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Production of Staphylococcal Enterotoxins D and R in Milk and Meat Juice by *Staphylococcus aureus* Strains

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Abstract

Seventeen *Staphylococcus aureus* strains were tested for production of staphylococcal enterotoxin D (SED) and staphylococcal enterotoxin R (SER) in milk and meat juice. SED was secreted in milk by 12 *S. aureus* strains at 6–54 ng/mL at 24 h and 9–98 ng/mL at 48 h. Another five strains secreted SED at 0.9–1.9 μg/mL at 24 h and at 1.2–2.4 μg/mL at 48 h. Strains producing high levels of SED in milk secreted 77–666 μg/mL of SED in meat juice at 24 h and 132–1225 μg/mL at 48 h. Strains producing lower amounts of SED in milk secreted 228–1109 ng/mL of SED at 24 h and 377–1782 ng/mL at 48 h in meat juice. Tested *S. aureus* strains produced SER in milk at 33–183 ng/mL at 24 h and 41–832 ng/mL at 48 h. Fourteen strains produced more SER in meat juice than in milk (17- to 232-fold and 15- to 269-fold more at 24 and 48 h, respectively). Three *S. aureus* strains secreted less than 74 ng/mL of SER in meat juice. Expression pattern of known enterotoxin regulators, that is, *agrA*, *sarA*, *hld*, *rot*, and *sigB*, was similar in selected strong and weak SED producers grown in both food matrices and could not explain differences in enterotoxin protein level. This suggests that enterotoxin regulation is more complex than previously thought. We demonstrated that in a number of tested *S. aureus* strains, production of SED and SER was significantly decreased in milk when compared with meat juice, supporting previous reports. However, certain strains secreted high amounts of SED and SER, irrespective of environment, likely contributing to higher food safety risk.

Keywords: *Staphylococcus aureus*, staphylococcal enterotoxins, SED, SER, expression, food

Introduction

Enterotoxin-producing *Staphylococcus aureus* is an important cause of foodborne intoxications worldwide. Dairy products, ham, sausages, and cooked meals are often incriminated in staphylococcal food poisoning (SFP) (Le Loir *et al.*, 2003). Staphylococcal enterotoxin D (SED) and staphylococcal enterotoxin R (SER) have been confirmed to exhibit emetic activity (Casman *et al.*, 1967; Ono *et al.*, 2008). They have previously been associated with SFP outbreaks, for example, SED was involved in an incident at a Swiss boarding school caused by consumption of soft cheese made from contaminated raw milk (Johler *et al.*, 2015). In turn, SER was potentially implicated in an SFP outbreak, which occurred at a lunch box shop in Fukuoka, Japan. In this study, *S. aureus* strains harboring pF5-like plasmids lacking the *sed* gene were indicated as a causative agent (Omoe *et al.*, 2003).

The *sed* gene is encoded along with *selj* and *ser* genes on a pIB485-like plasmid (Zhang *et al.*, 1998). *sed* promoter activity has been shown to be upregulated by Agr and SarA and downregulated by Rot and σ^B (Cheung *et al.*, 2004; Tseng *et al.*, 2004). Transcription of *sed* is moderately increased in the postexponential phase of growth, which was shown to result from an Agr-dependent decrease in Rot activity (Tseng *et al.*, 2004; Derzelle *et al.*, 2009). However, Yarwood and Schlievert (2003) suggested that SED is only partially upregulated by RNAIII and that it can be produced at high concentrations independently of Agr.

The gene encoding SER can be carried by a pIB485-like plasmid, together with *sed* and *selj* genes or by a family of pF5-like plasmids, which also contain *ses* and *set* genes, but not the *sed* gene (Omoe *et al.*, 2003; Ono *et al.*, 2008). The induction of *ser* expression was observed in the post-exponential growth phase and was sustained throughout the stationary phase, suggesting that *ser* might be controlled by Agr (Derzelle *et al.*, 2009).

Since most data on SED and SER expression come from studies conducted in microbial broths, our aim was to determine the production of SED and SER by *S. aureus* strains

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in milk and meat juice and evaluate expression of known enterotoxin regulators, that is, *agrA*, *sarA*, *hld*, *rot*, and *sigB*, during growth of *S. aureus* in both tested environments.

Materials and Methods

Bacterial strains and growth conditions

Seventeen *sed*- and *ser*-positive *S. aureus* strains were investigated (3 food, 13 human-derived isolates, and 1 reference strain) (Table 1). Enterotoxigenic reference *S. aureus* strains were kindly provided by Prof. Gerard Lina of the Centre National de Référence des Toxémies Staphylococciques, Faculté de Médecine, Lyon, France.

Ultra-high temperature 0.0% fat cow's milk (Mlekpol, Grajewo, Poland) and minced beef meat were purchased from a local store. Meat juice was obtained as described by Rantsiou *et al.* (2012) with an additional step introduced after material homogenization. It included supernatant sterilization at 121°C for 20 min and supernatant filtration through a 0.2-μm filter. *S. aureus* strains were grown in brain-heart infusion (BHI) broth (Biocorp, Warsaw, Poland) supplemented with 1% yeast extract (Biocorp) at 37°C, 230 rpm, overnight. Then, 100 mL of cow's milk or 100 mL of meat juice was inoculated with *S. aureus* BHI culture to obtain OD_{600} of 0.02 ($\sim 10^7$ colony-forming unit [CFU]/mL). Optical densities of BHI cultures were measured with Cary 100 UV-Vis Spectrophotometer (Varian, Inc.). Before inoculation, the BHI cultures were washed twice with phosphate-buffered saline to remove residual media. Cultures were incubated up to 48 h at 37°C with constant agitation at 230 rpm.

