Microbial metabolomics - techniques and applications; with a special focus on pathogens like *Staphylococcus aureus*

Microbial metabolomics - techniques and applications; with a special focus on pathogens like *Staphylococcus aureus*

M. Liebeke [1,2,3*]

Abstract

With the development of new functional genomics methods that can access the whole genome, transcriptome, proteome and metabolome more comprehensive insights in cellular processes are possible. Largely based on these advances, our knowledge about molecular constituents for many organisms is increasing at a tremendous rate. Until today, the genomes of several organisms including pathogen bacteria are already sequenced and pave the way for metabolic network constructions. With the help of microbial metabolomics (qualification and quantification of a huge variety of metabolites from a bacterium) deciphering of the bacterial metabolism is feasible. The metabolome pipeline or workflow encompasses the processes of sample generation and preparation, collection of analytical data, raw data pre-processing, data analysis and data integration. Cell sampling and metabolite extraction as well as analytical methods for metabolomics will be discussed in this review with specific interest in the application on pathogenic bacteria. Current developments and approaches for microbial metabolomics as well as drawbacks and pitfalls will be highlighted.

A special focus is given to applied metabolome studies on *Staphylococcus aureus*. This pathogen bacterium showed high attraction to be in the research focus for the interplay between metabolism and virulence.

Keywords

metabolomics, *Staphylococcus aureus*, metabolism, mass spectrometry (MS), nuclear magnetic resonance (NMR), gas chromatography (GC), liquid chromatography (LC)

[1]Institute of Pharmacy and [2]Interfaculty Institute for Genetics and Functional Genomics, Ernst-Moritz-Arndt-University of Greifswald, F.-L.-Jahnstr. 15, D-17487 Greifswald, Germany; [3]Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London, UK SW7 2AZ

[*]Corresponding author: Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London, UK SW7 2AZ, UK, Email: m.liebeke@imperial.ac.uk

Introduction

The complexity of biological systems poses an excellent scientific challenge to a comprehensive and integrative understanding of how components contribute to overall function. In recent years, advances in global genome, transcriptome, and proteome analysis provide access to these aspects of complexity. A direct link between such compositional data and the dynamic metabolic and physiological behavior of cellular systems is still not fully available. The `downstream` products of transcripts and proteins are the metabolites, which form the metabolome of an organism. The metabolome is defined as the quantitative complement of low-molecular-weight metabolites present in an organism under a given set of physiological conditions [1, 2]. Quantitative understanding of microbial metabolism and it's in vivo regulation requires knowledge of both extracellular and intracellular metabolites. Metabolomics terminology differentiates between quantitative analysis of both exometabolome and endometabolome referred as metabolite footprinting and metabolite fingerprinting, respectively [2, 3]. For an overview of terms and definitions and their relation among each other see **figure 1**.

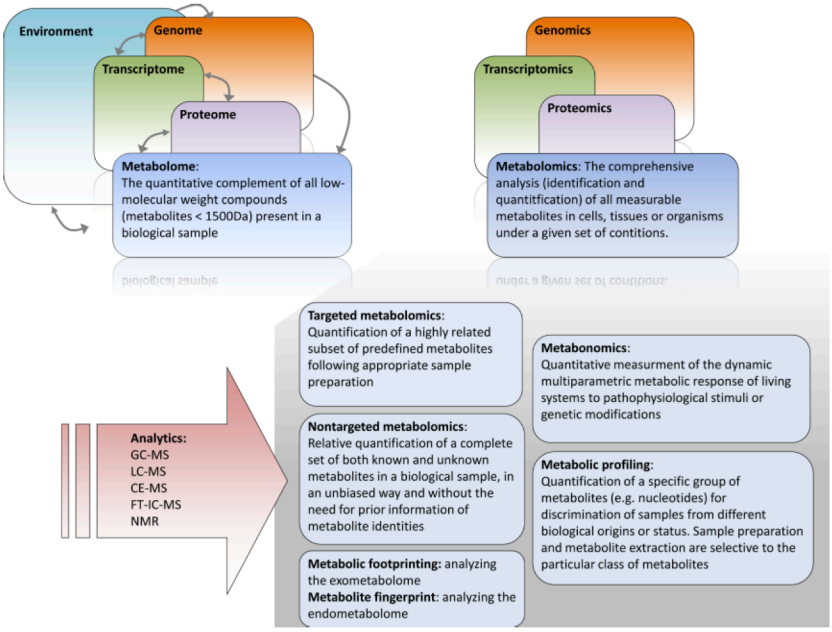

Fig.1 | Terms and definitions in the field of metabolomics. (Abbreviations: FT-IC- fourier transformation ion cyclotron mass spectrometry, MS- mass spectrometry, CE- capillary electrophoresis)

Constitution of a bacterial metabolome

The composition of the metabolome can vary considerably, depending on the analyzed organism. The plant kingdom comprises up to 200.000 primary and secondary metabolites [1, 4], less complex is the estimated *Saccharomyces cerevisiae* metabolome with 600 metabolites (<1500 Da) [5]. One basic question for microbial metabolome analysis is therefore: "How large is a bacterial metabolome?" Genome-scale models of bacterial metabolism gave hints for such metabolite numbers but refer only to calculations. Until now more than 20 constructed bacterial metabolic models exist [6], each show in average around 600 metabolites for diverse species. Based on genome annotations and model characteristics the metabolome differs between different calculations, e.g. *Bacillus subtilis* used 988 or 1138 compounds within the metabolic network [7, 8], whereas 537 remained after manual curation for analytical accessible metabolites [9]. In the gram-negative organism *Escherichia coli in silico* calculations result in 1039 compounds [10] and 694 metabolites after correction [9]. Genome-scale metabolic networks for the pathogen *Staphylococus aureus* posses over 700 metabolic reactions and calculated therefore 571 and 712 metabolites in two different calculations [11, 12]. Recently, a genome-scale comparison of 13 *S. aureus* strains showed a higher number of metabolic reactions (~1250) and metabolites (~1400) based on new reconstruction methodologies and whole-genome data [13]. In contrast, the bacterium with one of the smallest bacterial genome, *Mycoplasma genitalum* keeps 270 predicted metabolites [14]. Only limited data is available about the real metabolome of such pathogen bacteria. Perhaps, that is based on a focus of metabolic engineering in the past century driven by biotechnology implications, where the goal is to identify bottlenecks in biosynthesis routes in order to increase the flux from substrate to product [15].

To pinpoint a metabolite number for an organism, one has to consider the conditions which were observed during analysis. For that reason it is necessary to be aware about the fact that not every gene is always expressed and downstream products like proteins and metabolites do not exist under all given conditions, therefore calculated metabolite numbers are only reference values. There the next analytical task comes into account; different quantitative levels of metabolites were present in bacterial cells. For *E. coli* glutamate is the main metabolite ($9.6*10^{-2}$ mol$*$L^{-1}) and adenosine the less abundant measured metabolite ($1.3*10^{-7}$ mol$*$L^{-1}) [16] under glucose-fed conditions in exponential growth phase, resulting in five concentrations magnitudes between main solutes and less abundant ones. Complexity and varying distribution forces a sensitive and broad encompassing analytical method setting to decipher the bacterial metabolome.

