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'Omics' for microbial food stability: Proteomics for the development of predictive models for bacterial spore stress survival and outgrowth

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ABSTRACT

Bacterial spores are ubiquitous in nature. They are stress resistant entities that are a concern to microbiological food stability due to their environmental stress resistance. In addition germinating and outgrowing spores at undesired times and places pose a significant health burden. The challenge is amplified due to the heterogeneous germination and outgrowth behaviour of isogenic spore populations. We discuss the role of different 'omics' techniques, proteomics in particular, to study spore biology in detail. With examples, the use of label-based and label-free quantitative proteomics approaches in understanding the spore physiology is demonstrated. Also the need of genomics, single cell analyses and analysis of cellular physiology is discussed briefly. Certainly accurate comprehensive data obtained from omics methods and molecular physiology will underpin the development of robust molecular models of bacterial spore germination and outgrowth.

1. Introduction

The food processing industries face an ever-increasing demand for safe and mildly processed food to attain optimized product quality. Yet, mild processing also requires a more detailed insight into the risks of microbial resistance against, often combined, food preservation methods. Estimation of risks posed by pathogenic microorganisms in food is part of the Microbiological Risk Assessment (MRA) discipline. This assessment includes four sub-processes: Hazard identification, hazard characterization, exposure assessment and risk characterization (Brul et al., 2012). In particular for a thorough hazard characterization, academic and industrial researchers use more and more state of the art 'Omics' tools such as genome-wide sequencing tools (Genomics), genome-wide transcript (Transcriptomics) as well as protein analyses (Proteomics), assessment of the metabolic profile of microorganisms (Metabolomics) and in some cases microbial flux analysis (Fluxomics) to understand the mechanistic basis of cellular responses to changes in their environment. From the microbiological food stability and safety point of view, the details made available by such studies are important clues for the development and improvement of food preservation strategies. Generally, diseases and spoilage caused by spore formers are associated with thermally processed foods, as heat kills the vegetative cells but allows survival and subsequent growth of spore-forming organisms. Their presence thus poses a big challenge to the food industries. Spore stress resistance is in part due to their multi-layered structure and the ability to germinate under favorable environmental conditions. In addition the germination process itself is heterogeneous in the sense that not all spores in a population may germinate at the same time. Modelling inactivation of food-borne pathogens has been one of the first achievements in predictive microbiology. Inactivation models were initially focused on the destruction of *Clostridium botulinum* spores in low acid canned foods (Hersom and Hulland, 1980; Jay, 1992) but due to the paucity of molecular data and hazard of the toxin produced by vegetative *C. botulinum* cells, the models had to be by necessity conservative. Nevertheless, due to their practical usefulness the principles on which these models were grounded was used also for the generation of predictive models of microbial stability for the less hazardous but (much)more thermal resistant spoilage spore formers. It may come as no surprise that due to their conservative nature such models for these, generally Bacillus, species, still leave significant room for improvement where it concerns the trade of between the provision of the desired product quality whilst providing the necessary microbial product stability. Nowadays using 'omics' tools comprehensive molecular data can be gathered also in food

Abbreviations: LC-FT-IR-MS/MS, liquid chromatography coupled Fourier transform infra-red tandem mass spectrometry; MRA, Microbial Risk Assessment; EFSA, European Food Safety Authority; ISO, International Organization for Standardization; SDS-PAGE, sodium-dodecyl-sulphate poly-acrylamide gel electrophoresis; 2D-DIGE, two dimensional gel electrophoresis; PSM, peptide spectrum matches; SWATH, sequential windowed acquisition of all theoretical fragment ion mass spectra; DDA, data-dependent acquisition; PAI, protein abundance index; (em)PAI, exponentially modified protein abundance index; AUC, area under the curve; SILAC, stable isotope labelling by amino acids in cell culture; iTRAQ, isobaric tags for relative and absolute quantification; TMT, tandem mass tags; AQUA, absolute quantification; QconCAT, quantitative concatemer; CDGS, chemically defined growth & sporulation medium.

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microbiology and hence underpin the predictive models for microbial behaviour with biologically meaningful mechanistic parameters (Wells-Bennik et al., 2016). In this review we mainly focus on applications of proteomic tools for food safety research. Genomics and microscopic live-imaging techniques are also discussed as supportive tools where necessary. In the sections to follow the different proteomics techniques and implications thereof for spore biology and modelling are discussed.

2. Spore formers in the food chain and application of genomics

In order to minimize or eliminate the problems caused by the spores, it is very important to detect and estimate the amount of spores in the food sample. Efficient removal of spores is possible when simple and quick spore detection and removal systems are available. To build models and systems it becomes necessary to understand the heterogeneous properties of spores as well as to gain the knowledge about the entire sporulation and germination pathways. As ubiquitous as spores are, they manage to enter the food chain at many different points. The European Food Safety Authority (EFSA) agglomerated reports of *Bacillus cereus* foodborne poisoning cases showing that 10^5–10^6 cells or spores/g of food clearly can cause foodborne poisoning and in rare cases, 10^3 spores/g of food caused illness. Primarily infection may occur from the soil where the raw product is obtained from, which generally contains 10^3 to 10^5 spores per gram of soil (EFSA, 2005). This became apparent when genotypes matched between contaminating spores and those isolated from the originating farms. Furthermore, there is a possibility of secondary contamination occurring during processing of the raw product. The exosporium has been indicated to allow spores of many species to adhere to stainless steel surfaces such as those found inside milk silo tanks or processing equipment(s) (Lequette et al., 2011). Even occurrences in packaging materials have been reported (Pirttijärvi et al., 2000). Preventing contamination of a food product with spores therefore seems a rather difficult task. For this reason methods for proper detection of spore types in the end product are urgently needed.