Samples for ribonucleic acid (RNA) extraction and ELISA were collected at specified time points during each experiment. Cells were quantified by plating serial dilutions of bacteria onto BHI agar at 0, 3, 5, 8, 24, 32, and 48 h in 56, 237, 238, 214, and FRI1151m *S. aureus* strains (to determine growth curves in milk and meat juice) and at 24 and 48 h in remaining strains. pH was measured in each strain at 24 and 48 h of growth by FE20-FiveEasy™ pH meter (Mettler-Toledo, Greifensee, Swiss). All experiments were carried out in triplicate with two biological replicates.

Table 1. Source and Enterotoxin Gene Content of *Staphylococcus aureus* Strains Used in This Study

Strain	*Enterotoxin gene content*	*Source*
A900322	*sep*, *egc1*	Reference strain
FRI137	*sec*, *seh*, *sel*, *egc2*	Reference strain
CCM5757	*seb*, *sek*	Reference strain
FRI913	*sea*, *sec*, *see*, *sek*, *sel*, *tst*	Reference strain
FRI1151m	*sed*, *selj*, *ser*	Reference strain
56	*seb*, *sed*, *seh*, *selj*, *ser*	Food
214	*sea*, *sed*, *selj*, *ser*	Food
237	*sea*, *sed*, *seh*, *selj*, *ser*	Food
238	*sea*, *sed*, *selj*, *ser*	Food
253	*sea*, *sed*, *selj*, *ser*	Food
266	*sea*, *sed*, *selj*, *ser*	Food
272	*sea*, *sed*, *selj*, *ser*	Food
277	*seb*, *sed*, *egc1*, *selj*, *sek*, *ser*	Food
281	*sed*, *selj*, *sek*, *ser*	Food
427	*sed*, *egc1*, *selj*, *sep*, *ser*	Food
453	*sea*, *sed*, *selj*, *ser*	Food
508	*sed*, *selj*, *sep*, *ser*	Human
553	*sea*, *sed*, *egc1*, *selj*, *ser*, *tst*	Human
620	*sea*, *sed*, *selj*, *ser*	Human
641	*sea*, *sed*, *egc1*, *selj*, *ser*	Human
664	*sea*, *sed*, *selj*, *sek*, *ser*, *tst*	Human

Detection of enterotoxin genes

Detection of *sea-see* was performed according to Sharma *et al.* (2000). The *selu* gene was detected using the method described by Letertre *et al.* (2003). For the detection of the *tst* gene, the primers and conditions were described according to Monday and Bohach (1999). Detection of *sei*, *sem*, *sen*, *seo*, *seh*, *selj*, *sek*, *sel*, and *sep* was done as previously described (Bania *et al.*, 2006; Lis *et al.*, 2009). *S. aureus* reference strains served as polymerase chain reaction (PCR) controls.

Sandwich ELISA

Recombinant enterotoxins D (rSED) and R (rSER) were obtained by cloning *sed* and *ser* genes into pET-22b vector, as described previously by Lis *et al.* (2012). Regions encoding mature SED and SER were PCR amplified from *S. aureus* 214 strain. Cloning forward and reverse primers (listed in Table 2) carried the restriction sites for *NcoI* and *XhoI*, respectively. Enterotoxins were expressed in *Escherichia coli* Rosetta cells (Merck, Darmstadt, Germany) using IPTG induction (Sigma-Aldrich, St. Louis, MI) and purified on His-Select Cobalt Affinity Gel (Sigma-Aldrich) with on-column refolding. Rabbit polyclonal anti-SED antibody was purchased from Acris Antibodies (Herford, Germany). Rabbit polyclonal anti-SER antibody was obtained as described by Lis *et al.* (2012). Antibodies conjugated with biotin N-hydroxysuccinimide ester (Sigma-Aldrich) were used as secondary antibodies.

Samples for SED and SER detection were collected at 24 and 48 h of growth and stored at –20°C until analyzed. ELISA was performed as described earlier (Schubert *et al.*, 2016). The specificity of the ELISA was assessed using culture supernatants of *S. aureus* reference strains as controls for enterotoxins SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SElJ, SEK, SEL, SEM, SEN, SEO, SEP, and SER. The detection and quantification limits of the assays were 3 and 4 ng/mL for SED and 1.5 and 3 ng/mL for SER, respectively.

Enterotoxin concentration was measured with rSED and rSER as standards, using a 4-parameter logistic curve fit. Concentrations of recombinant enterotoxins were determined with the Bicinchoninic Acid Kit (Sigma-Aldrich). Data analysis was carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

RNA extraction and RT-qPCR

Bacteria for RNA isolation were collected at 5, 8, and 24 h of growth. RNA extraction, purification, cDNA synthesis, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were performed as described by Schubert *et al.* (2016). Primers used are listed in Table 2. *rpoB*, a housekeeping gene found to be stably expressed in milk, was used for cDNA normalization (Valihrach *et al.*, 2014). Transcript levels relative to *rpoB* were calculated according to Pfaffl (2001). Data analysis was carried out using Bio-Rad CFX Manager software.

