REVIEW

Proteome analysis of host–pathogen interactions: Investigation of pathogen responses to the host cell environment

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Received: March 28, 2011
Accepted: April 20, 2011

Infectious diseases are still a major health risk, and thus a better understanding of the interaction between human host cells and pathogenic microbes is urgently required. Since the interplay between both partners is highly complex, genome-wide analysis by OMICs approaches will likely make a major contribution to the elucidation of the pathophysiology of infection processes. In the concert of OMICs technologies, proteomics is particularly important because it reveals changes in the active players of the cell and has thus a close relationship to the phenotypic changes observed. While proteomic studies of in vitro-grown microbial pathogens are routinely established in many labs, in vivo proteomic approaches are still rare. Here, we will review the challenges and recent developments of proteomic analysis of microbial pathogens derived from cell culture or in vivo infection settings and summarize some lessons that have been learned from these studies.

Keywords:
Bacterial adaptation responses / Enrichment strategies / Infection biology / In vivo proteomics / Microbiology

1 Introduction

Due to the increasing emergence of resistances against antibiotics and the decreasing rate of approval of new antibacterial compounds, infectious diseases are still a major threat to human health [1]. Their impact is also enhanced by the changes in life style such as the increasing migration which contributes to spreading of infectious diseases worldwide. Thus, the pressure for the development of new antimicrobial chemotherapeutics and vaccination strategies is increasing. A comprehensive understanding of the intricate interplay between host and pathogen will substantially improve our ability to develop such new treatment concepts. Traditionally, the role of individual host proteins and bacterial factors in this interaction has been studied. However, even if these studies provide important insides in the role of particular components, this reductionist view has clear limitations due to functional redundancies and the likely involvement of hundreds if not thousands of genes in different stages of host–pathogen interaction [2]. With the tremendous advances in genomics and functional genomics technologies, these studies can now be performed at a genome-wide scale. Transcriptomics and proteomics screens for the response of the host to infections can be performed for animal models as well as human specimen at a routine basis. The analysis of the response of pathogens is much more challenging due to the limited availability of material from infection settings and the overwhelming amount of host RNA and proteins [2, 3]. Thus, many proteome studies of adaptation responses of pathogens still rely on the use of in vitro systems in which these pathogens are exposed to environmental challenges, such as variations in temperature, oxidative stress, or nutrient limitation that are thought to mimic stresses likely encountered by the

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Abbreviations: FACS, fluorescence-activated cell sorting; **IMS,** immunomagnetic separation; **MRM,** multiple reaction monitoring; **SMM,** shotgun mass mapping

pathogens in vivo [4–6]. These expression changes or variations in protein abundances are then compiled to signatures that can be used to describe particular physiological conditions or responses to the treatment with compounds, e.g., in order to define the mode of action of new antibacterial compounds by comparison to a set of established reference substances [7, 8].

However, even if these in vitro studies provide a comprehensive understanding of the physiology of pathogenic bacteria, yeasts and protozoan parasites and their adaptation responses, they at best only partially reflect the situation these pathogens experience during their encounter with animal or human host cells [2, 9–11]. Thus, there are increasing efforts to meet the challenges associated with the analysis of pathogen samples from infection settings.

In this review, we will provide a summary of the technical challenges and the progress that has been made in the proteome analysis of pathogens in cell culture or animal models and will also illustrate with selected example lessons that have been learned from these studies. We will also include the studies with yeasts and protozoan parasites but mainly focus on the analysis of bacterial pathogens.