To detect *B. cereus* and ensure food safety, current ISO guidelines (ISO 7932, ISO 21871) are based around incubation on agar plates for 24 h or even longer and subsequently confirming haemolytic properties (Schulten et al., 2000). These methods are efficient as a catch-all for any type of product tested, however they are time consuming. Developing a platform for direct detection of spores would allow for better, faster and ultimately cheaper detection methods. In order to develop such a platform, proper spore biomarkers would need to be identified. The problem becomes grave if the sporulating organism is a novel species. In such instances it is obligatory to perform genomics studies first wherein the genome is sequenced and properly annotated. Genomic techniques such as Illumina sequencing and ion torrents have now become affordable for these purposes. This can be seen from the fact that recently the genome of *B. weihenstephanensis* WSBC 10204, a psychrotolerant strain, was successfully sequenced using Illumina HiSEQ2500 sequencing technique. In this study, using genomics and proteomics methods a metabolic model was developed that indicated that the enzyme arginase, key to urea formation, was identified only in the cells grown at 6 °C (Fig. 1). Hence this process was identified as a putative antimicrobial target to interfere with low temperature proliferation of *B. weihenstephanensis* (Stelder et al., 2015). Also for known as well as the novel spore formers, the biochemical basis of the signalling mechanisms that operate when a dormant spore germinates largely remain to be uncovered; although the gene-expression processes operative during sporulation and germination have been described (Bassi et al., 2016; Eichenberger et al., 2003; Keijser et al., 2007; Ter Beek et al., 2008). Apart from other constituents such as water, di-picolinic acid, cortex peptidoglycan etc. the spore consists to a large extent of specific proteins. Hence spore proteins are good candidates for rapid detection and targets for strategies aimed at elimination of spores.

3. Qualitative proteomics

As an extension of the term genomics which refers to the study of the genome, i.e. all the genes present in a biological system, the term proteomics refers to the study of the proteome, i.e. all proteins present. Being the active molecules in a cell, information on which and how many proteins are present provides insight into the current physiological state of the cell. Mass spectrometry has long held a prominent if not defining position in this field (Aebersold and Mann, 2003) as the 21 amino acids which constitute the primary sequence of proteins are, with the exception of isomers leucine and isoleucine, distinguishable by their respective mass. Simply stated, by reducing the complex structures within a protein mixture to the primary “beads on a string”

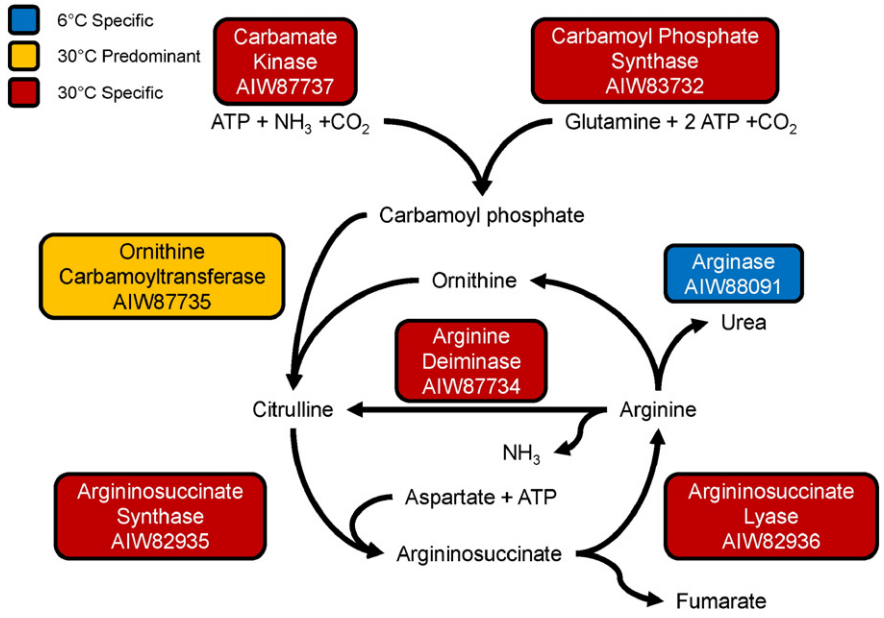

Fig. 1. - Model for differential expression of genes/identification of proteins from the urea cycle at different growth temperatures. Schematic representation of proteins involved in the urea cycle and their corresponding GenBank accession numbers. Most enzymes of the urea cycle were found to be present at 30 °C exclusively or predominantly. Only Arginase deviated from this trend, being identified only in isolates of cultures grown at 6 °C. [Reprinted with permission from (Stelder et al., 2015). Copyright (2015), American Chemical Society.]