Table 2. Primers Used for Cloning of *sed* and *ser* Genes and for RT-qPCR

Target	*Nucleotide sequence (5′-3′)*	*Amplicon length (bp)*	*Source*
Cloning			
sed	F: CATGCCATGGGCAAACATTCTTATGCAG R: CGGCTCGAGCTTTTCATATAAATAGATGTC	643	This study
ser	F: CATGCCATGGGCAAACCAGATCCAAGGCC R: CATGCTGGAGCATTGTAGTCAGGTGAAG	724	Lis *et al.* (2012)
RT-qPCR			
agrA	F: CCTCGCAACTGATAATCCTTATG R: ACGAATTTCACTGCCTAATTTGA	127	Valihrach *et al.* (2014)
hld	F: TAAGGAAGGAGTGATTTCAATGG R: GTGAATTTGTTCACTGTGTCGAT	90	Valihrach *et al.* (2014)
sarA	F: TTGCTTTGAGTTGTTATCAATGG R: TTTCTCTTTGTTTTCGCTGATGT	124	Valihrach *et al.* (2014)
sed	F: TCAATTTGTGGATAAATGGTGTAC R: TTTCCTCCGAGAGTATCATTAT	154	Derzelle *et al.* (2009)
ser	F: TCCTATTCCTTATTCAGAATACA R: GGGTATTCCAAACACATCTAAC	102	Derzelle *et al.* (2009)
sigB	F: TTCACCTGAGCAAATTAACCAAT R: ATCTTCGTGATGTGATTGTCCTT	145	Valihrach *et al.* (2014)
rot	F: TGCAGTATTTCAACCACACAC R: GTATCGTTAATGCGCCAGT	140	Sato'o et. al (2015)
rpoB	F: GACCTCTGTGCTTAGCTGTAATAGC R: GCGAACATGCAACGTCAAG	121	Duquenne *et al.* (2010)

RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

PCR efficiency for each primer pair was previously determined on genomic DNA from respective reference *S. aureus* strains by running serial 10-fold dilutions of the template (data not shown). Amplification efficiencies for all used primer sets were between 96% and 104%.

Statistics

Statistical significance of the results was assessed using the U Mann–Whitney test; $p < 0.05$ was considered statistically significant. Statistical analyses were performed using Statistica version 12 (StatSoft, Inc., Kraków, Poland).

Results

Bacterial growth

The growth curves were determined in milk and meat juice for five randomly selected *S. aureus* strains. *S. aureus* FRI1151m strain exhibited a significantly lower growth rate than the other strains in milk at all time points ($p < 0.05$). During growth in meat juice, higher bacterial counts were determined in *S. aureus* 56 strain at 24 and 32 h ($p < 0.05$) and in *S. aureus* 237 strain at 3 and 48 h ($p < 0.05$). Lower cell numbers were recorded for *S. aureus* 238 strain at 3, 8, and 24 h ($p < 0.05$) and for *S. aureus* FRI1151m strain at 32 and 48 h of culture ($p < 0.05$) (Fig. 1). All the 17 *S. aureus* strains reached 8.3–9.1 log CFU/mL at 24 h and 8.4–9.3 log CFU/mL at 48 h of culture in milk. During growth in meat juice, they reached 7.7–9.4 log CFU/mL at 24 h and 8.2–9.5 log CFU/mL at 48 h. Bacterial counts determined at 24 h were significantly higher in cultures conducted in milk than in meat juice ($p < 0.05$). No significant differences were noted in strains cultured both in milk and meat juice at 48 h.

In *S. aureus* strains grown in milk, the initial pH value of 6.86 was unaltered until 48 h of growth. The pH of meat juice decreased from 6.22 at the beginning of culture to 5.51–6.20 at 5 h of growth and subsequently increased reaching 6.38–8.11 at 24 h and 6.75–9.03 at 48 h. The pH values at 24 and

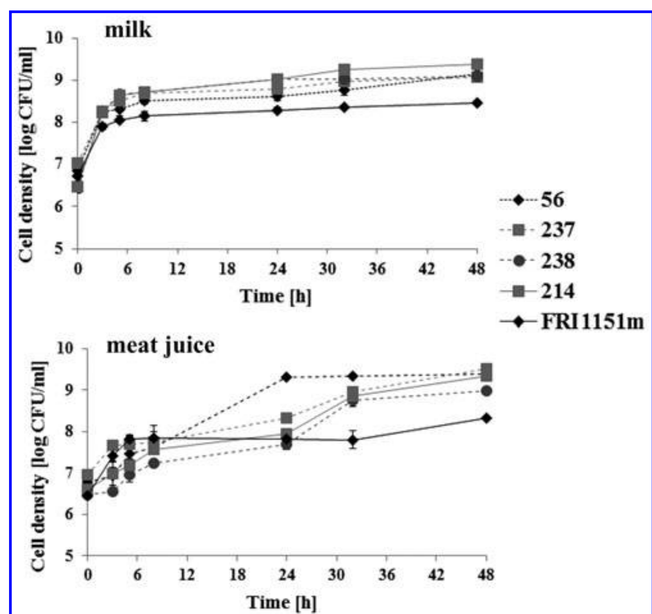

FIG. 1. Growth of *Staphylococcus aureus* strains producing high levels of staphylococcal enterotoxin D (SED) in milk, that is, FRI1151m and 214 (marked with ——), and randomly selected weak SED producers, that is, 56, 237, and 238 (marked with - - - -), determined in milk and meat juice.

Table 3. Production of SED and SER by *Staphylococcus aureus*, Determined by ELISA, and pH Values During Culture in Milk and Meat Juice