Analytical platforms in metabolomics

Recent advances in biochemical analysis with the establishment of metabolomics were done

with tremendous effort. Modern hyphenated techniques with powerful separation and sensitive detection devices are feasible to analyze a vast number of different metabolites. Common separation methods in metabolic research are gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE). All were mostly coupled to mass spectrometry (MS) devices to get mass/structural information out of the signals. Reviews about these analytical techniques were given recently by others [17-19]. The use of chromatography introduces further complications in systematic and random variance from sample to sample analyses. Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry with a very high resolution minimizes the requirement for using upstream chromatography for complex mixtures by applying direct infusion mass spectrometry (DIMS) and makes the problem more tractable [20] and paves the way for larger replicate numbers than

metabolome is much more complex as assumed in the past [27]. Therefore none of above mentioned techniques, even in conjunction with each other can generate data for a complete metabolome description [28, 29]. **Figure 2** depicts the complexity of the metabolome and some techniques to decipher it.

GC-MS metabolome analysis

The GC-MS application in metabolomics has a longstanding and successful development history. Volatiles as well as non-volatiles that can be volatilized by derivatization procedures are targets of this method. For most compounds a derivatization procedure is mandatory and adds more preparative steps to the analytical protocol. In most protocols, the polar groups and active hydrogen atoms, found in –OH, NH, –COOH, –SH, and other functional groups, were trimethylsilylated by N methyl-N-trimethylsilyltrifluoro-acetamide (MSTFA) or N,O-bis-trimethylsilyltrifluoroacetamide (BSTFA) to gain

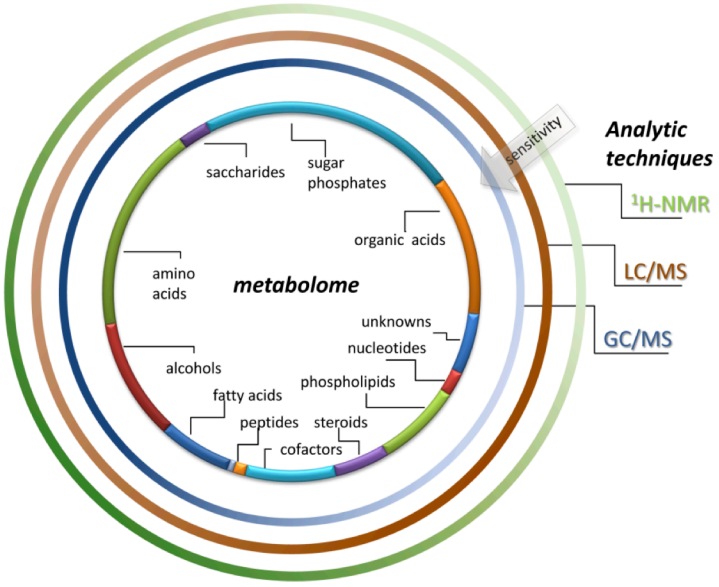

Fig.2 | Metabolomics scheme, inner circle represents different chemical classes of metabolites and a random distribution within a metabolome. Circles reflecting different common analytical techniques to qualify and quantify these classes, e.g. GC-MS (blue), LC-MS (orange) and ^{1}H-NMR (green). Without doubt, not one technique can solely discover the whole metabolome of an organism, therefore a color shade for each circle show the selectivity by intensity of color.

approaches with longer analysis time. This is needed for example for metabolome screening of huge gene knock out databases [21]. Another technique is nuclear magnetic resonance (NMR) spectroscopy; it provides fast and reproducible metabolite data and is one of the most reliable methods in the field of metabolomics [22-26]. The structural information gained for the chemical shift and signal coupling is one major advantage. But ^{1}H-NMR has its drawbacks as well, like limited capacity to reflect all signals of a complex mixture, like a cell extract has. Also, the detection limit is low compared to mass spectrometry. Today, it is obvious that the

more volatile compounds. By adding methoxyamine (MeOx) or ethoxyamine to the sample before silylation, the number of tautomeric forms of monosaccharides can be reduced while aldehyde or keto groups are converted to hydroxyamines or alkoxyamines. Many different protocols for the derivatization procedure of primary metabolites exist in the literature all with experiment-derived variations and originations of chromatographic peaks. These two fundamental issues could influence interpretation of metabolic data and therefore pathway interpretation [30, 31]. Recent developments in mass spectrometry and the combination of GC

Table 1 | Comparison of different metabolomics analytical techniques

Technology	Advantages	Disadvantages
GC-MS	- robust technology - retention time stability (enhanced using RTL) - quantitative (calibration) - good sensitivity (modest sample size needed) - high separation capacity - good quality software and databases for metabolite identification	- needs derivatization - sample is not recoverable - new metabolite identification is difficult - long chromatographic runs 20-60min
LC-MS	-flexible technology (potential to cover largest portion of metabolome) -high sensitivity (minimal sample size needed) -can be done without separation (direct injection)	- sample is not recoverable - quantification is elaborate - less robust instrumentation (compared to NMR and GC) - metabolite identification via peak trapping feasible
^{1}H-NMR	- quantitative - sample is recoverable - fast (2-10 min/sample), depends on sample size - no derivatization - robust instrumentation - structural information	- not very sensitive (high sample size needed) - no separation (fractions) - expensive instrumentation

with time-of-flight (TOF) detectors shortened the common analysis time dramatically. Higher scanning rates allow shorter chromatography (less than 15 min) with deconvolution of peaks by e.g. AMDIS software package (http://chemdata.nist.gov/mass-spc/amdis/) or software provided by commercial instrumentation (e.g., LECO ChromaTOF™) and makes GCxGC more feasible [32]. First insights via GCxGC into a bacterial metabolome were successfully applied [33]. To get the results out of two dimensional chromatography with mass spectral information new software is needed. A novel peak alignment algorithm was developed called DISCO (distance and spectrum correlation optimization)[34] and a software package where GC×GC chromatograms were used as contour plots which were then converted to gray-scale images and analyzed utilizing a workflow derived from 2D gel-based proteomics [35]. Progress is also made to shorten derivatization procedures through microwave irritation heating [36, 37] (see also **chapter 2**). Software and databases for peakfinding and identification are well developed in GC-MS analysis. There are commercially available huge metabolite databases (mass spectra of derivatized molecules) like NIST08 and Agilent®FiehnDB as well as free available ones like the golm metabolome database (GMDB) (see **table 4**). One drawback of GC-MS is the identification of unknown metabolites. Common profiles of bacterial or plant extract profiles have a majority of mass spectral tags (MSTs) which remain unidentified due to the lack of authenticated pure reference substances required for compound identification by GC-MS. Additional complications occur via derivatization. Exact masses for the metabolites or theirs fragments were altered by silyl-groups and prediction of molecular formula is therefore hampered. Hummel et al. provide a decision tree supported substructure prediction of metabolites out of the GMDB mass spectra collection which is an helpful tool for identification of unknowns [38].

