Research Paper

The aldehyde dehydrogenase AldA contributes to the hypochlorite defense and is redox-controlled by protein S-bacillithiolation in *Staphylococcus aureus*

Marcel Imbera,1, Vu Van Loia, Sylvia Reznikovb, Verena Nadin Fritscha, Agnieszka J. Pietrzyk-Brzezinska, Janek Prehn, Chris Hamilton, Markus C. Wahl, Agnieszka K. Bronowskab, Haike Antelmann,1

Abbreviations: ADH, aldehyde dehydrogenase; BSH, bacillithiol; BSSB, oxidized bacillithiol disulfide; CFU, colony-forming unit; CD, catalytic domain; Co-BD, coenzyme-binding domain; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FA, formaldehyde; H2O2, hydrogen peroxide; HOCl, hypochloric acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria Bertani; LMW thiol, low molecular weight thiol; MD, molecular dynamics; MG, methylglyoxal; MHQ, 2-methylhydroquinone; MPO, myeloperoxidase; MRSA, methicillin-resistant *Staphylococcus aureus*; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NaOCl, sodium hypochlorite; NEM, N-ethylmaleimide; OD300, optical density at 500 nm; RCR, reactive chlorine species; RES, reactive electrophilic species; ROS, reactive oxygen species; SCV, small colony variant; SID, subunit interaction domain; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

**Article Info**

**Abstract**

*Staphylococcus aureus* produces bacillithiol (BSH) as major low molecular weight (LMW) thiol which functions in thiol-protection and redox-regulation by protein S-bacillithiolation under hypochlorite stress. The aldehyde dehydrogenase AldA was identified as S-bacillithiolated at its active site Cys279 under NaOCl stress in *S. aureus*. Here, we have studied the expression, function, redox regulation and structural changes of AldA of *S. aureus*. Transcription of *aldA* was previously shown to be regulated by the alternative sigma factor SigmaB. Northern blot analysis revealed SigmaB-independent induction of *aldA* transcription under formaldehyde, methylglyoxal, diamide and NaOCl stress. Deletion of *aldA* resulted in a NaOCl-sensitive phenotype in survival assays, suggesting an important role of AldA in the NaOCl stress defense. Purified AldA showed broad substrate specificity for oxidation of several aldehydes, including formaldehyde, methylglyoxal, acetaldehyde and glycolaldehyde. Thus, AldA could be involved in detoxification of aldehyde substrates that are elevated under NaOCl stress. Kinetic activity assays revealed that AldA is irreversibly inhibited under H2O2 treatment in vitro due to over-oxidation of Cys279 in the absence of BSH. Pre-treatment of AldA with BSH prior to H2O2 exposure resulted in reversible AldA inactivation due to S-bacillithiolation as revealed by activity assays and BSH-specific Western blot analysis. Using molecular docking and molecular dynamic simulation, we further show that BSH occupies two different positions in the AldA active site depending on the AldA activation state. In conclusion, we show here that AldA is an important target for S-bacillithiolation in *S. aureus* that is up-regulated under NaOCl stress and functions in protection under hypochlorite stress.

**1. Introduction**

*Staphylococcus aureus* is a major human pathogen that causes local wound infections, but also life-threatening systemic and chronic infections, such as septicemia, endocarditis, necrotizing pneumonia and osteomyelitis [1–3]. Moreover, there is an increasing prevalence of hospital- and community-acquired methicillin-resistant *S. aureus* (MRSA) isolates that are often resistant to multiple antibiotics [4]. S.
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host-defense components [11,14

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However, the enzymatic pathways involved in detoxi-

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which is controlled by the MerR/NmlR-like regulator AdhR [35].

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During infections, S. aureus has to cope with the oxidative burst of activated macrophages and neutrophils, including reactive oxygen and chlorine species (ROS, RCS), such as hydrogen peroxide (H2O2) and the strong oxidant hypochloric acid (HOCl) [8–11]. HOCl is generated in

S. aureus – a pathogen that is frequently exposed to reactive oxidants and co-factor for redox-active compounds and co-factor for thiols activation state in

As ESKAPE pathogen by the

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All-atom molecular dynamics (MD) simulations suggest that the location of BSH in the AldA active site depends on the Cys activation state in

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RNA quality was assessed using the Nanodrop. Northern blot hybridizations were performed with the digoxigenin-labelled aldA-

2.2. RNA isolation and Northern blot analysis

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mutant proteins was performed by application of an imidazole gradient (0–500 mM) using His Trap™ HP Ni-NTA columns (5 ml; GE Healthcare, Chalfont St. Giles, UK) and the ÄKTA purifier liquid chromatography system (GE Healthcare) according to the instructions of the manufacturer. Purified proteins were extensively dialyzed against 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, and stored on ice until usage.