2 Technical challenges and current options for proteome analysis of pathogens from in vivo settings

Gel-based proteomic studies of bacterial pathogens have been performed since their introduction [12–15]. With the tremendous improvements in sequencing technologies allowing the determination of sequences of even numerous specific bacterial isolates at rather low costs and the improvements in MS-based technologies, it is now possible to apply proteomics on a large scale and comprehensively monitor bacterial proteins accomplishing a coverage of up to 80% if prefractionation strategies are employed [16–18]. If such studies include different proteomic subfractions such as the cytoplasm, membrane, and cell-wall proteins as well as secreted proteins, virulence factors involved in adaptation to host cells, adhesion, and invasion can also be captured. However, even if MS is now able to identify thousands of proteins/peptides in one measurement, large sample volumes are required to permit the extensive prefractionation and it is therefore still a challenge to investigate the proteomes of pathogens concomitantly with the host proteome at one time [3]. Due to smaller size and volume and limited amount of bacteria, bacterial proteins must be detected against an overwhelming background of host proteins. However, not only the low concentration of the bacterial proteins poses a challenge, but also the higher number of tryptic peptides from host proteins will interfere with those of bacterial origin during the ionization process [19]. Additionally, each cell type has unique biochemical properties and the preparation protocols for proteins vary for eukaryotic and prokaryotic cells. Therefore, the application of a universal preparation method will not permit the isolation of sufficient bacterial proteins if the pathogens had been internalized by human host cells. Rather, bacterial pathogens and host cells must be separated facilitating subsequent dedicated processing of bacterial samples.

2.1 Separation of pathogens from host cells/proteins

So far, three isolation protocols were mainly used to separate pathogens from host cells/proteins, namely centrifugation, immunomagnetic separation (IMS), and fluorescence-activated cell sorting (FACS). The most widely used method is the separation of bacteria or other pathogens such as *Candida albicans* via centrifugation after the lyses of the eukaryotic host cells by osmotic pressure [20, 21] or detergents such as Triton X-114 [22], which can be used to permeabilize unfixed eukaryotic cell membranes. In case of infected blood, bacteria can be separated from red blood cells by simple density gradient centrifugation [23]. In this study, Leghorn cross-chicken were infected with *Pasteurella multocida* x-73 and at the terminal stage of infection, about 4×10^{11} bacteria were detected in blood and subjected to centrifugation. Using this method, sufficient bacteria for further analyses by 1-D PAGE and 2-DE could be isolated even allowing analysis of the outer membrane proteome of this pathogen. In other studies, centrifugation was applied to separate *Salmonella*, *Listeria*, *Brucella*, *Mycobacteria*, *Yersinia*, *Campylobacter*, and *Legionella* [13, 15, 24–27] from their hosts for further analyses by 1-D or 2-DE. In some of these studies, pulse labeling with ^{35}S-methionine or ^{35}S-cysteine after infection of cycloheximide-treated host cells was employed to exclusively study the newly synthesized bacterial proteins via sensitive autoradiography [15, 20]. However, even if the large numbers of spots were monitored, only a few protein spots or bands could be identified by, e.g., N-terminal sequencing or peptide mass fingerprinting (PMF) in these early studies, due to the very low concentration of the isotopically labeled proteins and limits in sensitivity in MS. More recently, centrifugation and LC-MS/MS were combined in order to characterize about 1200 proteins from 10^9 *Porphyromonas gingivalis* cells after internalization by human gingival epithelial cells [28]. *Clostridium perfringens* could be successfully isolated and in a study by Sengupta and Alam via centrifugation in the presence of Percoll from peritoneal fluid of infected mice, which allowed the identification and quantitation of about 300 protein spots by 2-DE [29]. Furthermore, Shi et al. published a study, in which *Salmonella enterica* serovar Typhimurium was successfully isolated from infected RAW 264.7 macrophages via bacteria containing vacuoles (BCV) by combining sucrose-density centrifugation and LC-MS/MS analyses, which finally allowed the identification of 315 *S. enterica* serovar Typhimurium proteins of which 39 were strongly induced postinfection [30].

