage-encoded enterotoxins (sea and see).* The *sea* gene is carried by a polymorphic family of temperate bacteriophages.[25] The bacteriophage is inserted into the bacterial chromosome as a prophage and behaves like part of the bacterial genome. However, under environmental stress conditions, such as mild food preservation conditions, the prophage can be induced to replicate the phage genome and release

Table 1. Factors affecting *Staphylococcus aureus* growth and enterotoxin formation[4]

Factor	Optimal growth	Growth limits	Optimal SE production	SE production limit	Enterotoxin (s) reported affected	Notes to effect(s) on enterotoxin production	Examples of analysis of the specific factor in food products	References
Temperature	35–41°C	6–48°C	34–40°C	10–46°C	SEA, SEB, SEC, SED	Temperature seems to affect enterotoxin synthesis more that growth.	Milk Ham Egg products	44, 141–144, 169
pH	6–7	4–10	7–8	5–9.6	SEA, SEB, SEC, SED, SEE	Higher tolerance under aerobic compared with anaerobic growth conditions. Lactic acid particularly inhibits toxin formation. agr dependent regulation (SEC).	Ham Sausage	7, 75, 143–147, 169
a_w	0.99	0.83 ≥ 0.99	0.99	0.86 ≥ 0.99	SEA, SEB, SEC, SEH	SEB and SEC may be more sensitive than SEA and SEH. SEH enterotoxin production at a_w: 0.97 > 1 > 0.95.	Cured beef slurry Cured pork slurry Bacon Scrimp slurry Sausage	85, 90, 96, 142, 147–152
NaCl	0%	0–20%	0%	<12%	SEA, SEB, SEC	Raises temperature limit for SEA production. Low osmolality increases enterotoxin production. SEB production seems more strongly inhibited than growth.	Ham Sausage	85, 88, 96, 144, 153–156, 169
Oxygen	Aerobic	Anaerobic-aerobic	Aerobic	Anaerobic-aerobic	SEA, SEB, SEC, SEH	Increases yield of SEB up to 10-fold. 10% dissolved oxygen is optimal for SEB production.	Ham Prawn Sausage	98, 113, 157, 158, 159, 160, 161
Redox potential (Eh)	>+200 mV	≥200 to > +200 mv	>+200 mV	≥100 to > +200 mv	-	-	-	-
Lactococcus lactis	-	-	-	-	*sec*, *sel* (*sek*, *seg*, *seh*)	Strongly reduces transcription of *sec* and *sel* and sightly *sek*, *seg*, *seh*	Cheese	6, 107, 162
					sea	May favor the maintenance of *sea* in stationary phase.		

new bacteriophages.[52] Today, at least six completely sequenced *S. aureus* strains containing different *sea*-carrying prophages, Φ252B, ΦMu3, ΦMu50A, ΦNM3, ΦSa3ms and ΦSa3mw, have been found, all of which frequently carry the genes for enterotoxin A, staphylokinase and the complement inhibitor.[31,53-56] It was recently demonstrated that the transcription of *sea* is linked to some extent to the lifecycle of the SEA-encoding prophage,[57] in contrast to many other non-phage encoded enterotoxin genes such as *seb*, *sec* and *sed*. The polymorphic nature of the prophages has been found to affect the amount of SEA produced by the bacterial strain carrying the prophage.[26] Sequence analysis of the *sea* gene and its neighboring genomic regions have further indicated that SEA-producing strains can be grouped into two major groups, SEA_1 and SEA_2.[52] The endogenous promoter region, P_1, immediately upstream of *sea*, is found in both groups.[26] In addition, there may also be a second phage-related latent promoter, P_2, shown to express *sea* after prophage induction.[57] We have observed that *S. aureus* strains producing high amounts of

