Targeted proteomics for studying pathogenic bacteria

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List of abbreviations: antimicrobial resistance (AMR), data-dependent acquisition (DDA), data-independent acquisition (DIA), Sequential Windowed Acquisition of all THeoretical fragmentation spectra (SWATH), group A Streptococcus (GAS), *Mycobacterium tuberculosis* (Mtb), tuberculosis (TB), two-component systems (TCS), human serum albumin (HSA), fatty acid biosynthesis (FAB), factor H binding protein (fHbp), bacteriophage amplification detection (PAD), Staphylococcal enterotoxin A, B and D (SEA, SEB and SED), epsilon toxin (ETX), *Shigella dysenteriae* shigatoxin (STX1), *Escherichia coli* shigatoxin (STX2) and *Campylobacter jejuni* cytolethal distending toxin (CDT)

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Abstract

Mass spectrometry-based proteomics has been extensively used to map bacterial proteomes, which has led to a better understanding of the molecular mechanisms underlying bacterial infection and bacteria-host interactions. Quantitative proteomics using selected or parallel reaction monitoring is considered one of the most sensitive and specific quantitative mass spectrometry-based approaches and has significantly advanced proteome studies of pathogenic bacteria. Here, we review recent applications of targeted proteomics for bacteria identification, biomarker discovery and the characterization of bacterial virulence and antimicrobial resistance amongst others. Results of such studies are expected to further contribute to improve the fight against the most common human pathogenic bacteria.

Introduction

Bacterial infectious diseases are a major threat to human health as they cause a large and global burden of morbidity and mortality [1,2]. The emergence of antimicrobial resistant (AMR) bacteria dramatically reduces our arsenal of antibiotics to treat bacterial infections [3]. Understanding the physiology and the pathogenicity of bacteria is considered to be crucial to discover new targets for vaccines, diagnostics and drug development [4].

Proteins are the functional elements that perform and steer most of the biochemical reactions in a living organism. Proteins can be modified post-translationally by which their structure and function can be changed, and this information cannot be simply read from gene or transcript sequences. Proteomics is therefore complementary to genomics and transcriptomics. In the last two decades, mass spectrometry (MS)-based proteomics has become the method of choice to study proteins with high throughput and depth. Contemporary MS-based proteomics has advanced the field of

microbiology and this in several research domains, such as comprehensive mapping of bacterial proteomes and post-translational protein modifications (PTMs) [5–7], pathogen-host cell interactions [8–10], antimicrobial resistance [11–13] and biomarker discovery [7,14]. In addition to such research and development applications, MS-based MALDI-TOF analyses are now generally integrated in many hospital laboratories for routine identification of bacterial pathogens in clinical samples as well as for antibiotic resistance testing [15].

MS-based proteomics can be divided in two main categories: discovery and targeted proteomics. Discovery proteomics aims at the comprehensive identification of proteins in a sample [16]. This is most frequently achieved by so-called data-dependent acquisition (DDA) methods. In DDA, a mass spectrometer is set to select the most abundant precursor ions in a given mass spectrum for further fragmentation [17]. Despite that DDA is frequently used for discovery proteomics [18], one of its main limitations is the irreproducible precursor ion selection because of its stochastic sampling [19].

Contrary to discovery proteomics, targeted proteomics is a hypothesis-driven approach that relies on information about the analyte to monitor it with high specificity and sensitivity in large numbers of samples. A preselected group of peptides (for instance based on discovery proteomics data) can be selectively monitored yielding precise, sensitive and quantitative data for the targeted peptides, and thus their parent proteins [23]. **Figure 1** illustrates a typical workflow when applying targeted proteomics to study bacteria. A powerful aspect of targeted proteomics is its high reproducibility, as was demonstrated in intra- and inter-laboratory studies [24,25]. Highlighting its power, targeted proteomics was selected as method of the year 2012 by the journal Nature Methods [26]. Of note, following increased adoption of targeted MS in the field of biology and medicine, a workshop was held in 2013 at the National Institutes of Health with representatives from multiple communities to establish standardized criteria for successful development and application of a targeted MS assay. The participants tiered assays into three classes based on the precision required and extent of

analytical characterization. They also provided the experimental design parameters and assay characteristics required for each of the tier assays [27].

Data-independent acquisition (DIA) was first introduced in 2004 by Venable *et al*. Here, rather than selecting single peptide ions, all ions present in a certain m/z interval are co-fragmented [20]. By sequentially scanning contiguous intervals, the whole mass range can be covered. This Sequential Windowed Acquisition of all THeoretical fragmentation spectra (SWATH-MS) [21] or DIA was therefore suggested as a more comprehensive discovery method where MS2 data from all peptides were collected, thus allowing for the identification of increasing numbers of peptides. Despite the non-targeted way of collecting data, the analysis of DIA data can be performed in both a targeted manner as well as non-targeted (see below). Recently, DIA/SWATH-MS was employed to assess differential proteome signatures between pathogenic and non-pathogenic Rickettsia inside macrophages [28] and unraveled the ligands of *S. pneumonia* interacting with human brain endothelial cells [29].

Especially in samples where bacterial pathogens need to be studied at low abundance and in complex matrix such as blood, DDA analysis is hampered by its lower dynamic range, whereas targeted approaches are the methods of choice to overcome this problem (see also **Table 1**). While DIA/SWATH-MS is more used for quantitative clinical proteomics, SRM/MRM and PRM typically applied to quantify specific proteins or protein complexes. We here review recent applications of SRM/MRM and PRM, two established targeted acquisition methods in the field of pathogenic bacteria (**Table 2**).