The second elegant and promising method to isolate pathogens from host cells is the IMS-approach mentioned above, where, e.g., anti-IgG-coated Dynabeads^{TM} (Dynal, Oslo, Norway) are used in combination with antisera directed against specific pathogens to isolate the respective pathogens. Twine et al. used this strategy for isolation of *Francisella tularensis* from mouse spleen [31]. After incubation of the beads with homogenized cell lysates, bacteria could easily be enriched and purified, generating sufficient bacteria virtually free of contaminating host proteins for comprehensive 2-DE proteome analyses that identified 78 proteins with infection-associated changes in abundance. The ability to effectively remove host proteins is a particular advantage compared with simple centrifugation setups.

The third promising method to reisolate pathogens from their hosts is the well-established high-speed FACS method. As a prerequisite for this approach, it is necessary to express a fluorescence label in the pathogen, e.g. green fluorescence (GFP) or red fluorescence protein (DsRed), in order to facilitate the separation of host debris from pathogens. One of the pioneering studies relying on the combination of FACS and LC-MS/MS was published by Becker et al. [32]. Up to 700 *S. enterica* proteins could be mapped by MS after reisolation of bacteria from infected mouse tissue. These quantitative proteomics data together with network analysis indicated that metabolic redundancies limit the usefulness of additional metabolic enzymes as targets for antimicrobial compounds (see below and Section 2).

Paape et al. [33] used the FACS approach to isolate the protozoan parasite *Leishmania mexicana* from mice after enrichment of phagolysosomes by density gradient centrifugation. Combining 1-D gel-LC-MS and 2-DE analyses, 509 different proteins could be identified and classified [33]. Applying more sensitive LC-MS/MS techniques, the number of observed *L. mexicana* proteins could even be extended to 1764 [34]. In a third example, GFP-labeled *Staphylococcus aureus* was isolated after internalization by human bronchial epithelial cells and further analyzed by LC-MS/MS [35]. Sorted bacteria were collected via a novel filter technique [36] and digested directly using trypsin for shotgun mass mapping (SMM) [35]. As a result, using only 10^6 bacteria more than 500 proteins could be identified and quantified.

However, all these established methods for isolation of pathogens from host cells must still be improved to increase sample processing speed and thus reduce the risk of preparation bias as well as further reduce the level of host protein contaminations.

2.2 Post-isolation proteome analysis

Besides the optimal isolation strategy, the selection of the appropriate proteome analysis workflow is important. 2-DE and MS-centered shot gun approaches fundamentally differ; in that, the first allows protein centric separation and analysis, whereas the latter is peptide-centric. Since the number of available bacteria may dramatically vary from 10^6 to 10^{11} cells, both approaches can principally be followed, but gel-based approaches such as 2-DE can only successfully be applied if a minimum of about 10^8 cells can be isolated. This high number of intracellular bacteria can be provided for a number of pathogens including *Mycobacteria*, *Brucella suis*, *Pasteurella*, or *C. albicans* [11, 22, 23, 37, 38]. 2-DE can then be used to separate 300–500 unique proteins and has the advantage that each protein species is separated into distinct spots, which can be analyzed further via MS in order to obtain high sequence coverage and to identify post-translational modifications (PTMs) jointly associated with a particular proteins species. Once a master map has been created, 2-DE easily facilitates quantitative comparisons of proteomes from different stages of infection such as in vivo and in vitro cultivations. A particular advantage is the ability to monitor changes in protein synthesis, employing ^{35}S-methionine or ^{35}S-cysteine pulse-chase labeling approaches [15, 20].

However, often only a limited number of bacteria are accessible after infection of host cells. In such settings, gel-free MS-driven shotgun approaches are clearly preferred due to their greater sensitivity, which allow monitoring of 500–600 proteins from as few as 10^6 cells [35]. Peptide-centered MS-driven approaches can also capture membrane or surface-bound proteins, which play an important role in host–pathogen interaction because they mediate contact between infecting pathogen and its host. Dedicated analysis of membrane protein fractions has not been reported yet for bacteria recovered from infection settings probably because of lack of sufficient material for extensive purifications.

