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RESEARCH ARTICLE

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Importance of superoxide dismutases A and M for protection of *Staphylococcus aureus* in the oxidative stressful environment of cystic fibrosis airways

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Abstract

Staphylococcus aureus is one of the earliest pathogens that persists the airways of cystic fibrosis (CF) patients and contributes to increased inflammation and decreased lung function. In contrast to other staphylococci, *S. aureus* possesses two superoxide dismutases (SODs), SodA and SodM, with SodM being unique to *S. aureus*. Both SODs arm *S. aureus* for its fight against oxidative stress, a by-product of inflammatory reactions. Despite complex investigations, it is still unclear if both enzymes are crucial for the special pathogenicity of *S. aureus*. To investigate the role of both SODs during staphylococcal persistence in CF airways, we analysed survival and gene expression of *S. aureus* CF isolates and laboratory strains in different CF-related in vitro and ex vivo settings. Bacteria located in inflammatory and oxidised CF sputum transcribed high levels of *sodA* and *sodM*. Especially expression values of *sodM* were remarkably higher in CF sputum than in bacterial in vitro cultures. Interestingly, also *S. aureus* located in airway epithelial cells expressed elevated transcript numbers of both SODs, indicating that *S. aureus* is exposed to oxidative stress at various sites within CF airways. Both enzymes promoted survival of *S. aureus* during polymorphonuclear leukocyte killing and seem to act compensatory, thereby giving evidence that the interwoven interaction of SodA and SodM contributes to *S. aureus* virulence and facilitates *S. aureus* persistence within CF airways.

KEYWORDS

airway epithelial cells, cystic fibrosis, oxidative stress, persistence, polymorphonuclear leukocytes, *Staphylococcus aureus*, superoxide dismutase, virulence

1 | INTRODUCTION

Cystic fibrosis (CF) is a genetic disorder that affects more than 70,000 people of the Caucasian population worldwide (Cutting, 2015). In CF patients, the ion and water transport across epithelial membranes is dysregulated, which provokes the accumulation of a thick mucus in different organs, including the airways, and impairs important immune defence mechanisms, thereby facilitating bacterial infections (Bruscia & Bonfield, 2016; Strausbaugh & Davis, 2007).

Staphylococcus aureus is the most common and one of the first pathogens that can be isolated from the airways of CF patients (Cystic Fibrosis Foundation Patient Registry, 2018). Despite antistaphylococcal

treatment, *S. aureus* is able to persist over several years (Kahl et al., 1998; Schwerdt et al., 2018), causing inflammation (Sagel et al., 2009) and a decline in lung function (Junge et al., 2016). The long-term persistence of *S. aureus* might be facilitated by its ability to enter, replicate, and reside in professional phagocytes like macrophages (Li et al., 2017) and neutrophils (Gresham et al., 2000) and non-professional phagocytes as keratinocytes, fibroblasts, endothelial, and epithelial cells (Strobel et al., 2016), including also CF cells (Jarry & Cheung, 2006; Kahl et al., 2000). Furthermore, in some *S. aureus* CF isolates long-term persistence might be caused by enhanced production of nuclease, which was shown to facilitate bacterial escape from neutrophil extracellular traps (Herzog et al., 2019).

Polymorphonuclear leukocytes (PMNs) such as neutrophils are key players of the innate immune system that eradicate invading pathogens by either neutrophil extracellular trap formation (Brinkmann et al., 2004; Herzog et al., 2019), phagocytosis and intracellular degradation in oxidised phagosomes or release of reactive oxygen species (ROS) and reactive nitrogen species during oxidative burst (Nordenfelt & Tapper, 2011). Furthermore, PMNs contain high amounts of antimicrobial proteins that are secreted upon cell activation, among them the calcium-, manganese-, and zinc-sequestering protein complex S100A8/A9 (also called myeloid-related protein 8/14 or calprotectin), which plays an important role in maintaining the nutritional immunity in the host (Austermann, Spiekermann, & Roth, 2018; Corbin et al., 2008).

Invasion of non-professional phagocytes is regarded as a mechanism that protects *S. aureus* from the host immune system and antibiotic therapy (Garzoni & Kelley, 2009). However, also the intraepithelial location might be harmful to *S. aureus* as human bronchial and tracheal epithelial cells were shown to express the membrane-located nicotinamide adenine dinucleotide phosphate dual oxidase DUOX1 (Geiszt, Witta, Baffi, Lekstrom, & Leto, 2003) as well as the cytoplasmic enzyme xanthine oxidoreductase (Cantu-Medellin & Kelley, 2013; Papi et al., 2008), which contribute to extracellular and intracellular ROS formation, presumably also during CF lung disease (Pongnimitprasert et al., 2008). Imbalanced intracellular and extracellular redox systems (Ziady & Hansen, 2014) and enormous PMN infiltration (Konstan, Hilliard, Norvell, & Berger, 1994) additionally account for oxidative stressful conditions in the CF airways that seem to be detrimental to invading pathogens. However, *S. aureus* has developed a defence system comprising in contrast to other staphylococci two superoxide dismutases (SODs), SodA and SodM (Clements, Watson, & Foster, 1999; Valderas & Hart, 2001), metalloenzymes that abolish superoxide radicals and protect the bacterium from oxidative damage (Gaupp, Ledala, & Somerville, 2012). Importantly, while SodA is present in all staphylococci (Valderas, Gatson, Wreyford, & Hart, 2002) and seems to be exclusively dependent on its cofactor manganese (Clements et al., 1999), SodM is unique to *S. aureus* (Valderas et al., 2002) and was found to be cambialistic being active with either manganese or iron (Garcia et al., 2017). Additionally, although SodA is the main SOD in *S. aureus* with higher protein abundance (Treffon et al., 2018), under special conditions, for example *S. aureus* presence in inflamed tissues with S100A8/A9 availability, SodM seems to have a unique role for maintaining SOD activity (Garcia et al., 2017).

Recently, we identified SodM as a protein that was higher abundant in a late *S. aureus* isolate, which persisted 13 years in the airways of an individual CF patient, in comparison with the clonal first isolate (Treffon et al., 2018). Such results were confirmed by high expression of *sodM* in long-persisting *S. aureus* isolates of several CF patients compared with first isolates (Treffon et al., 2018). From these results, we predicted the importance of this enzyme for staphylococcal long-term persistence in CF airways.

In this study, we analysed survival and gene expression of the same *S. aureus* CF isolates and laboratory strains (Treffon et al., 2018) in different in vitro infection models and during growth in artificial sputum medium (ASM; Sriramulu, Lunsdorf, Lam, & Romling, 2005), thereby further exploring the role of SodM and SodA during chronic *S. aureus* infection within CF airways. Additionally, to compare our results with in vivo conditions, we characterised the CF habitat of *S. aureus* and its influence on the bacterial gene expression by measuring the levels of inflammatory (S100A8/A9) and oxidative stress (8-isoprostane) markers as well as transcription levels of *sodA*, *sodM*, and other genes involved in bacterial oxidative stress defence within CF sputum samples by RNA sequencing (RNA-Seq) analysis. Our findings indicate that *S. aureus* is not only exposed to ROS in CF sputa but also in the intracellular environment of airway epithelial cells. In both habitats, transcription of *sodA* and *sodM* was enhanced compared with control samples. Furthermore, *sodA* and *sodM* seem to act compensatory, as only the deletion of both SODs, but not the loss of one SOD reduced survival of *S. aureus* in the presence of PMNs. Therefore, we conclude that SodA and SodM contribute to bacterial survival and persistence in these niches.