Targeted acquisition methods

Targeted acquisition methods in proteomics can be roughly divided in two main approaches: selected reaction monitoring (SRM), also known as multiple reaction monitoring (MRM) [30–32], and parallel reaction monitoring (PRM) [33].

SRM is typically performed on a triple quadrupole instrument to monitor specific transitions (m/z-values of precursor/fragment ion pairs) (**Figure 2**), which results in increased reproducibility, selectivity and sensitivity compared to discovery-based approaches. Hybrid mass spectrometers such as the quadrupole-Orbitrap [34] and the quadrupole-TRAP instrument [35], made it possible for targeted quantification in so-called PRM mode (**Figure 2**). In this mode of operation, full MS/MS-spectra rather than selected transitions are acquired for predefined precursor ions, resulting in simultaneous monitoring of all product (fragment) ions [36]. Parallel reaction monitoring thus avoids the selection and optimization of the transitions, which makes PRM assay development easier. Another advantage of PRM over SRM is its higher selectivity due to the higher resolution and mass accuracy when Orbitrap analyzers are used, which results in an improved separation of the target peptides from background peptides [33,34]. Despite the lower selectivity of SRM, its higher sensitivity keeps SRM as the method-of-choice for projects for which sensitivity is key.

Quantification itself in targeted proteomics can be done label-free, but the accuracy of the quantification might then be insufficient due to the variation in signal intensities in LC-MS analyses [30] A more precise and accurate method relies on stable isotope-based quantification. Here, isotope labelled peptides are spiked as internal standards into the sample and quantification is based on the intensities of the analyte and the internal standard signals. Through the use of such isotopically labeled internal standards, both relative and absolute quantification of analytes can be performed.

The number of peptides that can be analyzed during a single LC-SRM run is typically limited to 50 to 100 [31]. Despite the speed of the currently available mass spectrometers, this number can only be reached when a so-called scheduled method is used. Here, a peptide is only monitored during the retention time interval it is eluting with a certain set tolerance. In this way, the sensitivity is greatly improved as the instrument can spend more time scanning the analyte-of-interest instead of scanning for all analytes at once. The smaller the retention time window for each analyte, the more

peptides can be monitored with a considerable sensitivity in a single LC-MS/MS run. The possibility of monitoring the peptide in the correct retention time window is highly dependent on the reproducibility of the chromatographic separation. To increase this reproducibility the use of indexed retention times (iRTs) was introduced [37]. This implies spiking the sample with standard peptides that have retention times spread over the whole peptide elution window. According to the relative retention time of the analyte to the retention time of these iRT peptides, an indexed retention time can be calculated. Hence, if the retention time of the iRT peptides is known, the retention time of the targeted peptide can be predicted. By using iRTs, the interval where the targeted peptide should be monitored can be adjusted on the fly [38]. Hence, this interval can be kept short and more peptides can be monitored. Moreover, the predicted retention time can be used in the data analysis afterwards to have an extra confirmation of the correctness of the monitored peptide. Another way of improving the sensitivity of both PRM and SRM methods is the introduction of IS-PRM [39] or iSRM. Here, the isotopically labeled peptide is used as a trigger for monitoring the light peptide. In this way the time on the mass spectrometer is used more efficiently and a higher sensitivity is reached.

While PRM and SRM only monitor a defined peptide list, DIA monitors all peptides present in the sample. Despite the higher complexity of data analysis, a targeted list can easily be adapted afterwards, while with a targeted acquisition method, a sample would need to be re-analyzed. DIA on the othed hand provides a permanent record of the content of the sample and can be re-analyzed as many times as needed without re-running the sample on LC-MS/MS. Opposed to PRM and SRM no isotopically labeled peptides are used in DIA for quantification. Here, a label-free approach is used providing less intense and less expensive method development. To increase the confidence of an identification again the use of iRTs is recommended [40]. DIA Data analysis can be performed in a targeted manner by using spectral libraries. These libraries are created through DDA analysis, which again comes with the limitations of DDA analysis. By fractionating a sample however,

the overall sensitivity can be highly increased and the under-sampling effect of DDA is highly decreased. Using such data as a spectral library for DIA increases the sensitivity of the DIA analysis as well. Untargeted ways of DIA analyses are done through different software tools such as DIA-Umpire [41] and Pecan [42].

SRM assay repositories for pathogenic bacteria

The global burden of diseases caused by pathogenic bacteria such group A Streptococcus (GAS) and *Mycobacterium tuberculosis* (Mtb) is high [43,44]. During an infection, pathogenic bacteria can rapidly regulate their proteome composition to adapt to their host environment and evade its immune defense. Quantitative and comprehensive proteome-wide analysis can considerably improve our understanding of these mechanisms and has been proven useful to study differences between disease causing and non-disease causing strains. SRM assays for 10,412 distinct peptides of the Gram-positive human pathogen GAS have been generated and used to build a proteome-wide SRM assay repository for this pathogen [6]. The authors showed transportability of the SRM assays across GAS and related species. The resource described in this study can be used to understand the biology of GAS and forms a basis for the construction of SRM assays for other pathogens. For example, Sjöholm and colleagues used this GAS SRM assay repository to select and rank peptides to study interactions between the pathogen and host cells [45].