Table 2. The staphylococcal enterotoxins

Enterotoxin	Variant	ORF length (bp)	Mature length (aa)	Molecular weight (Da)	Genetic backbone	References
SEA		774	233	27,100	Prophage	15, 25, 52, 163
	SEA_1	774	233	27,100	Prophage	
	SEA_2	774	233	27,100	Prophage	
SEB		801	239	28,336	SaPI	15, 59
SEC					SaPI	15, 28, 31, 38, 60, 164
	SEC_1	801	239	27,531	SaPI	
	SEC_2	801	239	27,531		
	SEC_3	801	239	27,563	SaPI	
	SEC_{bov}	816a	271[b]	27,618	SaPI	
	SEC_{sheep}[c]			27,517		
SED		777	228	26,360	Plasmid	15, 24
SEE		774	230	26,425	Prophage	15, 27
SEIG		777	233	27,043	egc	15, 29, 54, 57, 165
	$SEIG_2$	729[a]	242[b]		Prophage	
	$SEIG_V$	777	233	26,985	egc	
SEIH		726	218	25,210	scc[d]	15
SEI		729	218	24,298	egc	15, 29, 32
	SEI_v	729[a]	242[b]		egc	
SEIJ		806	245	28,565	Plasmid	15, 34, 39
SEIK		729	219	25,539	SaPI	15, 38, 54, 57, 166
	$SEIK_2$	729[a]	242[b]		Prophage	
SEIL		723	215	24,593	SaPI	15, 28, 38
SEIM		722	217	24,842	egc	15, 29
SEIN		720	227	26,067	egc	15, 29, 32
	$SEIN_v$[c]				egc	
SEIO		783	232	26,777	egc	15, 29
SEIP		783[a]	260[e]	27,000	Prophage	31, 48, 167
SEIQ		729[a]	242[e]	25,207	SaPI	38, 166
SER		600[a]	259[e]	27,049	Plasmid	34, 168
SES		774[a]	257[e]	26,217	Plasmid	168
SET		651[a]	216	22,614	Plasmid	168
SEIU		786[a]	261[e]	27,100	egc	32, 48
	$SEIU_v$	771	256[e]		egc	
SEIV		720	239[b]		egc	21

ORF, open reading frame. [a]ORF obtained from the National Center for Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov.ludwig.lub.lu.se/gene, March 25, 2010. [b]precursor aa length from NCBI, www.ncbi.nlm.nih.gov.ludwig.lub.lu.se/sites/entrez?db=Protein&itool=toolbar, March 25, 2010. [c]ORF length and aa sequence not found in NCBI. [d]R. Cao, unpublished data. [e]precursor aa length.

SEA belong to the SEA_1 group, and that strains producing low amounts of SEA belong to the SEA_2 group (unpublished data). Furthermore, a subgroup of the SEA_1 strains was also found to be associated with a stress-induced boost in SEA production as the second phage-related promoter, P_2, was activated. Enterotoxin E (SEE) is the toxin most similar to SEA, having 90% amino acid identity.[48] The *see* gene is situated on a defective prophage, in contrast to the prophage encoding *sea* and *see* expression appears to be unaffected by bacterial growth.[58]

agr-regulated enterotoxins (seb, sec and sed). The *seb* gene is carried on the *S. aureus* pathogenicity island, SaPI3,[59] while enterotoxin C (SEC) exists in multiple variants, C1, C3, Cbov, which are situated on SaPI4, SaPIn1/m1 and SaPIbov, respectively.[31,60] SaPIs are highly mobile phage-related staphylococcal pathogenicity islands that can integrate into specific sites (known as the *attC* sites) in the chromosome determined by the specificity of SaPI-encoded integrases. SaPI particles are released by phage-induced lysis, and can infect and integrate into a new host with very high frequency.[61] The *sed* gene is situated on a 27.6 kb penicillinase plasmid, pIB485, in *S. aureus*.[24] Despite being encoded by different mobile genetic elements, the expression of *seb, sec* and *sed* genes is induced during the transition