2.4. Construction of the *S. aureus* COL aldA deletion mutant and the complemented aldA and aldAC279S mutant strains

The *S. aureus* COL ΔaldA deletion mutant was constructed by allelic replacement via the temperature-sensitive shuttle vector pMAD as described [28]. Briefly, for construction of the plasmids pMAD-aldA, the 500 bp up- and downstream flanking gene regions of aldA were amplified using the primers aldA-pMAD-up-for/rev and aldA-pMAD-down-for/rev from *S. aureus* COL genomic DNA (Table S2). The aldA up- and downstream flanking regions were fused by overlap extension PCR and ligated into the BglII and *SalI* sites of plasmid pMAD. The pMAD constructs were electroporated into the restriction-negative and methylase-positive intermediate *S. aureus* RN4220 strain and further transferred to *S. aureus* COL by phage transduction using phage 80 [29]. Transductants were streaked out on LB agar with 10 µg/ml erythromycin and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 30 °C. Blue transductants with pMAD integrations were selected for plasmid excision by a heat shock as described [30]. Erythromycin-sensitive white colonies were selected on X-gal plates and screened for aldA deletions by PCR and DNA sequencing.

The complemented aldA and aldAC279S mutant strains were constructed using the pRB473 plasmid as described [31]. Briefly, aldA and aldAC279S sequences were amplified from plasmids pet11b-aldA and pet11b-aldAC279S using the primers aldA-pRB-for/BamH and aldA-pRB-rev-KpnI. The PCR products were digested with *BamH* and *KpnI* and inserted into the pRB473 plasmid that was digested using the same enzymes resulting in plasmids pRB473-aldA and pRB473-aldAC279S. The plasmids were transferred to the ΔaldA mutant via phage transduction as described [31].

2.5. AldA activity assays

AldA activity was monitored spectrophotometrically at 340 nm and 30 °C with the substrate and NAD⁺ as cofactor by the production of NADH using a CLARIOstar (BMG Labtech) spectrophotometer. The oxidation of different aldehyde substrates (formaldehyde, methylglyoxal, acetaldehyde and glycol aldehyde) was measured in an assay mixture containing 1.25 mM NAD⁺ and 2.5 µM AldA in reaction buffer (100 mM Tris-HCl, 1.25 mM EDTA, pH 7.5). After pre-incubation, the reaction was started by addition of the aldehyde substrates and NADH was removed from the binding site, its translational and rotational degrees of freedom were altered and the molecule has been re-docked to the protein, in order to check whether the docking procedure was able to reproduce the native binding mode, as observed in related crystal structures. After the positive verification, the BSH molecule was docked to both Q1 (holo-enzyme with NAD⁺) and Q2 (apo-enzyme without NAD⁺) sites detected by FTMap [34].

During the docking calculations, the BSH molecule was subjected to 50,000 cycles of molecular-mechanical energy minimisation at the protein-binding site. The number of maximum ligand orientations was 50,000. The constraint was the distance between sulfur atoms from the Cys279 thiol and the sulfur of BSH. The 25 best-scoring poses (BSH-protein complexes) were further analyzed by means of secondary re-scoring using SeeSAR [36] and validated with the Gaussian09 programme [42] using HF/6-31G* basis set.

The temperature was kept constant at T = 300 K by using velocity rescaling with a coupling time of 0.1 ps. The pressure was kept constant at 1 bar using an isotropic coupling to Parrinello-Rahman barostat with a coupling time of 0.1 ps [43]. A cut-off of 1 nm was used for all non-bonded interactions. Long-range electrostatic interactions were treated with the particle-mesh Ewald [44] method using a grid spacing of 0.1 nm with cubic interpolation. All bonds between hydrogens and heavy atoms were constrained using the LINCS algorithm [45]. Each of the systems were immersed in a cubic TIP3P water box containing ~115,000 atoms. Simulation units were maintained neutral by adding sodium and chloride counter ions (0.1 M concentration).

Prior to MD simulations, the systems undergone 50,000 steps of molecular mechanical energy minimisation. This was followed by 100 ps MD simulations, during which position constraints were used on all backbone atoms, heavy atoms of BSH and NAD⁺. After the following unrestrained equilibration phase (10 ns) the production runs were carried out for 50 ns, with an integration time step of 2 fs. The cut-off for non-bonded interactions was 0.1 nm. The atomic coordinates were saved every 100 ps. For the visual inspection of the results we used xmgrace [46] and UCSF Chimera [47] packages. Free binding energy calculations have been performed using the MMnPBSA.py program from AmberTools package [48]. Binding energies have been calculated between BSH and the protein at the two different binding sites, as in Q1 and Q2, for the last 25 ns of the simulation.
3. Results