2 | RESULTS

2.1 | *sodA* and *sodM* expression differ in *Staphylococcus aureus* cystic fibrosis isolates and laboratory strains

In our former study, we investigated the expression of *sodM* in several first and late *S. aureus* isolates cultured from CF airways and laboratory strains after 6 hr of growth in ASM (Treffon et al., 2018). To extend the knowledge about *sod* expression during *S. aureus* persistence in CF airways, we used the same *S. aureus* CF isolates and laboratory strains (Treffon et al., 2018; Tables S1 and S2) and determined transcription of the second staphylococcal SOD, *sodA*, by quantitative reverse transcription polymerase chain reaction (RT-qPCR) under the same growth conditions as before. Interestingly, by comparing *sodA* expression of the *S. aureus* isolates from each of the six CF patients, only for one patient (P12) we detected a significantly reduced expression of *sodA* in the late isolate compared with the respective first isolate (Figure 1). The late isolates of the remaining five patients had similar *sodA* transcription levels as their corresponding first isolates. Furthermore, the mean gene expression of *sodA* in the clinical isolates

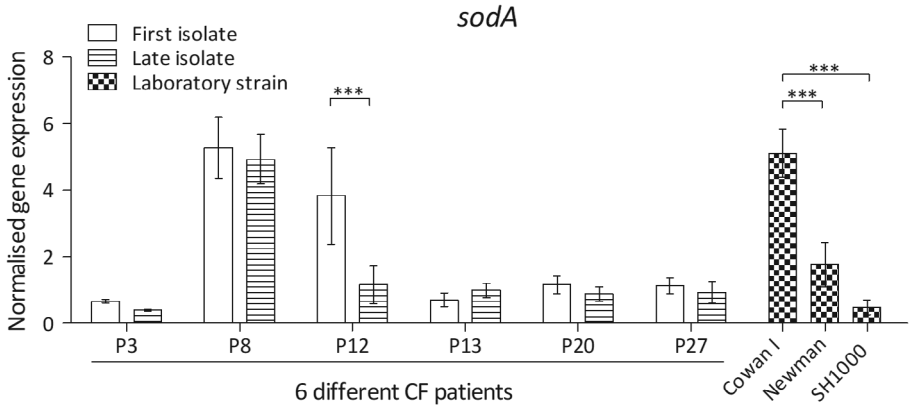
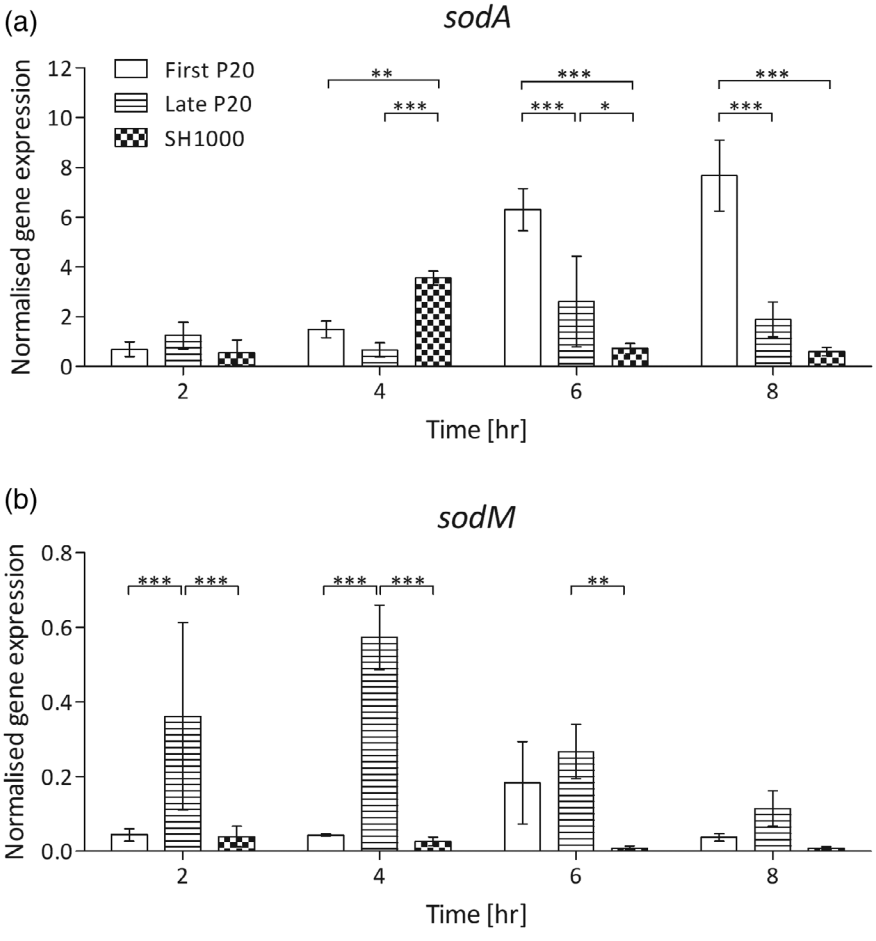


FIGURE 1 Expression analysis of *sodA* by quantitative reverse transcription PCR (RT-qPCR) in the first and late *Staphylococcus aureus* isolates of six cystic fibrosis (CF) patients (P3, P8, P12, P13, P20, P27) and in three *S. aureus* laboratory strains after cultivation of bacteria for 6 hr in artificial sputum medium (ASM). Data were normalised against expression levels of the housekeeping genes *aroE* and *gyrB*, which were set to one, and represent the mean of three independent replicates ± standard deviation (SD), analysed in technical duplicates. Statistical analysis was performed using an one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test for multiple comparisons (***$p \leq .001$)

FIGURE 2 Expression analysis of *sodA* (a) and *sodM* (b) by RT-qPCR in the first and late *Staphylococcus aureus* isolates of Patient 20 (P20) and the *S. aureus* laboratory strain SH1000 after cultivation of bacteria for 2, 4, 6, and 8 hr in ASM. Data were normalised against expression levels of the housekeeping genes *aroE* and *gyrB*, which were set to one, and represent the mean of at least three independent replicates ± SD, analysed in technical duplicates. Statistical analysis was performed using a two-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$)



was slightly lower than in the laboratory strains (normalised gene expression 1.84 vs. 2.46; Figure S1a). This is clearly in contrast to the transcription of *sodM* investigated in our previous study (Treffon et al., 2018), which was significantly enhanced in most long-persisting isolates compared with the corresponding first isolates and considerably higher in clinical isolates compared with laboratory strains (Figure S1b). Such data imply that SodM, but not SodA, seems to play a special role for the persistence of *S. aureus* in CF airways. However, in all *S. aureus* strains, the mean normalised expression of *sodA* exceeded the levels of *sodM* measured in our earlier study by factors between 1.2 and 59.9 (Table S3), indicating that SodA, but not SodM, is the main staphylococcal SOD.

To investigate growth phase dependent differences of *sod* expression in *S. aureus*, the first and late isolate of one CF patient (P20), which were already examined in earlier studies (Treffon et al., 2018; Windmüller et al., 2015), and one laboratory strain (SH1000) were cultured for up to 8 hr in ASM to perform RT-qPCR analysis with samples taken after 2, 4, 6, and 8 hr of growth. The time points of maximal gene expression were strain dependent (Figure 2). In accordance with our previous findings (Figure 1) and our former study (Treffon et al., 2018), after 6 hr of cultivation in ASM the clinical isolates expressed higher levels of *sodA* and *sodM* than the laboratory strain. Interestingly, throughout growth the first isolate of P20 revealed a higher *sodA* expression than the late isolate, whereas the late isolate showed a stronger *sodM* transcription compared with the first isolate (significant differences at 6 and 8 hr for *sodA* [Figure 2a] and 2 and 4 hr for *sodM* [Figure 2b]), indicating that at least in these two isolates both SODs could act compensatory, where a high expression of *sodA* might compensate a low expression of *sodM* and vice versa.

