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A proteomics workflow for quantitative and time-resolved analysis of adaptation reactions of internalized bacteria

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ABSTRACT

The development of a mass spectrometric workflow for the sensitive identification and quantitation of the kinetics of changes in metaproteomes, or in particular bacterial pathogens after internalization by host cells, is described. This procedure employs three essential stages: (i) SILAC pulse-chase labeling and infection assay; (ii) isolation of bacteria by GFP-assisted cell sorting; (iii) mass spectrometry-based proteome analysis. This approach displays greater sensitivity than techniques relying on conventional cell sorting and protein separation, due to an efficient combination of a filtration-based purification and an on-membrane digestion. We exemplary describe the use of the workflow for the identification and quantitation of the proteome of 10^6 cells of *Staphylococcus aureus* after internalization by S9 human bronchial epithelial cells. With minor modifications, the workflow described can be applied for the characterization of other host–pathogen pairs, permitting identification and quantitation of hundreds of bacterial proteins over a time range of several hours post infection.

1. Introduction

Infectious diseases are still one of the major challenges for the health care systems. In 2002 about 26% of all cases of death worldwide, totaling nearly 15 million people, were due to infectious diseases [1]. The development of new biomarker targets and therapies requires a thorough understanding of the pathophysiology of infectious diseases, particularly the intimate interplay between the pathogen and the host during the progression of disease. The introduction and development of the collection of functional genomics technologies (genomics, transcriptomics, proteomics, and metabolomics) commonly known as OMICS-technologies have paved the way for a new level of understanding, because they can provide a holistic view of the adaptive changes that take place during infection in both host and pathogen. While genomics and transcriptomics are routinely used to address infection-related questions at a system-wide level, limitations in sensitivity and specificity in the field of proteomics until very recently restricted the generation of complementing data sets at the protein level. Integration of these different OMICS-datasets will provide an integrated view from adaptation at the gene expression level through physiological and cellular adaptation.

With the development of the newest generation of high-accuracy mass spectrometers (MS) such as LTQ-Orbitrap XL MS, the perspective changed because gel-free protein identification and quantitation of complex samples in minute amounts down to the low picogram range became possible [2].

Compared to classical setups dealing with pure cell cultures or isolated tissue, the characterization of pathogenic bacteria from host–pathogen settings are even more difficult, because only extremely small amounts of sample are accessible for the infecting pathogen. While one challenge is the sensitivity of current proteomics techniques, an even greater problem is the selection of adequate pre-separation techniques for the low numbers of bacteria available in such experimental setups. Different approaches have

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been developed for isolation of bacteria from host cells such as sucrose gradient centrifugation [3], immunomagnetic separation [4], and FACS sorting via fluorescent proteins [5]. A common feature of these techniques is the requirement of large quantities of cells (>10^8 cells) for sample preparation and protein identification, making them unsuitable for experiments with limited biomass availability, such as characterization of internalized bacterial pathogens.

Recently, we introduced a workflow that allows time-resolved quantitative monitoring of the adaptive response of only 10^6 cells of *S. aureus* HG001 [6] carrying plasmid pMV158GFP [7] after internalization by 4×10^5 S9 human bronchial epithelial cells, a non-professional phagocytic cell line. Recent studies have already combined flow cytometry and 2-DE to investigate 10^8–10^9 internalized pathogens requiring a sorting time of several days [8]. In our approach, we gained an increase in sensitivity by optimizing an established workflow (Fig. 1) that combined pulse-chase labeling, FACS sorting via GFP, on-membrane washing [9] and digestion [10], high sensitivity nLC-MS/MS, and comprehensive data analysis (Fig. 2). The prior labeling of bacteria with SILAC allowed quantification even of low intense signals in a correct manner and discrimination of such low-abundance bacterial peptides (heavy) from host peptides (light), which is still crucial in high complex proteomics approaches. Using this approach, one can now investigate intracellular bacteria with initial starting material two to three orders of magnitude lower than what was usually required for a quantitative proteomics approach [11]. This workflow is not only suitable for the characterization of individual species but a modified version has also been applied to the study of a mixture of bacteria [10] and we can envision its application for the analysis of complex consortia in metaproteomics approaches.