Schmidt and colleagues used shotgun proteomics to generate a comprehensive protein abundance map of *E. coli* under 22 different experimental conditions. In a next step, the authors applied SRM to quantify a subset of 41 proteins within a concentration range covering more than four orders of magnitude, to establish a calibration curve for the determined MS-intensities of all identified proteins. With this workflow, the authors determined absolute copy numbers for more than 2,300 *E. coli* proteins across different conditions [46].

Since the introduction of targeted proteomics, efforts have been made to store SRM experimental data, such as the MRMaid database, which is a repository of published and experimentally validated SRM transitions, and the SRMAtlas, a resource of high-quality, complete proteome SRM assays [47,48]. Schubert and colleagues generated a Mtb proteome library that contains SRM assays for 97% of all annotated proteins of Mtb, and these assays are also stored in the SRMAtlas [49]. Given the extensive proteome coverage, this spectral library may also become a resource for DIA analysis.

Proteomics data repositories allow the research community to access, validate and reanalyze the available datasets and allow the results to be used in novel ways [50]. The availability of these SRM bacteria-related assays in proteomics repositories is relevant as they can be directly applied to many proteins and to any number of samples and conditions in the corresponding bacterial species or closely related species.

Monitoring bacterial metabolic processes

Campylobacter jejuni is one of the most common causes of gastroenteritis worldwide [51]. To understand the physiological changes in intracellular *C. jejuni*, Liu and colleagues studied its proteome at different time points after infection of cultured mammalian cells using shotgun proteomics and applied SRM to quantify nine metabolic bacterial enzymes that are central in the main respiration pathways. The authors showed that after internalization by the host cell, the bacteria undergo a significant metabolic downshift and down-regulate aerobic respiration. Such knowledge may help the development of novel antimicrobial strategies by targeting relevant metabolic pathways [8].

Targeted proteomics also contributed to advances in synthetic biology and metabolic engineering. The former allows designing and engineering new biological functions and systems [52], while the latter involves the engineering of metabolic pathways to synthetize specific products and/or improve cellular properties essential for effective and reliable performance in large-scale industrial

bioprocesses [53]. One example is the application of SRM to monitor production of enzymes involved in the mevalonate pathway in *E. coli* driven by several inducible and constitutive promoters, and to monitor tyrosine biosynthesis, a valuable intermediate for engineering chemicals and therapeutic molecules [54]. Batth and colleagues optimized and validated SRM assays for over 400 proteins from more than 20 major metabolic pathways in *E. coli* including glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway, mixed acid fermentation, many amino acid biosynthesis pathways and fatty acid biosynthesis. Their assays provide a resource to further characterize *E. coli* proteins in various pathways in different conditions, and for metabolic engineering of *E. coli* [55].

Bacterial pathogenicity mechanisms

When bacterial pathogens infect a host, they induce host innate immune responses which generally kill the infecting agent. To understand why some bacteria are capable to evade the host's immune response and to develop novel therapeutic strategies, it is crucial to understand the molecular mechanisms underlying bacterial pathogenicity and virulence.

SRM has significantly contributed to our insights into the regulatory mechanisms of bacteria, with the two-component systems (TCS) being key examples. TCSs are bacterial signaling mechanisms [56] that allow bacteria to deal with rapid changes in cellular or environmental conditions. Shotgun proteomics was followed by MRM to determine absolute levels of the Kdp(F)ABC complex and the KdpDE-TCS proteins, both involved in potassium transport [57]. To understand the biological function and dynamics of the Cpx-TCS, Surmann and colleagues used SRM to quantify its components in *E. coli*. In this study, the authors showed the importance of the Cpx system in modulating the acid stress response and the cell wall stability in *E. coli* [58]. In addition, several virulence factors that promote *Streptococcus pyogenes* colonization, immune evasion and spread have been identified using SRM [59]. The Gram-positive *S. pyogenes* is a common colonizer of the skin and upper respiratory tract and in most cases causes a relatively mild disease. However, invasive

strains can penetrate to deeper tissues and cause more severe conditions that are life-threatening [60]. Upon infection, *S. pyogenes* has the capacity to induce a powerful inflammatory response leading to vascular leakage at the site of infection. Lange and colleagues applied SRM to investigate how the bacteria regulate the expression of virulence proteins when exposed to plasma. The authors showed that four of the nine targeted proteins are significantly regulated in response to human plasma including the C5a peptidase that has been previously identified as a promising vaccine candidate [61]. Moreover, the same group performed label-free quantitative MS analysis of *S. pyogenes* adaptation to human plasma. Their data revealed that 10% of proteins either increase or decrease in abundance and, among them, the proteins involved in fatty acid biosynthesis (FAB) showed a significant decrease. *S. pyogenes* is known to induce a powerful inflammatory response leading to vascular leakage at the site of infection, and in response to that, the bacteria express surface proteins that bind several plasma proteins such as human serum albumin (HSA). These interactions might influence gene expression in *S. pyogenes* with implications for bacterial adaptation and virulence. Malmström *et al*. used SRM to prove that HSA-binding surface proteins of *S. pyogenes* influenced the expression of FAB proteins [62]. In addition, Sjöholm and colleagues combined shotgun proteomics with SRM to characterize the interaction network between human plasma and *S. pyogenes* bacterial surface proteins. The authors used two bacterial isolates belonging to the same serotype obtained from the same patient: one from the throat that led to asymptomatic pharyngitis and one from the leg that caused an invasive infection. Their analysis led to 36 proteins with differences in binding profile between the invasive and the non-invasive strain [63]. The same group used targeted proteomics to construct a stoichiometric host-pathogen surface density model. Their workflow relied on SRM analysis of a *S. pyogenes* wild-type strain and an M1-protein deficient mutant strain. The M1-protein is the most important virulence factor attached to the surface of *S. pyogenes* and is responsible for the binding of several plasma proteins. This model outlined the

topology and density of the host-pathogen protein interaction network on the bacterial surface, revealing a dense and highly organized protein interaction network [45].