2.2 | Both superoxide dismutase genes are highly expressed in the oxidised and inflammatory cystic fibrosis airways

In vivo, *S. aureus* was found to be located within thick mucus covering the CF lung (Ulrich et al., 1998). To elucidate the role of genes involved in the bacterial oxidative stress response during chronic staphylococcal airway infection of CF patients in comparison with colonisation-specific genes that are crucial for bacterial retention in the host, but not related to the bacterial oxidative stress response, we performed RNA-Seq analysis of four *S. aureus*-positive sputum samples of two individual CF patients (two samples per patient) and analysed the expression of *sodA* and *sodM*, the hydrogen peroxide-detoxifying catalase *katA* and alkyl hydroperoxide reductase *ahpCF*, the manganese transporter *mntABC* involved in the acquisition of the SOD cofactor manganese, the virulence and respiratory regulators *perR*, *sarA*, *sigB*, and *srrAB*, the clumping factors and fibrinogen-binding proteins *clfAB* and the fibronectin-binding proteins *fnbpAB*. The fold change of gene expression was determined by normalising the number of transcripts per million of each gene to the number of transcripts per million of the housekeeping genes *aroE* and *gyrB* in the respective sample. Genes with a fold change higher than one-fold increase were defined as strongly expressed genes. Interestingly, while in all sputum samples transcription of *sodM* was constantly strong (fold change between 3.5 and 6), the expression levels of *sodA* showed a great variability (fold change between 1.3 and 7.2; Figure 3). Importantly, also other genes involved in the oxidative stress response including *katA*, *perR*, *sarA*, *sigB*, *srrAB*, and especially *ahpCF* and *mntABC* were expressed several times higher than the housekeeping genes. Additionally, expression of most oxidative stress defence genes exceeded transcription of the colonisation-specific genes *clfAB* and *fnbpAB*.

The elevated expression of *sodA*, *sodM*, and other oxidative stress response genes in *S. aureus* located in CF airways compared with the housekeeping genes is most likely due to the presence of oxidative stress provoked by an antioxidants/oxidants imbalance (Ziady & Hansen, 2014) and the enormous accumulation of ROS-generating PMNs (Konstan et al., 1994; Nordenfelt & Tapper, 2011) in the CF patients' lung. While the presence of oxidative stress can be proven by measuring the levels of the lipid peroxidation product and oxidative stress marker 8-isoprostane (Antus, 2016), accumulation of PMNs can be detected by determining the concentration of the inflammatory host protein S100A8/A9, also termed "CF antigen," which makes up to 40% of the cytoplasmic proteins in PMNs (Dorin et al., 1987; Roth et al., 1992). To analyse the oxidative and inflammatory properties of CF airways, we analysed the levels of both markers in supernatants of sputa from CF patients with chronic *S. aureus* airway infection and healthy individuals by enzyme-linked immunosorbent assays (ELISA). In accordance with our assumption, we detected significant higher concentrations of both markers in sputum samples of CF patients compared with control samples (Figure 4). Interestingly, as supernatants that contained high levels of 8-isoprostane in most cases comprised also high concentrations of S100A8/A9, the presence of oxidative stress seems to directly correlate with the accumulation of

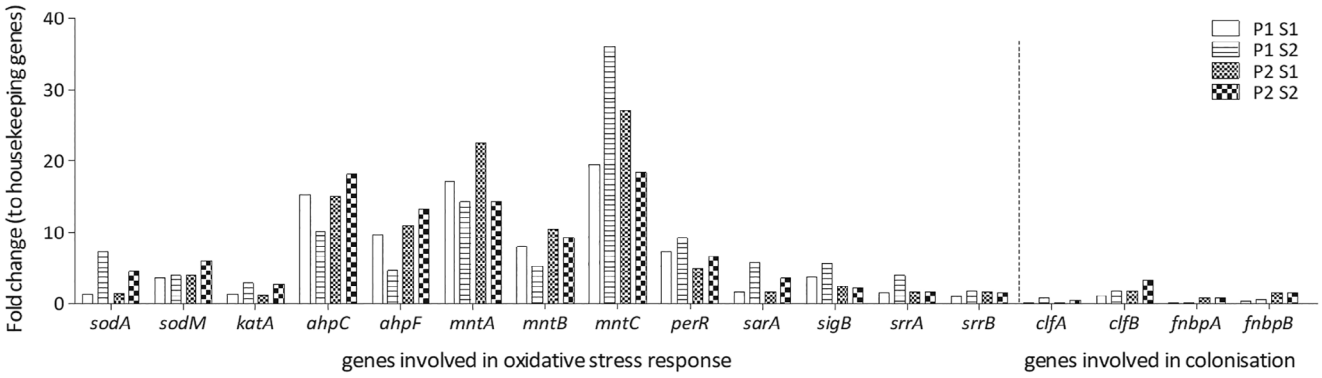


FIGURE 3 Expression of genes involved in staphylococcal oxidative stress response and colonisation in sputa of two different CF patients, analysed by RNA sequencing. RNA of two sputum samples (S1 and S2) per patient was extracted and sequenced on Illumina HiSeq 2,500 using the TruSeq SBS Kit v3-HS (50 cycles, single ended run). Expression levels are given as transcripts per million normalised to the expression levels of the housekeeping genes *aroE* and *gyrB*, which were set to one

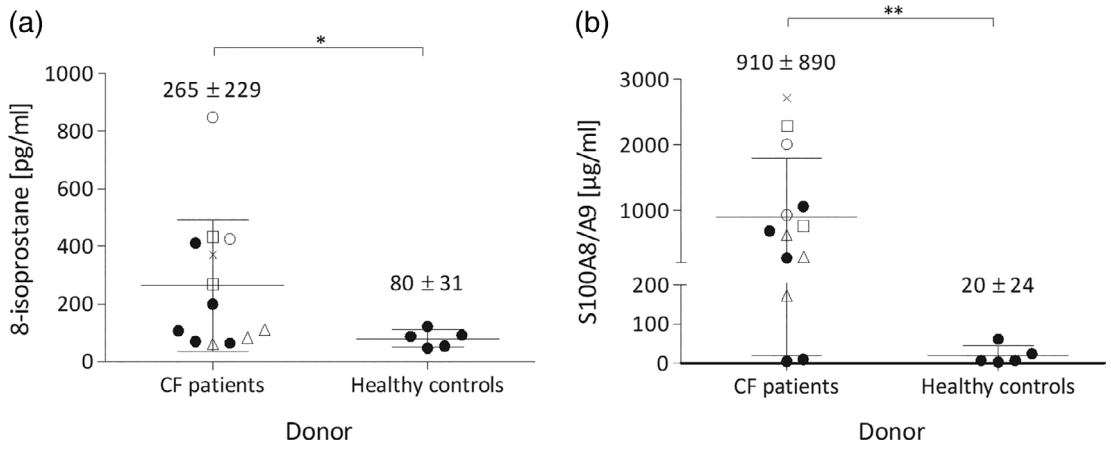


FIGURE 4 Concentration of the oxidative stress marker 8-isoprostane (a) and the polymorphonuclear leukocyte (PMN) marker S100A8/A9 (b) in sputum supernatants from nine cystic fibrosis (CF) patients and five healthy control individuals. Amounts of 8-isoprostane and S100A8/A9 were determined with enzyme-linked immunosorbent assays (ELISA). Each sample was measured either in two (8-isoprostane) or at least three (S100A8/A9) dilutions and technical duplicates. Each dot represents one sputum sample. Whereas black dots indicate single sputum samples of individual donors, clear dots, squares, and triangles represent sputum samples that were obtained during different clinical visits of three CF patients. Crosses mark the sputum sample S2 from CF Patient 2, which was part of the RNA sequencing analysis depicted in Figure 3. The mean marker concentrations of each group were averaged and are displayed above the data points ± SD. Statistical analysis was done applying a Welch's *t*-test for unequal variances (*$p \leq .05$, **$p \leq .01$)

PMNs and vice versa (Figure S2). Importantly, RNA-Seq analysis and ELISA of the second sputum sample (S2) of CF Patient 2 revealed high levels of *sodA* and *sodM* transcripts (Figure 3) as well as high concentrations of 8-isoprostane and S100A8/A9 (Figure 4, crosses), indicating that the presence of oxidative stress and/or PMNs in CF sputum could induce *sod* expression in *S. aureus*.

To proof that this is true for the clinical CF isolates, we cultivated the first and late isolate of Patient 20 as well as strain SH1000 as control in bacterial growth medium supplemented without or with the oxidative stressors paraquat or xanthine/xanthine oxidase (XXO) and analysed the expression of *sodA* and *sodM* by RT-qPCR. As expected, the transcription of both genes was enhanced in bacteria that were confronted with oxidative stress compared with those cultivated without paraquat or XXO (Figure S3). In fact, in the clinical CF isolates exposure to oxidative stress resulted in *sod* expression 2.1 to 4.6 times higher than the expression of the housekeeping genes (set to 1), which is comparable with RNA-Seq data of *S. aureus* located in CF sputum, where *sod* expression was 1.3 to 7.2 times higher than transcription of *aroE* and *gyrB* (Figure 3). Taken together, our data show that the airways of some CF patients represent an oxidised and inflammatory environment that can trigger the expression of *sodA*, and in particular of *sodM*, in *S. aureus* strains that colonise this niche.