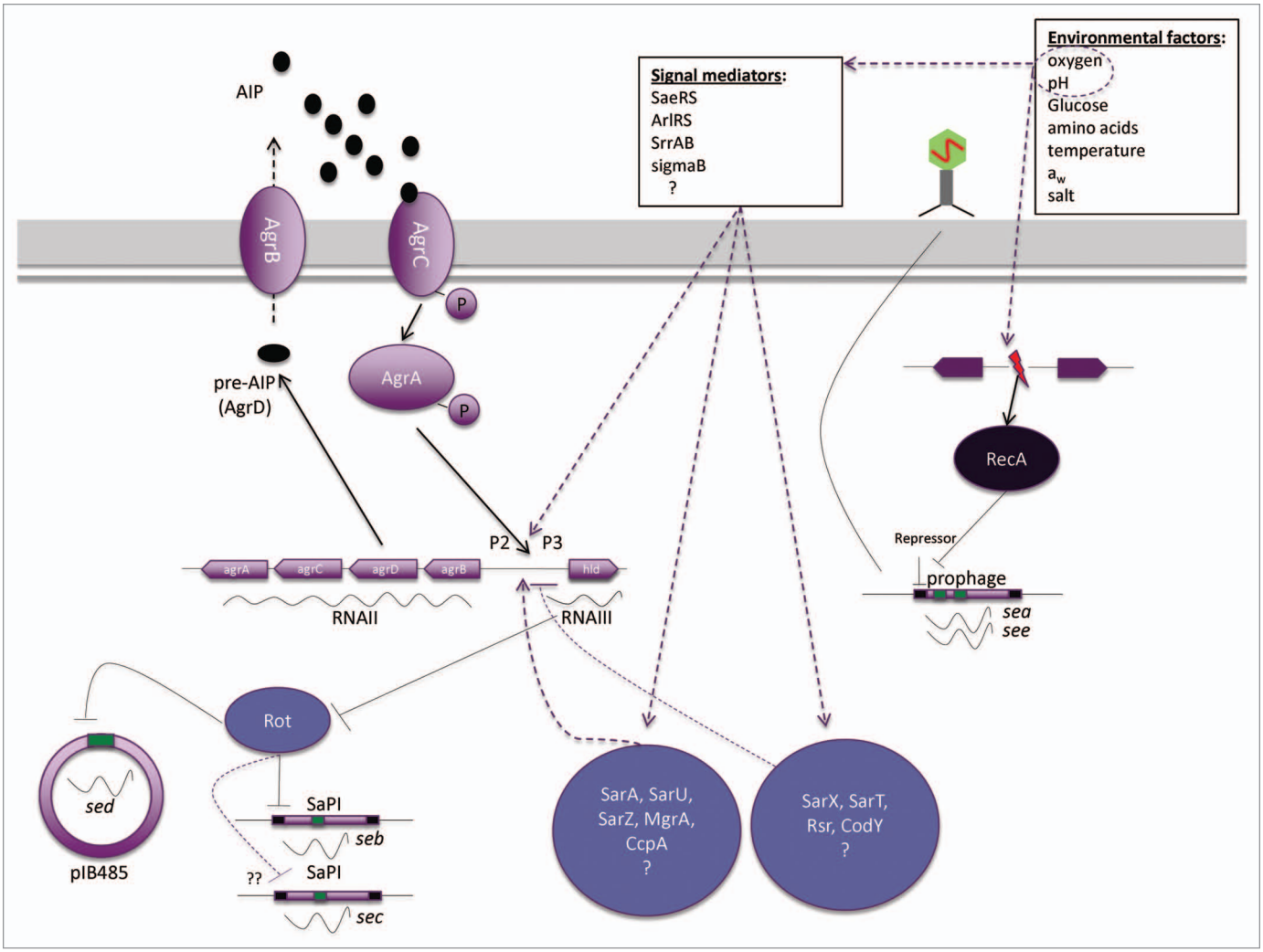

Figure 1. Regulation of *se* transcription in *S. aureus*. Black arrows represent identified direct regulation, while dashed purple arrows indicate potential regulatory pathways. The individual regulators and regulatory pathways are described in the text.[66] AIP, autoinducing peptide.

from the exponential to the stationary phase, an expression pattern characteristic of proteins encoded by genes regulated by the Agr regulatory system.[24,58,62-65] Yet, the two *se* genes encoded by SaPIs, *seb* and *sec*, undergo a much more drastic induction than the plasmid-encoded *sed*.[58]

The Agr signal transduction system is a quorum sensing system that allows *S. aureus* to respond to cell density, as recently reviewed by Thoendel et al.[66] Briefly, the *agr* locus generates two different transcripts, RNAII and RNAIII, driven by the promoters P2 and P3, respectively (**Fig. 1**). RNAII encodes the structural genes for the quorum sensing system *agrB*, *agrD*, *agrC* and *agrA*. AgrD and AgrB act to generate the quorum sensing molecule [autoinducing peptide (AIP)], which after reaching a threshold level stimulates activation of AgrC and AgrA, a two component regulatory system. Activated AgrA then upregulates the promoters P2 and P3, generating more RNAII and RNAIII transcripts. The P3 transcript, RNAIII, encodes delta-hemolysin but, more importantly, the RNAIII itself is the intracellular effector of gene regulation in the cell.[66-68] As the cell grows the intracellular level of RNAIII increases due to the autoregulatory circuit of the Agr system, leading to increased transcription of secreted virulence factors such as enterotoxins, and reduced transcription of a subset of genes encoding cell wall proteins.[69,70] Loss of the Agr signal transduction system is reported to result in substantial loss in the transcript level of *seb, sec* and *sed* and thus the corresponding SEB, SEC and SED production.[24,63,65,71]