3.1. The aldehyde dehydrogenase AldA is strongly oxidized at its active site Cys279 due to S-bacillithiolation under NaOCl stress in S. aureus

The aldehyde dehydrogenase AldA was previously identified as S-bacillithiolated at its catalytic active site Cys279 in S. aureus and Staphylococcus carnosus [26,32]. In addition, both aldehyde dehydrogenases, GapDH and AldA displayed the highest oxidation increase of 29% under NaOCl stress in S. aureus using the thiol-redox proteomics approach OxICAT [26]. The OxICAT method is based on thiol-labelling of the reduced AldA Cys279 peptide with light 12C-ICAT reagent, followed by reduction of the Cys279-SSB peptide and its labelling with heavy 13C-ICAT reagent [49]. The percentage oxidation of the Cys279 peptide of AldA under control and NaOCl stress is reflected by the mass spectra of the ICAT-labelled peptide pair as quantified in the previous study [26] (Fig. 1A). The strong 29% oxidation increase of the active site Cys279 is shown here again which is caused by S-bacillithiolation [26]. To confirm that AldA can be S-bacillithiolated also in vitro, we expressed and purified His-tagged AldA from E. coli extracts. Purified AldA was treated with H2O2 after pre-exposure to 10-fold excess of BSH and the reversible S-bacillithiolation of AldA was verified using BSH-specific Western blot analyses in the absence and presence of DTT (Fig. 1B). The S-bacillithiolated AldA band is denoted with AldA-SSB. Next, we were interested to study the expression, function, redox-regulation and structural changes of AldA under NaOCl and aldehyde stress.

3.2. Transcription of aldA is induced SigmaB-independently under thiol-specific stress conditions by formaldehyde, NaOCl and diamide in S. aureus COL

We used Northern blot analysis to study aldA transcription in S. aureus COL under different thiol-specific stress conditions, including sub-lethal doses of 1 mM NaOCl, 2 mM diamide, 0.75 mM formaldehyde, 0.5 mM methylglyoxal, 50 µM methylhydroquinone (MHQ) and 10 mM H2O2 (Fig. 2A). The Northern blot results revealed that aldA transcription is strongly induced in S. aureus COL wild type after exposure to formaldehyde, diamide and NaOCl stress, but less strongly under methylglyoxal stress (Fig. 2A). No significant induction of aldA was detected under MHQ and H2O2 treatment. These transcriptional results indicate that AldA could be involved in the hypochlorite stress defense or in detoxification of aldehydes. In previous microarray experiments, aldA was identified as member of the SigmaB general stress regulon, which responds to heat and salt stress (NaCl), MnCl2 and alkaline stress conditions in S. aureus [50,51]. The sigB-dependent promoter sequence was mapped in the aldA regulatory upstream region (GTTTAT-N14-GGATAA) as promoter U1137.SigB.M2 previously [52]. In the condition-dependent transcriptome of S. aureus NCTC8325-4 [53], the strongest aldA transcription was monitored during the stationary phase in rich LB and TSB medium as well as during plasma stress as visualized by the Aureowiki Expression data browser (http://genome.jouy.inra.fr/cgi-bin/aeb/viewdetail.py?id=NA_2184537_2185964_-1) [52].

To investigate whether the thiol-specific induction of aldA transcription by formaldehyde, diamide and NaOCl requires SigmaB, we performed Northern blot analysis with RNA isolated from a sigB deletion mutant in comparison to the wild type (Fig. 2B). The Northern blot results showed similar aldA transcriptional induction in the sigB mutant under NaOCl, diamide and formaldehyde stress compared to the wild type. Even a higher aldA transcription occurred under methylglyoxal stress in the sigB mutant. These results indicate that aldA transcription is subject to SigmaB-independent control mechanisms under thiol-specific stress conditions by an unknown thiol-specific transcription factor that remains to be elucidated. No additional SigA promoter was identified upstream of aldA previously [52], presumably because the conditions were different compared to our thiol-stress conditions. In previous studies, a refined consensus for SigA- and SigB-dependent promoter sequences was revealed based on 93% of S. aureus transcriptional units [52]. In the aldA regulatory region, a putative SigA-dependent promoter was identified upstream of the SigB promoter, which could drive the thiol-specific expression of aldA (Fig. 2B).