2.3 | Superoxide dismutases protect *Staphylococcus aureus* against killing through polymorphonuclear leukocytes

As indicated above, bacteria that infect the CF airways are confronted with high numbers of PMNs (Konstan et al., 1994) that eradicate pathogens by phagocytosis and oxidative burst (Nordenfelt & Tapper, 2011). Thus, the expression of oxidative stress defence proteins seems to be indispensable for bacterial survival upon exposure to these immune cells. To verify this hypothesis, we investigated the viability of the *S. aureus* laboratory strain SH1000, the SH1000 *sodA* and *sodM* deletion mutants MHKA (Δ*sodA*) and MHKM (Δ*sodM*), the complemented mutants MHKAc and MHKMc expressing either wild type *sodA* or *sodM* from plasmids, and the SOD double deletion mutant MHKAM (Δ*sodA*Δ*sodM*; Table S2) during challenge with PMNs isolated from healthy blood donors. However, in contrast to our expectations, we observed only a faintly reduced survival of the single deletion mutants compared with the wild type, indicating that the loss of either *sodA* or *sodM* does not represent a significant disadvantage for the viability of *S. aureus* under these conditions (Figure 5a). Nevertheless, complementation of MHKA and MHKM resulted in a significant better bacterial survival compared with the wild type and the single deletion mutants, most likely caused by the constitutively strong expression of the plasmids used for complementation, which raised the SOD transcription levels of the complemented strains highly over the SOD expression levels of the wild type (Table S4). This is of special interest, as during cultivation in ASM the complemented mutants revealed a reduced growth compared with wild type and deletion mutants (Figure S4). Thus, although a high expression of *sodA* or *sodM* seems to negatively affect bacterial growth, it apparently protects *S. aureus* from killing by PMNs. Importantly, in a separately conducted PMN killing experiment, the SOD double deletion mutant MHKAM was eradicated significantly faster by the immune cells compared with the wild type, giving evidence that only the loss of both SODs dramatically reduces the bacterial ability to resist PMN killing (Figure 5b). Therefore, we conclude that the expression of either *sodA* or *sodM* is essential for the survival of *S. aureus* during challenge with PMNs.

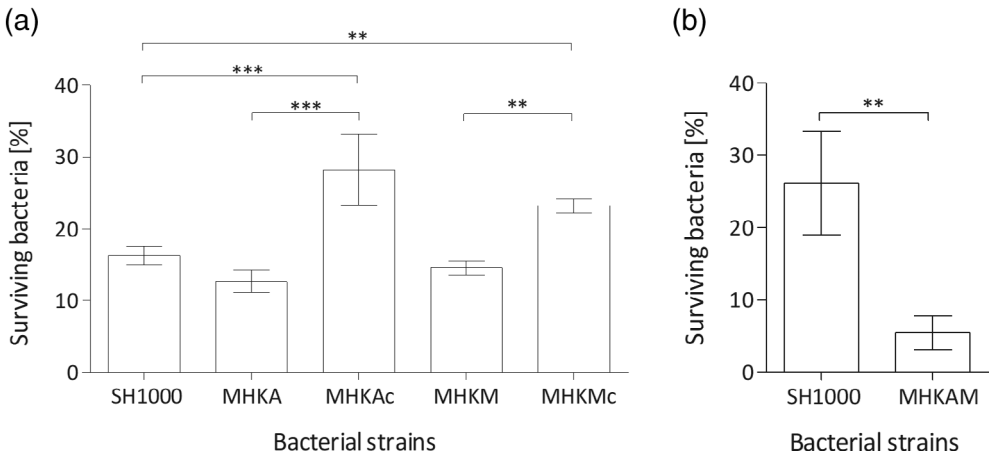


FIGURE 5 Bacterial survival after cultivation with PMNs from healthy blood donors. PMNs from healthy volunteers were isolated from whole blood samples and infected with the *Staphylococcus aureus* laboratory strain SH1000 and the SH1000-derived superoxide dismutase (SOD) mutants MHKA (Δ*sodA*), MHKAc (complemented MHKA), MHKM (Δ*sodM*), and MHKMc (complemented MHKM; a). In a separately conducted experiment, PMNs were infected with SH1000 and the SH1000-derived SOD double deletion mutant MHKAM (Δ*sodA*Δ*sodA*; b). After 2 hr of incubation, the surviving bacteria were plated on agar plates to determine colony forming units (CFU)/ml. The percentage of surviving bacteria was calculated by dividing CFU/ml after 2 hr of incubation with PMNs by CFU/ml of the inoculum used for infection. Data represent the mean of at least three independent replicates ± SD, analysed in technical duplicates. Statistical analysis was done applying a one-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons (a) or an unpaired Student's *t*-test (b; $^{**}p \leq .01$, $^{***}p \leq .001$)

To investigate, if a high expression of either *sodA* or *sodM* also facilitates true clinical *S. aureus* CF isolates to survive phagocytosis, we performed the PMN killing assay with first and late *S. aureus* strains isolated from the airways of six individual CF patients. As most of the late isolates had a similar *sodA* expression (Figure 1), but a higher *sodM* transcription (Table S3) compared with the corresponding first isolates, we expected that the late isolates will also survive better during immune cell challenge than their early counterparts as demonstrated for the defined double *sodAM* mutant. In contrast to our assumption, some late isolates tended to be killed faster by PMNs compared with the corresponding first strains (Figure S5), indicating that there must be additional other factors than *sod* expression with an impact on the survival of clinical *S. aureus* strains upon exposure to PMNs. However, only for one isolate pair, this difference was significant. But interestingly, all clinical *S. aureus* CF strains revealed a much higher mean survival rate in the PMN killing assay (range 30% to 80%, Figure S5) compared with the laboratory strains (mean survival of app. 20%, Figure 5) pointing at an early selection of *S. aureus* clones with resistance to PMN killing.

2.4 | Late *Staphylococcus aureus* cystic fibrosis isolates tend to invade airway epithelial cells at higher rates than corresponding first isolates

In the lung, next to immune cells *S. aureus* has to face airway epithelial cells, which are part of the first line of host defence against invading pathogens (Günther & Seyfert, 2018). Several studies demonstrated that *S. aureus* is able to evade the host immune system by invasion and persistence in these non-professional phagocytes (Kahl et al., 2000; Strobel et al., 2016), including also CF cells (Jarry & Cheung, 2006; Kahl et al., 2000), which is suggested to contribute to recurrent staphylococcal infections in CF patients. To analyse if the first and late *S. aureus* isolates from six different CF patients and the laboratory strains Cowan I, Newman and SH1000 invade airway epithelial cells and to elucidate, if bacterial invasion depends on the disease status of the host cell, we determined the amount of internalised bacteria 2 hr postinfection with either A549 (non-CF) or CFBE41o$^-$ (CF) airway epithelial cells by counting colony forming units (CFUs) of plated cell lysates and by staining intracellular bacteria with fluorescein-5-isothiocyanate (FITC) and propidium iodide (PI). Although a lower number of bacteria invaded CF cells compared with A549 cells, in both cell lines, *S. aureus* strains showed comparable invasion traits (Figure 6). Nevertheless, most significant differences in bacterial invasion could be detected in A549 cells (Figure 6a). The laboratory strains behaved as already demonstrated by Grundmeier et al. (Grundmeier et al., 2004), giving evidence that our experiments worked well: Strains Cowan I and SH1000 were highly invasive compared with strain Newman. Interestingly, in A549 cells, two of the clinical late isolates were significantly more invasive than the corresponding first isolates (Figure 6a). In contrast, the late isolate of Patient 12 (P12) showed the opposite profile and the long-persisting *S. aureus* CF strains of three other patients revealed no difference in invasiveness compared with the respective short-persisting isolate. These results were mostly confirmed by a different approach using flow cytometric investigation. Also here, higher invasive strains tended to be more internalised (FITC-positive particles [clear]) and killed less (FITC+PI-positive particles [grey]) than the less invasive strains (Figure S6). However, only a few of these

