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The phosphoproteome and its physiological dynamics in *Staphylococcus aureus*

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ABSTRACT

Phosphorylation events on proteins during growth and stress/starvation can represent crucial regulation processes inside the bacterial cell. Therefore, serine, threonine and tyrosine phosphorylation patterns were analyzed by two powerful complementary proteomic methods for the human pathogen *Staphylococcus aureus*. Using 2D-gel analysis with a phosphosensitive stain (Pro-Q Diamond) and gel-free titanium dioxide based phosphopeptide enrichment, 103 putative phosphorylated proteins with successfully mapped 68 different phosphorylation sites were found in the soluble proteome of *S. aureus*. Additionally, in a proof of concept study, 8 proteins phosphorylated on arginine residues have been identified. Most important for functional analyses of *S. aureus*, proteins related to pathogenicity and virulence were found to be phosphorylated: the virulence regulator SarA, the potential antimicrobial target FbaA and the elastin-binding protein EbpS. Besides newly identified phosphorylation sites we compared our dataset with existing data from literature and subsequent experiments revealed additional phosphorylation events on highly conserved localizations in FbaA. Differential analysis of phosphorylation signals on the 2D-gels showed significant changes in phosphorylation under different physiological conditions for 10 proteins. Among these, we were able to detect newly appearing signals for phosphorylated isoforms of FdaB and HchA under nitrosative stress conditions.

Introduction

Continuously changing stress and starvation conditions in their natural habitats have maintained an evolutionary pressure for bacteria toward the development of complex adaptational networks. For pathogenic bacteria, not only nutrient availability and physico-chemical parameters of their environment, but also host defense mechanisms are of importance and elicit adaptive and immune evasion responses. Deeper insights into these adaptive and evasive strategies are crucial for understanding microbial physiology in the host in general and pathogenicity in particular (Flannagan et al., 2009).

Regulatory proteins and hence the regulatory networks are often modulated by post translational modifications like phosphorylation and dephosphorylation events (Mijakovic and Macek, 2012; Ohlsen and Donat, 2010). Due to the negative charge, phosphorylation may trigger protein activation, inactivation and degradation by reorganization of the protein structure (Johnson and Barford, 1993). This modulation of protein activity is found in different processes such as stress responses, central carbon metabolism and cell growth, as well as in virulence of bacterial pathogens (Eymann et al., 2007; Madec et al., 2002; Truong-Bolduc et al., 2008). Regarding pathogenic processes, phosphorylation events play an important role in cell–cell interaction and adherence, invasion of bacterial infectors into host cells and changes in host cellular structure and function (Chao et al., 2010; Schmidl et al., 2010).

In the last years knowledge on protein phosphorylation has been gained in large-scale studies for a wide range of bacteria rendering insights into putative regulatory sites of post translational modification including phosphorylation. Whilst the first studies merely focused on phosphorylation of histidine and aspartate residues in two-component systems (Hoch, 2000) and of histidine residues

Abbreviations: HCD, higher C-trap dissociation; MAHMA NONOate, 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine; SCX, strong cation exchange chromatography; S/T/Y, serine/threonine/tyrosine; TE, Tris–EDTA (ethylenediaminetetraacetic acid) buffer.

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playing a key role in regulation of the phosphotransferase system (Deutscher et al., 2006), phosphoproteome studies targeting Ser/Thr/Tyr kinases (STYKs) and phosphatases were conducted not until the post-genome era. More recently, it has been proven that also phosphorylation of arginine residues plays a role in biological regulation and is accessible by current analytical methods (Elsholz et al., 2012; Fuhrmann et al., 2009; Ge and Shan, 2011; Macek and Mijakovic, 2011; Pereira et al., 2011).

Among virulent bacteria, *Staphylococcus aureus* is a major threat as a human pathogen due to its dominant role in nosocomial infections worldwide. Spreading antibiotic resistances and the ineffectiveness of current antibiotic treatment strategies for *S. aureus* (Otto, 2010) urgently require global investigations on pathogenicity and its regulation (Reyes et al., 2011; Young et al., 2012).

Proteins that are regarded as major determinants of pathogenicity for *S. aureus* are found mainly to be secreted and/or cell-wall associated (Bronner et al., 2004; Cheung et al., 2004). Here, expression and targeting is closely coupled to growth phase and inducing stimuli. Accordingly, regulation of virulence is tightly controlled by different transcription factors leading to the activation or repression of complete regulons (Cheung et al., 2008; Jelsbak et al., 2010; Mekalanos, 1992).

First studies on protein phosphorylation events involved in bacterial virulence focused on two component systems functioning as switches for regulons specific for the effectors of pathogenicity. Regulation of these systems is mainly characterized on molecular level by phosphorylation/dephosphorylation events on histidine and aspartate residues (Delaune et al., 2012; Dubrac and Msadek, 2004; Fournier and Hooper, 2000; Giraudo et al., 1999; Lina et al., 1998; Yarwood et al., 2001).

