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Distribution and infection-related functions of bacillithiol in *Staphylococcus aureus*

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ABSTRACT

Bacillithiol (Cys-GlcN-malate, BSH) serves as a major low molecular weight thiol in low GC Gram-positive bacteria including *Bacillus* species and a variety of *Staphylococcus aureus* strains. These bacteria do not produce glutathione (GSH). In this study, HPLC analyses were used to determine BSH levels in different *S. aureus* strains. Furthermore, the role of BSH in the resistance against oxidants and antibiotics and its function in virulence was investigated. We and others (Newton, G.L., Fahey, R.C., Rawat, M., 2012. Microbiology 158, 1117–1126) found that BSH is not produced by members of the *S. aureus* NCTC8325 lineage, such as strains 8325-4 and SH1000. Using bioinformatics we show that the BSH-biosynthetic gene *bshC* is disrupted by an 8-bp duplication in *S. aureus* NCTC8325. The functional *bshC*-gene from BSH-producing *S. aureus* Newman (NWMN_1087) was expressed in *S. aureus* 8325-4 to reconstitute BSH-synthesis. Comparison of the BSH-producing and BSH-minus strains revealed higher resistance of the BSH-producing strain against the antibiotic fosfomycin and the oxidant hypochlorite but not against hydrogen peroxide or diamide. In addition, a higher bacterial load of the BSH-producing strain was detected in human upper-airway epithelial cells and murine macrophages. This indicates a potential role of BSH in protection of *S. aureus* during infection.

Introduction

Staphylococcus aureus asymptomatically colonizes the skin and anterior nares of about one-quarter of the human population (Foster, 2004). However, it is an opportunistic pathogen that can cause a wide variety of diseases ranging from abscesses to life threatening systemic infections (Boucher and Corey, 2008; Lowy, 1998). During the last decades *S. aureus* has rapidly adapted to a broad range of antibiotics. Nowadays *S. aureus* has become a major threat as a nosocomial pathogen with resistances against last resort antibiotics (Livermore, 2000). Thus, there is an urgent need in developing new therapeutics. Since virulence factors are the molecular drivers of invasion, intracellular persistence and phagosomal escape, the understanding of the pathogenicity of *S. aureus* is of utmost importance for the treatment of infection with this menacing human pathogen.

Reversible thiol-disulfide exchange reactions play a central role in biology for donating and accepting electrons and controlling protein function (Ortenberg and Beckwith, 2003). Low molecular weight (LMW)-thiols are small molecules with diverse functions inside the cell. They can serve as proteinogenic amino acids (cysteine), carriers for acyl groups (Coenzyme A), detoxifiers of reactive oxygen and nitrogen species (ROS, RNS) or xenobiotics, or as buffer reagents to maintain the cellular redox-status (glutathione). However, the distribution of LMW-thiols differs among various organisms (Fahey, 2012). Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is the primary LMW-thiol in eukaryotes and most Gram-negative bacteria (Masip et al., 2006). The GSH-system together with the thioredoxin (Trx) and glutaredoxin (Grx) systems constitute the major thiol-dependent redox pathways in *Escherichia coli* cells (Holmgren, 1989). GSH with its enzymatic systems plays a key role in many physiological processes, like maintenance of the thiol-redox balance, protein folding, detoxification

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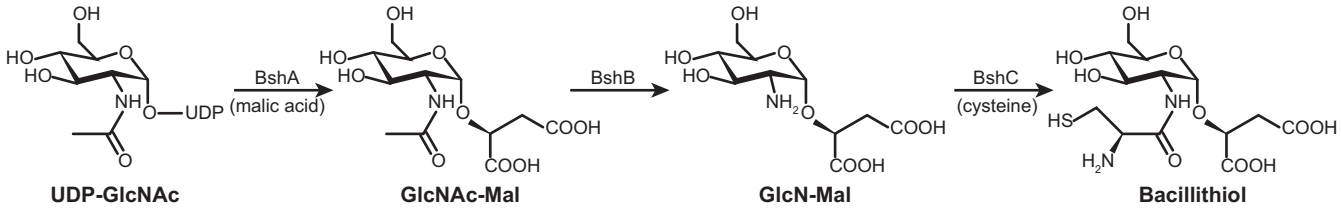

Fig. 1. Biosynthesis of bacillithiol (BSH). The pathway for BSH biosynthesis is shown as reported previously (Gaballa et al., 2010): malate is added to the UDP-activated GlcNAc by BshA, subsequently GlcNAc-Mal is deacetylated by BshB and cysteine is likely added by BshC.