In order to quantify the proteins identified by gel-free LC-MS/MS approaches, many strategies can be applied. Label-free quantification techniques include intensity analysis of peptide ions, MS/MS-spectral counting [39], or the exponentially modified protein abundance index (emPAI) [40]. If stable isotopes are included, the user has the choice between isotopic labeling via isotope-coded affinity tagging (ICAT) [41], isobaric tagging for relative and absolute quantitation (iTRAQ), isobaric peptide termini labeling (IPTL) [42], absolute quantitation using standard peptides (AQUA) [43], and metabolic labeling via stable isotope labeling with amino acids in cell culture (SILAC) [44]. The pro and cons of the different approaches have already been discussed extensively [45–49].

All chemical, postharvesting labeling protocols such as ICAT, iTRAQ, or isobaric peptide termini labeling can be used for such experiments if enough material is accessible. On the contrary, SILAC can only be used if the pathogen is auxotroph and the labeling pattern is not disturbed by bacterial metabolism. Furthermore, persistence in the host or host cells needs to be long enough to permit sufficient labeling of newly synthesized proteins. However, an

Table 1. Comparative display of selective examples of in vivo proteomics approaches exploring the adaptation of bacterial pathogens to the host niche

	Becker et al. [32]	Xia et al. [28]	Twine et al. [31]	Kruh et al. [50]	Schmidt et al. [35]
Host cells	Mouse spleen	Human gingival epithelial cells	Mouse spleen	Guinea pig lung	Bronchial epithelial cells
Pathogen	*S. enterica*	*P. gingivalis*	*F. tularensis*	*M. tuberculosis*	*S. aureus*
Labeling	GFP	–	–	–	GFP/SILAC
Separation	FACS	Centrifugation	IMS	None, crude extracts	FACS+filter
Number of pathogens	10^8	10^9	10^8–10^9	n.d.	10^5–10^6
Post-isolation analysis	LC-MS/MS	LC-MS/MS	2-DE	LC-MS/MS	LC-MS/MS
Protein identification	MS/MS	MS/MS	MS/MS	MS/MS	MS/MS
Quantitation	–	Intensity and spectral counting	Spot intensities	Spectral counting	SILAC
Number of proteins/spots	~700	1223	401 Spots	545	526
Network analysis	Biocyc	–	–	–	Voronoi tree map

adaptation of the traditional labeling scheme can also be used in which bacteria are labeled to saturation prior to exposure to the host. If concomitantly with the exposure to the host the heavy amino acids are withdrawn, both proteins newly synthesized upon exposure to the host (light variants) as well as protein degradation seen as decrease in heavy label can be explored [35].

Label-free quantification methods also seem to be promising for the quantitation of low-abundance proteins, since their sensitivity is remarkably high. However, three important aspects have to be considered before this method can be applied. First, it is necessary to rely on mass spectrometers with high accuracy such as FTICR or LTQ-Orbitrap and HPLC instruments with reliable retention times; second, enough material should be available to determine the protein concentrations before MS measurements in order to apply equally amounts of peptides; and third, the number of interfering peptides from the host cells should be reduced to a minimum in order to avoid false-positive identifications and ratios. Exponentially modified protein abundance index was already successfully applied for the quantification of *L. mexicana* [34]. In another remarkable study, two methods, intensity and spectral counts, were successfully used to quantify the proteins from about 10^9 intracellular *P. gingivalis* recovered from human gingival epithelial cells [28]. As a result, 385 were detected as overrepresented and 240 underrepresented compared with the control.

Kruh et al. [50] followed an entirely different workflow by homogenizing lung tissue of guinea pigs 30 or 90 days after infection with *Mycobacterium tuberculosis* and then subjecting the crude protein extracts directly to LC-MS/MS analysis without reisolation of bacteria. In total, 545 *M. tuberculosis* proteins (roughly 300 each at 30 and 80 days postinfection) were detected and then quantified by spectral counting. Surprisingly, only a small fraction of proteins was detected both at the early and at the late time points of infection and the observations made differed significantly from the previous in vitro studies (see below). However, for spectral count methods, a sufficient number of MS/MS spectra should be available, which is not often the case for low-concentrated samples.