Bacteria use protein secretion systems that can induce their uptake by host cells such as macrophages and neutrophils. In the case of *Mycobacterium marinum*, the Esx-1 system is a type VII secretion system that is required for lysis of the phagosomal membrane and thus the translocation of the pathogen into the cytosol [64]. Although several genes have been associated with Esx-1-mediated transport and virulence, the contribution of individual genes to export is largely unknown. Champion and colleagues used MRM to quantify the level of 13 Esx-1-associated proteins across 16 Esx-1-deficient *Mycobacterium marinum* strains and to define statistical rules for assigning novel substrates using phenotypic profiles of known Esx-1 substrates. Using this approach, the authors identified three additional Esx-1 substrates that might promote virulence in *M. marinum* [65]. In 2016, Peters *et al*. applied SRM on seven clinically relevant mycobacterial strains showing various degrees of pathogenicity and they revealed differential expression of 23 proteins implicated in virulence. Their data suggest strain-specific bacterial fitness in the W-Beijing lineage, which is of particular interest due to its increasing prevalence [66]. *Neisseria meningitidis* (meningococcus) is an encapsulated gram-negative bacterial pathogen responsible for significant morbidity and mortality worldwide by causing invasive meningococcal disease [67]. Factor H binding protein (fHbp) is a lipoprotein present on the surface of *N. meningitidis* that improves the survival of the bacteria in human blood by binding human factor H. fHbp is a component of Bexsero and Trumenba, two licensed vaccines against meningococcus B [68,69]. SRM was applied to quantify fHbp in a panel of 105 serogroup B meningococcal strains representative of the genetic diversity of *N. meningitidis* isolates. The data showed that variant 1 strains express more fHbp compared to variant 2 and 3 strains. In addition, SRM was applied to calculate the antigen density required for bacterial killing by anti-fHbp antibodies in human serum, which helps in studying the efficacy of vaccines in humans [70].

Antibiotic-resistance mechanisms

Bacteria have developed different mechanisms to become resistant to antibiotics. One of their most successful strategies is the production of enzymes that inactivate the antimicrobial activity of the antibiotic by hydrolyzing or by chemically modifying its structure. β-lactamases are produced by β-lactam resistant bacteria to hydrolyze the β-lactam ring of this class of antibiotics [71]. In the case of chloramphenicol resistance, acetyltransferases make the antibiotic ineffective through acetylation [72]. Another successful mechanism used by AMR bacteria is preventing the antibiotic to reach its intracellular or periplasmic targets by decreasing the permeability (influx) of the antibiotic or by upregulating efflux pumps, a bacterial machinery that exports the antibiotic out of the bacterial cell [73]. The methods currently used in clinical microbiology laboratories to determine the resistance of bacteria to antibiotics are based on *in vitro* growth of the bacteria in the presence of antibiotics by phenotypic disc diffusion or minimal inhibitory concentration dilution methods [74,75]. Targeted proteomics has proven to be a powerful tool to monitor the resistance of bacterial isolates to a number of different antibiotics.

Pseudomonas aeruginosa is an opportunistic pathogen causing acute and chronic infections in human [76]. The prevalence of multidrug-resistant strains of *P. aeruginosa* is increasing worldwide and represents a considerable therapeutic challenge [77]. Charretier and colleagues developed a SRM-based method as a rapid and reliable method to quantify proteins involved in antibiotic resistance in *P. aeruginosa* such as AmpC cephalosporinase, OprD porin and the four major efflux pumps [78]. The same research group also characterized methicillin-resistant *Staphylococcus aureus* strains using SRM by targeting two proteins, PBP2a and PBP2c, responsible for beta-lactam resistance [79]. Bacteriophage amplification detection (PAD) [80] combined with MRM has been applied to determine resistance to clindamycin or cefoxitin antibiotics in *Staphylococcus aureus*. Briefly, after phage amplification, MRM was used to monitor two phage capsid-derived peptides in

samples thought to contain *S. aureus*. The main advantage of this assay is the combination of high species-specificity by the bacteriophage and the ability to rapidly test against multiple antibiotics [81].

Next to the detection of antibiotic resistant bacteria, targeted proteomics has also allowed to understand the evolution of antibiotic resistance in bacteria. DDA followed by PRM-based quantification was used to compare protein levels in different lineages of *Mycobacterium tuberculosis* in the presence or absence of rifampicin. The authors found that DosR dormancy proteins were more abundant in typical Beijing strains compared to other strains prior to drug exposure. These proteins allow Beijing strains to persist for prolonged periods during rifampicin treatment, thus providing an evolutionary advantage of the Beijing genotype [82].