3.3. AldA plays important roles in the defense against NaOCl stress in S. aureus COL

Next, we analyzed the role of AldA in protection under NaOCl and aldehyde stress in S. aureus. It was previously shown that methylglyoxal...
is produced in E. coli cells treated with HOCl [20]. Thus, AldA could function in methylglyoxal detoxification under HOCl stress also in S. aureus. AldA harbors a conserved active site Cys279 which is essential for its catalytic activity [54–56]. The function of AldA and the conserved Cys279 under methylglyoxal, formaldehyde and HOCl stress was analyzed in growth and survival assays of an aldA deletion mutant and its aldA and aldAC279S complemented strains (Figs. 3, 4, S1 and S2). The growth of the aldA mutant was not affected under sub-lethal formaldehyde and methylglyoxal stress in comparison to the wild type (Fig. S1). In addition, no significant phenotypes of the aldA mutant and the aldA complemented strains were detected in survival assays after exposure to 4 mM methylglyoxal (Fig. 3AB) and 2 mM formaldehyde stress (Fig. S2). However, the aldA mutant was significantly impaired in growth after exposure to sub-lethal concentrations of 1.5 mM NaOCl stress (Fig. 4A). In survival assays, the aldA mutant showed also a strongly decreased survival after treatment with 3.5 mM NaOCl (Fig. 4C). This survival defect of the ΔaldA mutant could be restored back to wild type level in the aldA complemented strain, but not in the aldAC279S mutant (Fig. 4D). This indicates that AldA is involved in protection of S. aureus against NaOCl stress and that Cys279 is essential for AldA activity in vivo.

Fig. 2. Transcriptional induction of aldA under formaldehyde, methylglyoxal, NaOCl and diamide stress in S. aureus COL wild type (A) and in the sigB mutant (B). (A) RNA was isolated from S. aureus COL wild type under control conditions as well as after treatment with sub-lethal doses of 0.75 mM formaldehyde, 0.5 mM methylglyoxal, 1 mM NaOCl, 2 mM diamide, 10 mM H2O2 and 50 µM methylhydroquinone (MHQ) for 15 and 30 min and subjected to Northern blot analysis for aldA (SACOL2114) transcription. (B) For comparison of Northern blot analysis of aldA transcription between the wild type and the sigB mutant, RNA was isolated from S. aureus COL wild type and the sigB mutant after exposure to 0.75 mM formaldehyde, 0.5 mM methylglyoxal, 1 mM NaOCl and 2 mM diamide for 15 min. Transcription of aldA is similarly up-regulated under formaldehyde, NaOCl and diamide stress in the wild type (A) and in the sigB mutant (B) indicating a SigmaB-independent thiol-stress regulatory mechanism of aldA transcription. The known SigmaB-dependent promoter sequence and a putative SigA-dependent promoter in the aldA upstream regulatory region are shown below the Northern blot in (B). The methylene blue stain is the RNA loading control showing the abundant 16S and 23S rRNAs. The experiments were performed in 3 biological replicates.

Fig. 3. AldA is not essential for the survival of S. aureus under methylglyoxal stress. For the survival phenotype assays, S. aureus COL wild-type (WT), the ΔaldA deletion mutant (A) and the aldA and aldAC279S complemented ΔaldA mutants (ΔaldA pRB473-aldA and ΔaldA pRB473-aldAC279S) (B) were grown in RPMI until an OD500 of 0.5 and treated with 4 mM methylglyoxal. Survival assays were performed by spotting 10 µl of serial dilutions after 1–3 h of NaOCl exposure onto LB agar plates. The experiments were performed in 3 biological replicates.
3.4. AldA shows broad substrate specificity for oxidation of various aldehyde substrates, including formaldehyde and methylglyoxal in vitro

To study the function and substrate specificity of AldA in vitro, the catalytic activity was measured using different aldehyde substrates, including formaldehyde, methylglyoxal, glycolaldehyde and acetalddehyde in concentrations ranging from 0.5 to 100 mM. AldA activity was measured in a spectrophotometric assay in the presence of NAD+ as a cofactor with the different aldehyde substrates by monitoring the NADH production as absorbance increase at 340 nm. The AldA activity assays revealed increasing NADH production with increasing concentrations of all aldehyde substrates indicating that AldA has broad substrate specificities. Formaldehyde and methylglyoxal were oxidized by AldA, resulting in NADH generation. The AldA activity was inhibited with 0.3–1 mM H2O2 and the remaining AldA activity was measured in the spectrophotometric assay with 15 mM methylglyoxal as substrate. AldA activity was inhibited with 0.3–1 mM H2O2 and the remaining AldA activity was measured in the presence of BSH and H2O2. In this case, however, the activity of the oxidized AldA protein could be restored to 66% by DTT reduction in the presence of BSH and H2O2. These results indicate that the active site Cys279 of AldA is very sensitive to overoxidation by H2O2 in the absence of BSH. To assess the effect of S-bacillithiolation on AldA activity, the enzyme was pre-exposed to 0.3–0.5 mM BSH prior to oxidation with 0.3–1 mM H2O2 and the remaining AldA activity was measured in the spectrophotometric assay with 15 mM methylglyoxal as substrate. AldA activity was inhibited with 0.3–1 mM H2O2 after pre-treatment with 0.3–0.5 mM BSH (Fig. 6C). In this case, however, the activity of the oxidized AldA protein could be restored to 66% by DTT reduction indicating that AldA is subject to irreversible S-bacillithiolation in the presence of BSH and H2O2 (Fig. 6D). S-bacillithiolation of AldA and its reversibility with DTT was further confirmed in BSH-specific Western blots (Fig. 1B). These results suggest that S-bacillithiolation protects the AldA active site Cys279 against overoxidation and functions in redox-regulation of AldA activity in vitro.