Nevertheless, GC-MS metabolomics is favored in microbial metabolome investigations (metabolites <800 Da) [9, 39-42] because of its sensitivity, wide coverage of different compound groups like short carboxylic acids, monosaccharide's, alcohols, fatty acids, sugar-phosphates, sterols and more. All groups are analytically accessible only with variations in one technical set up.

LC-MS metabolome analysis

Another platform of chromatography coupled to MS is liquid chromatography (LC-MS). This method differs from GC-MS in distinct ways (see **table 1**). Based on different column and mobile phase chemistry, LC-MS analysis take place at lower temperatures and uses other ionization sources than GC-MS. Common sources like electrospray ionization (ESI) and less common like atmospheric pressure chemical ionization (APCI) do not require sample volatility which makes derivatization needless in most cases. Even though derivatization is helpful to improve ionization or chromatographic resolution in some cases[43]. HPLC is a versatile separation technique and a wide range of different applications are feasible. For bacterial metabolome analysis approaches like ion pairing (IP) methods [44-46], hydrophilic interaction liquid chromatography (HILIC) [47] and reversed phase (RP) [43] methods were used. All employed methods were specialized for very polar compounds of central metabolic pathways,

which have the greatest impact for bacterial physiological. Recently, the LC-MS methods were used more and more for absolute quantitative data generation [16] than for profiling projects. This work-intensive method needs tremendous efforts to generate calibration curves for each metabolite of hundreds in a day-to-day manner. A prerequisite is therefore a high standardized and robust analytical method to minimize errors. The defined aim is a number of metabolites per cell, in combination with other „omics" data in order to provide data for systems biology approaches.

One of the most important requirements in quantitative analysis by mass spectrometry is the use of suitable internal standards. The most similar compounds to the analytes are stable isotope labeled metabolites. Different approaches exist for their usage in microbial metabolomics. The isotope dilution approach [48] is one opportunity and the use of pure labeled standards is another one [49]. Besides those, a variety of internal standard compounds and methods exist in metabolome studies to standardizes and quantify data. Isotopic labeling can also be used for flux calculations based on tracer experiments and stoichiometric balancing. Fluxomics, very common in microbial physiology research is reviewed by others [50-52] and established technical protocols were described [53]. Mainly focused on central carbon metabolism, flux experiments were applied in biotechnology research [42, 54] and recently adopted to decipher the physiology of pathogen bacteria [55, 56]. These methods enable flux quantities and flux directions and give very helpful results for metabolic network interpretations.

NMR metabolome analysis

Nuclear magnetic resonance (NMR) spectroscopy is used for a variety of metabolomics approaches. Acquisition of ^{1}H, ^{13}C and ^{31}P spectra provides access to a large number of various metabolites. Based on the natural abundances and therefore depending acquisition times makes ^{1}H-NMR is the most preferred technique compared to other nuclei. As above mentioned, the two major drawbacks of NMR are missing separation of compounds and less sensitivity. The first problem is partly overcome by modern two-dimensional NMR experiments like J-resolved experiments which extend the spectral space. Sensitivity issues can be overcome by using NMR cryoprobes [57] or by using more biomass, which is often limited. Pattern recognition approaches were used for the study of different metabolome profiles finding discriminating metabolites between examined influence factors in bacteria [58, 59]. Using a more quantitative approach to study the extracellular pools and time-resolved concentration alterations, Behrends et al. show a vast number of conclusions out of the data from the metabolic footprint of *Pseudomonas aeruginosa* [60]. Quantification of metabolites by ^{1}H-NMR is really straight forward no need of calibration curves makes it preferable for fast and robust measurments in metabolomics. Using the metabolic footprint approach it was also feasible to characterize consumption behaivior of *B. licheniformis* growing in complex soil extract [61] (see also **chapter 1**) and to observe different phenotypes of *S. aureus* under changing conditions and to combine these data with e.g. proteome findings [62, 63]. Another popular application of NMR is ^{13}C-NMR, widely used for e.g. metabolic flux [64] or isotopologe studies [65]. These techniques were reviewed in detail [66].

Metabolome sampling

However, microbial metabolomics is not only about data generation, but primarily about translating metabolome data into biologically relevant information. For that purpose it is essential to provide representative snapshots of a bacterial metabolome. In particular a dataset has to present identical metabolite composition as that of the cells at the time-point of harvesting [67]. Concerning that demand, a metabolome analysis of a microorganism is much more challenging than that of proteomes and transcriptomes as the turnover of analytes is strikingly different. In common, proteins and mRNA changed after minutes or even hours but in enzymatic active solutions metabolites showed turnovers in the range of milliseconds and seconds. Therefore, accurate determination of intracellular metabolite levels requires well-validated procedures for sampling and sample handling. The first steps of sample treatment, like rapid sampling, quenching of metabolic activity, separation of extracellular medium (if possible) and metabolite extraction were shown as most crucial steps in microbial metabolomics.

Table 2 | Important characteristics of an microorganism for the development of a metabolome sampling protocol

Characteristic	Value		Question
Growth medium	- complex	- minimal, chemical defined	- washing/ no washing - Ionic strength of washing solution
Cultivation type	- suspension	- adherent (Intracellular?)	- filtering/scraping
Growth rate	- fast	- slow	- cell turnover rates, sampling apparatus or manual sampling
Accessible biomass/optical density	- high	- low (<mg)	- filtration problems, quenching solution problems
Phenotype	- Gram-positive	- Gram-negative	- susceptible to leakage, - cell wall thickness - mechanical disruption (bead mill, glass beads, ultrasonication, French pressure cell press) - or organic solvent leakage
	thermophil mesophil	psychrophil	- quenching with cold or hot solutions

Regarding recent data, the most challenging obstacles are **i)** complete and fast quenching/stopping of metabolism **ii)** powerful extraction of whole intracellular metabolite pool **iii)** avoidance of compound conversion or degradation during the process, ensuring enzyme inactivation or effects of the used solvents itself [41, 67-73] and **iv)** the obtained sample matrix should be in appropriate composition to the analytical method. In addition to that, it is known from various studies that a protocol for the generation of a metabolome sample is unique for each organism; perhaps even each strain needs an adopted protocol [67, 74, 75]. Many characteristics of the particular organism have to be regarded for protocol development (see **table 2**).