of ROS and RNS or providing reducing power for ribonucleotide or arsenate reduction (Holmgren, 1976; Masip et al., 2006; Messens and Silver, 2006). GSH is absent in most Gram-positive bacteria, archaea and amitochondrial eukaryotes (Fahey et al., 1984). The parasitic protozoa Leishmania and Trypanosoma use trypanothione that is composed of two GSH-molecules peptide-bonded at the glycinyl carboxylic groups with spermidine (Krauth-Siegel et al., 2003). Mycothiol (MSH; AcCys-GlcN-myo-inositol) is the major LMW-thiol in Actinomycetes (Jothivasan and Hamilton, 2008; Newton and Fahey, 2002). The LMW-thiol bacillithiol (Cys-GlcN-malate, BSH; Fig. 1) is present in most *Bacillus* species and *Staphylococcus* and was originally isolated from *Deinococcus radiodurans* (Newton et al., 2009). It has been shown that BSH confers resistance to the epoxide antibiotic fosfomycin and sodium hypochlorite in *Bacillus subtilis* (Chi et al., 2011; Gaballa et al., 2010; Newton et al., 2012). The resistance to fosfomycin relies on the BSH-dependent *S*-transferase FosB that adds BSH to fosfomycin in order to open the ring structure of the epoxide antibiotic (Sharma et al., 2011). The biochemistry of FosB has been analyzed for FosB-homologs of different *Bacillus* species and *Staphylococcus aureus* (Lamers et al., 2012). It has been shown that FosB uses BSH as preferred thiol-cofactor and functions only poorly with cysteine. BSH also functions in protection of protein thiols in different *Bacillus* species and *Staphylococcus carnosus* and controls the activity of the OhrR repressor in *B. subtilis* due to formation of mixed BSH protein disulfides (*S*-bacillithiolation) under hypochlorite stress (Chi et al., 2011, 2013). The results indicate that *S*-bacillithiolations are functionally similar to *S*-glutathionylations of eukaryotes. The role of BSH as electron donor has been recently investigated in *B. anthracis*, but BSH did not function in reduction of ribonucleotide reductase (Gustafsson et al., 2012).

S. aureus strains are very heterogenous for example in terms of their genetic composition and expression of virulence factors. We aimed for a comprehensive profiling of LMW-thiols in different *S. aureus* strains. Recently, Newton et al. (2012) showed that *S. aureus* NCTC8325 and its derivatives 8325-4 and SH1000 lack BSH. Our data on LWM thiol analysis were in progress at the same time when the paper was published. In this paper, we studied the genetic reasons for the failure of BSH synthesis in *S. aureus* NCTC8325 derivatives, reconstituted the BSH synthesis and analyzed the stress resistance as well as the survival of *S. aureus* inside different eukaryotic cells in the presence and absence of BSH. Our data show that the presence of BSH increases the resistance against fosfomycin and selected oxidants. Most strikingly, we demonstrate that the restoration of BSH synthesis effectively enhances the survival of *S. aureus* cells in a phagocytosis assay. As an unexpected result, we also found that *S. aureus* is able to accumulate GSH at the onset of the stationary phase.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used for physiological analyses were the *S. aureus*-strains: 8325-4, SH1000 and Newman. These strains were grown at 37 °C in a chemically defined medium (CDM) described previously (Pöther et al., 2009) with and without 1 mM glutathione, in Lennox LB (Invitrogen) or TSB (Oxoid) under vigorous agitation. Growth was measured by monitoring the optical density at 540 nm in LB and TSB and at 500 nm in CDM. *S. aureus* 8325-4 and SH1000 containing the plasmid pRB473-pXyl were grown in LB containing 10 μg/ml chloramphenicol and 1% xylose. For construction of these genetically manipulated *S. aureus* strains, *Escherichia coli* DH5α and *S. aureus* RN4220 were used and grown in LB with 100 μg/ml ampicillin (*E. coli*) or 10 μg/ml chloramphenicol (*S. aureus*). *S. aureus* 8325-4 was exposed to different stresses in LB at an optical density at 540 nm of 0.5. Compounds used for stress exposure were 1, 3 and 6 mM diamide, 2, 4 and 8 mM NaOCl, 10, 20 and 40 mM H_2O_2 or 2.75, 5.5 and 16.5 μM fosfomycin.

RNA preparation and Northern blotting

Total RNA was prepared as described by Fuchs et al. (2007). In brief, cells were harvested and treated directly with sodium azide to diminish metabolism. Subsequently cells were mechanically disrupted and RNA was isolated by acidic phenol extraction. The RNA was washed thoroughly, precipitated and resuspended in deionized water.

Digoxigenin-labeled RNA probes were synthesized as described earlier (Wetzstein et al., 1992) with oligonucleotide primers listed in Table 1. Two microgram samples of total RNA were used for Northern blots that were performed as described earlier (Chi et al., 2011).

Construction of BSH-complemented S. aureus 8325-4 and SH1000

An approximately 1.7-kb DNA-fragment of *S. aureus* Newman containing the ORF of NWMN_1087 (*bshC*) was amplified by PCR using primers bshC-comp-for-BamHI and bshC-comp-rev-EcoRI (Table 1). The purified fragment was digested with EcoRI and BamHI and ligated into the likewise digested vector pRB473-pXyl (gift from Knut Ohlsen, University of Würzburg, Germany). The vector pRB473-pXyl contains the *xylR*-gene (including operator and promoter) of *S. xylosus* within the multiple cloning site of pRB473 (Brückner et al., 1993). The vector was electroporated into *S. aureus* RN4220 and subsequently transduced with phage 85 into *S. aureus* 8325-4 and SH1000 resulting in *S. aureus* 8325-4 pRB-*bshC* and *S. aureus* SH1000 pRB-*bshC*. For control purposes *S. aureus* 8325-4 was transduced with the empty pRB473-pXyl vector without NWMN_1087 resulting in *S. aureus* 8325-4 pRB473-pXyl.

Isolation of thiols as their mBBr-derivatives

Cells were grown in TSB and 30 ml of exponentially grown cells (OD_{540} 1) or 10 ml of stationary phase cells, respectively, were harvested. Twenty milliliters of stationary phase cells grown in CDM were harvested. The isolation of thiols was performed as described previously (Pöther et al., 2009). In brief, cells were centrifuged, washed with 50 mM Tris–HCl (pH 8.0) and resuspended in 50%