peptide sequences through biochemical means, mass spectrometers can readily determine the order of the beads. By fragmentation into smaller bits of string, measuring the mass difference allows for determination of the identity of the next bead missing. In a bottom-up approach comparison of the determined sequences to all potential sequences coded on the genome is done whereby we can infer the current state of the proteome. Qualitative protein identifications for spores have been done in the past (Abhyankar et al., 2011; Driks, 1999; Henriques and Moran, 2007; Kuwana et al., 2002). In general membrane proteins with multiple membrane spanning domains are hardly detected because of their low abundance and poor solubility in aqueous media. This is true for spore inner membrane as well. Yet, to identify the spore inner membrane proteins a qualitative proteomics study in *B. subtilis* was successfully completed recently (Zheng et al., 2016). In this study (Fig. 2 (A) and (B)), proteins HtrC & YpeB were identified which may be at the basis of germination triggering SleB and thus cortex and coat lysis (Bernhards et al., 2015; Meaney et al., 2015). Hence this study can be beneficial to understand the interaction of inner membrane proteome with the cortex and coat proteome and its possible links with spore germination across different species of spore formers. Apart from the membrane proteins, cross-linked protein fraction from spore coats also challenges the proteomics research mainly due to the low abundance of cross-linked peptides, resistivity of proteins towards proteases etc. Nevertheless efforts are constantly being made in the field to tackle this issue. Such qualitative studies have been providing many diverse protein targets for building spore detection systems. As mentioned previously, in order to develop accurate models for Spore Risk Assessment, accurate quantitative data is needed. This can be exemplified using the model proposed by Wang et al. (2015), about the induction of spore memory by SpoVA channel gating. The SpoVA channel gating leads to Ca^{2+}-DPA release leading to spore germination. To understand the cooperativity of these channels and to build a predictive model there is a further need of accurate quantitative data about the presence of the various subunits of these channels in spores. This can be achieved by the quantitative proteomics approached described below. Additionally, a proper biomarker needs to be robust and specific to the system, be abundant enough to have reliable interaction with the sensor, and be accessible to the molecule it is supposed to bind to, be that an antibody or otherwise. Also therefore it is important to quantify the proteome of spores of food spoilage and pathogenic *Bacilli*, as well as to obtain information on protein localization within the spore.

4. Quantitative proteomics

Traditionally, information about protein abundance is acquired using gel-based approaches based on analytical principles commonly used in biochemistry. Typically a protein sample is resolved by two-dimensional SDS-PAGE. Subsequently comparing the intensity of staining between different gels gives a measure of protein abundance (Issaq and Veenstra, 2008). Using the more refined version of this approach called 2D-DIGE, two fluorescently stained samples are resolved on the same gel and fluorescence intensity is measured (Unlü et al., 1997). The role of mass spectrometry is then simply identifying the protein(s) present in each gel spot. Although this method does not require advanced mass spectrometric equipment and is therefore