Biomarker discovery and diagnostic applications

Early diagnosis of bacterial infections is crucial to timely start treatment with the correct antibiotics. A biomarker is defined as a measurable indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention [83]. The identification of novel biomarkers is of great interest for the development of novel diagnostics as well as therapeutics. MS-based proteomics approaches have emerged as leading technologies for the identification of potential disease-specific biomarkers in complex biological samples. A robust workflow typically begins with a discovery phase by shotgun proteomics in which a large number of proteins are screened. This phase is followed by verification and validation of a smaller number of candidate proteins using more accurate quantitative techniques such as targeted proteomics [84]. The advancements in the field of targeted proteomics have also led to the discovery of novel candidate markers for the diagnosis of several bacterial diseases.

Kruh-Garcia and co-workers developed MRM assays for 76 peptides representing 33 mycobacterial proteins that were previously shown to be present in exosomes isolated from TB patients' serum

samples. Twenty of these proteins were confidently identified in human sera. This study is the first that identified a unique and specific panel of *Mtb* potential biomarkers enriched in exosomes [85]. This preliminary list of *Mtb* protein candidate biomarkers was used to refine MRM assays in a further study [86] by adding isotope-labeled peptide standards, optimizing data analysis algorithm and including strains from different geographical origins. Interestingly, the authors found that Cfp2 peptides performed best in the South Africa cohort, while Mpt32 peptides underperformed as biomarkers in the Bangladesh cohort. MRM has also been used to quantify Antigen 85 complex (Ag85) and compare Ag85 levels among various clades of *Mtb*. The Ag85 complex, represented by Ag85A, B and C proteins in the mycobacterial secretome, has been suggested as a potential diagnostic and vaccine candidate for TB [87]. Kruh-Garcia and colleagues demonstrated by MRM that there is variability in Ag85B expression and secretion, and to a lesser extent Ag85A secretion, across lineages and within subclades. This study showed the importance of MRM in assessing quantitative variation between highly homologous proteins and how this technology can be used to guide bacterial biomarker selection [88].

The combination of targeted proteomics with other detection methods has been successfully applied to identify bacteria. A combinational approach targeting both the bacterial DNA and proteins using PCR and MRM respectively, has been applied to differentiate the highly virulent *Yersinia pestis* from the closely related less virulent *Yersinia pseudotuberculosis*. The detection of the former by only DNA-based molecular assays resulted in high number of false positives especially when assays were challenged with mixtures of clinical and environmental samples. Larson and colleagues first identified the YPO1670 gene as a chromosomal target unique to *Y. pestis*, a finding which was confirmed by PCR. In a next step, the YPO1670 protein was monitored by MRM on samples from *Y. pestis* strains. Their data confirmed that this chromosomally encoded protein is unique to *Y. pestis* and can be used as a reliable biomarker for this pathogen [89].

Chenau *et al*. combined the specificity and the sensitivity of two complementary methods, immunoaffinity capture and SRM for the direct detection (without prior culture) of *Y. pestis* in complex environmental and food samples. Immunoaffinity capture enables the isolation of intact bacterial cells using anti-Pla antibodies, while SRM was used to monitor and quantify three *Y. pestis*-specific protein markers (murine toxin, plasminogen activator and pesticin) [90]. Pierce and colleagues used a labeled PAD technique coupled to MRM to detect and quantify viable *Staphylococcus aureus* by monitoring three peptides representative of the major capsid head protein, a specific biomarker of bacteriophage 53 [91].

Monitoring food safety

Foodborne intoxications are an important cause of morbidity and mortality and result from eating food or drinking water contaminated with pathogens such as bacteria, parasites and viruses, or their toxins. The symptoms range from mild and self-limiting (vomiting and diarrhea) to life-threatening (such as sepsis, kidney and liver failure). Beyond the individual level, there is an economic burden of foodborne diseases particularly at the level of agriculture, health system and food industries [92]. Therefore, in food technology, monitoring the production process for possible bacterial contamination in the final food product is essential.

Targeted proteomics has improved studies on food quality and safety [93]. *Staphylococcus aureus* is one of the leading causes of foodborne illnesses when dairy products and meats are often contaminated with toxins such as Staphylococcal enterotoxin B (SEB) and Staphylococcal enterotoxin A (SEA). SEB is highly heat-resistant and is found in over 50% of enterotoxin food poisonings [94]. Bao and colleagues applied MRM to detect and quantify SEB in raw chicken. Briefly, after extraction, proteins were digested with trypsin and three tryptic peptides that are present at different locations of the SEB structure were selected as heavy-labeled internal standards for MRM measurements. Their method provided high accuracy of detection and quantification [95].

Andjelkovic and colleagues developed a method based on online solid-phase extraction coupled to MRM to develop a method that can identify the presence of SEB and SEA in different food matrices. To achieve this, the authors monitored two and three different labeled proteotypic peptides representative for SEA and SEB respectively in six blinded extracts (four milk samples and two buffer samples). Their results showed that the toxins were successfully detected in milk samples, and the detection limits of SEA and SEB were about 8 and 4 ng/g respectively, levels that are comparable or even lower than those achieved with most of the other identification methods [96].