Selected examples illustrating the applied separation and quantitation techniques are summarized in Table 1.

2.3 Are proteome analyses of only thousands of bacterial cells feasible?

Even if the technology has greatly improved, published data indicate that so far still large numbers of bacteria (>10^6) are required for a comprehensive protein profiling and quantitative analyses. However, in many infection settings, e.g. extended or chronic infections, the number of accessible bacteria is much lower and thus the question emerges if proteome analyses could also be envisioned with smaller sample sizes? Technical developments in three areas might help to overcome current limitations. First, when applying FACS separation, the use of a vacuum filter technique and on-membrane digestion was the most important steps for proteome analyses of as little as 10^6 *S. aureus* [35]. The sample volume was dramatically reduced compared with standard sorting techniques in which about 5 mL volume has to be collected for 10^6 cells. Further refinement of this approach currently facilitates the detection of about 300 proteins only from 10^4 to 10^5 *S. aureus* cells (Schmidt and Völker, unpublished data).

Second, ''direct MS.'' Usually, peptides will be measured by LC-MS/MS using the highest precursor ions of the corresponding MS spectra for tandem MS and this process is characterized by a stochastic selection. However, MS instruments and software are continuously improved and it is currently possible to directly select the fragment sets of precursor ions from predefined proteins/peptides in a complex mixture. Especially in samples that are typically contaminated with host proteins, the targeted (direct MS)

method would allow direct selection of pathogen specific peptides for MS/MS. The significance of directed MS methods has been shown in the study of Schmidt et al. [51] in which these methods have shown to be superior in terms of performance, sensitivity, and reliability.

Third, the most promising technique to detect low-abundance proteins is likely multiple reaction monitoring (MRM), which allows the detection of peptides at attomole level. The sensitivity of MRM instruments such as QQQ instruments is about 10–100 times higher than conventional high-sensitivity MS. Theoretically, this technology would allow the identification of about 300 proteins from only 10^3 *S. aureus* cells. The workflow of the method comprises three steps: First, the accessible proteome will be mapped by conventional LC-MS/MS from high amounts of pathogens, and the MS/MS spectra will be stored in a database. Second, based on this information, peptides uniquely identifying the proteins of interest will be selected for MRM, where exclusively those peptides will be measured. Third, the selected target peptides will be quantified in multiple replicates and employing isotopically labeled standard peptides for absolute quantification [52, 53]. The value of this approach has been convincingly demonstrated by Lange et al., who quantified low-abundance virulence factors from the cultures of *Streptococcus pyogenes* exposed to increasing amounts of plasma [54]. The data set recorded allowed the identification of virulence factors that significantly changed in abundance upon plasma exposure [54].

2.4 Are secreted proteins amendable to proteomic analysis of samples from in vivo settings?

Secreted proteins constitute a particularly important subgroup of the proteome, because by direct contact to host cells they can trigger host cell lysis as toxins but also influence adhesion, colonization, and invasion as well as diverting host responses, e.g. by inhibition of the immune system. Yet, this important protein group is largely lost using enrichment procedures for intact bacterial cells (centrifugation, IMS, and FACS). Lange et al. [54] demonstrated that secreted virulence factors can be monitored by MRM technology even in plasma. However, in this study *S. pyogenes* was exposed to plasma and crude protein extracts were directly measured. In internalization settings, secreted proteins can be captured by not only restricting MS analysis to the enriched bacteria but also investigating host cell crude protein extracts probably containing the secreted virulence factors. An alternative and probably by far more sensitive method would be the dedicated isolation of compartments containing the pathogens such as the BCVs or the phagolysosome, which would then by lysed and subjected to LC-MS/MS or MRM in addition to the measurement of the enriched bacterial fraction [55]. Analysis of BCVs and phagolysosomes has already been reported [11, 30] and has now to be combined with sensitive start of the art MS techniques.