One major influence factor on a successful sampling protocol is the constitution of the cell membrane/wall. This determines susceptibility to leakage and is the most pronounced difference between Gram-positive and Gram-negative organisms. Direct quenching in an organic solvent is not feasible for Gram-negatives and yeast cells. It led to a drastic loss of metabolites due to unspecific leakage [67, 70, 71, 76]. Recent developments showed a prevention of leakage by using organic solvent/water buffered and cell isotonic quenching solutions as well as appropriate washing solutions [69, 71, 72, 77]. Leakage while quenching is an unintended side effect. The main goal is the effective inactivation of enzymes, but that depends also on different characteristics of the organism and the quenching procedure. One point mentioned in **table 2** is the habitat of the bacterium comparing thermophilic, mesophilic and psychrophilic organisms. Depending on normal temperature for the particular bacterium the chosen quenching solution temperature could be critical for stopping enzymatic reactions. Subsequently, after quenching metabolite extraction has to be performed. Several methods exist for efficient extraction of different metabolites, but the literature is contradictory regarding the adequacy and performance of each technique or solvent. In consideration of above mentioned problems it is not surprising that a vast number of different sampling methods exist for popular organisms like *E. coli*, *S. cerevisiae* and other [75, 78]. Each protocol is optimized for the question applied and enhanced by new developments in analytical methods. For most pathogen bacteria like *Streptococci*, *Enterococci* and *Mycoplasma* defined sampling protocols are still lacking. For *Staphylococcus aureus* growing in shaking flasks a fast filtration method was developed [74, 79] (see also **chapter 3**), this protocol for example is not applicable for other phylogenetically Gram-positives bacteria like *Bacillus subtilis* [54] and adherent growing *Mycoplasma pneumonia* [80]. Sampling via filtration is for some microorganisms applicable but there exist also other procedures with high efficiency. An automated sampling device coupled to a stirred

Table 3|Proposed metabolic-marker, reflecting quality of the sampling protocol

Marker value	Calculation	Values		References
Adenylate Energy Charge AEC	([ATP]+ 0.5[ADP])/ ([ATP] + [ADP] + [AMP])	0.8-0.95	-aerobic, non-limited conditions	Atkinson [117]
Redox-Balance - catabolic reduction charge	[NADH[/([NAD]$^+$+[NADH])	0.03-0.07	-aerobic, non-limited conditions	Andersen [118]
Glycolysis ratio	[Glucose-6-phosphate]/ [Fructose-6-phosphate]	~4.2	-this ratio quickly drops if glucose influx stops before metabolism is arrested or samples warm up before extraction	Ewald et al. [119]

tank reactor was developed for monitoring intracellular metabolite dynamics by Schaefer et al. [77] (automated fast sampling devices were reviewed by Schädel et al. [81]). Also a filter-culture approach [82] and a fast centrifugation method (<15 sec) [54] were developed. Analysis of extracellular and intracellular metabolites (biased by unavoidable leakage) by summation of their concentrations is also a common approach [83].

During the development of a sampling and extraction protocol above mentioned problems have to be observed carefully. Leakage and optimal extraction of metabolites can be tested by well organized experimental setups. Much more challenging is the determination of some proposed metabolic-marker for successful sampling. These values need the determination of absolute quantified metabolites, e.g. adenosine nucleotides concentrations for the adenylate energy charge (AEC) calculation (see **table 3**). The AEC is the most common marker for effective quenching, mainly affected by the ATP concentration, the value drops fast below 0.8 if energy consuming enzymes were still active during sampling [67, 74]. An established protocol for microbial metabolomics should therefore be published with all procedures together with minimum required reporting standards as stated by the metabolomics standards initiative [84].

The variety environmental influence factors concerning metabolome investigations can be studied with a sufficient sampling protocol in hand. This was successfully applied for e.g. *E. coli* [85], *Pseudomonas putida* [41] and *Corynebacterium glutamicum* [40]. These studies based on a change in the medium composition showed significant alterations in the metabolome profile or fingerprint, indicating the importance of a controlled cultivation especially of the chosen growth medium. All studies showed high metabolite dynamics under applied changing conditions reflecting the fast turnover capability of the bacterial metabolome. First quantitative metabolome data for microorganisms are rare but coming up recently in large number [16, 41, 75, 86]. For example, the intracellular *E. coli* metabolome was dominated, on a molar basis, by a small number of abundant compound classes: amino acids (49%), nucleotides (mainly ribonucleotide triphosphates, 15%), central carbon metabolism intermediates (15%), and redox cofactors and glutathione (9%) as detected by targeted LC-MS [16].

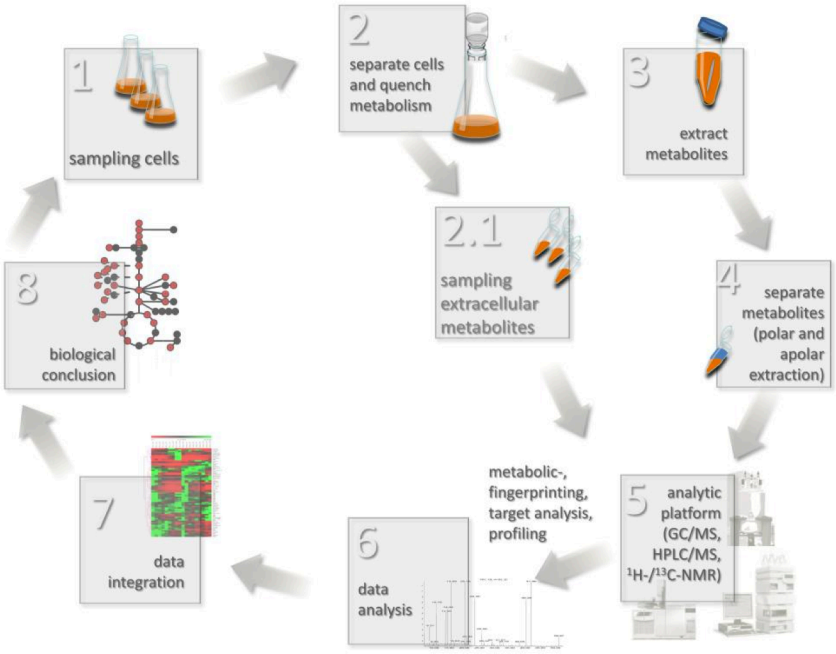

Fig.3 | Workflow for metabolome approaches, starting at cultivation of microorganisms under defined conditions **(1)**. Cells were separated from the growth medium and immediately quenched **(2)** for example by fast filtration and stop of metabolism with a cold organic solution followed by liquid N_2 freezing. Afterwards metabolites were extracted by different ways, simply with organic solvents like alcohols, acetonitrile and other, or by additional mechanical cell disruption via a beat mill or other procedures **(3)**. Extracts can further be separated by polar and apolar fractionation to simplify the sample matrix **(4)**. These extracts and the extracellular samples (yielded by easy filtration of growth medium) **(2.1)** were conducted to an analytical platform **(5)**. Samples can be used for profiling, quantification, metabolite fingerprints etc. and chromatograms or spectra were analyzed **(6)**. Obtained data were integrated in statistic models and visualization tools to validate information **(7)** for biological conclusions **(8)**.

As in this example, glutamine and nucleotides present often a main part of a bacterial metabolome, may based due to their central role in metabolism and diverse functions.