	SED, ng/mL				SER, ng/mL				pH			
	Milk		Meat juice		Milk		Meat juice		Milk		Meat juice	
Strain	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
FRI1151m	1452 ± 95	2278 ± 108	666,359 ± 13,237	1,225,196 ± 41,309	160 ± 17	168 ± 4	9947 ± 643	12,208 ± 678	6.67	6.79	7.14	8.03
56	33 ± 3	68 ± 6	1109 ± 95	1782 ± 84	141 ± 10	397 ± 42	9137 ± 361	25,339 ± 1375	6.81	6.71	6.94	8.18
214	1840 ± 59	2082 ± 95	119,290 ± 2339	298,028 ± 10,649	162 ± 13	183 ± 22	2668 ± 209	9093 ± 364	6.72	6.73	6.52	7.42
237	35 ± 8	71 ± 11	720 ± 40	787 ± 51	134 ± 16	165 ± 14	4196 ± 283	24,251 ± 1435	6.84	6.89	6.51	7.32
238	38 ± 11	63 ± 10	826 ± 43	1196 ± 49	141 ± 10	164 ± 14	5719 ± 135	14,277 ± 745	6.81	6.91	6.75	7.71
253	22 ± 2	25 ± 9	228 ± 13	583 ± 18	95 ± 12	282 ± 13	26 ± 1	34 ± 3	6.79	6.82	6.81	7.37
266	42 ± 7	71 ± 7	248 ± 35	536 ± 34	116 ± 10	157 ± 17	3526 ± 375	13,337 ± 815	6.85	7.03	6.38	7.09
272	23 ± 1	47 ± 3	717 ± 38	1143 ± 17	100 ± 7	104 ± 12	3456 ± 294	11,677 ± 432	6.94	7.17	6.77	7.97
277	30 ± 3	40 ± 9	264 ± 35	768 ± 45	33 ± 7	41 ± 7	10 ± 1	13 ± 4	6.87	6.99	6.88	8.25
281	6 ± 1	9 ± 2	958 ± 33	1300 ± 31	82 ± 8	101 ± 8	13,318 ± 2302	27,272 ± 1998	6.74	6.63	6.91	8.18
427	936 ± 78	2395 ± 139	200,146 ± 5025	274,378 ± 8984	128 ± 16	832 ± 33	6798 ± 581	12,527 ± 1385	6.82	6.56	7.58	8.53
453	1934 ± 34	2363 ± 118	172,012 ± 3258	181,197 ± 4336	183 ± 19	253 ± 16	6931 ± 249	9242 ± 501	6.84	6.94	8.11	9.03
508	13 ± 2	18 ± 2	1000 ± 25	1304 ± 49	102 ± 7	108 ± 8	14,618 ± 921	18,636 ± 942	6.75	6.76	7.04	7.95
553	1101 ± 69	1153 ± 44	77,072 ± 2634	132,580 ± 6078	132 ± 16	142 ± 17	2684 ± 160	3396 ± 163	7.15	7.17	7.27	7.77
620	54 ± 11	98 ± 10	839 ± 22	856 ± 27	50 ± 4	118 ± 11	11,609 ± 390	15,019 ± 1234	6.97	6.93	7.10	7.91
641	33 ± 6	53 ± 3	1010 ± 68	1128 ± 56	75 ± 16	193 ± 26	4220 ± 347	7860 ± 354	6.95	7.08	7.90	8.90
664	16 ± 2	26 ± 3	280 ± 32	377 ± 27	54 ± 5	98 ± 8	66 ± 4	74 ± 6	6.98	6.8	6.46	6.75

48 h of bacterial growth in milk and meat juice are listed in Table 3.

Production of SED by S. aureus *strains*

Twelve of 17 *S. aureus sed*- and *ser*-positive strains, whose gene content is presented in Table 1, produced relatively small amounts of SED in milk with mean levels of 29 ng/mL at 24 h and 49 ng/mL at 48 h of culture. Five strains (FRI1151m, 214, 427, 453, and 553) secreted significantly higher amounts of SED in milk ($p < 0.005$) with mean levels of 1.2 μg/mL at 24 h and 2.4 μg/mL at 48 h. They were designated as strong SED producers. Significantly more SED was produced when *S. aureus* strains were grown in meat juice ($p < 0.05$). Strong producers of SED in milk secreted SED in meat juice with mean levels of 247 μg/mL at 24 h and 422 μg/mL at 48 h of growth. These amounts were significantly higher than for the remaining strains ($p < 0.005$), which produced SED in meat juice with mean levels of 683 ng/mL at 24 h and 980 ng/mL at 48 h.

Strong SED producers secreted between 65 and 459 times and between 77 and 538 times more SED in meat juice than in milk at 24 and 48 h, respectively, whereas the remaining *S. aureus* strains produced between 6 and 171 times and between 8 and 141times more SED in meat juice than in milk at 24 and 48 h, respectively (Table 3).

The bacterial numbers reached by tested strains in meat juice at 24 and 48 h as well as in milk at 24 h were not related to higher or lower SED expression. In turn, weak SED producers cultured in milk grew to higher densities at 48 h than strong SED producers ($p < 0.05$).

Production of SER by S. aureus *strains*

Tested *S. aureus* strains produced moderate amounts of SER in milk with mean levels of 111 ng/mL at 24 h and 206 ng/mL at 48 h of growth. Among them, 2 strains expressed less than 100 ng/mL of SER at 48 h, 11 strains produced between 100 and 200 ng/mL, and 4 strains secreted more than 200 ng/mL in milk. Fourteen *S. aureus* strains produced significantly higher levels of SER in meat juice after 24 and 48 h ($p < 0.005$). SER production by these strains in meat juice ranged from 2.7 to 14.6 μg/mL after 24 h and 3.4 to 27.3 μg/mL after 48 h. They produced between 17- and 232 times and between 15 and 269 times more SER in meat juice than in milk at 24 and 48 h, respectively. Three *S. aureus* strains secreted less than 74 ng/mL of SER in meat juice (Table 3).

The cell numbers reached by tested strains in meat juice and milk at 24 and 48 h were not related to higher or lower SER expression.