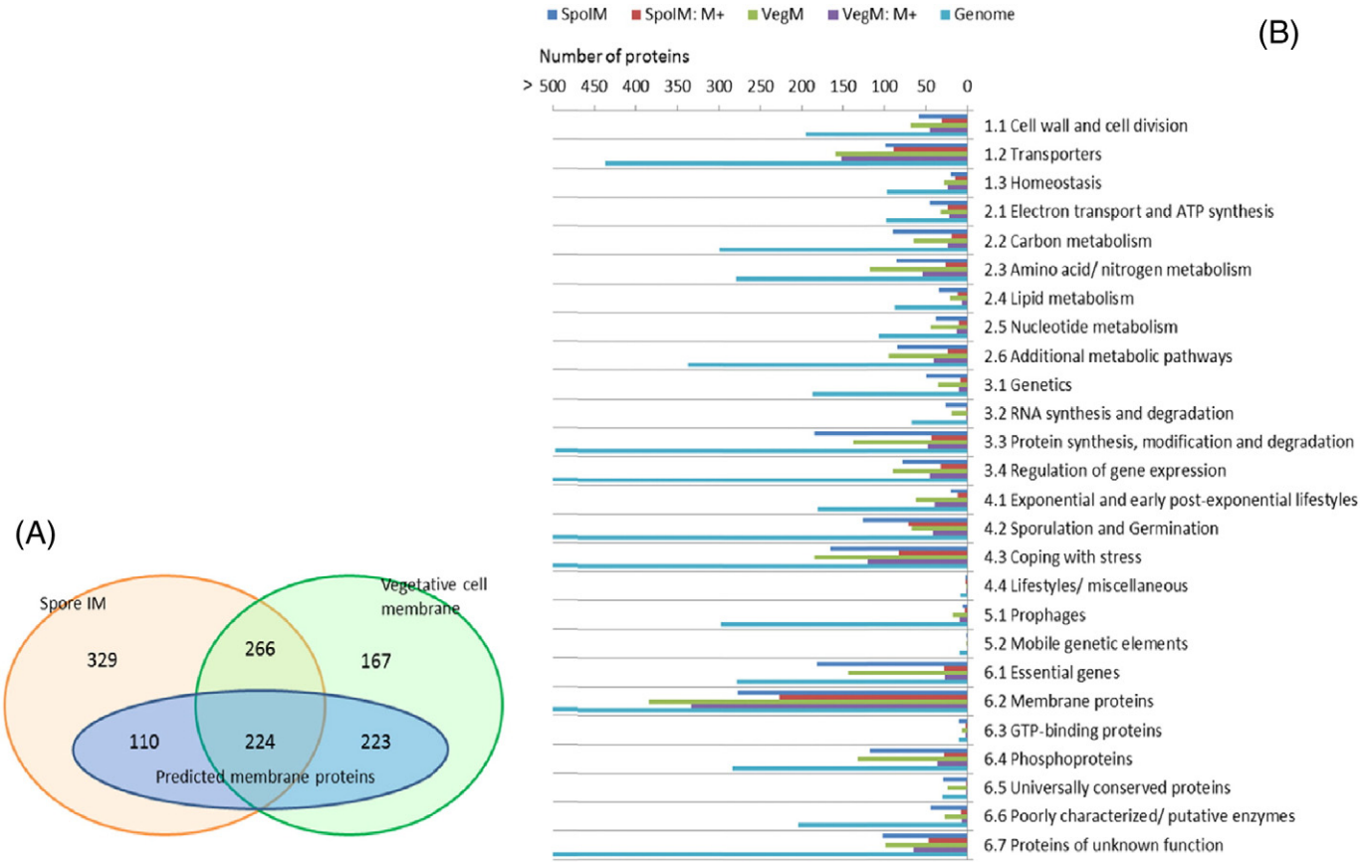

Fig. 2. (previous page)– (A) Venn diagram of proteins identified from the *B. subtilis* spore inner membrane (IM) isolation and vegetative cell membrane fraction. (B) Categorization of identified proteins according to *SubtiWiki*. The six main categories are (1) cellular processes, (2) metabolism, (3) information processing, (4) lifestyles, (5) prophages and mobile genetic elements, and (6) groups of genes. SpoIM, identified in spore IM; VegM, identified in vegetative cell membrane; M+, predicted to be membrane proteins using the following programs or databases: PSORTb 3.0, Phobius,BOMP, PRED-LIPO, LocateP and Inter-ProScan 5 [Reprinted with permission from (Zheng et al., 2016). Copyright (2016), American Chemical Society].

accessible to a wide audience, there are severe limitations. For instance, considering a situation where the protein sample is complex, a likely situation given the thousands of potential gene products encoded on a typical genome, multiple proteins may be present in the same gel spot. Furthermore, considering that the stain generally binds non-specifically to protein, potentially with a different affinity for any given one, correctly ascribing the observed stain intensity to a singular protein is also difficult. With major advances in mass spectrometric techniques our ability to properly ionize peptides and accurately determine the mass of analytes has taken dramatic leaps. On-line coupling of liquid chromatography further expanded this potential as it enables resolution of more and more complex samples in a single analysis (Aebersold and Mann, 2003). As such, this enabled the development of a huge number of methods to acquire quantitative information directly from the MS data. The quantification is mainly done via two approaches, Relative quantification or Absolute quantification, which are based on whether and when an isotopically labelled reference is introduced (Fig. 3). The preference of approach then depends on the research question and the nature of the sample being analyzed. The relative quantification allows a proteome wide analysis of changes in the proteome under different experimental conditions while under absolute quantification calculation of molecular numbers or study of stoichiometry of the selected target proteins can be performed. Relative quantification can be done by label-free approaches or by metabolic and/or chemical labelling approaches whereas absolute quantification makes use of synthetic peptides or proteins as external reference. The following paragraphs will provide an overview of available techniques and the type of situation in which they are most applicable. Readers are referred to the in-depth reviews of these techniques for explicit details (Bantscheff et al., 2012; Bantscheff et al., 2007; Ong and Mann, 2005; Zhu et al., 2010).

4.1. Label-free methods for relative quantification

The intensity of the signal measured by the mass spectrometer depends on the physical properties of the molecule analyzed, the most notable being ionization efficiency. As these properties vary widely between peptides and are affected by the properties and quantity of other molecules present in the sample, it is very difficult to correlate signal intensity to the number of molecules present with a one-size-fits-all calculation. Though mass spectrometry is inherently not a quantitative technique, after interpretation of the MS/MS data there are some parameters which may be used as an indication of abundance at the protein level. For instance, the most abundant proteins are generally the top identifications with the highest score and the highest number of peptide spectrum matches (PSM). In a spectral counting approach, the number of PSMs is used to compare protein expression levels between different states (Liu et al., 2004). The major benefit is that this requires minimal extra work in method development in order to get a quantitative interpretation of an entire proteome. A downside is that in order to acquire reliable data this approach requires thorough and proteome-wide resolution along with multi-replicate analysis of the proteome under investigation. Though recently data independent acquisition is on the rise with technologies such as SWATH garnering ever greater attention (Arnhard et al., 2015; Rosenberger et al., 2014; Tate and Hunter, 2009), most LC–MS/MS experiments use a data dependent acquisition (DDA). This means that of all peptides detected in MS^1 there is only a limited number selected for fragmentation. This number depends on the duty cycle of the instrument and performance of the LC system. In DDA the mass spectrometer chooses the top 'X' peptides of the MS^1 survey spectrum to fragment based on criteria predefined by the experimenter and disregards the rest. Generally these criteria entail