Data mining and metabolite identification

To date, metabolomics generates enormous data sets with the use of state of the art techniques and high-throughput approaches. Handling of these data requires a comprehensive and well adapted bioinformatics infrastructure. Using laboratory information management systems (LIMS), data storage with backup systems are essential tools in a metabolomics laboratory. Further, data extraction out of a vast number of chromatograms or spectra needs full attention and is often one major pitfall in metabolomics studies [87]. A variety of vendor software as well as open source software is available for peak-finding, -deconvolution, -integration etc. (review by Katajama et al. [88] and web source overview http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/Peak_Alignment/). Firstly non-biological effects like column changes, temperature differences and solvent differences for gradients should be corrected, in terms this affects shifts in retention time of peaks and baseline as well as detector response. Therefore it is essential to include a variety of internal standards into each sample to correct analysis in a standardized manner [89]. Secondly biological variability must be taken into account. This includes biomass (often as cell dry weight

Table 4 | Metabolomics software for data-handling of LC-MS, GC-MS and ^{1}H-NMR analysis as well as databases with detailed metabolite information (e.g. spectra, nominal mass, chemical shift)

Software for data-processing	LC-MS (MS^n)	GC-MS	NMR	References
BinBase http://fiehnlab.ucdavis.edu/projects/binbase_setupx/	+	+	-	[120, 121]
MetaboAnalyst http://www.metaboanalyst.ca	+	+	+	[122]
MetAlign http://www.metalign.wur.nl/UK/	+	+	-	[123]
Mzmine, mzmine2 http://mzmine.sourceforge.net/	+	+	-	[124]
XC/MS, XC/MS2 http://metlin.scripps.edu/download/	+	+	-	[125, 126]
AMDIS http://chemdata.nist.gov/mass-spc/amdis/	+	+	-	
Metabolite databases/Spectra libraries	**LC-MS (MS^n)**	**GC-MS**	**NMR**	**References**
Biological Magnetic Resonance Data Bank http://www.bmrb.wisc.edu/	-	-	+ >300	[127]
fiehn lib http://fiehnlab.ucdavis.edu/Metabolite-Library-2007/	+	+ >1.000	-	[120]
golm metabolome database http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/gmd.html	-	+	-	[128]
Human metabolome data base (HMBD) http://hmdb.ca/	+ >8.000	+ >2.000	+ >400	[129]
KNApSAcK - Comprehensive Species-Metabolite Relationship Database http://kanaya.naist.jp/KNApSAcK/	- >50.000	-	-	
MassBank http://www.massbank.jp/	+ >50.000	-	-	
Metlin http://metlin.scripps.edu/	+ >20.000	-	-	[130]
National Institute of Standards and Technology (NIST)	+ >150.000	+	+	
Pubchem http://pubchem.ncbi.nlm.nih.gov/	- >10.000.000	-	-	

measured), cell number and cell volume for concentration calculations, e.g. molar number of metabolite per µL of cell volume [16] or growth rate.

After all, one ends up with a list of metabolite values, with a significant number of unknown compounds. Handling of these is a major task in metabolome analysis. It is therefore essential sharing open access databases with as much as possible information about measured compounds [27]. In **table 4** some software examples and databases are shown. The analysis results should be in peak lists that include names of metabolites, entries of unknown compounds with unique nomenclature, quantitative data and information about the peak identification as well as quantification. With the ability to produce extreme large datasets, the problem to extract information out of these matrixes rises. Therefore the application of unbiased statistical data analysis tools could help. Multivariate statistical tools help to generate hypotheses by reducing mathematically the vast number of parameters in data sets and visualizing the clustering behavior of parameters. These could be helpful for finding e.g. biomarker or significant changes between samples of different origin (e.g. between aerobic/anaerobic conditions) or samples from different strains [90]. Details about different approaches and statistical models like principal component analysis or orthogonal-partial least square analysis were given in recent publications [90-92].

Metabolome studies of Staphylococcus aureus

Pathogenic bacteria are a major cause of human death and disease and cause infections such as cholera, sepsis, typhoid fever and tuberculosis. Some organisms, such as staphylococci present a class of commensal bacteria with hugh potential to burden humans. Its fast developing multi-resistance against antibiotics makes these bacteria so called "super bugs"[93]. A recent overview implicated the enormous importance to decipher the metabolism with regards to its influence on staphylococcal virulence [94]. Previous work shows for example the impact of the activity of tricarboxylic acid cycle (TCA) enzymes on the virulence of *S. aureus* [95, 96]. However, there is a lack of comprehensive qualitative and quantitative metabolomic data compared to large sets of genomic, transcriptomic and proteomic data available for *S. aureus* [97, 98]. Mainly based on ^{1}H-NMR exometabolome investigations, it was shown that *S. aureus* has an altered metabolism after deletion of single genes for TCA enzymes [63, 95]. Deletion of succinate dehydrogenase gene (*sdh*) provoked an accumulation of succinate in the medium and pinpoints the interruption of the TCA with consequences for biofilm production and aerobic growth. Amino acid consumption was decreased in the Δ*sdh* strain as a consequence of TCA interruption. Another study showed the impact of the presence or absence of the redox-sensing regulator Rex on the *S. aureus* metabolome [62]. Rex has an impact on fermentation pathway activity under aerobic and anaerobic conditions. Exometabolome data complemented proteome results of this study, e.g. an increase in formiate and ethanol producing enzymes was followed by respective metabolite accumulation. Relevance for Rex activity linked to virulence was given by the fact that lactate was produced under aerobic conditions in the mutant strain, which indicates a higher lactate dehydrogenase (Ldh) activity as in the wild-type strain. Interestingly, it has been shown that the activity of Ldh enables *S. aureus* to resist the innate immune response [99]. The findings of Pagels et al. [62] suggest that deactivation of Rex might be crucial for this phenomenon.

Results from Chatterjee et al. [100] propose that ClpC, as a part of the Clp ATPase/Clp protease system in *S. aureus* is involved in the expression regulation of genes and/or proteins of gluconeogenesis, the pentosephosphate pathway, and other central pathways. These changes in carbon metabolism result in alterations of the intracellular concentration of free NADH and e.g. fatty acid composition. In combination with proteome data the implemented genome-scale pathway analysis reveals strong evidence for ClpC as a critical factor in staphylococcal metabolism, stress regulation, and late-stationary growth phase survival. The change to stationary phase, initiated by glucose starvation was examined in more detail by a metabolome and proteome study [101] (see also **chapter 6**). The presented data consist of time-resolved metabolite and protein changes from *S. aureus* COL grown in chemical defined medium. Applying GC-MS and LC-MS as well as metabolic footprinting by ^{1}H-NMR, it was possible to detect 94 metabolites from *S. aureus*. These metabolome data where compared

with the results of 2D-gel based proteomics for more than 500 proteins. Interestingly, the correlation between both ,,omics"-technologies was high in central pathways but failed for some distinct examples. Especially the amino acid pools showed remarkable changes in concentrations (>100 fold changes), whereas the maximum detected protein change was about 2-10 fold comparing exponential growing versus stationary phase cells.