The RNAIII-mediated impact on the transcription of *seb* and *sed* is indirect and is dependent on the presence of a functional Rot (repressor of toxins), which is a member of the Sar family of transcriptional factors of *S. aureus*. Rot binds to promoter regions, as shown for the *seb* promoter, thereby repressing the transcription of genes.[72-74] When the Agr system is induced during post-exponential growth RNAIII basepairs with *rot* mRNA. This mediates translational repression of *rot* mRNA, and subsequently lowers the amount of cellular Rot.[72] It is not known whether the activation of *sec* is regulated by the *agr* system via the RNAIII-Rot interaction, but it has been reported that SEC_1 and SEC_2 are produced during different growth

phases, and the regulation of the enterotoxins of this biotype (C) may differ.[64]

Numerous transcriptional regulators affect the function of the *agr* system, and may in theory indirectly affect the levels of *seb, sec* and *sed* transcription (**Fig. 1**).[66] These regulators respond to various environments and stresses also known to affect enterotoxin synthesis.[66,75-78] The two-component system SrrAB and CodY are two of the interesting regulatory candidates known to directly control *agr* expression. SrrAB is involved in the adaptation to anaerobic growth, and inhibits RNAIII expression by binding to the P2 and P3 promoters. CodY is a transcriptional regulator, whose DNA binding ability is controlled by the cellular GTP pool, thus sensing nutrient availability.[77-80] SarA, another member of the Sar family of homologs, positively affects *agr* transcription and its DNA-binding activity to P2 is dependent on cellular redox conditions and pH and has been demonstrated to positively control *seb* and *sec*.[76,81,82] In accordance with the above observations, dissolved oxygen level, nutrient availability and pH have been shown to influence the formation of *agr*-regulated enterotoxins (**Table 1**). SaeRS, another two-component system, suggested to act downstream of *agr,* has been shown to positively regulate *sec* expression.[83] It has been proposed that SaeRS responds to several environmental stimuli, including high glucose and salt levels, low a_w and low pH.[84]

The enterotoxins B, C and D are, however, only partially upregulated by the Agr system and can be produced independently of *agr*.[85,86] Although SarA is required for full *agr* loci transcription, SarA has also been shown to regulate *seb* transcription independently of RNAIII, and the alternative sigma factor, sigmaB, has been reported to reduce *seb* expression, possibly by repressing both the *agr* system and a second unidentified inducer.[87,88] Notably, many of the environmental conditions known to repress *seb* transcription, such as high salt content and alkaline conditions, are also known activators of sigmaB.[86,89]

The non-classical enterotoxins (SElG–SElV). Regarding regulation of the non-classical enterotoxins, results from a kinetic study indicate that the expression of the majority of the newly described *se* genes is not controlled by the *agr* system.[58] Data from this study show that only the transcript level of *seh, ser* and *sel* increases in the post-exponential phase, which implies possible regulation by the Agr regulatory system. *seh* mRNA was found to undergo a much more drastic induction than *ser* and *sel*, and activation of *seh* took place earlier in the growth cycle than the classical *agr*-controlled *seb* and *sed* genes. This expression pattern is consistent with results reported by Sakai et al.[90] showing that maximal SEH production takes place in the late exponential phase, while SEB is mainly produced in the stationary phase.[58,70,90] The transcript level of other investigated *se* genes either remained unchanged during growth (*sej, sek, seq*, *sep*), or decreased slightly (*seg, sei, sem, sen, seo, seu*) after exponential growth. Most of the *se* genes with unchanged transcription are phage-encoded, and may therefore be regulated by the processes that govern lysogeny. In contrast, the *se* genes that showed a slight decrease in transcript level during growth are encoded by the *egc* operon and, notably, these enterotoxins could not be detected using two-dimensional gel electrophoresis.[58,91] It is still unclear whether the non-classical enterotoxins are responsible for food poisoning, and so far SEH is the only non-classical enterotoxin detected in foods responsible for food poisoning.[92,93]

Impact of Environmental Factors on SE Production

SFP is often associated with growth in protein-rich food such as meat and dairy products.[15,94] These products are highly complex matrices compared with broth, with respect to, e.g., microbial content, salt, pH, nutrient availability, oxygen availability and temperature.[95] Generally, growth of *S. aureus* is necessary for enterotoxin production, although enterotoxin production does not always accompany growth, and in a few cases toxin production has been observed in non-replicating cell cultures, most recently by Wallin-Carlquist et al.[8] in ham products.[96-98] Studies have been performed to identify key parameters that prevent or stimulate enterotoxin production in laboratory media and in diverse food products, and a multifaceted network of environmental and genetic factors seems to regulate enterotoxin production.[6,8,42] Some of the identified effects of environmental conditions on enterotoxin production are listed in **Table 1**.

