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Analysis of *Staphylococcus aureus* proteins secreted inside infected human epithelial cells

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ABSTRACT

Staphylococcus aureus, an opportunistic pathogen is able to invade into and persist inside non-professional phagocytic cells. To do so, this bacterium possesses a wide range of secreted virulence factors which enable attachment to the host as well as intracellular survival. Hence, a monitoring of virulence factors specifically produced upon internalization might reveal targets for prevention or therapy of *S. aureus* infections. However, previous proteome approaches enriching *S. aureus* from lysed host cells after infection did not cover secreted virulence factors.

Therefore, we used density gradient centrifugation and mass spectrometry to identify *S. aureus* HG001 proteins which were secreted into compartments of infected human bronchial epithelial S9 cells. Because shotgun mass spectrometry revealed only few bacterial proteins amongst 1905 host proteins, we used highly sensitive and selective single reaction monitoring mass spectrometry as an alternative approach and quantified 37 bacterial proteins within the *S. aureus* containing host cell compartment 2.5 h and 6.5 h post infection. Among them were secreted bacterial virulence factors like lipases, pore forming toxins, and secreted adhesins which are usually hard to detect from infected sample material by proteomics approaches due to their low abundance. *S. aureus* adapted its proteome to improve its response to oxidative and cell wall stress occurring inside the host, but also, increased the amounts of some adhesins and pore-forming toxins, required for attachment and host cell lysis.

1. Introduction

The Gram-positive bacterium *Staphylococcus aureus*, an opportunistic pathogen, colonizes about 30% of the healthy human population without causing clinical symptoms. However, it can also lead to diseases ranging from milder skin infections to severe diseases with high morbidity and mortality such as toxic shock syndrome, endocarditis, pneumonia or septicemia (Lowy, 1998). Some *S. aureus* strains developed resistance to a broad range of antibiotics such as methicillin (for example MRSA). They constitute potent nosocomial pathogens that cause diseases which are difficult to treat, and until now no effective vaccine exists against them. *S. aureus* can colonize and thrive in different niches because it encodes a multitude of mostly cell wall-bound and secreted virulence factors. This virulence factor repertoire comprises proteins involved in adhesion and invasion, proteins enabling acquisition of nutrients, spreading in the host as well as degradation of host cells, and, furthermore, proteins that help bacteria to evade the immune response (Hecker et al., 2010). Also, this pathogen is able to invade and persist in non-professional phagocytic cells (Garzoni and Kelley, 2009; Ellington et al., 1999; Sinha and Herrmann, 2005; Kintarak et al., 2004; Haslinger-Löffler et al., 2005), and thus, to hide from the host's immune system.

Cell wall-bound virulence factors, so-called adhesins, help *S. aureus* to adhere to host cell molecules in order to facilitate colonization which can be regarded as first important step of an infection (Gordon and Lowy, 2008). Members of this group are among others fibronectin-binding proteins A and B (FnbA, FnbB), elastin-binding protein (EbpS), clumping factor A and B (ClfA, ClfB) (Clarke et al., 2009), and the iron-regulated surface determinant proteins (IsaA, IsdB, IsdC, and IsdH) (Kim et al., 2010). But also secreted proteins such as the staphylococcal

Abbreviations: MS, mass spectrometry; p.i., post infection; SRM, single reaction monitoring; SCC, *S. aureus* containing compartments

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coagulase can mediate adhesion between *S. aureus* and host cell molecules. Furthermore, *S. aureus* secretes pore-forming, cytolytic toxins like alpha-hemolysin (Hla), the bicomponent gamma-hemolysin (HlgBC) or leukocidin-like proteins (LukS, LukF) (DuMont and Torres, 2014) to damage membranes and to lyse neutrophils after ingestion which is an effective strategy against elimination by the innate host defense (Otto, 2014). *S. aureus* also possesses a broad repertoire of secreted proteins which manipulate host proteins in order to survive inside the host, such as lipases Lip and Geh, and proteases like aureolysin (Aur) (Sieprawska-Lupa et al., 2004; Hu et al., 2012). Besides its capability of adhesion and manipulation of the host for escaping the immune response, *S. aureus* was furthermore recognized to be able to persist inside non-professional phagocytic host cells (Garzoni and Kelley, 2009; Lâm et al., 2010). Intracellular persistence requires strategies for intracellular survival. Avoidance of lysosomal killing, replication inside the *S. aureus* containing compartments (SCC, e.g., endosomes, phagosomes, or phagolysosomes), and potential outbreak from SCC leading to cytosolic replication of bacteria and, finally, to host cell apoptosis constitute such strategies which were recently reviewed (Fraunholz and Sinha, 2012).

Thus, analysis of *S. aureus* inside intracellular compartments and especially determination of the abundance of its secreted virulence factors upon infection is crucial to gain a better insight into mechanisms of host-pathogen interaction and may identify putative intervention points for treatment. In former studies, we were able to characterize adaptive changes of pathogen and host after internalization using fluorescence-supported or magnetic separation of bacteria from infected human respiratory epithelial cells like S9 or A549 cells upon lysis with a detergent (Surmann et al., 2015; Depke et al., 2014). However, these protocols did not allow profiling of proteins released by internalized bacteria except for some cell-associated precursors, because soluble bacterial molecules that were not attached to the bacterial cells were lost during enrichment.