2. Materials and methods

2.1. Reagents, chemicals and media

- Acetone (ice-cold, stored at −20 °C; Merck, cat No. 1.00014.1011).
- Acetic acid (AA; Merck, cat. No. 1.00063.2511).
- Acetonitrile (ACN; Roth, cat. No. T195.2).
- Alexa Fluor® 568 phalloidin (Invitrogen, cat. No. SKU#A12380).
- Amino acid mixture group A (Promocell; customer formulation), alanine, valine, leucine, isoleucine; 50 mM each, cat. No. C-97027).

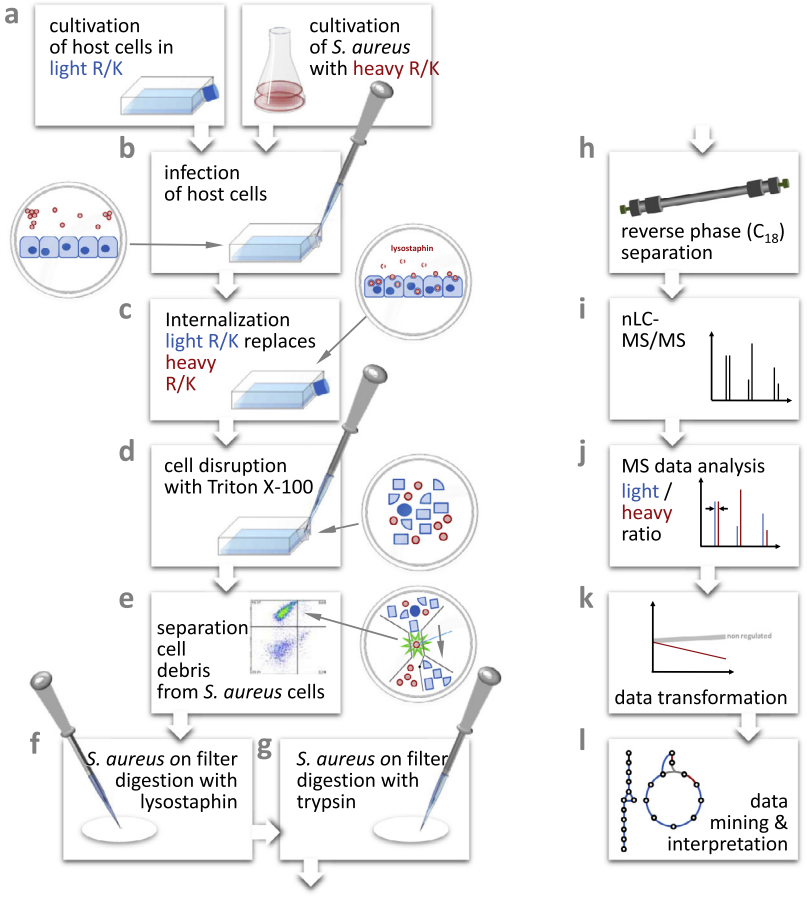

Fig. 1. General workflow to identify and quantify proteins from internalized *S. aureus* HG001 cells in a time-resolved manner. (a) S9 human bronchial epithelial cells were grown to confluence in eMEM containing light arginine and lysine. In parallel *S. aureus* was cultivated to exponential growth phase in pMEM, containing the $^{13}C_6$ heavy isotopes of arginine and lysine. (b) The *S. aureus* culture was diluted with eMEM to a MOI of 25 and then transferred to the host cells. (c) After 1 h of internalization remaining non-internalized bacteria were killed by lysostaphin treatment. After internalization, bacteria can only incorporate light counterparts of the heavy amino acids into their proteins. (d) Samples were taken hourly, host cells were disrupted by Triton X-100 and internalized *S. aureus* were released. (e) At each point in time, GFP-positive bacteria were separated from cell debris via FACS and sorted on a low protein binding filter device. (f) and subjected to proteolytic digest first by lysostaphin. (g) followed by trypsin. (h) Tryptic peptides were purified by ZipTip and (i) measured via nLC–LTQ-Orbitrap XL MS. (j) Identification and determination of peptide ratios were performed with the Elucidator software package. (k) Protein light *vs.* heavy ratios with a more than twofold change relative to the median over the time course were defined as up- or downregulated. Proteins with a smaller deviation of their ratio from the average ratio of all proteins were defined as non-regulated. (l) Regulated proteins were interpreted in the context of their role in cellular physiology.