3.5. AldA is redox-regulated and protected by protein S-bacillithiolation under H2O2 stress in vitro

We were interested whether S-bacillithiolation inhibits AldA activity and protects the active site Cys279 against overoxidation in vitro. Using the spectrophotometric assay, AldA activity was measured after oxidative stress with 15 mM methylglyoxal as substrate and NAD+ as coenzyme by monitoring NADH generation at 340 nm. Treatment of AldA with 0.5–1 mM H2O2 resulted in a strong inactivation of its enzymatic activity (Fig. 6A). Inactivation of AldA with H2O2 alone was irreversible since AldA activity could not be restored after treatment with 10 mM DTT (Fig. 6B). These results indicate that the active site Cys279 of AldA is very sensitive to overoxidation by H2O2 in the absence of BSH. To assess the effect of S-bacillithiolation on AldA activity, the enzyme was pre-exposed to 0.3–0.5 mM BSH prior to oxidation with 0.3–1 mM H2O2 and the remaining AldA activity was measured in the spectrophotometric assay with 15 mM methylglyoxal as substrate. AldA activity was inhibited with 0.3–1 mM H2O2 after pre-treatment with 0.3–0.5 mM BSH (Fig. 6C). In this case, however, the activity of the oxidized AldA protein could be restored to 66% by DTT reduction indicating that AldA is subject to irreversible S-bacillithiolation in the presence of BSH and H2O2 (Fig. 6D). S-bacillithiolation of AldA and its reversibility with DTT was further confirmed in BSH-specific Western blots (Fig. 1B). These results suggest that S-bacillithiolation protects the AldA active site Cys279 against overoxidation and functions in redox-regulation of AldA activity in vitro.

3.6. Structural comparison of AldA with other aldehyde dehydrogenases

We were further interested in the structure and the structural changes of AldA upon S-bacillithiolation. A crystal structure of S. aureus AldA (denoted as saAldA) has been determined by the Midwest Center for Structural Genomics (PDB 3TY7). For understanding the enzyme’s catalytic mechanism, we performed structural homology searches for saAldA with the DALI server [58] (http://ekhidna.biocenter.helsinki.fi/dali_server/) and the PDBFold (SSM) server (http://www.ebi.ac.uk/msd-srv/ssm/). saAldA shows high homology to many other aldehyde dehydrogenases (ADHs) from bacteria, plants and humans. The root-mean-square deviations (r.m.s.d.) and sequence similarities of AldA’s homologs are listed in Table S3. In contrast to the tetrameric bacterial ADHs (pfAMSDH, saBADH,
ADH, pa BADH), sa AldA is a dimeric enzyme and thus more similar to plant ADHs that are also active as dimer (Fig. 7A). Regardless of the oligomerization state, the overall fold of a subunit is highly conserved among all ADH enzymes. Similarly as in other ADHs, a sa AldA subunit is composed of a coenzyme (NAD\(^+\))-binding domain (Co-BD; residues 1-122, 137-244 and 439-464), a catalytic domain (CD; residues 245-438) and a subunit interaction domain (SID; residues 123-136 and 465-475; Fig. 7A). Reduced AldA (2.5 \(\mu\)M) was incubated with different concentrations of aldehyde substrates ranging from 10 to 100 \(\mu\)M in reaction buffer (100 mM Tris HCl, 1.25 mM EDTA, pH 7.5). The oxidation of the aldehydes was measured in the presence of NAD\(^+\) as coenzyme and NADH generation was monitored at 340 nm using a spectrophotometer. The results are from 3 replicate experiments. Error bars represent the SEM.

### Fig. 5. Purified AldA shows broad substrate specificity towards various aldehydes in vitro. The catalytic activity of the aldehyde dehydrogenase AldA was analyzed with increasing concentrations of different aldehyde substrates, including (A) formaldehyde (FA), (B) methylglyoxal (MG), (C) acetalddehyde (AA) and (D) glycol aldehyde (GA).