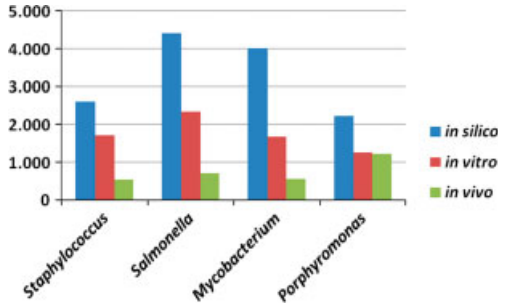
2.5 Can PTM of bacterial proteins be addressed in vivo?

PTMs play an important role in the interplay between the host and the pathogen. Numerous virulence factors of bacterial pathogens have been found to be covalently modified with, e.g., carbohydrate residues, which have an impact on protein structure and function such as fine tuning of cell–cell recognition, adherence, and signaling [56]. Besides glycosylation, phosphorylation, acetylation, and oxidation on methionine and cysteine may play an important role in the pathogenesis. In various studies, methionine sulfoxide reductase (MsrA) has been shown to increase in level during internalization [35, 57], probably indicating increased oxidative modification/damage of proteins. Methionine sulfoxide reductase is a repair enzyme that reduces methionine sulfoxide residues in proteins, which is an important step after oxygen stress.

PTMs occurring in bacterial proteins in vivo have not been specifically addressed by proteomics screens so far. Since analytical workflows for the analysis of protein phosphorylation, acetylation, and oxidative damage are in place [58–61], they can, in principal, also be applied to bacteria reisolated from infection settings. However, given the low cell numbers available in such experiments and the fact that at any given time only a fraction of a protein is modified, e.g. by phosphorylation, such analyses are challenging and thus only useful with enriched samples.

2.6 Coverage of the proteome of in vivo samples

Bacteria are sufficiently simple to allow a comprehensive coverage of the expressed proteome under in vitro conditions [16, 17, 62]. However, this extensive coverage often requires prefractionation and separate the analysis of proteomic subfractions (cytosol, membrane proteins, cell-wall proteins, and secreted proteins) that are subsequently assembled [16]. Bacterial samples reisolated from eukaryotic host cells are available only in limited amounts and thus usually preventing extensive prefractionation and therefore only the most abundant proteins can be covered. The best coverage to date has been accomplished for *Porphyromonas gingivalis* where more than 1200 proteins were detected in the samples reisolated from human epithelial cells [28]. For other examples selected in Fig. 1, between 500 and 700 proteins can be displayed [32, 35, 50], which still provide a reasonable coverage of metabolism and in addition many proteins of thus far undefined function. Since pathogens are under strong selection pressure in their interaction with the host, these proteins likely fulfill important functions and thus proteomics provides promising targets for further detailed studies (see below).



	Staphylococcus	Salmonella	Mycobacterium	Porphyromonas
in silico	2,600	4,400	4,000	2,227
in vitro	1,700[16]	2,343[62]	1,668[64]	1,245[28]
in vivo	526[35]	700	545[50]	1,223[28]

Figure 1. Comparative display of theoretically predicted proteins and proteins observed in in vitro and in vivo proteomics analyses. Data on predicted and in vitro and in vivo observed proteins are derived from the following references: *Staphylococcus* [16, 35], *Salmonella* [62], *Mycobacterium* [50, 64], *Porphyromonas* [28].