Wallin-Carlquist et al.[7,8] have recently studied SEA and SED formation in boiled and smoked ham. Notably, a prolonged *sea* expression and SEA formation were observed over the course of a week, instead of a short-term growth-associated *sea* expression[8] and unexpectedly *sed* expression followed the same general pattern as the prophage-encoded SEA in both ham products, as the genes are regulated differently.[7] The difference in the enterotoxin expression pattern observed for *S. aureus* on the ham products and in liquid culture is probably related to the different physiological states of the staphylococci. On the ham, *S. aureus* forms a biofilm, while in the culture the bacteria are planktonic. An active *agr* quorum sensing system is known to limit biofilm formation in *S. aureus*, and the activation of the *agr* system has been connected with the dispersal of *S. aureus* from an established biofilm upon glucose depletion.[99] A second peak in *sed* expression observed in boiled and smoked ham could be due to glucose levels running low in the meat, activating the *agr* system, initiating the detachment of *S. aureus* from the biofilm, consequently inducing *sed* expression. Furthermore, recent studies show that mature biofilms are acidic environments.[100-102] Low pH can cause prophage induction, leading to increased *sea* expression, explaining the prolonged *sea* expression observed on boiled and smoked ham. Enhanced transcription of phage-encoded virulence genes upon prophage induction has been demonstrated for Panton-Valentine leukocidin in *S. aureus,* streptococcal pyrogenic exotoxin C and a DNase in *Streptococcus pyogenes*, as well as the Shiga and Shiga-like toxins in *Escherichia coli*.[57,103-106] Cretenet and colleagues[6] recently used a transcriptomic approach to study virulence expression in a cheese matrix under the influence of *Lactococcus lactis*, showing that *sea* expression was slightly increased in this acidic environment. Another possible explanation of a second boost in *sea* expression may be that the temperate phage is activated by oxidative stress via RecA-mediated response.

In the study by Cretenet and colleagues[6] the expression of *sec* and two other genes that may be *agr*-controlled, *seh* and *sel*

in *S. aureus,* isolated from a cheese matrix, was also followed. Using a transcriptomic approach, expression data revealed that both the dynamics and the levels of *sec, seh* and *sel* expression differed notably from those observed during growth in a chemically defined medium (CDM). The expression levels were significantly lower in the cheese matrix than in the CDM, and the post-exponential induction, characteristic of *agr*-regulated genes, was absent. Low water activity in the cheese matrix correlated with a reduction in *saeRS* level and was suggested to be responsible for, at least, the reduced *sec* expression in the cheese matrix.[6] The expression of the *se* genes was also studied in the cheese matrix in the presence of *L. lactis*.[107] In these mixed cultures, *L. lactis* has previously been reported to reduce the expression of *sec, sel* and *seh*, and for *sec* the reduction was suggested to be partly due to reduced activity of SarA and the *agr* system.[107] The presence of *L. lactis* in the cheese matrix also downregulated the expression of RNAIII and *sarA*, while the *rot* level was increased. However, although the activity of the *agr* system was reduced in the presence of *L. lactis* and a decrease in *sec, seh* and *sel* levels had previously been observed in mixed cultures with *L. lactis*, both *seh* and *sel* were upregulated in the cheese matrix by the presence of *L. lactis*, while the *sec* level was not affected by its presence.[6] These results illustrate that, despite the observation of similar expression patterns of *sec, seh* and *sel* in CDM and in mixed cultures in laboratory studies, the effects of environmental conditions in a food matrix are dependent on the type of enterotoxin, and that the regulatory organization is multifactorial.

Risk Assessment and Predicitive Microbiology to Control Enterotoxin-Producing *S. aureus* in Foods

In this section, predictive microbiology models and risk assessment of *S. aureus* are reviewed with the objectives of illustrating different approaches and highlighting challenges in relation to the findings presented in previous sections. The focus is on staphylococcal growth and enterotoxin production; other characteristics, such as antibiotic resistance, are not addressed.