### Fig. 6. Inactivation of AldA of *S. aureus* in response to H2O2 in the absence and presence of BSH in vitro. Reduced AldA (30 \(\mu\)M) was oxidized with 0.3-1 mM H2O2 for 5 min in the absence (A, B) or presence of BSH (C, D) in reaction buffer (100 mM Tris HCl, 1.25 mM EDTA, pH 7.5). The AldA activities were measured with 15 mM methylglyoxal as substrate and NAD\(^+\) as coenzyme by monitoring NADH production at 340 nm using a spectrophotometer. To analyze the irreversible inactivation of AldA by H2O2 alone, AldA was treated with 1 mM H2O2 without BSH followed by reduction with 10 mM DTT (C). The reversibility of AldA S-bacillithiolation with 0.3 mM H2O2 and 0.3 mM BSH is shown after DTT-reduction resulting in 66% of regeneration of AldA activity (D). The S-bacillithiolation of AldA and its reduction using DTT was further confirmed in BSH-specific Western blot analysis as shown in Fig. 1B. P-values were calculated as follows: \(p < 0.001\); \(p < 0.0001\); and \(p < 0.00001\). The results are from 3 replicate experiments. In all graphs, mean values are shown, error bars represent the SEM and p-values are calculated using a Student’s unpaired two-tailed t-test by the graph prism software.
adopt two alternative conformations, a “resting” and “attacking” (Fig. 7C), depending on the enzyme activation state. In the apo-enzyme structure, the Cys residue is in the resting conformation, whereas upon NAD^+ binding the Cys thiol moiety rotates away from the nicotinamide part of NAD^+ and is closer to the substrate-binding pocket [54–56]. The Cys residue serves as a nucleophile during catalysis, leading to a covalent thioester-enzyme adduct with the substrate \[54,55,57\]. The conserved glutamate residue then serves as a base to activate a water molecule for hydrolysis of the thioester-enzyme intermediate \[55,59\]. In addition to the Cys and glutamate residues, there are two other conserved residues, a lysine (K156 in saAldA) and a glutamate (E455 in saAldA), that are involved in a proton relay that allows the deprotonation of E245, and, as a consequence, proton abstraction from the hydrolytic water [56].

Another common feature of the ADHs is the presence of a cation-binding site located in the Co-BD (Fig. 7B, D). Co-BD is formed by the three main chain carbonyl groups of an isoleucine/valine (I25 in saAldA), a glutamate/aspartate (E91 in saAldA) and a glutamate residue (E173 in saAldA) \[60–62\]. The cation bound at this site is usually sodium or potassium, and it was reported that the enzyme activity is...
slightly higher in the presence of sodium [60]. In the saAldA structure, a magnesium ion is present at this site, most likely because magnesium was the only cation present in the crystallization solution. The role of the cation-binding site is to maintain the structural integrity of the protein and to stabilize a loop involved in binding of NAD⁺ [60–62].

The available saAldA structure represents the apo-enzyme. In contrast, the structures of plant ADHs and of pBaADH contain the coenzyme NAD⁺. In the case of pFAMS DH, the structures of pFAMSDH/NAD⁺/intermediate complexes are also available [55]. Comparison of the apo, NAD⁺, NAD⁺/intermediate states shows that binding of the coenzyme or the formation of the intermediate does not influence the secondary structure elements within the enzyme, while rearrangements are observed in the side chains of residues involved in catalysis [54,55]. In the ADHs, the NAD⁺ is bound in the hydrophobic pocket of the Co-OD. Only the nicotinamide nucleotide moiety is turned towards a negatively charged pocket, in which the catalytic cysteine residue is located (Fig. 7B). NAD⁺ is engaged in only few polar contacts with the enzyme [54,62].

Although the overall structure, the active site and the cation-binding site are highly conserved among the ADHs, these enzymes show broad substrate specificities and the amino acid residues involved in substrate binding are different among the ADHs. Nevertheless, even a single ADH is able to use many different aldehydes as substrates. For example, sIAMADH can oxidize many different aldehydes [62]. Thus, differences in the substrate-binding residues determine differences in the still comparatively broad substrate spectra of the enzymes.

3.7. S-bacillithiolation of the AldA active site depends on the Cys activation state as revealed by molecular dynamics simulation