A combination of transcriptome and intracellular metabolome data was performed for *S. aureus* in a comparative study between wild-type strain and a serine/threonine kinase (PknB) and a respective phosphatase (Stp) mutant strains [102]. Differences in nucleotide metabolism were detected with both ,,omics"- techniques in the Δ*pknB* strain. Untargeted analysis of polar compounds by ion-pairing LC-MS showed remarkable differences in cell wall metabolism between these Δ*pknB*, Δ*stp* and wild-type strain [103](see also **chapter 7**). The findings showed an influence of this system on the peptidoglycan biosynthesis and pave the way for new investigations for e.g. antibiotic target searching regarding these systems. The urgent search for new anti-staphylococcal drugs and the elucidation of mode of action for new promising substance was basis of the study from Liu et al. [104]. They used GC-MS analysis to investigate the effect of frequently used and new antibiotics on the intracellular metabolome of *S. aureus*. It was possible to group the profiles of known substances into the same mode of action (MOA) classification in a statistical approach. The other way around, a prediction of the MOA from new compounds is entirely conceivable. This preliminary study shows the feasibility of metabolomics to elucidate MOA of new compounds and should be part of ongoing work in microbial metabolomics of pathogens. A very recent study shows the impact of staphylococcal secondary metabolites on virulence regulation. Wyatt et al. [105] isolated nonribosomal dipeptides (aureusimines) which act as direct regulators for virulence and may offer novel leads for anti-infective drugs.

The above mentioned studies are only examples for the tremendous effort of metabolome investigations on staphylococci. In summary, the last years in the post genomic era, have made substantial contribution for the understanding of bacterial physiology with the development and combination of new analytical as well bioinformathical methods. Especially studies of proteomics or transcriptomics in combination with metabolome analysis gave deeper insights into the complex regulatory network of e.g. staphylococci.

Future prospects

As stated in the introduction, deciphering the metabolome of a bacterium needs more than one technique. This issue of developing new methods must be part of metabolomics research in future. Furthermore, it is of crucial importance to analyze cell metabolism in the natural environment. This is promising for the generation of authentic physiological data. Some efforts to solve this problem were done in the areas of isotopologue analysis [55, 56], desorption electrospray ionization mass spectrometry (DESI) [106] and single cell analysis [107-109]. For human pathogens with obligate or facultative intracellular lifestyle, metabolism is strongly dependent on the host cell constitution. Recent data show that *in vivo* metabolism is remarkably different to *in vitro* conditions [110], e.g. growing in complex media within shaking flasks compared to cells grown in their natural habitat. Vice versa the host cells adapts to the intracellular present bacteria possibly with metabolic alterations [111]. Studying this interplay is of enormous interest for understanding pathogenicity or persistence of such bacteria. For example, *Listeria monocytogenes* replicates in the cytosol of murine macrophages. It was shown by in vivo ^{13}C-pertubation techniques that central carbon metabolism is modulated by the virulence regulator PrfA (positive regulatory factor A) [65] and different to that of extracellular bacteria growing in a defined glucose containing culture medium [56]. These studies analyzed the isotopic allocation of amino acids incooperated in the proteins of cells. Quenching of the cells is therefore not essentially important, since proteins were hydrolyzed to single amino acids and analyzed. More challenging is a real metabolomics approach adapted to intracellular living microorganisms and it will be one future task in many laboratories focusing on bacteria with intracellular lifestyle. The most crucial steps are (**i**) appropriate quenching of both cell types (**ii**) separating cells in acceptable time (**iii**) harvesting enough cells for metabolome profiling.

In contrast to these analytical problems, the bioinformatic interpretation to combine all multi-

major challenge. For bacterial multi-,,omics" studies a review is given by Zhang et al. [112]. As one example Protecs a comprehensive and powerful storage and analysis system for,,omics" data was build up [113]. This is an important step to explore multi-,,omics" data sets in microorganisms.

With respect to the above mentioned examples for future experiments the field of metabolomics needs new analytical techniques to cover a broader range of metabolites and to be more sensitive. Special applications for compound classes e.g. the huge variety of lipids (lipidomics) [114, 115] need more progress since these compound classes are important for comprehensive metabolome conclusions as recently shown for eukaryotic stem cell differentiation [116]. Finally, the questions about the complete composition and quantities of the bacterial metabolome are still unanswered but metabolomics is on the way to fill these gaps. The extension of our knowledge over the metabolome of bacteria will improve the description of their metabolic network and cellular phenomena in general.