Predictive microbiology models for staphylococcal growth and enterotoxin production. One difficulty when estimating human exposure to a hazard or evaluating the safety of a production process is to quantify changes in the number of microorganisms or the amount of toxin in the food at the stage of food production or processing of interest. Predictive models are useful tools that can be used to estimate these changes, depending on the properties of the microorganism, and the nature of the food and the way it is handled, stored and processed.[108] However, predictive models cannot be used as the sole determinant of product safety.[109] If important decisions are to be made based on the results of a predictive model it must be validated in the food of interest.

Predictive models are based on data describing changes in numbers of microorganisms or levels of toxin fitted to primary models. Secondary models describe the effects of environmental factors, such as temperature and pH, on the parameters in primary models, e.g., the maximum specific growth rate. A summary of different models is provided by van Gerwen and Zwietering.[110] Tertiary models are predictive models implemented in user-friendly software. Tertiary *S. aureus* models available free on the internet include the growth and survival models in the US FDA pathogen modeling program (PMP) and the growth model in the ComBase modeling toolbox, the ComBase Predictor.[111] In addition, growth and inactivation curves from published studies are available in the ComBase database. There are also several kinetic models describing the physico-chemical effects of the food or the environment on *S. aureus* growth rates.[112,113] Available kinetic tertiary models can be used to predict growth and lag time based on various input values, for example, temperature, pH, water activity, nitrite concentration and aerobic/anaerobic conditions (PMP) (ComBase Predictor) and temperature in various types of meat (THERM).[114] An alternative approach is to describe the boundary between growth and no growth,[95] or survival/death,[115] the time before growth,[116] or the probability of growth, as a function of environmental parameters. Such models can be useful in the design of food processes to prevent growth and enterotoxin production. For instance, Stewart et al.[116] developed a time-to-growth model to study the effects of different humectants used to achieve shelf-stable intermediate-moisture foods. Similarly, Valero et al.[95] developed a model for the probability of growth and found an abrupt transition of the interface between growth and no growth at low temperatures, where optimal levels of pH and water activity were required for growth. Obeso et al.[115] described the effect of initial lytic phage titers and initial *S. aureus* contamination of pasteurized milk on the probability of *S. aureus* survival at different temperatures.

Food contamination is often incidental, and by few cells. Consequently, new approaches have emerged based on the study of individual cells to derive distributions of growth parameters for use in predictive models.[117] For instance, Sado Kamdem et al.[118] studied the effect of the concentration of fatty acids at two pH values on the distribution of division times among single cells of *S. aureus*. Considerable variation in division times was observed at the single cell level, which was masked when studies were performed at the population level. This emphasizes the difficulty in making predictions, especially under conditions of stress and at low levels of contamination. Variation has also been observed at strain level.[119] The generation times of 34 *S. aureus* strains isolated from poultry and cultured in chicken broth at 17°C ranged from 2 to 17 h.[120] These results emphasize the need to address biological variation and the usefulness of probabilistic approaches to predictive modeling using distributions of growth parameters instead of single fixed values. Vora et al.[121] used a probabilistic simulation approach to evaluate the effect of contamination level of *S. aureus* on the survival/gradual decline in intermediate-moisture foods. They reported no effect of initial contamination levels but both simulations and observations indicated a wide variation in decline rates, including occasional increases in population. Interactions with other microorganisms, present in food or added, may have profound effects on *S. aureus* growth (as exploited by the use of starter cultures in fermented foods) and thus also on enterotoxin production.[122] For example, Le Marc et al.[123] developed a kinetic model that described the inhibitory effect of a starter culture of

lactic acid bacteria on *S. aureus* growth in milk when the lactic acid bacteria had exceeded a critical density.

Although several studies of the kinetics of the production of different enterotoxins or expression of enterotoxin genes exist, very few predictive models of enterotoxin production are available. Fujikawa and Morozumi[124] developed a model based on observations that SEA was detectable at levels greater than 6.5 log10 cfu ml^{-1}, and increased linearly during the whole growth curve in a sterile milk medium. The rate of SEA production increased linearly with temperature from about 15 to 32°C, and was described by the following equation:

$$p = 0.0376 \times t - 0.559 \quad (1)$$

where p is the rate of SEA production (ng $ml^{-1}h^{-1}$) and t is the temperature (°C). SEA was still produced at temperatures above 32°C, but the rate of increase with temperature leveled off. Thus, there is a lack of predictive staphylococcal enterotoxin models.