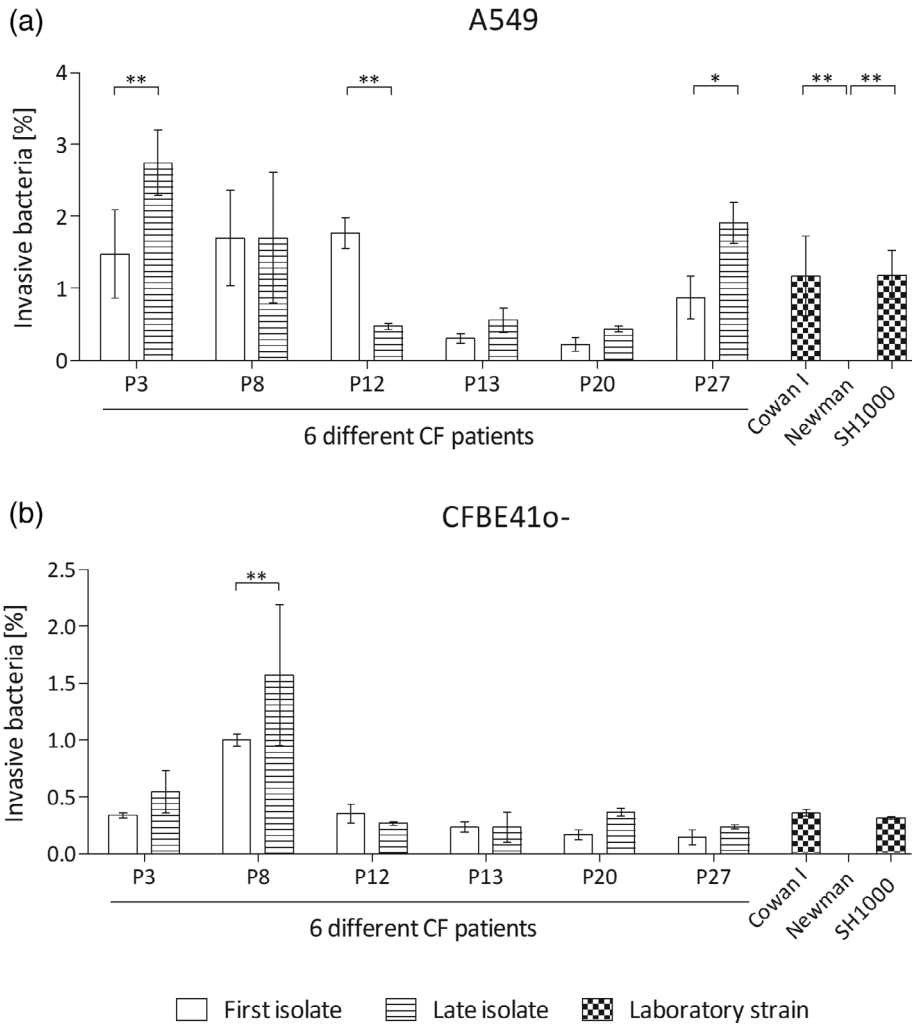


FIGURE 6 Invasion of the first and late *Staphylococcus aureus* isolates of six cystic fibrosis (CF) patients (P3, P8, P12, P13, P20, P27) and three *S. aureus* laboratory strains in A549 (a) and CFBE41o$^-$ cells (b). Epithelial lung cells were infected with *S. aureus* and incubated for 2 hr, followed by eradication of extracellular bacteria. Cells were lysed and intracellular bacteria were plated on agar to determine number of internalised bacteria by counting CFU. Percentage of invasive bacteria was calculated by dividing CFU/ml of internalised bacteria by CFU/ml of the inoculum used for infection. Data represent the mean of at least three independent replicates ± SD, analysed in technical duplicates. Statistical analysis was done using an one-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons (*$p \leq .05$, **$p \leq .01$)

differences were significant. Furthermore, for the isolates of Patient 12 (P12) the CFU-determination assay and the flow cytometric analysis revealed contradictive results, indicating that although both assays address the intracellular bacterial survival, their outcome can be different and has to be interpreted carefully. Nevertheless, these data demonstrate that, independent of the disease status of the host cell, at least some long-persisting *S. aureus* CF isolates are more invasive and survive better in respiratory airway epithelial cells than the corresponding short-persisting clones, which could have contributed to their extended persistence in the CF airways.

2.5 | Upregulation of *sodA* and *sodM* after internalisation in cystic fibrosis airway epithelial cells

Airway epithelial cells are able to form intracellular and extracellular ROS due to the expression of membrane-located dual oxidases (Geiszt et al., 2003) and the cytoplasmic xanthine oxidoreductase system (Cantu-Medellin & Kelley, 2013; Papi et al., 2008). In addition, several studies indicate that bacteria, internalised in epithelial cells, are exposed to oxidative stress (Battistoni et al., 2000; Boncompain et al., 2010). As in CF, severe intracellular and extracellular redox imbalance can occur (Ziady & Hansen, 2014), especially CF airway epithelial cells might represent oxidative stressful compartments, which could be detrimental to invading bacteria that do not express a proper oxidative stress defence system. To elucidate the role of SodA and SodM for the intraepithelial survival of *S. aureus*, we analysed the invasion and intracellular survival of the SOD deletion mutants MHKA, MHKM, and MHKAM in CFBE41o$^-$ and A549 cells and investigated *sodA* and *sodM* transcription in certain clinical CF isolates and laboratory strains in the extracellular and intracellular environment of CFBE41o$^-$ cells. Unexpectedly, the invasion assay revealed neither clear survival advantages nor disadvantages of the SOD mutants during infection of both cell lines (data not shown). Nevertheless, the transcription analysis of *sodA* and *sodM* in four clinical *S. aureus* isolates and three laboratory strains after 1 or 2 hr of intracellular residence in the extracellular and intracellular environment of CFBE41o$^-$ cells demonstrated that upon 1 hr of intracellular residence in the CF cells, in all examined clinical isolates transcription of *sodM*, but not *sodA*, was enhanced compared with the expression in extracellular bacteria (Figure 7). Accordingly, at this time point, mean fold changes