The Gram-positive, spore forming *Bacillus anthracis* is classified as a category A agent, the highest rank of potential bioterrorism agents. Contamination by *B. anthracis* can occur by contaminated food ingestion or aerosol inhalation. Detection of *B. anthracis* is challenging because of its high genetic similarity with *B. cereus* and *B. thuringiensis*. Therefore, Chenau and colleagues developed an approach to reliably detect and unambiguously discriminate *B. anthracis* from closely related strains in complex environmental samples (milk and soil). Their approach consists of combining immunocapture of intact spores, followed by extraction and subsequent proteolysis of proteins, and finally targeted MRM detection of proteotypic peptides from the small acid-soluble spore protein (SASP-B) isoform specific to *B. anthracis*. This approach was shown to specifically detect *B. anthracis* in a mixture of different Bacillus species [97]. The same group conducted comparative proteomics of *B. anthracis*, *B. cereus* and *B. thuringiensis* spores to identify proteoforms unique to *B. anthracis*. The authors first combined the data from both bottom-up and top-down approaches with those from DNA sequencing. This resulted in the identification of 11 candidate markers that are unique to *B. anthracis*. In a further step, out of these 11 proteins, four peptides representatives for four proteins were selected to be monitored in LC-SRM mode. The obtained results confirmed the high specificity of the identified four markers to *B. anthracis* [98].

Immune extraction combined with PRM was developed for the detection and absolute quantification of three toxins: *Ricinus communis* toxin ricin, SEB and *Clostridium perfringens* epsilon toxin (ETX) in

human biofluids (urine, serum and plasma) and food matrices (milk and tap water). At least 7 peptides were targeted for each toxin (43 peptides in total) with a quadrupole-Orbitrap instrument. Quantification was performed using stable isotope-labeled toxin standards spiked before immunocapture [99]. In the same context, the researchers developed a LC-SRM method for absolute quantification of eight toxins: ricin, ETX, SEA, SEB SED, shigatoxins from *Shigella dysenteriae* and entero-hemorragic *Escherichia coli* strains (STX1 and STX2) and *Campylobacter jejuni* cytolethal distending toxin (CDT) in food matrices. Overall, the data showed high sensitivity of the assay developed with results below the toxin concentrations expected to be detected in the event of intentional food poisoning [100].

Conclusions and future outlook

Applications of targeted proteomics in the field of pathogenic bacteria have expanded greatly in recent years because of the specificity, reproducibility and multiplexing capability of this approach compared to immunological assays. SRM and PRM have thus emerged as powerful tools for sensitive and reliable quantification of proteins associated with bacterial infection, especially in the context of antibiotic resistance and clinical diagnosis. Results obtained with these approaches have improved our understanding of virulence mechanisms of different bacterial species and of host-pathogen interactions. SRM/MRM has been the method-of-choice in most studies reviewed here, very likely because of its high sensitivity and reproducibility for quantifying (low abundance) proteins. On the other hand, when analyzing samples in complex matrices, high resolution analysis and accurate measurements are deemed necessary and hence PRM is recommended. The application of PRM in pathogenic bacteria is still in its infancy, though method development is well documented and data analysis is similar to SRM [101,102]. Another factor to consider is the time required for the development of an assay. When rapid screening of patient samples is needed, PRM is recommended as there is no need to optimize transitions as SRM/MRM assays. The sensitivity provided by triple

quadrupole mass spectrometers will probably keep the research community in favor for using SRM however, we suspect that PRM will be increasingly implemented due to its higher selectivity and its more straightforward method development.

One of the major limitations of SRM and PRM is the rather limited number of proteins that can be measured. A new promising approach for larger-scale protein quantification is the DIA-based targeted method such as SWATH-MS. To our opinion this strategy will not yet overtake SRM and PRM as the method-of-choice for targeted proteomics because when compared to SRM and PRM, DIA holds lower sensitivity and specificity, and data analysis requires more sophisticated bioinformatics tools because of its complexity. Nonetheless, we expect that by using improved MS instruments, in the future, one should achieve high-throughput analysis of several hundreds of proteins in targeted mode, improve the sensitivity for measuring low abundant proteins in complex biological samples, thus enabling implementation of targeted proteomics in clinical laboratory settings.

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Conflict of interest statement

The authors have declared no conflict of interest.

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Figure legends

Figure 1. Schematic representation of a typical workflow to study bacteria proteomes, comprising (a) the identification of candidate proteins of interest, (b) MRM assay development and (c) quantitative MRM analysis of study a high number of samples.