Several protocols have been published to study intracellular pathogens' maturation and stage-specific changes in phagosomal protein content by isolating bacteria-containing phagosomes (Herweg et al., 2015). These protocols include affinity immune-precipitation (Vorwerk et al., 2015), magnetic bead-based assays (Steinhäuser et al., 2013) as well as different density gradient centrifugation procedures (Lührmann and Haas, 2000), and combinations of these methods. Proteome profiles of pathogen containing intracellular compartments and/or their respective microbes upon separation from infected host cells have been published before including those containing *Mycobacterium avium*, *Mycobacterium tuberculosis*, *Leishmania major, Legionella pneumophilia, Chlamydia trachomatis,* and *Legionella hackeliae* (Desjardins, 2003; Mattow et al., 2006; Sturgill-Koszycki et al., 1994; Shevchuk et al., 2009; Aeberhard et al., 2015). Recently, a mass spectrometry-based approach was used to analyze secreted bacterial virulence factors and host proteins in infected tissue from human nasal polyps colonized by *S. aureus* (Schmidt et al., 2017).

In the study presented here, we isolated intact SCCs containing complete bacteria as well as proteins secreted by these bacteria into the compartmental lumen in order to identify soluble bacterial effectors promoting intracellular survival after internalization of *S. aureus* HG001 (Herbert et al., 2010) by S9 cells (Zeitlin et al., 1991; Flotte et al., 1993). First, we profiled the complete sample by nanoLC-MS/MS in an iterative manner and detected only few staphylococcal proteins against the background of about 1900 host proteins.

To improve the sensitivity for bacterial proteins, and especially cell wall-bound or secreted virulence factors, we reanalyzed the same samples using highly sensitive and selective single reaction monitoring (SRM) and then identified 45 pathogen specific proteins involved in the virulence of *S. aureus* at least at one point in time and quantified changes in abundance of 37 of them 2.5 h and 6.5 h post infection (p.i.)

To our knowledge, we report for the first time the enrichment and quantification of secreted virulence-associated staphylococcal proteins captured from isolated SCCs at different times after infection.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. aureus HG001 (Herbert et al., 2010) constitutively expressing plasmid pMV158GFP-encoded (Nieto and Espinosa, 2003) green fluorescence protein (GFP) was used for proteome analyses in this study. For fluorescence microscopy strain HG001 pJL-sar-cer (Liese et al., 2013) expressing the cyan fluorescent protein (CFP) was used. Cultivation prior to cell culture infection was performed as described before in prokaryotic minimal essential medium (pMEM) (Surmann et al., 2014). Serial dilutions of bacterial cultures were incubated at 37 °C and 220 rpm overnight in medium containing 20 µg/mL tetracycline for pMV158GFP or 10 µg/mL erythromycin for pJL-sar-cer. Before infection of the host cells an exponentially growing culture was inoculated in fresh pMEM to an optical density at 600 nm (OD_{600}) of 0.05 and cultivated in a water bath with linear shaking (150 strokes per minute) at 37 °C until OD_{600} 0.4.

2.2. Eukaryotic cell culture

The human bronchial epithelial S9 cell line (ATCC® number CRL-2778) (Zeitlin et al., 1991; Flotte et al., 1993) was cultivated in eukaryotic minimal essential medium (eMEM) at 37 °C in saturated air humidity with 5% CO_2 as described before (Surmann et al., 2014). S9 cells expressing yellow fluorescent protein (YFP)-tagged lysosome-associated membrane protein 1 (LAMP1-YPF) obtained from Martin Fraunholz, Würzburg, were used to visualize late endosomal/lysosomal compartments by fluorescence microscopy and cultivated as described above. YFP-positive cells were selected by adding 3 µg/mL blasticidin (Blasticidin S-Hydrochlorid BioChemica, AppliChem, Darmstadt, Germany) to the medium (Giese et al., 2009). With a FACSAria III-u high-speed cell sorter (Becton Dickinson Biosciences, San Jose, CA, USA) YFP-positive S9 cells were enriched by cell sorting with an 85 µm nozzle and 85 psi sheath pressure. The emission of YFP was detected in the FITC-channel (exc 488 nm/em 530/30 nm). YPF-positive cells were cultured further on as described above in eMEM supplemented with blasticidin.

2.3. Internalization and isolation of compartments containing S. aureus and its secreted proteins

S9 cells were infected with *S. aureus* HG001 pMV158GFP as described previously (Surmann et al., 2014). Briefly, confluent S9 cells grown in eMEM were infected with exponentially growing bacteria at OD_{600} 0.4 in pMEM diluted in eMEM to a multiplicity of infection (MOI) of 25 and buffered with 2.2 g/L $NaHCO_3$ (PAN Biotech, Aidenbach, Germany). After 1 h incubation at 37 °C and 5% CO_2 in a humid atmosphere, medium was removed and replaced by eMEM containing 10 µg/mL lysostaphin which killed non-internalized bacteria. At 2.5 h and 6.5 h p.i. medium was removed, and cell layers were washed twice with phosphate buffered saline (PBS). Cells were scraped and resuspended in sucrose buffer consisting of 8.55% sucrose, 20 mM HEPES pH 6.5 in HPLC grade water containing protease inhibitor 1:20 (Complete EDTA-free Protease Inhibitor Cocktail Tablets, Roche, Berlin, Germany; one tablet dissolved in 50 mL extraction solution according to instructions, aliquots were frozen at −20 °C until usage).

All steps of the isolation of SCC were carried out on ice. All cells of one point in time were pooled and centrifuged at 4 °C, 2000 ×*g* for 10 min. The cell pellet was resuspended in 500 µL 8.55% sucrose buffer and lysed using a 21 gauge syringe needle (10 times up and down). Lysed cells were centrifuged at 4 °C, 500 x*g* for 7 min. Supernatant was transferred into a clean vial, and the remaining pellet was resuspended again in 500 µL 8.55% sucrose buffer. Lysis was repeated as before with a 20 gauge syringe needle. After centrifugation, both supernatants were combined and centrifuged twice (4 °C, 500 ×*g* for 7 min). In between,