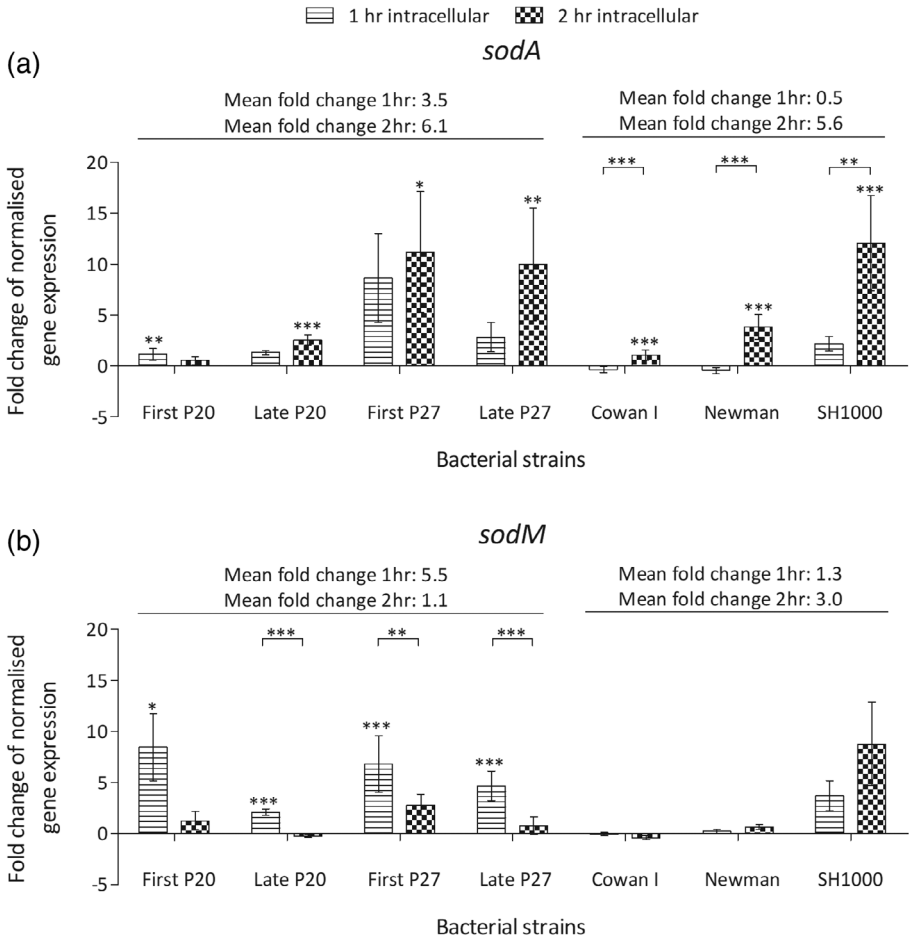


FIGURE 7 Fold changes in gene expression of *sodA* (a) and *sodM* (b) in bacteria internalised for 1 and 2 hr in CFBE41o$^-$ cells compared with gene expression in extracellular bacteria, which were in contact with epithelial cells, analysed by RT-qPCR. Cystic fibrosis (CF) epithelial lung cells were infected with clinical first and late *Staphylococcus aureus* isolates of Patients 20 (P20) and 27 (P27) or *S. aureus* laboratory strains Cowan I, Newman, and SH1000 and incubated for 1 hr. Afterwards, cultivation medium was collected and used for the analysis of the gene transcription in extracellular bacteria. For investigation of the gene expression in intracellular persisting bacteria, remaining extracellular bacteria were killed and CF cells were lysed directly (1 hr of bacterial intracellular persistence) or cultivated for another hour with persistence medium before lysis (2 hr of bacterial intracellular persistence). The intracellular bacteria were collected, and the gene expression was examined by RT-qPCR. All samples were analysed in technical duplicates and at least three independent replicates. Expression values of the target genes were normalised against expression levels of the housekeeping genes *aroE* and *gyrB*, which were set to one. The graph shows the fold changes of the normalised gene expression in internalised bacteria compared with the normalised gene expression in extracellular bacteria that were in contact with the epithelial cells, which was set to zero. Statistical analysis was done on the basis of the normalised gene expression data (Table S5), applying a two-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons (*$p \leq .05$, **$p \leq .01$, ***$p \leq .001$). Mean fold changes of *sodA* or *sodM* expression in CF isolates or laboratory strains that were internalised for 1 or 2 hr in the CF cell line are indicated above the respective columns

of *sodM* expression in CF isolates were higher compared with changes of *sodA* expression (5.5 vs. 3.5, respectively). In contrast, in the laboratory strains, 1 hr postinfection neither *sodA* nor *sodM* expression was enhanced compared with the transcription in extracellular bacteria. However, although expression of *sodM* in clinical isolates diminished at 2 hr postinfection, transcription of *sodA* was significantly enhanced at the 2 hr time point in clinical isolates and laboratory strains compared with the expression in extracellular bacteria, pointing at a role of *sodM* during earlier time points and for *sodA* during later time points after invasion. In summary, our findings suggest that although the deletion of SODs apparently does not reduce the intraepithelial survival of *S. aureus*, the bacterium presumably is exposed and reacts to ROS inside airway epithelial cells by upregulation of *sodA* and *sodM*, which seem to be important during different phases of bacterial intracellular residence.

3 | DISCUSSION

Due to strong infiltration by PMNs (Konstan et al., 1994) and an imbalance of the redox system (Ziady & Hansen, 2014), the CF airways represent an oxidative stressful and challenging environment for invading pathogens like *S. aureus*. Nevertheless, this bacterium is able to persist in this niche for up to several years (Kahl et al., 1998; Schwerdt et al., 2018), which demonstrates its impressive adaptive capacity. To combat oxidative stress, *S. aureus* possesses two metal-

dependent SODs, SodA (Clements et al., 1999) and SodM (Valderas & Hart, 2001). In contrast to SodA, which is present in all staphylococci, SodM is unique to *S. aureus* (Valderas et al., 2002). It has a cambialistic character with regard to its metal cofactor and can function with manganese or iron, which is important to maintain its activity in inflamed areas such as CF airways, where manganese is sequestered by S100A8/A9, and therefore, SodM is considered to contribute to the bacterial subversion of the host nutritional immunity (Garcia et al., 2017). However, as it is still not clear, if both SODs are crucial for the bacterial pathogenicity (Clements et al., 1999; Garcia et al., 2017; Karavolos, Horsburgh, Ingham, & Foster, 2003; Mandell, 1975), we further investigated the role of these enzymes during *S. aureus* infection in CF.

SodA was detected prior to SodM and was described as the major staphylococcal SOD (Clements et al., 1999). Our observation that during cultivation in ASM, gene expression of *sodA* is several times higher than *sodM* transcription in *S. aureus* CF isolates and laboratory strains emphasises this assumption. Nevertheless, as *sodM*, and not *sodA*, is significantly stronger expressed in clinical CF isolates compared with laboratory strains (Treffon et al., 2018), we suggest that especially SodM plays a crucial role for the staphylococcal infection of CF airways. Evidence that both SODs are important for *S. aureus* in vivo is given by RNA-Seq analysis of four CF sputum samples, which revealed elevated transcription of *sodA*, and in particular of *sodM*, in all samples compared with the expression of the housekeeping genes *aroE* and *gyrB*. Importantly, while the expression levels of *sodA* in CF sputum resembled the *sodA* transcription during in vitro cultivation of *S. aureus* in ASM, amounts of *sodM* transcripts in sputum (Figure 3) exceeded *sodM* expression in ASM immensely (Figure 2, Table S3). Interestingly, also in a former study, where the transcriptome of *S. aureus* colonising the anterior nares of healthy individuals and *S. aureus* in vitro cultures in brain heart infusion or synthetic nasal medium was analysed (Chaves-Moreno et al., 2016), higher *sodA* and *sodM* transcription in vivo compared with in vitro was detected (Figure S7a). Although in *S. aureus* nose isolates, *sodA* was constantly strong expressed, expression levels of *sodM* varied greatly among the samples. This contrasts our findings in CF sputum, where *sodM* expression was constantly high and *sodA* transcription differed strongly between the samples (Figure 3, Figure S7b), which highlights the role of *sodM* for *S. aureus* colonising CF sputum. However, in CF sputum along with *sodA* and *sodM* also other genes involved in oxidative stress defence including *katA*, *perR*, *sarA*, *sigB*, *srrAB*, and especially *ahpCF* and *mntABC* seem to be essential for *S. aureus*, as they were expressed several times higher than the housekeeping genes *aroE* and *gyrB*, too. *katA* and *ahpCF* are compensatory acting peroxidases that are under control of the peroxide-responsive repressor PerR and detoxify hydrogen peroxide (Cosgrove et al., 2007; Horsburgh, Clements, Crossley, Ingham, & Foster, 2001). As hydrogen peroxide is a byproduct of superoxide radical degradation by SODs, enhanced expression of both peroxidases makes sense in an oxidative stressful environment, where SODs are highly active. Also, *mntABC* was shown to play an essential role in the resistance of *S. aureus* towards oxidative stress, as this operon encodes for an uptake system for manganese, which is an important cofactor of SodA and SodM and itself has ROS-detoxifying activity (Radin, Zhu, Brazel, McDevitt, & Kehl-Fiea, 2019). Thus, the high transcription levels of *mntABC* detected in *S. aureus* located in CF sputum seem to mirror the oxidative stressful conditions the bacterium has to face in the CF airways.

In contrast to most oxidative stress defence genes, transcription of the colonisation-specific genes *clfAB* and *fnbpAB* in CF sputum was very low, indicating that *S. aureus* trapped in CF mucus is rarely exposed to host cell membranes. These colonisation specific genes seem to play a more essential role for *S. aureus* colonising the human nose, as several *S. aureus* nose isolates investigated by Chaves-Moreno et al. transcribed high amounts of *clfAB* and *fnbpAB* (Figure S7a). Interestingly, also in the nose, *S. aureus* seems to cope with ROS, as fold changes of oxidative stress defence gene expression in *S. aureus* nose isolates were almost comparable with fold changes of oxidative stress defence gene transcription in *S. aureus* colonising CF sputum (Figure S7).