References

[1] O. Fiehn, *Plant Mol Biol* **2002**, *48*, 155.
[2] S. G. Oliver, M. K. Winson, D. B. Kell, F. Baganz, *Trends Biotechnol* **1998**, *16*, 373.
[3] D. B. Kell, M. Brown, H. M. Davey, W. B. Dunn, I. Spasic, S. G. Oliver, *Nat Rev Microbiol* **2005**, *3*, 557.
[4] R. Mungur, A. D. Glass, D. B. Goodenow, D. A. Lightfoot, *J Biomed Biotechnol* **2005**, *2005*, 198.
[5] J. Forster, I. Famili, P. Fu, B. O. Palsson, J. Nielsen, *Genome Res* **2003**, *13*, 244.
[6] M. Durot, P. Y. Bourguignon, V. Schachter, *FEMS Microbiol Rev* **2009**, *33*, 164.
[7] Y. K. Oh, B. O. Palsson, S. M. Park, C. H. Schilling, R. Mahadevan, *J Biol Chem* **2007**, *282*, 28791.
[8] C. S. Henry, J. F. Zinner, M. P. Cohoon, R. L. Stevens, *Genome Biol* **2009**, *10*, R69.
[9] M. J. van der Werf, K. M. Overkamp, B. Muilwijk, L. Coulier, T. Hankemeier, *Anal Biochem* **2007**, *370*, 17.
[10] A. M. Feist, C. S. Henry, J. L. Reed, M. Krummenacker, A. R. Joyce, P. D. Karp, L. J. Broadbelt, V. Hatzimanikatis, B. O. Palsson, *Mol Syst Biol* **2007**, *3*, 121.
[11] S. A. Becker, B. O. Palsson, *BMC Microbiology* **2005**, *5*, 8.
[12] M. Heinemann, A. Kummel, R. Ruinatscha, S. Panke, *Biotechnol Bioeng* **2005**, *92*, 850.
[13] D. S. Lee, H. Burd, J. Liu, E. Almaas, O. Wiest, A. L. Barabasi, Z. N. Oltvai, V. Kapatral, *J Bacteriol* **2009**, *191*, 4015.
[14] P. F. Suthers, M. S. Dasika, V. S. Kumar, G. Denisov, J. I. Glass, C. D. Maranas, *PLoS Comput Biol* **2009**, *5*, e1000285.
[15] M. J. van der Werf, *Trends Biotechnol* **2005**, *23*, 11.
[16] B. D. Bennett, E. H. Kimball, M. Gao, R. Osterhout, S. J. Van Dien, J. D. Rabinowitz, *Nat Chem Biol* **2009**, *5*, 593.
[17] S. G. Villas-Boas, S. Mas, M. Akesson, J. Smedsgaard, J. Nielsen, *Mass Spectrom Rev* **2005**, *24*, 613.
[18] A. Scalbert, L. Brennan, O. Fiehn, T. Hankemeier, B. S. Kristal, B. van Ommen, E. Pujos-Guillot, E. Verheij, D. Wishart, S. Wopereis, *Metabolomics* **2009**, *5*, 435.
[19] Jun Han, Raju Datla, Sammy Chan, C. H. Borchers, *Bioanalysis* **2009**, *1*, 1665.
[20] S. C. Brown, G. Kruppa, J. L. Dasseux, *Mass Spectrom Rev* **2005**, *24*, 223.
[21] K. Nakahigashi, Y. Toya, N. Ishii, T. Soga, M. Hasegawa, H. Watanabe, Y. Takai, M. Honma, H. Mori, M. Tomita, *Mol Syst Biol* **2009**, *5*, 306.
[22] J. P. Grivet, A. M. Delort, J. C. Portais, *Biochimie* **2003**, *85*, 823.
[23] J. L. Griffin, *Curr Opin Chem Biol* **2003**, *7*, 648.
[24] N. V. Reo, *Drug Chem Toxicol* **2002**, *25*, 375.
[25] C. Ludwig, M. R. Viant, *Phytochem Anal* **2010**, *21*, 22.
[26] O. Beckonert, H. C. Keun, T. M. Ebbels, J. Bundy, E. Holmes, J. C. Lindon, J. K. Nicholson, *Nat Protoc* **2007**, *2*, 2692.
[27] T. Kind, M. Scholz, O. Fiehn, *PLoS One* **2009**, *4*, e5440.
[28] J. M. Buscher, D. Czernik, J. C. Ewald, U. Sauer, N. Zamboni, *Anal Chem* **2009**, *81*, 2135.
[29] J. L. Griffin, *Philos Trans R Soc Lond B Biol Sci* **2006**, *361*, 147.
[30] H. Kanani, P. K. Chrysanthopoulos, M. I. Klapa, *J Chromatogr B Analyt Technol Biomed Life Sci* **2008**, *871*, 191.
[31] F. Xu, L. Zou, C. N. Ong, *J Proteome Res* **2009**, *8*, 5657.
[32] J. W. Allwood, A. Erban, S. de Koning, W. B. Dunn, A. Luedemann, A. Lommen, L. Kay, R. Loscher, J. Kopka, R. Goodacre, *Metabolomics* **2009**, *5*, 479.
[33] S. Yang, M. Sadilek, R. E. Synovec, M. E. Lidstrom, *J Chromatogr A* **2009**, *1216*, 3280.
[34] B. Wang, A. Fang, J. Heim, B. Bogdanov, S. Pugh, M. Libardoni, X. Zhang, *Anal Chem* **2010**, *82*, 5069.
[35] H. G. Schmarr, J. Bernhardt, *J Chromatogr A* **2010**, *1217*, 565.
[36] K. A. Kouremenos, J. J. Harynuk, W. L. Winniford, P. D. Morrison, P. J. Marriott, *J Chromatogr B Analyt Technol Biomed Life Sci* **2010**, *878*, 1761.
[37] M. Liebeke, A. Wunder, M. Lalk, *Anal Biochem* **2009**, *401*, 312.
[38] J. Hummel, N. Strehmel, J. Selbig, D. Walther, J. Kopka, *Metabolomics* **2010**, *6*, 322.
[39] M. M. Koek, B. Muilwijk, M. J. van der Werf, T. Hankemeier, *Anal Chem* **2006**, *78*, 1272.
[40] S. Strelkov, M. von Elstermann, D. Schomburg, *Biological Chemistry* **2004**, *385*, 853.
[41] M. J. van der Werf, K. M. Overkamp, B. Muilwijk, M. M. Koek, B. J. C. van der Werff-van der Vat, R. H. Jellema, L. Coulier, T. Hankemeier, *Molecular Biosystems* **2008**, *4*, 315.
[42] J. Becker, C. Klopprogge, H. Schroder, C. Wittmann, *Appl Environ Microbiol* **2009**, *75*, 7866.
[43] W. C. Yang, M. Sedlak, F. E. Regnier, N. Mosier, N. Ho, J. Adamec, *Anal Chem* **2008**, *80*, 9508.
[44] J. M. Buescher, S. Moco, U. Sauer, N. Zamboni, *Anal Chem* **2010**, *82*, 4403.
[45] L. Coulier, R. Bas, S. Jespersen, E. Verheij, M. J. van der Werf, T. Hankemeier, *Anal Chem* **2006**, *78*, 6573.
[46] W. Lu, M. F. Clasquin, E. Melamud, D. Amador-Noguez, A. A. Caudy, J. D. Rabinowitz, *Anal Chem* **2010**, *82*, 3212.
[47] S. U. Bajad, W. Lu, E. H. Kimball, J. Yuan, C. Peterson, J. D. Rabinowitz, *J Chromatogr A* **2006**, *1125*, 76.
[48] L. Wu, M. R. Mashego, J. C. van Dam, A. M. Proell, J. L. Vinke, C. Ras, W. A. van Winden, W. M. van Gulik, J. J. Heijnen, *Anal Biochem* **2005**, *336*, 164.
[49] B. D. Bennett, J. Yuan, E. H. Kimball, J. D. Rabinowitz, *Nat Protoc* **2008**, *3*, 1299.

[50] Y. J. Tang, H. G. Martin, S. Myers, S. Rodriguez, E. E. Baidoo, J. D. Keasling, *Mass Spectrom Rev* **2009**, *28*, 362.

[51] C. Wittmann, *Microb Cell Fact* **2007**, *6*, 6.

[52] N. Zamboni, U. Sauer, *Curr Opin Microbiol* **2009**, *12*, 553.

[53] N. Zamboni, S. M. Fendt, M. Ruhl, U. Sauer, *Nat Protoc* **2009**, *4*, 878.

[54] R. J. Kleijn, J. M. Buescher, L. Le Chat, M. Jules, S. Aymerich, U. Sauer, *J Biol Chem* **2009**, *285*, 1587.

[55] A. Schmid, W. Neumayer, K. Trulzsch, L. Israel, A. Imhof, M. Roessle, G. Sauer, S. Richter, S. Lauw, E. Eylert, W. Eisenreich, J. Heesemann, G. Wilharm, *J Biol Chem* **2009**, *284*, 12165.

[56] E. Eylert, J. Schar, S. Mertins, R. Stoll, A. Bacher, W. Goebel, W. Eisenreich, *Mol Microbiol* **2008**, *69*, 1008.

[57] D. Carrieri, K. McNeely, A. C. De Roo, N. Bennette, I. Pelczer, G. C. Dismukes, *Magn Reson Chem* **2009**, *47 Suppl 1*, S138.