Expression of sed *and* ser *genes and selected genes involved in their regulation*

The level of transcripts was assessed in all five *S. aureus* strains producing the highest levels of SED in milk, that is, *S. aureus* FRI1151m, 214, 427, 453, and 553, and six randomly selected weak SED producers, that is, *S. aureus* 56, 237, 238, 266, 281, and 508.

sed transcripts were detected in all tested *S. aureus* strains. A rise of relative level of *sed* RNA was observed between 8 and 24 h of culture in milk in *S. aureus* FRI1151m, 281, and

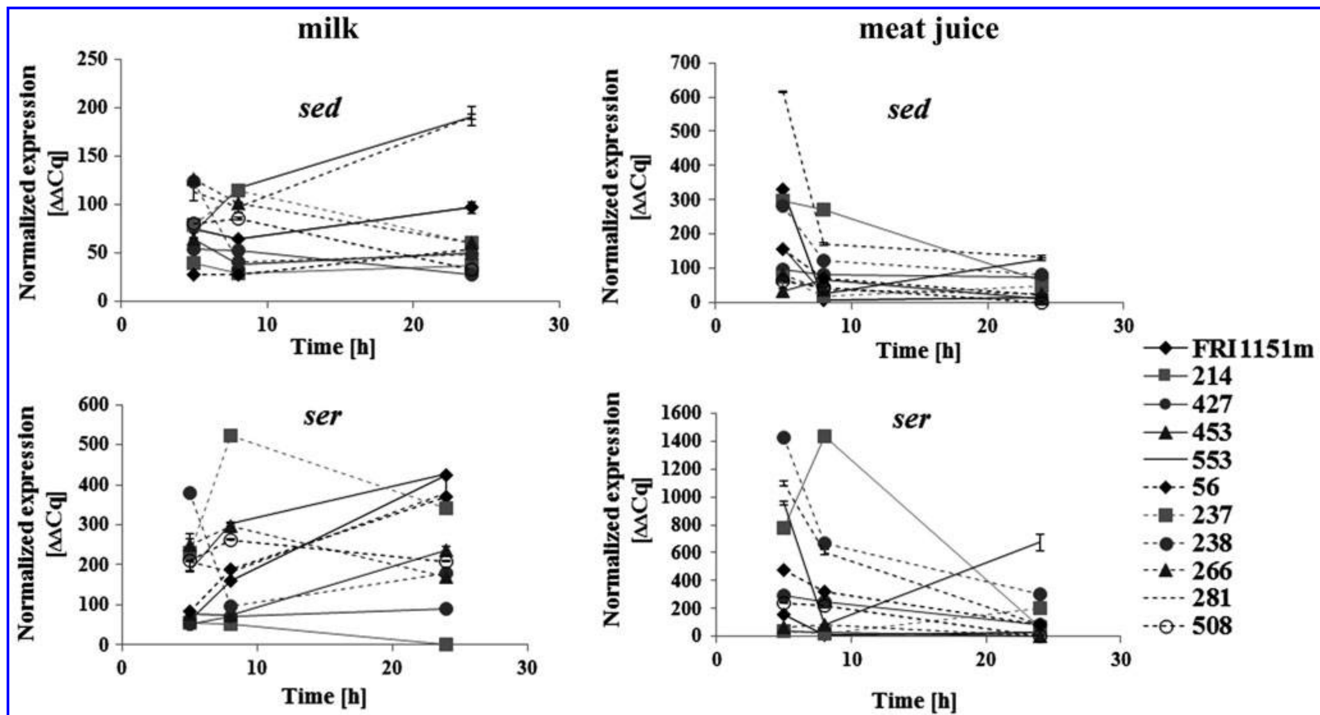

FIG. 2. Relative *sed* and *ser* ribonucleic acid (RNA) levels in strong SED producers in milk, that is, *Staphylococcus aureus* FRI1151m, 214, 427, 453, and 553 strains (marked with ——), and randomly selected weak SED producers, that is, *S. aureus* 56, 237, 238, 266, 281, and 508 strains (marked with - - - -), determined in milk and meat juice.

553 ($p<0.05$). The highest *sed* levels during bacterial growth in meat juice were noted at 5 h in *S. aureus* FRI1151m and 281 and at 8 h in *S. aureus* 214 and 281 ($p<0.05$) (Fig. 2).

The relative level of *ser* was upregulated between 5 and 8 h of growth in milk in *S. aureus* FRI1151m, 56, 237, and 553 and between 8 and 24 h in *S. aureus* FRI1151m, 56, 238, 281, and 553 ($p<0.005$). There was a significant downregulation of *ser* RNA between 5 and 8 h of culture in meat juice in *S. aureus* FRI1151m, 56, 238, 281, and 553 ($p<0.05$) and between 8 and 24 h in *S. aureus* 214, 238, and 281 ($p<0.005$). Relative level of *ser* transcript was the highest at 24 h in *S. aureus* 553 grown in meat juice ($p<0.05$) (Fig. 2).

The highest *agrA* levels during bacterial growth in milk were observed at 5 h in *S. aureus* 56, 237, and 453 ($p<0.005$). There was a significant upregulation of *agrA* RNA between 8 and 24 h of culture in milk in *S. aureus* 281 ($p<0.05$). The relative level of *agrA* was downregulated from 5 to 8 h of growth in meat juice in *S. aureus* 56, 214, 237, 238, and 281 ($p<0.005$), followed by a further drop up to 24 h in *S. aureus* 214 ($p<0.05$). Contrarily, the level of *agrA* RNA was upregulated between 8 and 24 h of culture in meat juice in *S. aureus* 56, 281, and 427 ($p<0.005$) (Table 4).

The relative level of *sarA* was upregulated from 8 to 24 h of growth in milk in all tested strains, except *S. aureus* 427 and 508 ($p<0.005$). The expression of *sarA* RNA in *S. aureus* strains grown in meat juice revealed a similar pattern as in cultures conducted in milk. Most strains, except *S. aureus* 214, 453, and 508, showed significant upregulation of *sarA* RNA between 8 and 24 h of growth ($p<0.005$), whereas a drop in *sarA* level was noted in *S. aureus* 214 and 453 ($p<0.005$) (Table 4).

The *rot* level was downregulated from 5 to 8 h of culture in milk in *S. aureus* 238 ($p<0.05$) and from 8 to 24 h in *S. aureus* 266 ($p<0.05$). There was a significant increase in *rot* level between 8 and 24 h of growth in milk in *S. aureus* 56, 214, 281, and 453 ($p<0.05$). The highest levels of *rot* during bacterial growth in meat juice were observed at 5 h in *S. aureus* FRI1151m and 281 ($p<0.005$). Relative level of *rot* was upregulated from 8 and 24 h of culture in meat juice in *S. aureus* 281, 427, and 553 ($p<0.005$) (Table 4).