As transcription levels of *sodA* and *sodM* in CF sputum (Figure 3) were similar to *sod* expression in *S. aureus* cultivated in vitro with oxidative stress (Figure S3), we suggest that bacteria located in CF mucus are exposed to oxidative stress, most likely caused by inflammation. There are already several studies that investigated the levels of the oxidation product 8-isoprostane and the inflammatory biomarker S100A8/A9 in CF sputum according to the disease state of the CF patient. Interestingly, while elevated 8-isoprostane levels were only detected in CF sputum collected during exacerbation (Gray et al., 2010), S100A8/A9 was present at high concentrations in sputum expectorated from CF patients with either stable disease or exacerbation (Reid, Misso, Aggarwal, Thompson, & Walters, 2007; Roth et al., 1992). By measuring high concentrations of both markers in the supernatant of sputum expectorated by CF patients with stable disease and chronic *S. aureus* infection, we demonstrated that oxidative stress and PMN accumulation can also occur in CF patients with stable disease and might be linked to chronic infection with *S. aureus*.

As our statistical analysis revealed a direct correlation between S100A8/A9 and 8-isoprostane, we suggest that PMNs represent a prominent source of ROS in the CF airways. Importantly, as indicated by the results of our RNA-Seq and ELISA analysis of sputum sample S2 from CF Patient 2 and the RT-qPCR investigation of bacteria cultivated with paraquat and XXO, high concentrations of 8-isoprostane, and the presence of oxidative stressors seem to directly account for high expression levels of both SODs in *S. aureus*, giving evidence that the SOD gene expression is induced by oxidative stress, which so far was demonstrated in vitro (Karavolos et al., 2003), but not in vivo. In addition, a high expression of *sods* and other oxidative stress defence genes, including peroxidases and manganese transporters, in inflamed CF sputum characterised by elevated levels of the antimicrobial PMN protein complex S100A8/A9, which was shown to inhibit bacterial SODs due to sequestration of the SOD-cofactor manganese (Kehl-Fie et al., 2011), could represent an immune evasion mechanism that enables *S. aureus* to outcompete nutritional immunity.

Due to their detoxifying character (Karavolos et al., 2003; Valderas & Hart, 2001), SODs should protect *S. aureus* from eradication by ROS-generating PMNs (Nordenfelt & Tapper, 2011) and airway epithelial cells (Cantu-Medellin & Kelley, 2013; Pongnimitprasert et al., 2008). Interestingly, our PMN killing assay demonstrated that, although the loss of both SODs strongly reduces the staphylococcal survival during exposure to PMN, the deletion of one SOD does not affect the viability of *S. aureus* as strong as expected. Additionally, overexpression of either *sodA* or *sodM* seems to enhance the bacterial survival in the presence of PMNs significantly, despite the fact that it is slightly growth limiting. We therefore assume that at least the expression of one SOD is sufficient and apparently necessary for the survival of *S. aureus* during PMN killing and suggest a compensatory role of SodA and SodM, which might enable *S. aureus* to counterbalance the loss of one SOD. Congruent with our data, a supportive activity of SodM was also described by Valderas et al., who showed that a *S. aureus sodA* deletion mutant was unaffected by the addition of paraquat during the late exponential growth phase, when *sodM* expression was maximal (Valderas & Hart, 2001). Our results extend these findings and give evidence that this unique compensatory relation between SodA and SodM essentially supports the fight of *S. aureus* against the host immune system. However, the results of the PMN killing assay with clinical *S. aureus* CF strains isolated from the airways of CF patients show that most likely early selection of *S. aureus* clones with enhanced resistance to PMN killing and other factors that promote the survival of *S. aureus* during phagocytosis occurred, which should be addressed in further studies. In general, *S. aureus* CF isolates seem to be well adapted to the challenge with PMNs as the survival rate of clinical strains was much higher compared with that of the used laboratory strains.

In contrast to the results of Battistoni et al., who observed a slightly reduced survival of an *Escherichia coli sodC* deletion mutant and an enhanced viability of *sodC* overexpressing *E. coli* strains compared with the respective wild type upon infection of HeLa cells (Battistoni et al., 2000), we detected neither clear advantages nor disadvantages during the invasion and intracellular survival of the staphylococcal SOD deletion mutants in CF and non-CF airway epithelial cells, indicating that in *S. aureus*, (a) the loss of one SOD can be counterbalanced by the other SOD, or (b) both SODs do not play essential roles in these processes. Nevertheless, additional to the findings of Michalik et al., who detected a higher abundance of SodA and SodM in *S. aureus* that resided in human bronchial epithelial cells compared with nonadherent bacteria (Michalik et al., 2017), our study revealed an upregulation of *sodA* and *sodM* in intracellular compared with extracellular bacteria and, with regard to our aforementioned results, we assume that intraepithelial bacteria are exposed to ROS, which was also demonstrated for *Chlamydia trachomatis* infecting HeLa cells (Boncompain et al., 2010).

In the intracellular environment of CFBE41o$^-$ cells, *sodM* seems to play a role during early and *sodA* during late time points after invasion. This observation could be explained by the presence of S100A8/A9 in the cytoplasm of stimulated epithelial cells (Kato, Kouzaki, Matsumoto, Hosoi, & Shimizu, 2017), which might inactivate SodA and boost the expression of *sodM* 1-hr postinfection (Garcia et al., 2017). However, further experiments are required to proof this hypothesis.

Apart from new aspects regarding the staphylococcal *sod* expression in epithelial cells, our invasion analysis revealed that some of the long-persisting CF isolates tended to be more invasive and able to survive the intraepithelial residence than the corresponding first isolates. This indicates that invasion of host cells might represent an adaptive mechanism that facilitates staphylococcal long-term persistence in the CF airways. Recently, Tan et al. came to the same conclusion as they detected that late *S. aureus* CF isolates have an improved ability to persist intracellularly for up to 6 days in CFBE41o$^-$ cells compared with their corresponding early isolates (Tan et al., 2019). In addition to their investigation, our study shows that the differences in the intraepithelial survival of early and late *S. aureus* CF isolates (a) cannot only be observed in CF cells but do also occur in airway epithelial cells without cystic fibrosis transmembrane conductance regulator defect and (b) can be measured already a few hours postinfection.

Summarised, our results shed new light on the function of SodA and especially SodM for *S. aureus* colonising the respiratory tract of CF patients. We acquired new ex vivo and in vitro evidence that both SODs seem to act compensatory, protect the bacterium from oxidative stress in the CF mucus, and arm the pathogen for its fight against PMNs. The upregulation of *sodA* and *sodM* in bacteria upon invasion of airway epithelial cells might support their intracellular residence and thereby, promote staphylococcal long-term persistence. Thus, the unique interaction of the two SODs seems to contribute to the successful chronic staphylococcal infection of CF airways.

4 | EXPERIMENTAL PROCEDURES

4.1 | Ethical statement

The study was approved by the local ethical committee, approval number 2010-155-f-S, and all patients signed the informed consent form.

4.2 | Bacterial strains and growth conditions

In this study, we analysed the same *S. aureus* isolates and laboratory strains listed in Treffon et al. (Treffon et al., 2018; Tables S1 and S2). The clinical strains were isolated as described recently (Hirschhausen et al., 2013). The laboratory strains Cowan I, Newman, and SH1000 were used for comparative analysis. For specific investigation of the role of SODs during staphylococcal infection, some experiments were performed with the SH1000 deletion mutants MHKA (Δ*sodA*), MHKM (Δ*sodM*), and MHKAM (Δ*sodA*Δ*sodM*; Karavolos et al., 2003) as well as the complemented SOD deletion mutants MHKAc (Δ*sodA* + pCN68.2*sodA*) and MHKMc (Δ*sodM* + pCN68.2*sodM*; Table S2), which were generated by introducing plasmids containing the respective wild type gene, following the

instruction of the In-Fusion® HD Cloning Plus Kit (Takara Bio). Mutants were grown in medium without addition of antibiotics to exclude any influence on bacterial gene expression and behaviour. The stability of the genetic modification under these conditions was confirmed by RT-qPCR analysis (data not shown). Growth of SH1000 and the mutants in ASM without antibiotics is depicted in Figure S4.