3 Lessons of the in vivo proteome studies

In contrast to the numerous proteome studies of bacteria cultivated in in vitro laboratory settings, only very limited studies have so far been performed from pathogens reisolated from cell culture or animal models. An emerging theme from these studies with various pathogens either bacteria, yeasts, or protozoan parasites is that laboratory conditions mimic the encounter with human or animal host cells only to a limited extend. This observation is not surprising because a multitude of factors contribute to the interplay between host and pathogen and this collection of factors cannot be captured in vitro. Cell-culture infection settings have been successfully applied as simplified models for host–pathogen interactions for a number of organisms including *P. gingivalis*, *S. aureus*, *M. tuberculosis*, *C. albicans*, and *L. mexicana* [11, 22, 28, 33–35]. These studies revealed extensive differences between in vitro cultivation and pathogens in contact with human host cells, e.g. for *P. gingivalis* where close to 50% of all proteins detected differed in abundance [28]. Commonly, metabolic enzymes required for fast growth were present at lower level, indicating reduced growth rates and enzymes for biosynthetic routes such as amino acid biosynthesis were reduced in the cell-culture settings, probably because of the availability from the host cells/media [22, 35]. However, these cell-culture models are confined to interaction with a single host cell type and entirely miss contributions of other cell types, e.g. invading immune cells.

Kruh et al. have provided convincing evidence that none of the in vitro studies adequately reflects the in vivo situation [50]. Sampling bacteria from guinea pig lungs at 30 and 90 days postinfection, the authors recognized that the two protein fractions overlapped only to a very limited extend, probably indicating highly dynamic temporal expression patterns during infection. Only in vivo OMICs studies can capture the different populations that are derived during prolonged infection processes, whereas in vitro studies are limited to the analysis of a single clonal population [50]. However, the different subpopulations existing in patients pose a challenge have all to be targeted in the treatment of tuberculosis. Furthermore, the in vivo proteomics study revealed a shift in nutrient acquisition from carbohydrates to lipids as major sources of carbon and energy. Thus, *M. tuberculosis* did not seem to be nutrient limited in the lung contrary to the expectations from in vitro studies [50].

For *S. enterica*, the in vivo proteome studies have been carried a step further by integrating the data with metabolic network analysis and extensive testing of *S. enterica* mutants for the defects in established mouse infection models [32]. An intriguing outcome of this study was that only a limited number of metabolic enzymes are essential for establishing a successful infection by *S. enterica*. Apparently, due to functional redundancy, *S. enterica* can easily overcome the impact of single deletions or resort to the utilization of other nutrients available in the nutrient-rich environment of the host [32]. Unfortunately, this limits the prospective of exploiting new metabolic enzymes as targets for new antimicrobials because most essential pathways are already targeted by antibiotics currently used [2]. Becker et al. could also prove a selective over-representation of essential enzymes under in vivo conditions compared with other proteins, thus providing supporting evidence that the proteins with so far undefined function discovered in vivo might probably be promising new targets [32]. Finally, by combining proteomics data, metabolic network construction and phenotypic screening of mutants, a convincing strategy for prioritization of candidates for the followup studies was provided, which should be implemented in other studies as well [2, 32].

4 Outlook

Due to the advances in cell separation and MS, in vivo proteome studies are now feasible. However, sensitivity and coverage of specific subfractions such as the secretome still need to be improved. These proteomics studies will provide long lists of proteins, differentially expressed proteins, in the different host environments and it will be a challenge to prioritize the targets for detailed followup analysis. Interpretation of such large-scale data sets will likely profit from integration with other OMICs data, which has already started for in vivo approaches [22]. Integration of time-resolved data from expression profiling, proteomics and also metabolomics of both sides, the pathogen and the host will likely lead to a new level of understanding of the interplay between host and pathogen and provide options for the discovery for new targets for antimicrobial strategies and vaccine development. However, such multi-OMICs approaches will also

require a considerable effort for new analysis tools that integrate and provide easy access and visualization of data from multiple laboratories, such as the efforts started by the National Institute for Allergy and Infectious Diseases (NIAID)[63].

The authors acknowledge funding from the Deutsche Forschungsgemeinschaft through SFBTRR34 and GRK840, the BMBF through funding of the ZIK-FunGene and the Alfried Krupp Foundation. Furthermore, the authors are grateful for the excellent work of all the members of the Infection Biology Group of the Department of Functional Genomics.

The authors have declared no conflict of interest.

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