Overview of microbial risk assessments of *S. aureus*. Risk assessments of *S. aureus* encompass a range of approaches from illustrative examples[125] and partial risk assessments,[126] to quantitative microbial risk assessments (QMRA) based on probabilistic modeling.[127] Food products assessed include milk,[128,129] skim milk,[130] unripened raw-milk cheese,[131] pork-based Korean food,[127] kimbab,[132,133] home-cooked foods[126] and cream-filled baked goods.[10] The results of risk assessments are equally varied. For model unripened raw milk cheese production Lindqvist et al.[131] estimate that the probability for unsatisfactory concentrations of *S. aureus* (>6 log10 cfu g^{-1}) is ~4.5 x 10^{-2} in a high pH cheese and for on farm production of pasteurized drinking milk in the UK Barker et al.[129] estimate that the filler tank contains significant levels of toxin with probability ~2.9 x 10^{-5}.

The hazard identified in risk assessments is *S. aureus* in general, or enterotoxigenic strains explicitly and/or the enterotoxin (SEA, or not specified). Although growth and subsequent toxin production can be prevented by storing "potentially hazardous" foods below 7°C and 10°C, respectively, poor personal hygiene and handling practices and inadequate refrigeration of foods have been identified as the main factors contributing to staphylococcal foodborne disease. As illustrated in the risk assessments, foods not stored below growth temperatures for sensory reasons (e.g., kimbab) or processing that includes steps under growth permissive conditions (e.g., cheese, milk) are also of concern.

The hazard characterization step has focused on food poisoning symptoms, and has not addressed the issues of particularly susceptible populations or immunity. For toxigenic microorganisms dose-response relations are essentially that of a chemical toxin, i.e., a threshold model.[134] Due to knowledge gaps various levels of enterotoxin have been used as the threshold. In several studies, levels of bacteria in the food were used as a proxy for potentially hazardous doses based on reported levels of *S. aureus* required for the detection of enterotoxin. Threshold levels for enterotoxin of 20,[127] 94[128] and 20 or 100 ng[130] per serving have been used. These levels are based on outbreak data. Threshold levels expressed as the number of *S. aureus* bacteria of 5 to 8 log CFU per g have been used.[e.g.,131] Kim et al.[127] used a constant relation between toxin production and cell numbers under the conditions they evaluated based on an equation developed from milk data by Soejima et al.[130]:

$$Tox = 0.9300751 \times C - 6.662092 \quad (2)$$

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

Exposure assessments have described initial contamination of the starting ingredients and their changes, mostly growth but also inactivation, due to cooking and during production, holding and storage. In contrast, consumer handling and consumption have not been described in any detail, and exposure is assessed per g or per serving. Exposure assessments based on measurements of enterotoxin in food and subsequent calculations have been reported in, for example, reference 130, but it is more common to rely on initial data on *S. aureus* levels and prevalence followed by modeling of the effect of processes on changes in *S. aureus* levels. Two studies have assessed enterotoxin production based on predicted numbers of *S. aureus* using either the model of Fujikawa and Morozumi[124] and 15°C as the temperature limit for toxin production,[128] or **Equation 2**.[127]

Risk characterization has been based on the number of *S. aureus* (CFU) or the concentration of enterotoxin (ng) per g or per serving. Sensitivity and scenario analyses in these studies have identified the initial contamination levels together with temperatures and storage/holding times[127,130,131,133] and pH[131] as having the greatest impact on the assessment endpoints. In one study, the assumption concerning the threshold level for the number of *S. aureus* cells required for hazardous levels of enterotoxin to be produced contributed most to the uncertainty in the risk estimate.[131] This highlights the importance of filling the knowledge gap concerning the relationships between growth, survival and enterotoxin production in various foods and in dose-response relationships. This shortcoming is also reflected in the lack of predictive models for risk assessment and evaluation of process safety. Thus, safety is commonly evaluated based on predicted levels of *S. aureus* that have been associated with enterotoxin production, and more seldom in terms of the predicted enterotoxin level or the actual measured value. This may be a limitation in view of the dynamic and complex interplay between growth, gene expression, metabolism and enterotoxin levels and the potential uncoupling between cell numbers and the amount of enterotoxin produced, as reviewed in this manuscript. Potentially stronger models that describe the production of enterotoxin in conditions that correspond to food matrices could have the biggest impact on estimates of risk for SFP.