For the gene expression analysis of bacterial in vitro cultures, bacteria were incubated for 2 hr (early logarithmic growth phase), 4 hr (midlogarithmic growth phase), 6 hr (early stationary growth phase), and 8 hr (stationary growth phase) in ASM (Sriramulu et al., 2005) at 37°C and 160 rpm under aerated conditions. The main bacterial culture was set to a starting OD_{578nm} of 0.1, using a pre-culture for inoculation. Bacteria that were used for the infection of eukaryotic cells were grown for 17 hr (late stationary growth phase) in ASM at 37°C and 160 rpm under aerated conditions, starting from an OD_{578nm} of 0.1. Subsequently, bacteria were washed with D-PBS (D8537, Sigma-Aldrich), set to an OD_{578nm} of 1 (in case of the PMN killing assay to an OD_{578nm} of 4) in RPMI-1640 medium (R7388, Sigma-Aldrich) supplemented with 2 mg/ml $NaHCO_3$ (Sigma-Aldrich) and treated with a sonifier in order to separate cell clumps. Additional information about the bacterial isolation procedure, the analysed clinical isolates, the complementation of the mutants and bacterial treatment procedures for FITC-labeling and cultivation with oxidative stressors are given in the supporting information.

4.3 | RNA isolation and quantitative reverse transcription polymerase chain reaction

Isolation of RNA, synthesis of cDNA, and RT-qPCR were performed as described recently (Treffon et al., 2018) with some modifications. Further information is provided in the supporting information.

4.4 | RNA extraction and sequencing analysis of cystic fibrosis sputa

Sputa (S1, S2) of two CF patients (CF Patients 1 and 2), colonised chronically with *S. aureus*, were collected at two different time points each during 2016 and stored at −80°C until processing. Samples were gently mixed with an equivalent volume of Sputolysin (10%) and incubated for 30 min at 37°C on a ThermoMixer. RNA extraction, library construction, sequencing analysis, and data processing were conducted as described in Chaves-Moreno et al. (Chaves-Moreno et al., 2016) with some modifications. Details are given in the supporting information.

4.5 | Determination of 8-isoprostane and S100A8/A9 in sputum supernatants

Sputum samples of CF patients were received during routine medical check-up, and healthy individuals expectorated sputum after physical exercise. If necessary, sputa were diluted with a NaCl solution. All samples were centrifuged at 3,000 × *g* and 4°C for 15 min, and the supernatant was used for further analysis. The amounts of 8-isoprostane and S100A8/A9 were determined with the 8-Isoprostane ELISA Kit (No. 516351, Cayman Chemical) following the instructions of the manufacturer and a sandwich ELISA as described previously (Frosch et al., 2000), respectively.

4.6 | Polymorphonuclear leukocyte killing assay

PMNs were isolated from whole venous blood of healthy donors using PolymorphPrep™ (Progen). Details are given in the supporting information. PMNs and bacteria, grown as described above, were set to a concentration of 2×10^7 cells/ml and 5×10^8 CFU/ml, respectively. To start the reaction, 250 µl of PMNs were transferred into a 1.5 ml reaction tube containing 100 µl of RPMI medium (supplemented with 2-mg/ml $NaHCO_3$ and 2% of heat-inactivated foetal bovine serum (FBS) [Fiebig]) and 50 µl of 1:10 diluted guinea pig complement (Cedarlane). 100 µl of the bacterial solution (multiplicity of infection of 10) were added to reach a total volume of 500 µl. The tubes were fixed on a rotator (IKA® Loopster digital) running slowly at 10 rpm and incubated at 37°C and 5% CO_2. After 2 hr, a small volume of the reaction was diluted in water in order to lyse PMNs, and the solution was further diluted in D-PBS, plated on Tryptic Soy Agar (TSA) plates and incubated overnight at 37°C for CFU/ml determination of bacteria after cultivation with PMNs. Additionally, to the reaction tube with PMNs, 100 µl of bacteria were placed into a second tube containing 400 µl of RPMI. This solution (not incubated) was directly serially diluted in D-PBS, plated on agar plates and incubated overnight at 37°C to determine CFUs of the reaction at time point t_0.

4.7 | Cultivation and maintenance of human epithelial lung cells

We performed infection assays using A549 airway epithelial cells, established from cancerous lung tissue of a Caucasian male, and the bronchial epithelial cell line CFBE41o$^-$ (provided by Dr. D. Gruenert, University of California, San Francisco [Gruenert, Willems, Cassiman, & Frizzell, 2004]), derived from a ΔF508-homozygous CF patient. The cells were cultivated in RPMI-1640 medium, supplemented with 2 mg/ml $NaHCO_3$ and 10% FBS (Biochrom) at 37°C and 5% CO_2. Medium was changed at least every third day, and cells were splitted depending on their density. Although A549 cells were cultivated without coating, CFBE41o$^-$ cells were seeded in cultivation flasks and well plates that were treated with a coating solution, consisting of 0.1 mg/ml BSA (AppliChem), 30 µg/ml Collagen I (Corning®), and 10 µg/ml Fibronectin (Sigma-Aldrich), solved in LHC basal medium (ThermoFisher). Detachment of confluent cells was achieved by 15 min of incubation with Trypsin/EDTA (GE Healthcare).

4.8 | Airway epithelial cell culture infection model

Three days prior to the experiments, A549 cells were seeded at a density of 40,000 cells/cm^2, whereas CFBE41o$^-$ cells were seeded at a density of 30,000 cells/cm^2 and cultivated to confluency. Cells were cultivated in 12-well plates (Corning) for invasion assays with subsequent CFU/ml determination or flow cytometric analysis and in 75 cm^2 cell culture flasks (Greiner Bio-One) for gene expression analysis of intracellular bacteria. For each experiment, the cells were seeded in one additional well or flask that was used for cell number determination at the day of the experiment. At the day of experiment, the cells were washed with D-PBS and covered with invasion medium, consisting of RPMI supplemented with 2 mg/ml $NaHCO_3$ and 10 mg/ml human serum albumin (Kedrion). Subsequently, the host cells were infected with different *S. aureus* strains, using a multiplicity of infection of 100, and incubated at room temperature for 15 min to allow sedimentation of bacteria. Afterwards, eukaryotic and prokaryotic cells were incubated at 37°C and 5% CO_2. After several hours (as indicated), cells were washed two times with D-PBS and killing of extracellular bacteria was conducted twice by incubation of cells for 30 min with RPMI medium that was supplemented with 2 mg/ml $NaHCO_3$ and 20 μg/ml lysostaphin (AMBI Products). The supernatants of the second killing step were plated on agar to control the toxic effect of lysostaphin. After applying two further washing steps, cells were lysed in water (A549) or 0.09% Triton X-100 (CFBE41o$^-$) to release the intracellular bacteria or covered with persistence medium (RPMI supplemented with 2 mg/ml $NaHCO_3$, 1% Antibiotic/Antimycotic solution (PAA Laboratories), and 2% MycoKill AB (GE Healthcare)) for further cultivation. Cell lysates were pipetted through a syringe and treated different, depending on the experiment that was performed. A detailed description of the procedures of the invasion assay with subsequent CFU/ml determination, flow cytometric investigation, or gene expression analysis of intracellular bacteria is given in the supporting information.

4.9 | Statistical analysis

Statistical analysis was done applying either a one-way or two-way analysis of variance with subsequent Bonferroni's post hoc test for multiple comparisons, a Welch's *t*-test for unequal variances or an unpaired Student's *t*-test (*$p \le .05$, **$p \le .01$, and ***$p \le .001$) using the software GraphPad Prism 5.

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AUTHOR CONTRIBUTIONS

BCK and JT designed the study. BCK obtained the funding. JT performed and evaluated the majority of experiments and wrote the paper. DCM and DHP conducted and evaluated the RNA-Seq analysis of CF sputum and described the procedure in the Material and Methods part. SN gave theoretical and practical support with the cell culture assays. TV and JR analysed the S100A8/A9 concentration in sputum supernatant. All authors reviewed the paper.

CONFLICTS OF INTEREST

All authors declare to have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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