Figure 1

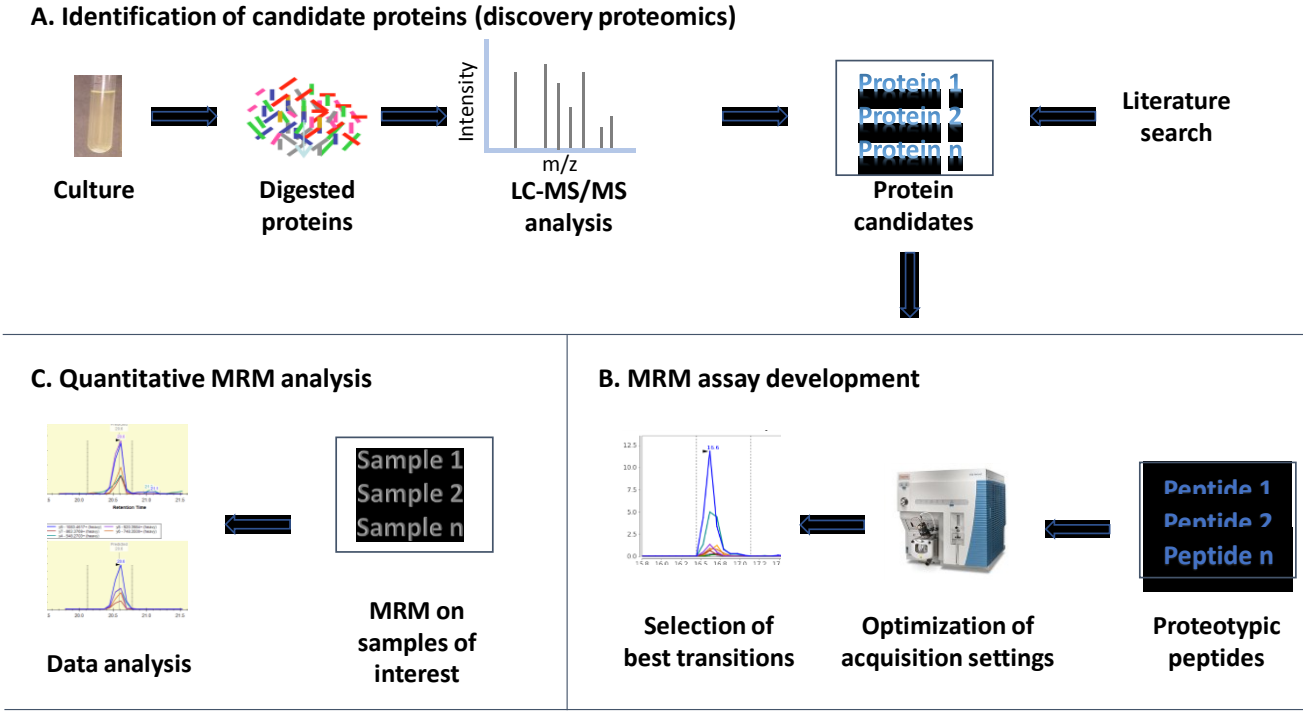

Figure 2. Schematic representation of targeted data acquisition approaches. (a) SRM/MRM is typically performed on triple-quadrupole instruments where a predefined precursor ion is selected in the first quadrupole (Q1), fragmented in the second quadrupole (Q2) that serves as a collision cell and then specific fragment ions are monitored individually in the third quadrupole (Q3). (b) PRM can be performed on quadrupole-orbitrap or QTOF instruments where Q1 selects the precursor ion that is then transferred to the higher energy collision-induced dissociation (HCD) cell for fragmentation. All fragment ions are then detected in the mass analyzer.

Figure 2

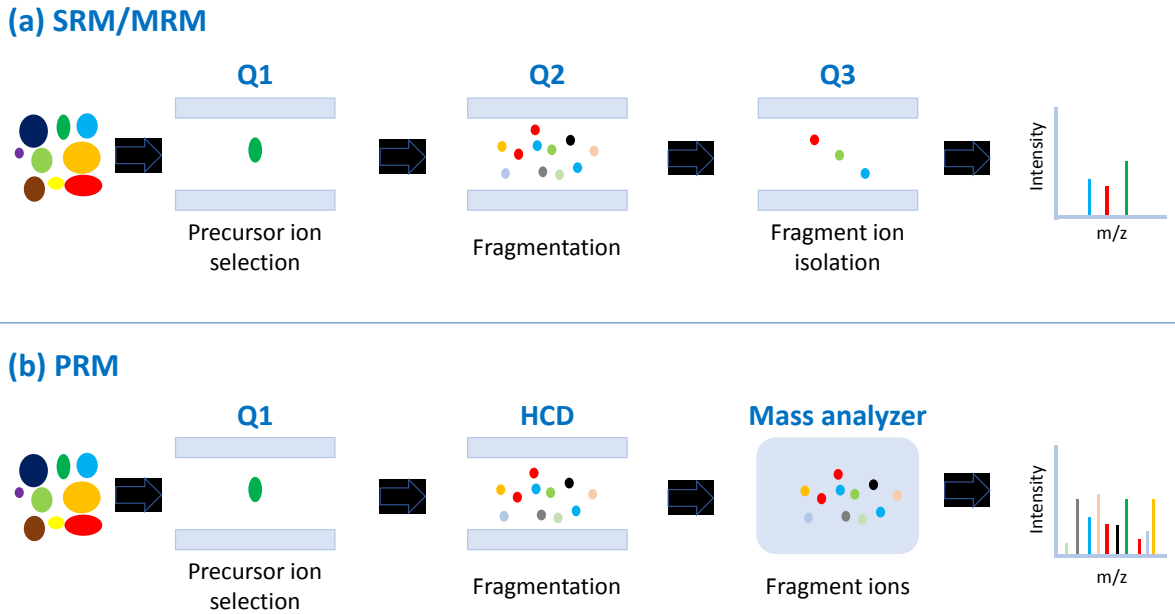

Table 1: Comparison of commonly used acquisition methods used for proteome studies.