Next, we analyzed the structural changes of AldA upon S-bacillithiolation and used molecular docking and molecular dynamics simulations to model BSH into the active site of the apo- and holoenzyme structures (Fig. 7EF). The structure of saAldA apo-enzyme (PDB 3TV7) was superimposed with the NAD⁺ binding structure from Pseudomonas fluorescens pFAMS DH (PDB 4I1W) to model the NAD⁺ cofactor into the AldA active site pocket (Fig. 7C). We further noticed that in the saAldA dimeric structure, the loop composed of residues 438-459 is not present which was modelled into the saAldA holo-enzyme structure based on the structure of pFAMSDH (Fig. 7F). This loop in the saAldA holo-enzyme structure may interfere with the location of BSH at the active site. To model the S-bacillithiolated active site Cys279 in the saAldA apo- and holoenzyme structures, we applied an adapted molecular docking algorithm based on Steric Clashes-Alleviated Receptor (SCAR) approaches [63], which takes into account the possibility of bond formation between ligand and receptor. Molecular docking and atomistic molecular dynamics simulation of the covalent BSH enzyme complex resulted in two best-scoring poses for BSH in the apo-enzyme (Q2) or holo-enzyme complex (Q1) (Fig. 7EF). However, no overlap between BSH and the loop (aa438-459) in the holo-enzyme structure was found and there was still room for an aldehyde substrate. Interestingly, these two different BSH positions in the AldA active site depend on the Cys279 activation state in the presence or absence of the NAD⁺ cofactor (Fig. 7EF). In the apo-enzyme structure, Cys279 bound to BSH is still in ‘resting’ position (Q2), while Cys279 is in the ‘attacking’ position in the holo-enzyme (Q1). Thus, the location of BSH in the active site pocket depends on the Cys279 activation state in the presence or absence of NAD⁺. The Q2 pose of BSH at the apo-enzyme without NAD⁺ seems to be energetically more favorable since Q2 had much better energy score (-19.7 +/- 10.0 kJ/mol) compared to the apo-enzyme without BSH (Fig. 7F). This further confirms that BSH can undergo disulfide formation with the active site Cys279 at different positions without major conformational changes.

4. Discussion

S. aureus is a major human pathogen of hospital and community-acquired infections, ranging from local skin infections to life-threatening systemic and chronic infections. During infections, S. aureus is exposed to ROS, RCS and RES that are produced as first line of defense by activated macrophages and neutrophils or can be also encountered as consequence of antibiotics treatment [10,11,64]. Thus, the understanding of the adaptation mechanisms of S. aureus to infection conditions to avoid killing by ROS, RCS and RES is important for the discovery of new drug targets to combat multi-resistant S. aureus infections.

In our previous work, we have identified the aldehyde dehydrogenase AldA as one of the most strongly oxidized proteins in the thiol-redox proteome in S. aureus, which showed a 29% oxidation increase under NaOCl stress using the OxIGAT analysis [26]. AldA uses a conserved active site Cys279 that was modified by S-bacillithiolation under NaOCl stress. Apart from AldA, the glyceraldehyde-3-phosphate dehydrogenase Gap was identified as S-bacillithiolated at its active site Cys151 under NaOCl stress. Thus, it is interesting to note that two functionally related aldehyde dehydrogenases are targets for oxidation at their active site Cys residues that both function in aldehyde oxidation. In this study, we demonstrated that AldA is specifically induced under thiol-specific stress conditions, such as NaOCl, diamide and formaldehyde stress. Expression of aldA was previously shown to be regulated by the alternative sigma factor SigmaB in response to heat shock, salt stress caused by NaCl and MnCl₂ as well as alkaline shock [50,51]. Here, we have shown that the thiol-specific expression of aldA occurs SigmaB-independently. Thus, aldA seems to be double-controlled by SigmaB and another thiol-stress sensing regulator to allow adaptation to general stress and starvation as well as thiol-stress conditions.

SigmaB has been previously shown to play an important role under infection conditions and controls biofilm formation and several virulence factors, such as adhesins [65,66]. The SigmaB regulon was induced after internalization of S. aureus by bronchial epithelial cells and required for intracellular growth as demonstrated by transcriptomics and proteomics [53,67,68]. Moreover, SigmaB has been implicated as central regulator in long-term persistence in human osteoblasts and controls the small colony variant (SCV) phenotype of persistent S. aureus infections [69,70]. Thus, it might be possible that adaptation of S. aureus from acute to chronic and persistent infections requires SigmaB and AldA to cope and adapt to the stationary phase and thiol-specific stress conditions inside macrophages and neutrophils. This adaptation to thiol-stress conditions is particularly important for S. aureus to survive under conditions of long-term persistent and chronic infections.

In this work, we have shown that AldA is an important member of the SigmaB regulon that provides protection under NaOCl stress.
conditions as shown in survival assays. However, the thiol-specific induction of aldA transcription seems to be SigA-dependently since the same induction level was observed in the sigB mutant under thiol-stress. A putative SigA-promoter was observed upstream of the SigB-promoter indicating that aldA transcription might be controlled by SigB and SigA containing RNA polymerase (RNAP) from adjacent promoters. The stronger aldA induction in the sigB mutant under methylyglyoxal stress could be explained by a higher affinity of SigA for the RNAP core enzyme compared to SigB and the lack of sigma factor competition in the sigB mutant [71]. Moreover, the thiol-stress-specific induction of aldA transcription might require additional transcriptional regulators that remain to be elucidated. In future studies, we also aim to investigate if AldA plays a role for the intracellular growth as well as persistence or chronic infections in S. aureus, which could require detoxification of toxic aldehydes to allow long-term survival.

