# Effect of sodium nitrite and regulatory mutations Δagr, ΔsarA, and ΔsigB on the mRNA and protein levels of staphylococcal enterotoxin D

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Abstract: Staphylococcal food poisoning results from ingestion of enterotoxins produced by Staphylococcus aureus. Staphylococcal enterotoxin D (SED) is one of the most common toxins detected in S. aureus strains associated with intoxications. The effect of sodium nitrite on enterotoxin production has been only partly investigated, despite its wide usage in meat products. In addition, the factors influencing SED regulation are unclear. The aim of this study was to determine the effect of sodium nitrite on sed transcription and SED production, as well as the effect of regulatory mutations on SED protein levels. Temporal sed mRNA and SED protein levels were compared in LB and LB supplemented with 150 mg/L nitrite, and SED protein levels between wild type (wt) and isogenic regulatory mutants (Δagr, ΔsarA, ΔsigB) under control and sodium nitrite conditions. Relative sed mRNA levels of wt strains were higher in late stationary phase in the presence of nitrite compared to control conditions. However, SED protein levels were decreased in the presence of nitrite. In LB, Δagr mutants showed SED levels similar to the wt, while ΔsarA mutants exhibited reduced and ΔsigB mutants increased SED levels compared to the wt. In LB with sodium nitrite, SED levels of mutant strains were reduced similar to the wt strains, except for two Δagr mutants, in which SED levels were increased in the presence of nitrite. Overall, strain-specific variation with regard to the effect of regulatory mutations was observed. In addition, the data suggests that SED regulation may not be as tightly dependent on Agr as previously described.

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# Effect of Sodium Nitrite and Regulatory Mutations Δ*agr*, Δ*sarA*, and Δ*sigB* on the mRNA and Protein Levels of Staphylococcal Enterotoxin D

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Running Head: Effect of sodium nitrite on SED expression

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**ABSTRACT**

Staphylococcal food poisoning results from ingestion of enterotoxins produced by *Staphylococcus aureus*. Staphylococcal enterotoxin D (SED) is one of the most common toxins detected in *Staphylococcus aureus* strains associated with intoxications. The effect of sodium nitrite on enterotoxin production has been only partly investigated, despite its wide usage in meat products. In addition, the factors influencing SED regulation are unclear. We aimed to determine the effect of sodium nitrite on *sed* transcription and SED production, as well as the effect of regulatory mutations on SED protein levels. Temporal *sed* mRNA and SED protein levels were compared in LB and LB supplemented with 150 mg/l nitrite, and SED protein levels between wild type (wt) and isogenic regulatory mutants (Δ*agr*, Δ*sarA*, Δ*sigB*) under control and sodium nitrite conditions. Relative *sed* mRNA levels of wt strains were induced in late stationary phase in the presence of nitrite compared to control conditions. However, SED protein levels were reduced in the presence of nitrite. In LB, Δ*agr* mutants showed SED levels similar to the wt, while Δ*sarA* mutants exhibited reduced and Δ*sigB* mutants increased SED levels compared to the wt. In LB with sodium nitrite, SED levels of mutant strains were reduced similar to the wt strains, except for two Δ*agr* mutants, in which SED levels were increased in the presence of nitrite. Overall, we observed strain-specific variation with regard to the effect of regulatory mutations. In addition, our data suggests that SED regulation may not be as tightly dependent on Agr as previously described.

**Keywords:** *Staphylococcus aureus, sed* expression, enterotoxin D formation, sodium nitrite, regulatory response

## INTRODUCTION

*Staphylococcus (S.) aureus* can give rise to the various diseases such as local and systemic infections and toxin-mediated diseases. Staphylococcal food poisoning is an intoxication caused by staphylococcal enterotoxins preformed in food. On average, 240 000 cases are estimated to occur yearly in the US (1), and 3000 cases are reported yearly in the EU (2).

Sodium nitrite ($NaNO_2$) is a widely used food additive contributing to the preservation, red meat color, and cured flavor of various meat products such as bacon, ham, and sausages. The mechanisms underlying its bactericidal and bacteriostatic action are not thoroughly understood, but inhibition of oxygen uptake, uncoupling of oxidative phosphorylation, and inhibition of metabolic enzymes have been described (3). The red color of the meat is retained when myoglobin and hemoglobin react with nitric oxide resulting from the reduction of nitrite. In *Clostridium botulinum*, sodium nitrite has been shown to inhibit growth by interfering with the formation of iron-sulfur clusters (4–6). The desired cured meat flavor is obtained with relatively low levels of nitrite (50 mg/kg) (7).