	DDA	**DIA**	**SRM/MRM**	**PRM**
Instruments typically used	Different types	Q-TOF,Q-Orbitrap	Triple quadrupoles	Q-TOF, Q-Orbitrap
Acquisition mode	Non-targeted	Non-targeted	Targeted	Targeted
Sensitivity	Medium	Medium	Very high	High
Selectivity	Medium/high*	High	Medium	Very high
Reproducibility	Low	High	Very High	Very High
Multiplexing	High	High	Medium	Medium
Workflow development time	Short	Medium	High	Medium
Data analysis complexity	Low	High	Low	Low
Isotopically labeled peptides for quantification required	Yes/no	No	Yes	Yes
Dynamic range	Low	Medium	High	High

* depends on the instrument used

Table 2. Reviewed studies applying targeted data acquisition by SRM/MRM or PRM with their main findings and level of quantification.

Bacteria species	Method	Main findings	Quantification	Reference
Repositories				
group A Streptococcus	SRM	SRM assays for 10,412 distinct peptides	Not applicable	[6]
Escherichia. coli	SRM	Absolute copy numbers for more than 2,300 *E. coli* proteins across different conditions	Absolute	[37]
Mycobacterium tuberculosis	SRM	Mtb SRM assay library for 97% of all annotated proteins	Absolute	[40]
Metabolism				
Campylobacter jejuni	SRM	*C. jejuni* undergoes a significant metabolic downshift and down-regulates aerobic respiration during host cell infection	Relative	[8]
Escherichia. coli	SRM	Absolute quantification of enzymes involved in the mevalonate pathway and identification of proteins involved in tyrosine pathway after strain engineering	Relative	[45]
Escherichia. coli	SRM	SRM assays for over 400 proteins representatives of more than 20 major metabolic pathways optimized and validated	Absolute	[46]
Pathogenicity				
Escherichia. coli	MRM	Kdp(F)ABC complex and KdpDE-TCS proteins are both involved in potassium transport	Absolute	[48]
Escherichia. coli	SRM	CpxA, CpxR and CpxP reduce acid stress response	Absolute	[49]
Streptococcus pyogenes	SRM	Several virulence proteins are regulated in response to human plasma	Relative	[52]
Streptococcus pyogenes	SRM	HSA-binding surface proteins of *S. pyogenes* influenced the expression of FAB proteins	Relative	[53]
Streptococcus pyogenes	SRM	36 plasma proteins differentially present in invasive versus non-invasive strains	Relative	[54]
Streptococcus pyogenes	SRM	Construction of a stoichiometric host-pathogen surface density model	Absolute	[36]
Mycobacterium marinum	MRM	Quantification of 13 Esx-1-associated proteins across 16 Esx-1-deficient strains and identification of 3 additional Esx-1 substrates that might promote virulence	Relative	[56]
Mycobacterium tuberculosis	SRM	23 virulence proteins differentially expressed reflecting various degrees of pathogenicity between strains	Relative	[57]
Neisseria meningitidis	SRM	Quantification of fHbp in 105 serogroup B meningococcal strains showed that variant 1 strains express more fHbp compared to variant 2 and 3 strains	Absolute	[61]
Antibiotic resistance				
Pseudomonas aeruginosa	SRM	Proteins AmpC, OprD and 4 major efflux pumps quantified to detect resistance	Relative	[69]
Staphylococcus aureus	SRM	Methicillin-resistant strains detected by targeting the proteins PBP2a and PBP2c	Not applicable	[70]
Staphylococcus aureus	MRM	MRM combined with bacteriophage amplification allows specific detection of clindamycin and cefoxitin resistance	Not applicable	[72]
Mycobacterium tuberculosis	PRM	DosR dormancy proteins are more abundant in Beijing strains allowing to persist for prolonged periods during rifampicin exposure	Relative	[73]
Biomarker discovery				
Mycobacterium tuberculosis	MRM	Twenty unique Mtb biomarkers are enriched in	Relative	[76]

		exosomes of human sera		
Mycobacterium tuberculosis	MRM	Identification of peptides as potential makers that are associated with specific geographical locations	Relative	[77]
Mycobacterium tuberculosis	MRM	Ag85B and Ag85A are differentially expressed and secreted across lineages and subclades	Absolute	[79]
Yersinia pestis	MRM	YPO1670 is a candidate biomarker for *Yersinia pestis*	Not applicable	[80]
Yersinia pestis	SRM	Murine toxin, plasminogen activator and pesticin monitored in environmental and food samples	Relative	[81]
Staphylococcus aureus	MRM	Species detection based on simultaneous quantification of the major capsid head protein, a specific biomarker of bacteriophage 53	Relative	[82]
Food safety				
Staphylococcus aureus	MRM	Accurate detection and quantification of SEB in raw chicken meat	Relative	[86]
Staphylococcus aureus	MRM	SEB and SEA identified in food matrices	Absolute	[87]
Bacillus anthracis	MRM	SASP-B isoform specific to *B. anthracis* monitored in complex environmental samples	Relative	[88]
Bacillus anthracis	SRM	4 protein markers were shown to be highly specific for *B. anthracis*	Relative	[89]
a)Multiple species	PRM	SRM assays developed for detection and absolute quantification of ricin, SEB and ETX in human biofluids	Absolute	[90]
b)Multiple species	SRM	SRM assays developed for detection and absolute quantification of ricin, ETX, SEA, SEB, SED, STX1, STX2 and CDT in human biofluids	Absolute	[91]

a) *Ricinus communis, staphylococcus aureus* and *Clostridium perfringens*. b) *Ricinus communis, staphylococcus aureus, Clostridium perfringens, Shigella dysenteriae*, *Escherichia coli* and *Campylobacter jejuni*.