Advances in risk assessments. It is clear that the amount of information and understanding relating to the biology of *S. aureus* and to SFP, is increasing rapidly. Novel molecular techniques, in particular, provide improved understanding of virulence and survival mechanisms, etc., as well as providing opportunities for improved detection and improved typing of *S. aureus* and SEs. However, this increase in available information is not fully reflected by developments in risk assessment and several challenges remain.

Established risk assessments focus on enterotoxigenic strains of *S. aureus* in order to estimate hazard strength but, in general, they do not discriminate other (sub)types; for example, types based on serology, on toxin genes or on the molecular markers that are commonly used in epidemiology.[e.g.135] For risk assessments type discrimination establishes a heterogeneous population that requires an assignment of (exponentially) increasing numbers of parameters to give a complete quantitative description. It seems inevitable that improved risk assessments will use clustering of types, based on hazard potential, but appropriate clusters are currently unknown. Toxin type appears most appropriate for clustering of *S. aureus* hazards and, in particular, the toxin gene regulation mechanisms are crucial,[e.g.136] however, quantitative models for inclusion in risk assessments have not been established. For some hazards, such as those associated with dairy products, there is an indication that small groups of toxin types, e.g., A, G and I, could identify populations that can be treated as homogeneous for risk assessments.[137]

Similar considerations, related to a reduction in complexity, surround assessments for hazards, such as those in dairy products, where *S. aureus* populations co-exist with other bacteria. Predictive models for population kinetics do not routinely account for complex competing populations and, in many cases, the precise mechanism of competition remains unclear. For hazards associated with *S. aureus*, improved risk assessments will include the role of coexisting bacteria but, in order to avoid overwhelming complexities, will distill the relevant interaction properties rather than describe detailed coupled dynamics. This approach is currently being used to establish the role of starter cultures in virulence expression of *S. aureus* and hence on hazards associated with *S. aureus* in cheese.[e.g.,107]

Current quantitative risk assessments for staphylococcal food poisoning include very generic information concerning the production of toxins, whereas molecular approaches highlight an increasing diversity of toxin types and regulation mechanisms. Sensitivity analysis indicates that, for endpoint measures related to enterotoxin, a population threshold for the initiation of toxin production is more significant than a temperature threshold or rate parameters.[129] This indicates that quantitative details for the population sensing mechanisms, and its relationship with the environment and with toxin types, will be necessary to inform improved risk assessments. Molecular methods have been used to identify differential expression of the staphylococcal enterotoxin genes during cell growth[58] and at explicit points during cheese making;[138] the details of transcription for SE genes can potentially inform improved models for toxin production and, hence, improved risk assessments.

Generally, risk assessments of the hazards associated with *S. aureus* have concentrated on the dynamics of cell populations and toxin production, whereas during the past decade research has been dominated by the association between virulence factors and accessory genetic elements, such as plasmids, prophages and pathogenicity islands.[e.g.,139] Currently the mechanism for including mobile genetics and horizontal transfer, into risk assessment methodology is uncertain but it is clear that this will be crucial for improved quantitative understanding of hazards associated with *S. aureus*. The association between toxin genes and mobile elements that supply antibiotic resistance, which are more widely studied, will contribute to this development.[140]

Future Perspectives

Foodborne diseases caused by bacteria present a constantly evolving challenge, and although a great deal is known about these bacteria, we are still not able to control them. Consumer trends and demands for fresh, minimally processed food are the driving force for the development of new innovative methods of food processing and preservation. Knowledge concerning pathogenic virulence may improve our understanding of foodborne diseases, allowing new solutions to the problem to be developed. This review has described how different food parameters influence SE formation and the importance of in situ studies. The traditional view is that SE production is correlated with bacterial growth, i.e., the more cells, the more toxin and the number of bacteria is usually counted to determine whether a food product is safe for human consumption. However, bacterial growth and SE production may be decoupled, and the behavior of *S. aureus* in food environments may be very different from that of pure bacteria in liquid cultures. This highlights the importance of performing studies in food matrices if the results are to be applied to real food products. A better understanding of how bacterial growth and virulence expression are related and regulated by environmental factors and food preservatives will provide safer food products and give rise to new approaches to disease prevention and control in the future, through the improvement of quantitative risk assessments.

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