Despite the wide utilization of sodium nitrite in food preservation, its effect on *S. aureus* growth and enterotoxin gene expression has been only partially investigated and regulatory mechanisms controlling staphylococcal enterotoxin D (SED) production in the presence of nitrite are unclear. Previous studies have shown that *S. aureus* growth is not affected by nitrite concentrations causing growth retardation in *Clostridium botulinum* or *Listeria monocytogenes* (8–10). However, an influence of pH on growth inhibition by sodium nitrite has been demonstrated in several bacterial species (11, 12). $NaNO_2$ was shown to inhibit growth and production of SEA at pH values below 7.0 (13), which corresponds to pH levels encountered in most meat products supplemented with sodium nitrite. It has been reported that nitrite concentrations of up to 200 mg/l did not affect *S. aureus* growth or SEB production (14). In

contrast, in sausages supplemented with sodium nitrite (c = 154 mg/kg), no SEA and SED formation was detected by ELISA despite *S. aureus* growth to $10^7$ CFU/g (15).

Production of plasmid encoded SED is regulated by several regulatory elements including accessory gene regulator (Agr), staphylococcal accessory regulator (SarA), sigma factor B (SigB), and repressor of toxins (Rot). Agr is a two-component quorum sensing system activated by increased cell density. Upon activation, the transcription of cell wall-associated proteins is repressed and exotoxin transcription is increased (16). DNA binding protein SarA regulates virulence gene transcription via Agr-dependent and independent mechanisms (17) increasing expression of several exotoxins such as *seb* and *tst* (18). Alternative sigma factor SigB is activated post-translationally by several environmental stresses and functions antagonistically to Agr (19). Rot is a global regulator repressing transcription of several exotoxins (20, 21). Most studies investigating the effect of regulatory mutations have however been conducted using derivatives of strain NCTC8325 harboring an 11-base deletion in *rsbU*, a gene encoding an indirect positive regulator of SigB (22). Since a defect in the *sigB* operon has been shown to affect global regulators Agr, Sar, and Rot, results generated using NCTC8325 derivatives may not be representative (23–26).

In this study, we aimed to determine the effect of sodium nitrite on *sed* transcription and SED production, as well as the effect of regulatory mutations (Δ*agr*, Δ*sarA*, Δ*sigB*) on SED protein levels levels in three different *S. aureus* strains originating from food poisoning outbreaks and an infection.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *S. aureus* strains used in this study are listed in Table 1. Isogenic mutant strains were constructed by transduction using phage 80α as previously

described (27, 28). Strains were grown in LB broth (Difco laboratories, Detroit, MI) (29) and in LB supplied with sodium nitrite ($NaNO_2$) (Pacovis AG, Stetten, Switzerland). Nitrite concentration of 150 mg/l ($a_w$ = 0.98) was chosen to correspond to the maximum amount generally added in meat products in the EU (30). Growth phases of all strains under control and $NaNO_2$ conditions were determined by viable cell counts using plate count agar (Sigma-Aldrich, Stockholm, Sweden), with incubation at 37°C for 18-24 h.

Single colonies were transferred from 5% sheep blood agar to 5 ml of LB broth and grown for 18 h (37°C, 225 rpm). Aliquots of 1 ml of the overnight cultures were centrifuged with Eppendorf 5424 (6000 × g for 10 min) and washed twice with 0.8% NaCl (Merck, Darmstadt, Germany) to remove residual media components. LB and LB supplemented with $NaNO_2$ were inoculated with $10^{-3}$ dilution of washed overnight culture to result in approximate cell density of 5 × $10^3$ CFU/ml and incubated at 37°C, 225 rpm. For RKI2Δ*sarA*, the $10^{-2}$ dilution was used instead of the $10^{-3}$ dilution to account for an extended lag phase in this strain. Culture supernatant samples for ELISA were harvested by centrifugation (14000 × g for 1 min) in two hour intervals until 12 h, and after 24 h. Two independent cultivations were performed for all strains to gain two independent samples of each strain, condition, and time point.