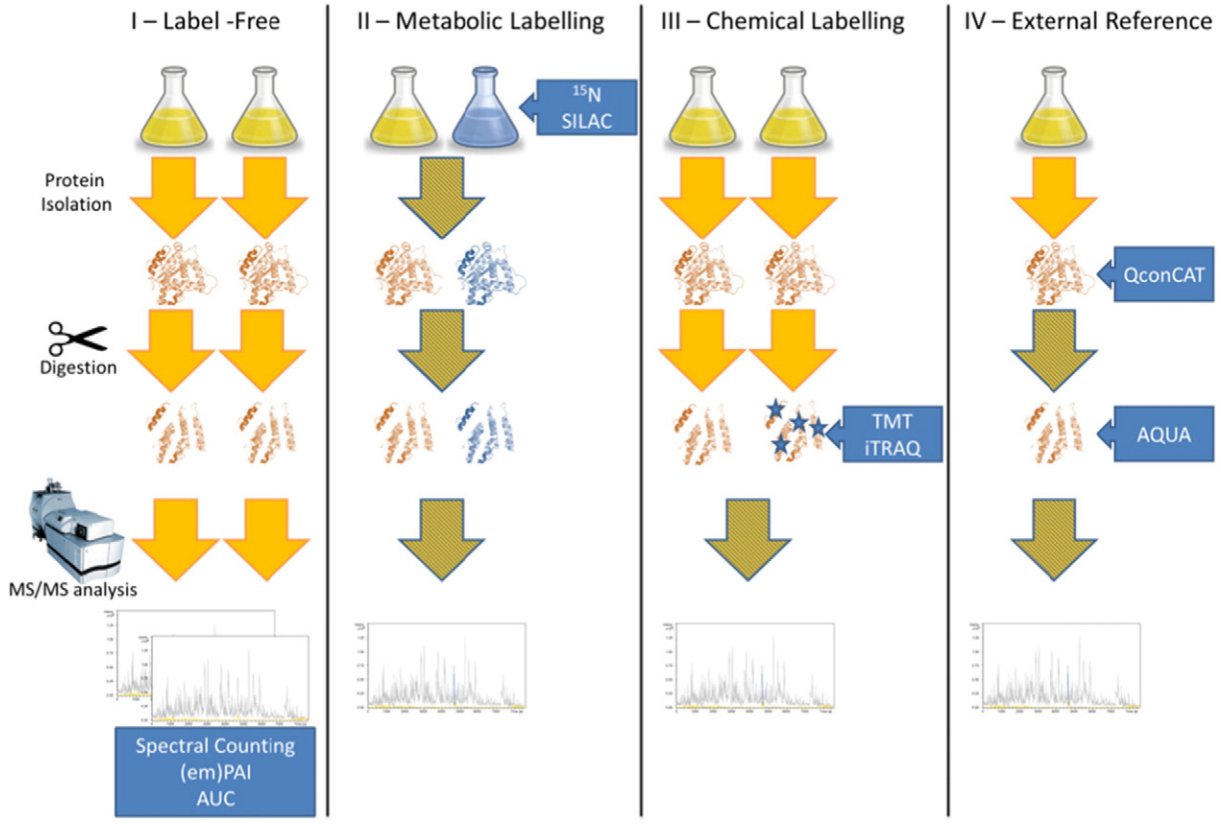

Fig. 3. - Quantitative proteomics strategies. (I) Label free quantitation using exponentially modified Protein Abundance Index ((em)PAI) scoring and labelling strategies with (II) metabolic labelling and Stable Isotope Labelling by Amino acids in Cell culture (SILAC), (III) use of labelled Tandem Mass Tags (TMT) and isobaric Tags for Relative and Absolute Quantification (iTRAQ) tags and (IV) spiking of synthetic peptides or proteins of known concentration. Please refer to the list of Abbreviations for the details.

a specification of the maximum number of peptides to fragment per cycle, minimal signal intensity for selectable peptides and time frame during which a previously selected mass is excluded. This implies that the robustness of the experimental procedure prior to MS analysis plays a role in whether a given peptide is included or discarded. The spectral counting approach is best suited for analysis of proteins across a limited dynamic range, which are easily isolated in large quantities to allow for multiple replicate analyses. The data obtained gives an indication of relative protein abundance between isolates.

Beyond spectral counting, group of Rappsilber (Rappsilber et al., 2002) first introduced the concept of a Protein Abundance Index (PAI) for label-free quantitative interpretation of LC–MS/MS data. This makes use of the general correlation between protein abundance and the number of peptides sequenced per protein. By also considering the number of peptides that are generally identifiable for a given protein it is possible to correct for differences between proteins. For example, larger proteins may give rise to more matching peptides being identified simply because it can be cut up into more fragments than a smaller protein. These approaches can be used to estimate relative protein abundance within a sample and have been successful in determining the stoichiometry of proteins. Exponentially modified PAI ((em)PAI) has even been shown to be sufficiently accurate to determine absolute abundance of 54 proteins in a whole cell lysate. However, as determinants of absolute abundance PAIs appear to be limited to estimates that may deviate 3–5 fold from the true value (Ong and Mann, 2005). Furthermore, complex samples spanning a large dynamic range of protein abundance are less well suited to being quantified by an (em) PAI approach as saturation may occur for high abundant proteins. This limits the use of PAIs to indicators for further development of experimental strategies rather than being useful endpoint absolute quantitative analyses.