To study the function of AldA and its redox-regulation under NaOCl stress in vitro, we purified the enzyme and determined its catalytic activities towards oxidation of various aldehydes. We could show that AldA has broad substrate specificities to oxidize formaldehyde, methylglyoxal, glycol aldehyde and acetaldehyde to their respective acids. The question arises about the physiological aldehyde substrate for AldA under in vivo conditions that are produced under infection conditions, such as under hypochlorite stress. Methylglyoxal was previously shown to be produced at higher levels under HOCI stress in E. coli [20]. Moreover, the gloA-nemRA operon was induced under methylyglyoxal and HOCI stress, which functions as important HOCl and methylglyoxal defense mechanism [19–22]. The FMN-dependent oxidoreductase NemA functions in detoxification of various electrophiles, such as aldehydes, N-ethylnamaleimide and quinones and its up-regulation under HOCI stress indicates the link between HOCl and aldehyde stress. In our work, we could also show that AldA responds to aldehydes, diamide and NaOCl and hence could be involved in methylglyoxal detoxification in S. aureus as well. However, in growth and survival assays, no phenotypes of the aldA mutant were detected under formaldehyde and methylglyoxal stress. Since AldA showed broad substrate specificity towards various aldehydes in vitro, its natural substrates could be different aldehydes that remain to be elucidated.

Of note, AldA shares strong 57% sequence similarity to betaine dehydrogenases from S. aureus, Pseudomonas aeruginosa and Spinacia oleracea. These enzymes function in oxidation of the toxic betaine aldehyde to glycine betaine which is a well-known compatible osmoprotectant [72,73]. Glycine betaine can be either taken up upon osmotic stress or synthesized from exogenously provided choline in a two oxidation steps via choline dehydrogenase (BetA) and betaine dehydrogenase (BetB) which are conserved in B. subtilis [72,73] and S. aureus [54]. The human tissues are rich sources of choline and betaine and thus, S. aureus encounters toxic aldehydes produced from choline during colonization and internalization. For some bacteria, the importance of the choline oxidation pathway for survival and virulence has been already demonstrated [73,74]. Of note, AldA is also induced under high osmolarity conditions provoked by NaCl stress in a SigmaB-dependent manner [50]. This could point to a possible function in the osmostress and thiol-stress response in S. aureus which remains to be elucidated. However, we could not detect AldA activity for oxidation of betaine aldehyde as substrate in vitro, indicating a different function of AldA in S. aureus (data not shown).

The catalytic activity of AldA depends on a highly conserved Cys279 active site which we identified as S-bacillithiolated under NaOCl stress in S. aureus [26]. Interestingly, this nucleophilic active site Cys residue was previously found oxidized to a mixed disulfide with beta-mercaptoethanol during protein crystallization of related betaine aldehyde dehydrogenases [54,74]. These results confirm the redox-sensitivity of the active site Cys of AldA as shown in this work. Our results have further demonstrated that S-bacillithiolation functions in redox-regulation and inactivation of AldA activity under H2O2 stress. In the absence of BSH, the active site Cys279 was very sensitive to over-oxidation as shown by its irreversible inactivation. In the presence of BSH, Cys279 was protected against overoxidation by the S-bacillithiolation as shown for the glyceraldehyde-3-phosphate dehydrogenase GapDH in S. aureus [26]. Both enzymes use a similar catalytic mechanism for the NAD+-dependent oxidation of the aldehyde substrate to generate the acid product [54,55,57]. In the catalytic mechanism of aldehyde dehydrogenase, the active site Cys was shown to adopt two conformations: the “attacking” or “resting” conformation depending on the presence or absence of the NAD+ cofactor. We used molecular docking and molecular dynamic simulations to model the S-bacillithiolated active site in the presence and absence of NAD+. In the apo-enzyme structure, BSH was bound to Cys279 in the resting state (Q2) position and occupied the cofactor-binding pocket. In the presence of NAD+, Cys279 was modified in the attacking state position (Q1) and BSH was repositioned close to the substrate-binding site.

In our previous docking approach with BSH at the Cys151 active site of GapDH, we found similar locations of BSH in the apo-enzyme and holo-enzyme structures related to the resting and attacking state. Thus, the highly flexible active site and the redox-sensitivity of the nucleophilic Cys residues facilitate their fast oxidation to the mixed disulfides with BSH. In both structural models, S-bacillithiolation of GapDH and AldA did not require major structural changes, which further explains their preferred formation of the BSH mixed disulfides. This flexible BSH position may ensure that catalytic active and resting AldA and GapDH enzymes can both be protected against overoxidation under NaOCl stress to ensure fast regeneration and reactivation of the enzymes.

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Author disclosure statement

No competing financial interests exist.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2018.02.001.

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