Samples for RNA extraction were harvested by centrifugation (8000 × g for 5 min) in early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary phase (T4). Cell pellets were resuspended in 500 µl RNA protect bacteria reagent (Qiagen, Hombrechtikon, Switzerland), incubated at room temperature for 5 min and harvested again by centrifugation (3000 × g for 5 min). Cell pellets were stored at -80°C before RNA extraction.

**RNA extraction and reverse transcription.** Cell lysis, RNA extraction, and reverse transcription were performed as previously described (31). For each sample, 100 ng of RNA was

converted to cDNA. The reverse transcription reaction was performed twice for each sample. RNA integrity numbers determined by Bioanalyzer (Agilent Technologies, Waldbronn, Germany) ranged from 7.1 to 9.1.

**Quantitative Real-time PCR (qPCR).** Single peaks in the melting curve analyses and single product bands on agarose gels confirmed target-specific amplifications for all primers (Table 2). qPCR experiments were performed using Light-Cycler-480 (Roche). A total reaction volume of 10 μl was used, including 4 μl cDNA template (dilution 1:100), optimized concentration of each primer, and the LightCycler480 SYBR Green I master mix (Roche). Water (no template) and RT minus samples were used as controls. An inter-run calibrator sample was included as a control for variation in cDNA synthesis and amplification. PCR cycling conditions included 8 min at 95°C, 45 amplification cycles (95°C for 10 s, the respective annealing temperature for 15 s, 72°C for 20 s, 78°C for 1 s with a single fluorescence measurement), a melting curve (60-95°C at 2.2°C /s and a continuous fluorescence measurement), and a final cooling step. Standard curves based on genomic DNA were generated to determine the efficiency of target gene amplification for each strain. All samples were amplified in triplicates. Expression levels of *sed* were normalized using *rho, gyrB*, and *proC* reference genes selected as previously described. (31). The effect of sodium nitrite on *sed* expression was assessed by comparing relative expression ratios between control and sodium nitrite conditions at the same time point in early exponential, mid-exponential, early stationary, and late stationary phase.

**ELISA.** ELISA analysis of SED was performed according to the protocol for SEA as previously described (32) with minor modifications: SED instead of SEA affinity-purified sheep polyclonal antibodies (Toxin Technology, Inc.; Sarasota, FL) were used. Each sample was

142 analyzed in three technical replicates. The reliability of the technical replications was verified
143 using Cronbach's Alpha (0.990).
144
145 **Statistical analysis.** Statistical analysis was performed using SPSS Statistics 22 (SPSS Inc.,
146 Chicago, IL). Results were considered significant at $p < 0.05$. Growth parameters such as
147 exponential phase growth rate and maximum cell density were determined using DMFit 3.0 (33)
148 and compared using Student's t-test. For qPCR data, log transformed relative expression ratios
149 were compared using one-way ANOVA. For ELISA data, log transformed SED levels between
150 different growth conditions (LB or LB supplemented with sodium nitrite) were compared using
151 three-way mixed design ANOVA. SED levels in wild type (wt) and isogenic regulatory mutants
152 were compared using one-way ANOVA.
153
154 **RESULTS**
155 **Impact of sodium nitrite on growth, SED production, and *sed* transcription in *S.***
156 ***aureus* wt strains.** Growth behavior and SED production of *S. aureus* strains RKI1, RKI2, and
157 SAI48, in LB and LB supplemented 150 mg/l nitrite are presented in Figure 1. Growth rates
158 ($\Delta 0.07 \pm 0.08$ lg CFU $ml^{-1}$ $h^{-1}$; $p = 0.44$) and maximum cell densities in stationary phase ($\Delta 0.20 \pm$
159 0.60 lg CFU/ml; $p = 0.54$) between the strains were similar under both growth conditions.
160 SED was detectable by ELISA after 6 h of incubation at a cell density of around $10^6$
161 CFU/ml (Fig. 1). SED levels gradually increased over time with the highest increase detected
162 between 8-10 hours, corresponding to late exponential growth phase.
163 Under control conditions, highest SED level detected was reached at 24 h in the three
164 examined strains. RKI2 exhibited highest SED levels, followed by RKI1 and SAI48. SED protein
165 levels were generally lower in the presence of nitrite compared to control conditions. The

reduction was most pronounced between 8 to 12 h in all three strains and statistically significant in RKI1 (at 8 h and 10 h), RKI2 (at 12 h), and SAI48 (at 10 h and 12 h). Three-way mixed design ANOVA identified a significant main effect of sodium nitrite on SED levels produced [$F(1, 9) = 68.933$, $p = 0.00$] and a significant interaction for strain × time × growth condition [$F(44, 36) = 6.597$, $p = 0.00$] as shown in Table 3.