Later, the role of S/T/Y-phosphorylation events in *S. aureus* leading to altered virulence gained attention. Here, an important example of phosphorylation-mediated regulation is the global regulator MgrA that is controlled by the eukaryotic-like protein kinase PknB. Post translational modification of MgrA is a key event in the control of expression of efflux pumps involved in drug resistance (Truong-Bolduc and Hooper, 2010; Truong-Bolduc et al., 2008). Furthermore, PknB was shown to be important for the resistance against a large range of antibiotics and for the full expression of virulence in *S. aureus* (Debarbouille et al., 2009).

As protein phosphorylation is assumed to play a major role in regulation of infection-related processes, the present work addresses phosphorylation patterns and its changes under different physiological conditions in *S. aureus* COL. To achieve this goal, we combined complementary approaches and first visualized phosphorylation patterns on 2D-gels and determined phosphorylation sites by mass spectrometry of excised and enzymatically digested protein spots. Second, we selectively enriched phosphopeptides without prior electrophoretical separation (gel-free) and analyzed them by mass spectrometry.

Taken together, we could identify 108 phosphorylated proteins with 76 different phosphorylation sites on serine, threonine, tyrosine and arginine residues. Furthermore, we followed changes in phosphorylation patterns on the 2D-gels for four different stress and starvation conditions. Furthermore, we followed changes in phosphorylation patterns on the 2D-gels for four different stress and starvation conditions. We report phosphorylation of proteins functioning as virulence factors or being regarded as possible targets for antimicrobial therapy. Additionally, we propose that these phosphorylation events are conserved within prokaryotes. Most interestingly, our quantitative data suggest a mechanism of swift regulation of metabolic fluxes by protein phosphorylation under nitrosative stress conditions to ensure supply with energy and to ensure detoxification of metabolic by-products.

Materials and methods

Bacterial strains and culture conditions

S. aureus COL (Shafer and Iandolo, 1979) was grown aerobically at 37 °C with vigorous shaking in synthetic medium (Gertz et al., 1999) with the following modifications: glycine and MOPS were omitted; all amino acids were added to a final concentration of 1 mM. Cultures for the gel-free approach targeting S/T/Y phosphorylations were grown aerobically to an optical density of 1.5–2.0 at 500 nm, which corresponds to the late exponential and transient growth phase. Cultures for the gel-free approach targeting arginine phosphorylations were grown aerobically in Luria Bertani (LB) medium to an optical density of 0.5 at 540 nm, which corresponds to the exponential growth phase.

Cell cultures were stressed at mid-log phase (OD_{500} = 0.5) and samples were taken at three distinct time points (before treatment, 15 min and 45 min after exposure to stress conditions). NO stress experiments were performed according to Hochgräfe et al. (Hochgräfe et al., 2008) by adding MAHMA NONOate [6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine; Sigma] serving as a NO donor to a final concentration of 500 μM (Hochgräfe et al., 2008). Salt stress was imposed by addition of 8% NaCl (w/v) to the culture. The thiol-specific oxidant diamide was used at a concentration of 1 mM to trigger oxidative stress. Glucose deprivation was achieved by limiting glucose concentration to 0.05% (w/v) causing a growth arrest at an optical density of 0.9. Here, samples were taken at mid-exponential phase (OD_{500} = 0.5), at the transition from exponential growth to stationary phase and 3 h after transition. All experiments were carried out in three biological replicates.

Sample preparation, 2D-PAGE, fluorescence staining, image analysis and quantitation

The bacterial cells were harvested on ice, centrifuged (10 min, 8000 rpm, 4 °C) and washed three times with TE buffer. The cell pellets were resuspended in a buffer containing 8 M urea/2 M thiourea and 10 mM NaF (as phosphatase inhibitor) and disrupted using a ribolyzer as described earlier (Becher et al., 2009). The soluble protein fraction was obtained by removal of the cell debris by centrifugation and the protein concentration was determined using Roti-Nanoquant (Roth, Karlsruhe, Germany). 2D-gel electrophoresis, phospho-staining (Pro-Q diamond, Invitrogen, Carlsbad, CA), Flamingo™ fluorescent gel staining (Biorad, Hercules, CA)) and picking of the 2D-gel spots were done according to Eymann et al. (2007). Image analysis, spot quantitation and determination of putative phosphorylated spots were performed with DECODON Delta2D 4.0 software (Decodon GmbH, Greifswald, Germany; http://decodon.com).

Identification of proteins and phosphate containing peptides from 2D-gel approach by MS

The manually picked gel pieces were digested and prepared for mass spectrometric analysis according to Schmidl et al. (2010). LC–MS/MS analysis was carried out using a nanoACQUITY UPLC system (Waters, Milford, MA) coupled online to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Concentration and desalting of peptide samples was achieved by using a trap column (Symmetry C18, 5 μm, 180 μm inner diameter × 20 mm, Waters, Milford, MA) with subsequent elution onto an analytical column (BEH130 C18, 1.7 μm, 100 μm inner diameter × 100 mm, Waters) by a binary gradient of buffer A (0.1% (v/v) acetic acid) and B (99.9% (v/v) acetonitrile, 0.1% (v/v) acetic acid) over a period of 45 min with a flow rate of 400 nl/min. The LTQ