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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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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 (Δ*agr*, Δ*sarA*, Δ*sigB*) 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, 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, 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.

1. Introduction

Staphylococcus (S.) aureus can give rise to various diseases such as local and systemic infections and toxin-mediated diseases. Staphylococcal food poisoning is an intoxication caused by pre-formed staphylococcal enterotoxins in food. On average, 240,000 cases are estimated to occur yearly in the US (Scallan et al., 2011), and 3000 cases are reported yearly in the EU (European Food Safety Authority, 2015).

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 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 (Duncan & Foster, 1968; Pierson & Smoot, 1982; Reddy, Lancaster, & Cornforth, 1983). The desired cured meat flavor is obtained with relatively low levels of nitrite (50 mg/kg) (Mac Donald, Stanley, & Usborne, 1980). The mechanisms underlying its bactericidal and bacteriostatic action are not fully understood, but inhibition of oxygen uptake, uncoupling of oxidative phosphorylation, and inhibition of metabolic enzymes have been described (Tompkin, 2005).

Despite the wide utilization of sodium nitrite in food preservation, its effect on *Staphylococcus aureus* growth and enterotoxin gene expression has been only partially investigated and regulatory mechanisms controlling staphylococcal enterotoxin D (SED) production in the presence of sodium nitrite are unclear. Previous studies have shown that *S. aureus* growth is not affected by nitrite concentrations causing growth retardation in *C. botulinum* or *Listeria monocytogenes* (Buchanan & Solberg, 1972; Lövenklev, Holst,

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Borch, & Rådström, 2004; Nyachuba, Donnelly, & Howard, 2007). However, an influence of pH on growth inhibition by sodium nitrite has been demonstrated in several bacterial species (Castellani & Niven, 1955; Tarr, 1941). Sodium nitrite was shown to inhibit growth and production of staphylococcal enterotoxin A (SEA) at pH values below 7.0 (Tompkin, Ambrosino, & Stozek, 1973), 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 staphylococcal enterotoxin B (SEB) production (McLean, Lilly, & Alford, 1968). In contrast, in sausages supplemented with nitrite (c = 154 mg/kg), no SEA and SED formation was detected by ELISA despite *S. aureus* growth to 10^7 CFU/g (Bang, Hanson, & Drake, 2008).

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 (Bronner, Monteil, & Prévost, 2004). DNA binding protein SarA regulates virulence gene transcription via Agr-dependent and independent mechanisms (Chien, Manna, Projan, & Cheung, 1999) increasing expression of several exotoxins such as *seb* and *tst* (Chan & Foster, 1998). Alternative sigma factor SigB is activated post-translationally by several environmental stresses and functions antagonistically to Agr (Novick, 2003). Rot is a global regulator repressing transcription of several exotoxins (Tseng & Stewart, 2005; Tseng, Zhang, & Stewart, 2004). 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 (Gertz et al., 1999). 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 (Bischoff, Entenza, & Giachino, 2001; Cassat et al., 2006; Hsieh, Tseng, & Stewart, 2008; Lauderdale, Boles, Cheung, & Horswill, 2009).

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 (*Δagr*, *ΔsarA*, *ΔsigB*) on SED protein levels in three different *S. aureus* strains originating from food poisoning outbreaks and an infection.

2. Materials and methods

2.1. 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 (Charpentier et al., 2004; Sihto, Tasara, Stephan, & Johler, 2015). The strains were grown in Luria Bertani broth (LB, Difco laboratories, Detroit, MI) (Bertani, 1951) 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 (EC., 2011). The growth phases of all strains under control and sodium nitrite conditions were determined by viable cell counts using plate count agar (Sigma–Aldrich, Stockholm, Sweden), with incubation of the plates 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 an 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 sodium nitrite 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 (14,000 × g for 1 min) at 2 h 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 for a duration of several hours to several weeks before being used for RNA extraction.

2.2. RNA extraction and reverse transcription

Cell lysis, RNA extraction, and reverse transcription were performed as previously described (Sihto, Tasara, Stephan, & Johler, 2014). For each sample, 100 ng of RNA was converted to cDNA. The reverse transcription reaction was performed twice for each sample. RNA integrity numbers were determined by Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and ranged from 7.1 to 9.1.

2.3. Quantitative real-time PCR (qPCR)

Single peaks in the melting curve analyses and single product

Table 1
S. aureus wt and mutant strains used in this study.

Strain ID	Relevant characteristics	Source (reference)
RKI1	strain associated with foodborne outbreak, CC8/t648, *sea*+, *sed*+, *sej*+	Robert Koch Institute, Germany
RKI2	strain associated with foodborne outbreak, CC8/t008, *sea*+, *sed*+, *sej*+	Robert Koch Institute, Germany
SAI48	strain isolated from *S. aureus* infection, CC5/t002, *sec*+, *sed*+, *sej*+	Institute of Medical Microbiology, University of Zurich, Switzerland
RKI1Δ*agr*	RKI1 with *ermB* replacing *agr*	(Sihto et al., 2014)
RKI1Δ*sigB*	RKI1 with *ermB* replacing *sigB*	(Sihto et al., 2014)
RKI1Δ*sarA*	RKI1 with *tetL* replacing *sarA*	(Sihto et al., 2014)
RKI2Δ*agr*	RKI2 with *ermB* replacing *agr*	(Sihto et al., 2014)
RKI2Δ*sigB*	RKI2 with *ermB* replacing *sigB*	(Sihto et al., 2014)
RKI2Δ*sarA*	RKI2 with *tetL* replacing *sarA*	(Sihto et al., 2014)
SAI48Δ*agr*	SAI48 with *ermB* replacing *agr*	(Sihto et al., 2014)
SAI48Δ*sigB*	SAI48 with *ermB* replacing *sigB*	(Sihto et al., 2014)
SAI48Δ*sarA*	SAI48 with *tetL* replacing *sarA*	(Sihto et al., 2014)