Temporal relative *sed* expression levels of strains RKI1, RKI2, and SAI48 generally increased continuously from early exponential phase to late stationary phase under control conditions and in the presence of nitrite (Fig. 2). Strain-specific differences in *sed* expression levels were observed, with RKI2 generally exhibiting higher *sed* expression levels, similar to the SED levels determined by ELISA. Relative *sed* expression in RKI2 was statistically significantly higher compared to SAI48 ($p = 0.01$) and RKI1 ($p = 0.00$) under control conditions in late stationary phase (T4). Similarly, under nitrite conditions RKI2 showed statistically significantly higher *sed* expression levels compared to SAI48 in early exponential and mid-exponential phase (T1-T2, $p < 0.02$). A tendency towards induced *sed* expression under nitrite conditions was observed in all strains from mid-exponential to late stationary phase (T2-T4). In two strains (RKI1, SAI48), *sed* expression was significantly higher in T4 in the presence of nitrite compared to control conditions ($p < 0.04$), in contrast to the reduced SED protein levels in the presence of nitrite determined by ELISA.

**Impact of regulatory mutations on growth and SED production in LB and LB with sodium nitrite.** Regulatory mutant strains grew similar to their parental wt strains under control and $NaNO_2$ conditions (Fig. 3). In addition, SED levels gradually increased over time. When grown in LB, all three Δ*agr* mutants exhibited SED production levels similar to isogenic wt strains throughout all growth phases. Interestingly, SED levels were elevated at 24 h in RKI2Δ*agr* compared to RKI2 wt. In Δ*sarA* mutants of SAI48 and RKI1, SED levels were

decreased compared to the wt ($p$ = 0.03 at 8 h), while loss of SarA showed no effect in RKI2. In Δ*sigB* mutants of SAI48 and RKI1, SED levels were higher in early and late stationary phase. The increase in SED levels was statistically significant in Δ*sigB* mutants at 24 h ($p$ = 0.03) (Table 4). At 24 h, SED levels were 3.1-fold higher in SAI48Δ*sigB* and 2.5-fold higher in RKI1Δ*sigB* compared to their respective wt strains. In contrast, SED levels of RKI2Δ*sigB* remained in the same level as in wt.

In regulatory mutants, SED production was reduced under $NaNO_2$ conditions similar to wt strains (Fig. 3, Table 3). However, in SAI48Δ*agr* and RKI1Δ*agr*, a trend towards increased SED production under $NaNO_2$ conditions was observed.

## DISCUSSION

Nitrate and nitrite are widely used in meat, fish, and cheese products to inhibit bacterial growth, maintain the color of the meat, and create the cured flavor. In this study, the effect of sodium nitrite and regulatory mutations on *sed* expression was determined in three *S. aureus* strains and their isogenic regulatory mutants. Using the maximum average concentration of nitrite in meat products, the growth rate and maximum cell density of the *S. aureus* strains were only slightly affected. This observation is in agreement with previous studies showing no growth inhibition by sodium nitrite in concentrations < 200 mg/l (8, 15). While sodium nitrite had only a modest effect on growth, *sed* mRNA and SED protein levels were notably affected. On the transcriptional level, relative *sed* mRNA levels were significantly increased in the presence of nitrite in late stationary phase compared to the control conditions. On the protein level, SED production was nonetheless decreased in wt strains and most regulatory mutants in the presence of nitrite in late exponential and early stationary growth phase. These findings demonstrate that

213 *sed* transcription levels do not always reflect extracellular SED protein levels as previously
214 shown for *sea* and *sec* (34, 35).