The level of *hld* was upregulated between 8 and 24 h of growth in milk in all tested strains, except *S. aureus* FRI1151m, 56, and 266 ($p<0.005$), in which the *hld* level was unaltered. Relative level of *hld* RNA increased from 5 to 8 h of culture in meat juice in *S. aureus* 214 ($p<0.05$) and decreased in *S. aureus* 56, 237, 281, 508, and 553 ($p<0.05$). There was a significant upregulation in *hld* level between 8 and 24 h of growth in meat juice in *S. aureus* 281 ($p<0.05$) and downregulation in *S. aureus* 214, 238, and 453 ($p<0.05$). *hld* transcripts were not detected in *S. aureus* 266 grown in milk and *S. aureus* FRI1151m and 266 cultivated in meat juice (Table 4).

Relative level of *sigB* was upregulated from 5 to 8 h of culture in milk in *S. aureus* 238 and 266 ($p<0.05$). There was a drop in *sigB* level between 8 and 24 h of growth in milk in *S. aureus* 214 ($p<0.05$) and its increase in *S. aureus* 237 and 281 ($p<0.005$). The highest levels of *sigB* during bacterial growth in meat juice were observed at 5 h in *S. aureus* 214 and 281 ($p<0.005$). The level of *sigB* RNA was upregulated from 8 to 24 h of culture in meat juice in *S. aureus* 281, 453, and 553 ($p<0.005$) and downregulated in *S. aureus* 56, 214, 237, 238, 266, and 508 ($p<0.005$) (Table 4).

Discussion

Milk and milk products are considered to be of particular significance as an staphylococcal enterotoxin (SE) source. In

Table 4. Relative RNA Levels of *agrA*, *sarA*, *rot*, *hld*, and *sigB* Involved in Enterotoxin Regulation Determined in *Staphylococcus aureus* FRI1151m, 56, 214, 237, 238, 266, 281, 427, 453, 508, and 553