A third label-free method has gained popularity with the rise of higher resolution instruments which can avoid overlap between peaks of different peptides of similar nominal mass. Though the intensity of the signal measured by the mass spectrometer cannot directly be converted to an absolute value of peptide abundance, there is a linear relation between signal and molecular concentration. By ensuring that the analyses and sample processing are reproducible, an area-under-the-curve (AUC) approach can then be used to compare the relative signal intensities between runs. These ratios can then be related to relative protein abundance in different samples using sophisticated software (Silva et al., 2006; Wang et al., 2003, 2006). This AUC approach can even be extended to determining absolute values by introducing an external reference protein in a known quantity. This can be used to determine a universal signal response (counts/mol) based on the observation of a linear relation between the average signal response of the top three most intense peptides and protein abundance. However, these calculations are restricted to proteomes of limited complexity and dynamic range due to the requirement of three peptide hits per protein (Silva et al., 2006). The label-free quantitation methods have been recently applied in elucidating the phosphoproteome of germinating spores (Rosenberg et al., 2015) and for investigation of protein abundance in the cellular proteome and secretome of *B. weihenstephanensis* (Stelder et al., 2015).

4.2. Labelled reference based relative quantitation approaches

Beyond label free approaches there are several techniques which take advantage of mass difference, the exact property mass spectrometers were designed to measure. Making use of stable isotopes which have the same physicochemical properties yet differ in mass, molecules are made distinguishable by MS. Many variations on this principle have been developed with the biggest difference between approaches being the point when the isotopic label is added. They can be subdivided based on whether the labelled reference is generated in situ through metabolic labelling, attached to isolated protein material through chemical labelling or, are generated and added externally as displayed in Fig. 3.

4.2.1. Metabolic labelling

For proteome wide quantitative approaches in a system where the sample is easy to grow, i.e. grown in cell culture, introducing isotopic labels metabolically allows for the creation of entirely labelled cells. Quantitative data is then acquired by mixing "heavy" cells grown in one condition with "light" cells grown in another condition. After isolating proteins and performing MS analysis, calculating isotope ratios for each identified peptide will show the relative protein abundance between the two conditions compared. A major benefit of these approaches is that sample and reference may be mixed and processed as one which eliminates technical variation that may occur during sample treatment.

There are several options as to which label to use depending on the metabolic accessibility of the organism of interest. In case the organism of interest does not require complex nitrogen sources, generally the cheapest and easiest solution is ^{15}N-labelling. Here, cells are grown using a medium containing a single nitrogen source, usually an ammonium salt, for several generations during which incorporation of the label occurs naturally (Oda et al., 1999; Paša-Tolić et al., 1999; Washburn et al., 2002). As the number of nitrogen atoms per protein can vary MS data can become relatively complicated, however the power of modern mass spectrometers and accompanying data analysis programs make this only a minor limitation as long as a high degree of label incorporation is ensured. In one such application, metabolic labelling was used to study the spore outer layer maturation in terms of inter-protein cross-linking in *B. subtilis* (Abhyankar et al., 2015). In this work the spore coat protein digestion efficiency of unlabeled young spores was studied in relation to the protein digestion efficiency in mature 8 day old labelled spores and it was observed that the ^{14}N/^{15}N ratio (young vs. mature spores) decreased as the spores became older, inferring from this a possible increase in protein cross-linking in more mature spores. The tyrosine- and lysine-rich coat proteins CotC, CotG, CotU, CotY and CotZ were found to be refractive to protein digestion. In addition the thermal resistance of young spores was found to be less than the mature spores (Fig. 4). Finally this study also suggested that the heterogeneity in spore germination may also be caused by cross-linked coat polymers needed to be broken during germination (Abhyankar et al., 2015). In addition with a metabolic labelling approach it is possible to focus on the protein turn-over of germinating spores (manuscript under preparation) again giving an opportunity to exploit protein targets for spore outgrowth inhibition and hence improved preservation of food.

Alternatively, the SILAC (Stable Isotope Labelling by Amino acids in Cell culture) approach aims at incorporation of one or more amino acids per peptide containing various isotopes (Ong et al., 2002; Ong and Mann, 2006). Which amino acids are used generally depends on the protease deployed to digest the sample. As the gold standard protease trypsin cuts at arginine or lysine, replacing these two amino acids with an isotopically labelled variant ensures every tryptic peptide contains at least one labelled amino acid. As labelling needs to be complete so as not to misinterpret the isotope ratios obtained it is generally considered a requirement to have (mutant) strains which are auxotrophic for the amino acids used to label. A big advantage of SILAC over ^{15}N-labelling is that there are more experimental setups in which it may be applied. SILAC may be multiplexed for instance, by using normal (Arg^0/Lys^0), medium (Arg^6/Lys^4), and heavy forms (Arg^{10}/Lys^8) of these labelled amino acids to be able to compare three conditions in one experiment. Alternatively a response to perturbation may be investigated by pulsing with the heavy amino acids in tandem which will label only newly synthesized protein (Schwanhausser et al., 2009). For instance the SILAC approach used for a phosphoproteome study in *B. subtilis* lead to successful detection of 2264 proteins and 177 phosphorylation sites, and quantification of 1666 proteins and 64 phosphorylation sites in five phases of cellular growth (Ravikumar et al., 2014). Thus the information obtained through both ^{15}N-labelling and SILAC shows quantitative