215 Interestingly, none of the Δ*agr* mutants tested in this study showed reduced SED
216 production compared to its isogenic wt. Agr is the most studied regulatory element in *S. aureus*
217 and has been regarded as one of the main positive regulators involved in the expression of several
218 enterotoxins including SED (36). More recent studies indicate however that the importance of
219 Agr may have been overestimated due to the use of SigB deficient derivatives of strain
220 NCTC8325. The lack of SigB activity appears to result in increased RNAIII expression and
221 subsequent overactivation of the *agr* system (26). Previous studies showing decreased production
222 of SEB, SEC, and SED in Δ*agr* mutants (37–39) were conducted using a strain designated as
223 ISP546 (40) representing a derivative of NCTC8325. This notion is also consistent with Schmidt
224 et al. suggestion that Agr is an inducer of *seb* only if the *sigB* operon is not functional, based on
225 the contradictory effect of Δ*agr* mutation in NCTC8325 and the Newman strain with an intact
226 *sigB* operon (41). The post-exponential increase in *sed* transcription has been reported to
227 indirectly result from reduction of Rot activity by the Agr system rather than from a direct effect
228 of Agr (20).

229 Decreased *sed* promoter activity in Δ*sarA* mutants has been previously reported by Tseng et
230 al. (20). In our study, we observed a tendency towards decreased SED levels in two out of three
231 Δ*sarA* mutants, with a statistically significant reduction in late exponential growth phase. For
232 SEB, Chan et al. showed reduced protein levels of the enterotoxin in Δ*sarA*, Δ*agr,* and
233 Δ*agr*Δ*sarA* mutants (18).

234 In our study, two of three Δ*sigB* mutants exhibited statistically significantly increased SED
235 levels in late stationary phase. This is in agreement with previous studies reporting increased *seb*
236 and *sed* promoter activity in Δ*sigB* mutants of parental strains harboring an intact *sigB* operon (20,

41). Schmidt et al. also demonstrated that the effect of loss of SigB is less pronounced in derivatives of NCTC8325 compared to the Newman strain (41). For SEB, increased production in a Δ*sigB* mutant of *S. aureus* strain COL has been demostrated also on protein level (42).

Interestingly, strain RKI2 generally deviated from the other two tested strains in terms of regulation of SED production. We hypothesized that this may be due to sequence variation, but sequencing of *sed* genes and *sed* promoter regions of the examined strains did not reveal any sequence variation related to differences in SED regulation (data not shown).

These findings suggest strain-specific differences in *S. aureus* enterotoxin gene regulation. This underlines the findings of other studies demonstrating the importance of a multiple strain investigation approach when studying regulatory elements (24, 43, 44). This is the first study investigating the effect of regulatory mutations (Δ*agr*, Δ*sarA*, Δ*sigB)* on SED mRNA and protein levels in several *S. aureus* strains other than derivatives of strain NCTC8325.

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**Figure legends**

**FIG 1** Growth and SED production of three wild type *S. aureus* strains (RKI1, RKI2, SAI48) in LB and LB supplemented with nitrite (150 mg/l). Cell harvesting time points for RNA extraction are designated as T1, T2, T3, and T4. Error bars represent one standard deviation of the mean. Statistically significant changes in SED production between LB and LB + $NaNO_2$ at the same time point are marked by an asterisk ($p < 0.05$).

**FIG 2** Relative *sed* expression ratios of three wild type *S. aureus* strains (RKI1, RKI2, SAI48) in early exponential (T1), mid-exponential (T2), early stationary (T3), and late stationary phase (T4) in LB and in LB supplemented with nitrite (150 mg/l). Expression ratios are represented relative to the calibrator sample. Error bars represent one standard deviation of the mean. Statistically significant changes in *sed* expression between LB and LB + $NaNO_2$ at the same time point are marked by an asterisk ($p < 0.05$).

**FIG 3** Growth and SED production of three wild type *S. aureus* strains (RKI1, RKI2, SAI48) and their isogenic regulatory mutants (Δ*agr,* Δ*sarA,* Δ*sigB*) in LB and LB supplemented with nitrite (150 mg/l). Error bars represent one standard deviation of the mean. Statistically significant changes in SED production between wt and isogenic mutant at the same time point and condition are marked by a plus symbol (+) and statistically significant changes in SED production between LB and LB + $NaNO_2$ at the same time point are marked by an asterisk (*) ($p < 0.05$). A) RKI1; B) RKI2; C) SAI48