		Normalized expression [ΔΔCq]					
		Milk			*Meat juice*		
Gene	*Strain*	*5 h*	*8 h*	*24 h*	*5 h*	*8 h*	*24 h*
agrA	FRI1151m	0.7 ± 0.0	0.6 ± 0.0	1.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
	56	14.7 ± 0.0[a]	6.9 ± 0.2	4.2 ± 0.1	20.2 ± 0.2[a]	6.4 ± 0.3[a]	14.7 ± 0.2
	214	6.6 ± 0.0	4.8 ± 0.0	4.3 ± 0.2	18.9 ± 0.2[a]	15.0 ± 0.3[a]	4.1 ± 0.0
	237	22.3 ± 0.3[a]	0.1 ± 0.0	2.1 ± 0.1	8.4 ± 0.1[a]	2.8 ± 0.0	2.9 ± 0.0
	238	1.2 ± 0.0	7.2 ± 0.0	4.3 ± 0.0	10.4 ± 0.2[a]	5.9 ± 0.0	3.8 ± 0.0
	266	0.4 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.0 ± 0.0
	281	4.7 ± 0.1	6.2 ± 0.0[a]	18.6 ± 0.7	22.4 ± 0.6[a]	2.2 ± 0.0[a]	9.2 ± 0.0
	427	3.2 ± 0.0	2.8 ± 0.1	1.6 ± 0.0	4.0 ± 0.0	3.3 ± 0.1[a]	15.8 ± 0.3
	453	11.4 ± 0.1[a]	9.0 ± 0.1	3.7 ± 0.0	2.0 ± 0.0	3.8 ± 0.1	1.0 ± 0.0
	508	4.4 ± 0.0	4.4 ± 0.0	1.7 ± 0.0	2.7 ± 0.0	2.6 ± 0.0	2.6 ± 0.0
	553	5.4 ± 0.2	2.9 ± 0.0	5.5 ± 0.0	5.0 ± 0.1	1.3 ± 0.0	2.4 ± 0.0
sarA	FRI1151m	7.5 ± 0.0	4.2 ± 0.1[a]	13.2 ± 0.0	1.3 ± 0.0	0.9 ± 0.0[a]	2.7 ± 0.0
	56	7.3 ± 0.7	6.9 ± 0.2[a]	13.5 ± 0.6	4.6 ± 0.1	2.7 ± 0.1[a]	13.5 ± 0.1
	214	6.5 ± 0.3	6.3 ± 0.1[a]	12.0 ± 3.0	2.9 ± 0.0	8.8 ± 0.1[a]	2.8 ± 0.0
	237	10.2 ± 0.0	2.7 ± 0.0[a]	25.7 ± 0.1	3.6 ± 0.0	1.2 ± 0.0[a]	2.7 ± 0.1
	238	2.9 ± 0.0	11.8 ± 0.0[a]	32.6 ± 0.1	7.9 ± 0.3	2.7 ± 0.0[a]	10.8 ± 0.1
	266	14.1 ± 0.1	8.1 ± 0.4[a]	21.7 ± 0.1	0.9 ± 0.0	0.9 ± 0.0[a]	2.7 ± 0.0
	281	23.0 ± 0.5	23.8 ± 0.0[a]	128.1 ± 2.1	15.4 ± 0.0	1.9 ± 0.0[a]	6.8 ± 0.2
	427	8.0 ± 0.0	7.0 ± 0.1	5.9 ± 0.1	1.2 ± 0.0	2.6 ± 0.0[a]	9.7 ± 0.0
	453	18.6 ± 0.1	17.4 ± 0.2[a]	48.4 ± 0.5	4.2 ± 0.0	4.4 ± 0.1[a]	3.6 ± 0.0
	508	14.8 ± 0.1	13.2 ± 0.2	13.9 ± 0.1	2.2 ± 0.0	1.1 ± 0.0	1.0 ± 0.0
	553	12.9 ± 0.0	11.5 ± 0.1[a]	27.7 ± 0.3	5.5 ± 0.1	0.8 ± 0.0[a]	4.9 ± 0.1
rot	FRI1151m	1.6 ± 0.2	2.6 ± 0.1	5.3 ± 0.1	8.7 ± 0.1[a]	1.1 ± 0.0	0.9 ± 0.0
	56	1.5 ± 0.0	2.4 ± 0.0[a]	4.1 ± 0.1	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0
	214	1.7 ± 0.0	1.6 ± 0.1[a]	5.7 ± 0.0	3.2 ± 0.0	4.1 ± 0.0	1.5 ± 0.0
	237	3.6 ± 0.1	4.1 ± 0.1	4.3 ± 0.1	2.0 ± 0.0	1.7 ± 0.1	1.4 ± 0.0
	238	5.8 ± 0.0[a]	1.9 ± 0.1	3.5 ± 0.1	4.4 ± 0.1	3.7 ± 0.0	3.0 ± 0.0
	266	3.4 ± 0.1	4.0 ± 0.2[a]	2.3 ± 0.2	1.5 ± 0.0	1.6 ± 0.0	1.7 ± 0.1
	281	3.7 ± 0.3	3.4 ± 0.1[a]	9.4 ± 0.2	7.2 ± 0.1[a]	2.2 ± 0.0[a]	5.4 ± 0.1
	427	2.0 ± 0.1	1.7 ± 0.0	1.4 ± 0.1	2.2 ± 0.1	2.2 ± 0.1[a]	4.1 ± 0.0
	453	1.5 ± 0.0	1.5 ± 0.0[a]	3.9 ± 0.0	2.8 ± 0.0	4.2 ± 0.0	2.4 ± 0.0
	508	2.0 ± 0.0	2.2 ± 0.1	2.1 ± 0.1	1.1 ± 0.0	1.8 ± 0.0	1.0 ± 0.0
	553	2.7 ± 0.1	4.9 ± 0.2	3.9 ± 0.3	1.9 ± 0.0	1.5 ± 0.0[a]	11.4 ± 0.5
hld	FRI1151m	0.6 ± 0.0	0.5 ± 0.0	1.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	56	8.2 ± 0.1	11.5 ± 0.1	12.0 ± 0.1	21.4 ± 1.1[a]	16.1 ± 0.2	23.5 ± 0.2
	214	1.8 ± 0.0	2.5 ± 0.1[a]	28.3 ± 0.5	16.6 ± 0.1[a]	121.2 ± 0.7[a]	7.0 ± 0.2
	237	6.0 ± 0.1	1.5 ± 0.0[a]	9.2 ± 0.0	39.8 ± 0.2[a]	18.7 ± 0.1	16.8 ± 0.2
	238	0.2 ± 0.0	12.8 ± 0.1[a]	19.8 ± 0.1	27.9 ± 0.2	27.0 ± 0.0[a]	9.0 ± 0.0
	266	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	281	4.0 ± 0.0	6.5 ± 0.2[a]	43.3 ± 0.2	31.5 ± 0.9[a]	15.3 ± 0.1[a]	34.6 ± 0.1
	427	9.3 ± 0.2	6.7 ± 0.0[a]	19.1 ± 0.1	16.2 ± 0.2	17.0 ± 0.3	21.7 ± 0.0
	453	11.6 ± 0.1	11.7 ± 0.1[a]	18.1 ± 0.5	24.2 ± 0.8	18.2 ± 0.0[a]	1.0 ± 0.0
	508	4.0 ± 0.1	5.6 ± 0.1[a]	10.7 ± 0.1	17.5 ± 0.0[a]	9.4 ± 0.1	10.1 ± 0.0
	553	6.8 ± 0.0	6.5 ± 1.1[a]	11.0 ± 0.1	57.1 ± 0.2[a]	5.2 ± 0.0	6.4 ± 0.0
sigB	FRI1151m	2.4 ± 0.1	2.5 ± 0.2	2.9 ± 0.1	5.7 ± 0.1	1.1 ± 0.0	1.9 ± 0.1
	56	2.8 ± 0.1	4.4 ± 0.2	6.4 ± 0.2	6.0 ± 0.2	4.6 ± 0.2[a]	2.3 ± 0.0
	214	4.9 ± 0.1	4.8 ± 0.1[a]	1.6 ± 0.2	16.8 ± 0.5[a]	16.8 ± 0.3[a]	1.7 ± 0.0
	237	5.5 ± 0.1	3.3 ± 0.1[a]	6.0 ± 0.2	9.3 ± 0.3	4.0 ± 0.2[a]	1.0 ± 0.0
	238	3.3 ± 0.1[a]	5.6 ± 0.2	4.8 ± 0.1	7.8 ± 0.1	6.1 ± 0.1[a]	1.3 ± 0.0
	266	6.9 ± 0.9[a]	11.7 ± 0.2	11.4 ± 0.3	9.4 ± 0.2	4.5 ± 0.2[a]	1.6 ± 0.0
	281	5.4 ± 0.1	5.8 ± 0.1[a]	15.8 ± 0.6	20.6 ± 0.6[a]	5.5 ± 0.3[a]	13.5 ± 0.3
	427	3.0 ± 0.1	4.2 ± 0.2	5.0 ± 0.1	6.2 ± 0.3	4.5 ± 0.2	5.3 ± 0.1
	453	6.2 ± 0.2	6.4 ± 0.1	5.0 ± 0.2	6.2 ± 0.1	6.4 ± 0.3[a]	10.3 ± 0.5
	508	5.4 ± 0.1	5.9 ± 0.2	5.5 ± 0.1	3.4 ± 0.1	3.9 ± 0.1[a]	2.1 ± 0.6
	553	6.4 ± 0.1	7.3 ± 0.3	7.1 ± 0.1	9.7 ± 0.2	2.1 ± 0.1[a]	7.6 ± 0.1

[a]Indicates points where significant changes in gene expression were noted ($p < 0.05$).

the European Union, only some milk-derived products are examined for enterotoxin content when the number of coagulase-positive staphylococci exceeds 10^5 CFU/g in a product (http://eur-lex.europa.eu). A number of SFP cases in the United Kingdom and France have also been associated with meat-based food products. In the United States, red meat was recognized as the main source of SE transmission (Le Loir *et al.*, 2003).