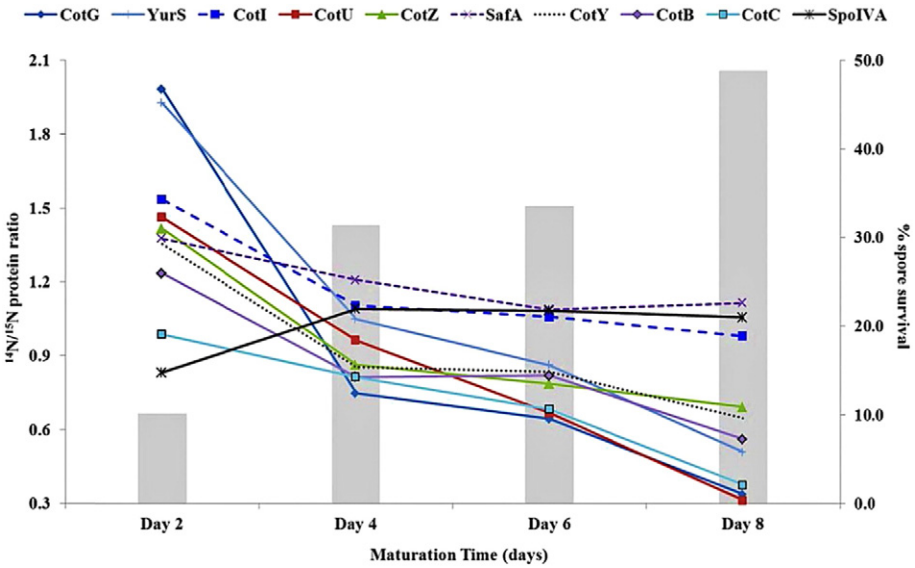

Fig. 4-. Isotope ratios, averaged over the identified peptides, from the crust, outer coat and inner coat proteins against the % survival of thermally stressed young to old spores. The primary Y-axis shows the change in the digestion extent of proteins as a function of $^{14}N/^{15}N$ ratios (line plots) while the secondary Y-axis shows the increase in the thermal resistance of spores as estimated by % survival rate (grey bars) of thermally stressed spores on tryptic soy agar plate after incubation for 24 h at 37 °C. Compared to younger spores reduced digestion extent for crust and outer coat proteins and higher thermal resistance (with initial heat inactivation at 70 °C for 30 min and thermal stress at 80 °C for 10 min; $P < 0.05$) is seen in more mature (day 6, day 8)spores when compared to younger (day 2) spores. Proteins CotG, YurS, CotI, CotU, CotB, CotC are localized in the outer coat while SafA, SpoIVA are inner coat proteins. CotY and CotZ are located in the crust. [Reprinted with permission from (Abhyankar et al., 2015), Copyright (2015), Elsevier].

changes in protein expression on a proteome wide scale which can be fed to the Risk Assessment models.

4.2.2. Chemical labelling

In case culturing the organism of interest is difficult, relative quantification strategies using chemical labelling may provide a solution. There are many different chemical labels available but the underlying principle is the same (Gygi et al., 1999; Ross et al., 2004; Thompson et al., 2003; Wiese et al., 2007). Analyte proteins are chemically modified with a different isotope label being used for each condition studied. The most prominent target is the free primary amine group found on lysine residues and the N-terminus as these are relatively abundant and thus lead to high label incorporation levels. As modifying the lysine at which tryptic cleavage should take place would interfere with digestion, modification generally takes places on the peptide level though there are cases where intact proteins are modified as well (Schmidt et al., 2005).

The most prominent methods used are isobaric tags for relative and absolute quantification (iTRAQ) (Wiese et al., 2007), and tandem mass tags (TMT) (Thompson et al., 2003). The nature of the tag introduced is different with iTRAQ being based on N-methylpiperazine derivatization, whereas TMT uses a small peptide as a tag but the eventual strategy is the same. After a peak has been selected in MS^1, fragmentation of the labelled peptides will release the tag which will split into a reporter group and a mass balance group. While the mass balance group is discarded, reporter ions of different masses will be detected in the MS/MS spectrum. Comparison of the intensity of the reporter ions then gives quantitative information. Using a variety of tags even allows for multiplexed analysis. Both methods provide an elegant solution to the problem that chemical modification may affect retention on the LC column by making tags isobaric so that differentially labelled peptides co-elute. The drawback to these methods in that the quantitative information is obtained from MS/MS spectra. Thus the same issue of DDA mentioned above arises in that only the top X peptides in a survey spectrum are selected and therefore quantified. This restricts application of these approaches to proteomes of limited complexity. Readers are referred to the studies performed on *B. subtilis* cells (Reddy et al., 2015) and *B. anthracis* spores (Jagtap et al., 2006) to understand the applications of iTRAQ methodology.

4.3. Absolute quantification

External labelled references consist of isotopically labelled peptides either synthesized in vitro in the case of AQUA i.e. Absolute quantification (Gerber et al., 2003) or concatenated into a synthetic protein which can be coded onto a plasmid and produced separately in the case of QconCAT or Quantitative concatemer (Beynon et al., 2005). In AQUA method, the synthetic peptides are spiked into the sample after protein digestion step. As the amount of reference peptide introduced is known, the resultant isotope ratios can then directly be converted to absolute abundance of the protein(s) of interest. To get reliable quantification results, proper quantification of the reference and the linearity of the response need to be assured. It is here that QconCAT gets the big advantage over AQUA as several peptides may be included in one protein. It is important to note that in the QconCAT approach the synthetic protein is spiked into the sample at the pre-digestion step. Depending on the number of Q-peptides selected per protein of interest around 25 proteins may be quantified using a single QconCAT. This severely reduces the amount of work required to verify each peptide. As they are present in the synthetic reference protein in a 1:1 ratio, concatenation of peptides of interest also provides direct stoichiometric information which is useful to determine ratios of interacting proteins. A unique benefit over other quantitative proteomics approaches is that the information obtained allows for relatively accurate determination of absolute protein abundances which can be related to copy numbers per cell. The potential downside of these external reference approaches is that they are targeted methods capable of quantifying only part of the proteome unless a large number of references are used (Brownridge et al., 2011). This limits their application to smaller sub-proteomes.