SED is a well-characterized toxin, and strains that produce this toxin have been involved in numerous SFP cases (Wieneke *et al.*, 1993; Kérouanton *et al.*, 2007). Since SED and SER are encoded on the same plasmid in *S. aureus*, contribution of both emetic toxins to SFP is not easy to define. According to Omoe *et al.* (2003), specific *S. aureus* Fukuoka clones harbor plasmids from which the *sed* gene is absent. These strains can secrete up to 200 ng/mL of SER in broth culture and were implicated in SFP case in Japan (Omoe *et al.*, 2003). It could thus be suggested that SER can contribute to food poisoning independently of SED. However, further study by the same team demonstrated that the Fukuoka strains encode two more emetic toxins, namely SES and SET, which complicate interpretation of the role of SER in SFP (Ono *et al.*, 2008).

Milk has previously been observed as an unfavorable environment for expression of staphylococcal enterotoxins (Valihrach *et al.*, 2013, 2014; Hunt *et al.*, 2014; Schubert *et al.*, 2016). Most studies published to date reported on staphylococcal strains carrying the *sec* gene. Based on these results, it was suggested that SEC production in milk should not account for high food safety risk (Hunt *et al.*, 2014). We recently demonstrated significant downregulation of SEC and SEH expression in milk in a number of *S. aureus* strains harboring the *seh* gene (Schubert *et al.*, 2016).

In this study, we demonstrate that other staphylococcal toxins, namely SED and SER, are expressed by *S. aureus* strains at lower levels in milk when compared with meat juice. Maximal SED concentration in milk produced by these strains did not exceed 100 ng/mL, while SER did not exceed 400 ng/mL. However, some strains included in our collection were able to secrete over 2 μg/mL of SED in milk. Meanwhile, strains producing low amounts of SED in milk were able to secrete up to 1.3 μg/mL of SED in meat juice, while strong SED producers secreted more than 650 μg/mL of SED into this medium.

Strong enterotoxin producers were also identified within SEH-producing strains, and high-level SEH production was related to a decrease of milk pH during culture, which was not observed in weak SEH producers (Schubert *et al.*, 2016). High-level SED production, observed in a number of our strains, was not related to modification of milk pH by *S. aureus* up to 48 h of culture. Relationship of milk pH and enterotoxin production seems not to apply to all toxins, indicating different regulation of their expression. The pH of cultures carried out in meat juice consequently decreased at 5 h of growth, then increased up to 48 h, reaching different values depending on the strain.

The decline of pH in our cultures can result from an accumulation of fermentative end products (Regassa *et al.*, 1992). Weak acids can pass the bacterial membrane and dissociate in the cytoplasm, which results in decrease of internal pH. To withstand unfavorable conditions, *S. aureus* can modify the pH of its environment. It was speculated that it can be achieved by metabolizing acids and producing diacetyl, pyrazine, and ammonia (Rode *et al.*, 2010). Thus, the pH variation observed in our meat juice cultures can result from differences in levels and types of end products secreted by a given strain.

Expression of virulence genes in *S. aureus*, including enterotoxins, is controlled by transcriptional regulators such as the *sarA* family and *sigB*, as well as two-component systems, for example, *agr* and *sae*RS (Novick, 2001). We examined expression of five genes previously shown to be involved in *sed* gene regulation; however, we could not establish a clear relationship between the enterotoxin protein level and expression of investigated regulators in strong and weak SED producers. Moreover, in a number of our strains, enterotoxin RNA profiles seemed not to reflect respective protein levels. It was recently shown that *sed* RNA level may not be convergent with SED level, suggesting that yet unexplored ways of enterotoxin regulation may exist (Sihto *et al.*, 2016).

Using mutational analysis of global regulators, Sihto *et al.* (2016) also demonstrated that SED expression may not be tightly controlled by Agr and that the effect of global regulators on SED may vary between strains. Schelin *et al.* (2011) demonstrated that enterotoxin production can be altered by the food matrix. Nonetheless, very few authors reported on molecular changes in *S. aureus* grown in milk. Lammers *et al.* (2000) investigating the response of *S. aureus* to the milk environment indicated four groups of genes specifically expressed in milk. They were involved in cell wall synthesis, nucleotide synthesis, transcriptional regulation, and carbohydrate metabolism. No changes in genes potentially involved in enterotoxin regulation were mentioned.

Conclusions

We demonstrated that in a number of tested *S. aureus* strains, SER was produced at higher levels than SED in milk and meat juice. With most strains tested in this study, production of SED and SER was significantly decreased in milk when compared with meat juice. However, certain *S. aureus* strains were still able to secrete considerable amounts of SED and SER in milk. No clear relationship of enterotoxin protein level and expression of known regulators could be established. Altogether, our results indicate that enterotoxin production in meat is likely to pose higher risk for food safety than enterotoxin production in milk, suggesting that an area of routine food control should be expanded. Nevertheless, food safety risk related to enterotoxin production in milk should still be considered important.

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Disclosure Statement

No competing financial interests exist.

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