Considering the methods introduced above for quantitative investigations of spore proteome, several complications due to the nature of the system studied become apparent. First of all, label-free approaches are ill-suited due to the difficulty in isolating all the proteins from the spore due to their insoluble and crosslinked nature. Second, metabolic labelling in *B. cereus* has proven difficult due to metabolic preferences of the bacterium. Metabolic ^{15}N-labelling was attempted using ammonium salts as a sole nitrogen source in a medium based on Chemically-defined Growth & Sporulation medium (CDGS), however this severely decreased

sporulation efficiency (unpublished results). The primary nitrogen source in regular CDGS is glutamate, which is a preferred nitrogen source for *B. cereus*, appears to be required for efficient sporulation (de Vries et al., 2004, 2005; Kennedy et al., 1971). However, using glutamate as a means of introducing full ^{15}N-labelling is expensive. SILAC labelling requires auxotrophic strains which are not available in *B. cereus*. And finally, chemical labelling approaches are once again ill-suited due to the crosslinked nature of the spore proteins which may prevent proper labelling.

These biological complications may be circumvented by introducing a labelled reference externally. Furthermore in the interest of obtaining protein copy numbers per spore absolute quantitative approaches such as AQUA or QconCAT would give unique insight into interactions within the spore coat. Considering the scope of the spore specific proteome is quite small, estimated at <200 proteins (Henriques and Moran, 2007), a significant part of which may be covered using only a single QconCAT. In fact, the abundance of 21 spore proteins of *B. cereus* has been determined recently by Stelder and colleagues (manuscript under review) using a QconCAT approach. This is the first time absolute quantification of spore proteins is reported and marks the first application of QconCAT in a heterogeneous system paving the way for further quantitative studies.

5. Need of single-cell/spore studies

All the above mentioned proteomics approaches can be applied to spore albeit at a population level. Though these studies are comprehensive the inherent differences between different spores of single population cannot yet be pointed out. Preservatives like weak organic acids (e.g. sorbic acid), have been studied for their effects on spore germination and outgrowth, initially primarily at the population level as single cell tracing molecular analyses were generally not sensitive enough (Ter Beek et al., 2008). The individual analyses on cell physiology thus become crucial to frame the genomics/proteomics data into the queries on cellular heterogeneity. One such method, called SporeTracker, developed by Pandey and colleagues (Pandey et al., 2013, 2015a; Ter Beek et al., 2011) allows non-destructive marking of individual cells or spores and further usage of user defined macros in ImageJ (extension to ObjectJ) to analyze cell growth as well as the spore germination & outgrowth. In this approach the cell growth rate measurements are based on the pixel area calculation of cell(s) or spores. In case of spores the system allows one to study germination by changes in the pixel intensities of phase-dark spores. This system has made it possible for researchers to monitor single spores (in a population) and has helped explaining the stochastics of time to germination & outgrowth both with and without stress conditions as well as under the effect of certain antimicrobial compounds raging from well-known weak organic acids to tea leaves extract (Pandey et al., 2015a, 2015b). The success of this method was defined when it revealed a new event in spore germination - the 'burst time' (Pandey et al., 2013). Going further using this approach it could also be confirmed that at lower pHi (internal cell pH) values germinating spores have lower (out)growth rate (Ter Beek et al., 2015; van Beilen and Brul, 2013). Efforts have also been to understand the effects of salt, sorbic acid and temperature on *B. cereus* spore germination at a single spore level using flowcytometry (van Melis et al., 2014). The latter method has as major advantage that many more single spores/cells can be interrogated compared to the use of live-imaging. However, with the former the sequence of events in single spores can best be established, providing more deterministic biochemical information. The biggest advantage of single cell analysis is that it has opened avenues for the analyses at the single spore level that will prove instrumental in modelling effects of preservatives during outgrowth through their impact on the intra-spore and intracellular pH as well as on spore protein biochemistry. Thus establishment of single cell/spore level proteomics is also a contemporary need.

6. Conclusions

Summarizing, the genomics and proteomics techniques have developed to such an extent that it is routinely possible to comprehensively analyze the complete spore genome & proteome thereby establishing a link between the observed behaviour of spores under different antimicrobial stresses to its fundamental basis. A deconvolution study using labelled or label-free techniques in a quantitative proteomic assessment of protein synthesis and degradation during sporulation and germination is foreseen. Here the prime focus will be on those elements that can help to build risk assessment models for use to the food industries. Although the use of proteomics for analyzing the cross-linked proteins and proteomics of single spores is challenging, to make the quantitative proteomics data truly beneficial and readily applicable to building the risk assessment models, efforts need to be combined and use must be made of the state-of-the art technology available in many academic laboratories and industries alike.

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