[58] A. F. Boroujerdi, M. I. Vizcaino, A. Meyers, E. C. Pollock, S. L. Huynh, T. B. Schock, P. J. Morris, D. W. Bearden, *Environ Sci Technol* **2009**, *43*, 7658.

[59] E. L. Gjersing, J. L. Herberg, J. Horn, C. M. Schaldach, R. S. Maxwell, *Anal Chem* **2007**.

[60] V. Behrends, T. M. Ebbels, H. D. Williams, J. G. Bundy, *Appl Environ Microbiol* **2009**, *75*, 2453.

[61] M. Liebeke, V. S. Brozel, M. Hecker, M. Lalk, *Appl Microbiol Biotechnol* **2009**, *83*, 161.

[62] M. Pagels, S. Fuchs, J. Pané-Farré, C. Kohler, L. Menschner, M. Hecker, P. J. McNamarra, M. C. Bauer, C. v. Wachenfeldt, M. Liebeke, M. Lalk, G. Sander, C. v. Eiff, R. A. Proctor, S. Engelmann, *Molecular Microbiology* **2010**, *76*, 1142.

[63] R. Gaupp, S. Schlag, M. Liebeke, M. Lalk, F. Gotz, *Journal of Bacteriology* **2010**, *192*, 2385.

[64] A. Rantanen, J. Rousu, P. Jouhten, N. Zamboni, H. Maaheimo, E. Ukkonen, *BMC Bioinformatics* **2008**, *9*, 266.

[65] W. Eisenreich, J. Slaghuis, R. Laupitz, J. Bussemer, J. Stritzker, C. Schwarz, R. Schwarz, T. Dandekar, W. Goebel, A. Bacher, *Proc Natl Acad Sci U S A* **2006**, *103*, 2040.

[66] T. W. M. Fan, A. N. Lane, *Progress in Nuclear Magnetic Resonance Spectroscopy* **2008**, *52*, 69.

[67] C. J. Bolten, P. Kiefer, F. Letisse, J. C. Portais, C. Wittmann, *Analytical Chemistry* **2007**, *79*, 3843.

[68] E. Kimball, J. D. Rabinowitz, *Anal Biochem* **2006**, *358*, 273.

[69] S. G. Villas-Boas, P. Bruheim, *Anal Biochem* **2007**, *370*, 87.

[70] A. B. Canelas, C. Ras, A. ten Pierick, J. C. van Dam, J. J. Heijnen, W. M. Van Gulik, *Metabolomics* **2008**, *4*, 226.

[71] M. Faijes, A. E. Mars, E. J. Smid, *Microb Cell Fact* **2007**, *6*, 27.

[72] J. Spura, L. C. Reimer, P. Wieloch, K. Schreiber, S. Buchinger, D. Schomburg, *Anal Biochem* **2009**, *394*, 192.

[73] C. Wittmann, J. O. Kromer, P. Kiefer, T. Binz, E. Heinzle, *Analytical Biochemistry* **2004**, *327*, 135.

[74] H. Meyer, M. Liebeke, M. Lalk, *Anal Biochem* **2010**, *401*, 250.

[75] A. B. Canelas, A. Ten Pierick, C. Ras, R. M. Seifar, J. C. van Dam, W. M. van Gulik, J. J. Heijnen, *Anal Chem* **2009**, *81*, 7379.

[76] C. Wittmann, J. O. Kromer, P. Kiefer, T. Binz, E. Heinzle, *Anal Biochem* **2004**, *327*, 135.

[77] U. Schaefer, W. Boos, R. Takors, D. Weuster-Botz, *Anal Biochem* **1999**, *270*, 88.

[78] M. R. Mashego, K. Rumbold, M. De Mey, E. Vandamme, W. Soetaert, J. J. Heijnen, *Biotechnology Letters* **2007**, *29*, 1.

[79] X. H. Wu, H. L. Yu, Z. Y. Ba, J. Y. Chen, H. G. Sun, B. Z. Han, *Biotechnol J* **2010**, *5*, 75.

[80] E. Yus, T. Maier, K. Michalodimitrakis, V. van Noort, T. Yamada, W. H. Chen, J. A. Wodke, M. Guell, S. Martinez, R. Bourgeois, S. Kuhner, E. Raineri, I. Letunic, O. V. Kalinina, M. Rode, R. Herrmann, R. Gutierrez-Gallego, R. B. Russell, A. C. Gavin, P. Bork, L. Serrano, *Science* **2009**, *326*, 1263.

[81] F. Schadel, E. Franco-Lara, *Appl Microbiol Biotechnol* **2009**, *83*, 199.

[82] M. J. Brauer, J. Yuan, B. D. Bennett, W. Lu, E. Kimball, D. Botstein, J. D. Rabinowitz, *Proc Natl Acad Sci U S A* **2006**, *103*, 19302.

[83] H. Taymaz-Nikerel, M. de Mey, C. Ras, A. ten Pierick, R. M. Seifar, J. C. van Dam, J. J. Heijnen, W. M. van Gulik, *Anal Biochem* **2009**, *386*, 9.

[84] M. J. van der Werf, R. Takors, J. Smedsgaard, J. Nielsen, T. Ferenci, J. C. Portais, C. Wittmann, M. Hooks, A. Tomassini, M. Oldiges, J. Fostel, U. Sauer, *Metabolomics* **2007**, *3*, 189.

[85] V. M. Boer, C. A. Crutchfield, P. H. Bradley, D. Botstein, J. D. Rabinowitz, *Mol Biol Cell* **2009**, *21*, 198.

[86] E. Marcellin, L. K. Nielsen, P. Abeydeera, J. O. Kromer, *Biotechnol J* **2009**, *4*, 58.

[87] L. Burton, G. Ivosev, S. Tate, G. Impey, J. Wingate, R. Bonner, *J Chromatogr B Analyt Technol Biomed Life Sci* **2008**, *871*, 227.

[88] M. Katajamaa, M. Oresic, *J Chromatogr A* **2007**, *1158*, 318.

[89] H. Redestig, A. Fukushima, H. Stenlund, T. Moritz, M. Arita, K. Saito, M. Kusano, *Anal Chem* **2009**, *81*, 7974.

[90] L. W. Sumner, E. Urbanczyk-Wochniak, C. D. Broeckling, *Methods Mol Biol* **2007**, *406*, 409.

[91] D. S. Wishart, *Methods Mol Biol*, *593*, 283.

[92] J. Trygg, E. Holmes, T. Lundstedt, *J Proteome Res* **2007**, *6*, 469.

[93] D. J. Payne, *Science* **2008**, *321*, 1644.

[94] G. A. Somerville, R. A. Proctor, *Microbiol Mol Biol Rev* **2009**, *73*, 233.

[95] Y. Zhu, E. C. Weiss, M. Otto, P. D. Fey, M. S. Smeltzer, G. A. Somerville, *Infect Immun* **2007**, *75*, 4219.

[96] Y. Zhu, Y. Q. Xiong, M. R. Sadykov, P. D. Fey, M. G. Lei, C. Y. Lee, A. S. Bayer, G. A. Somerville, *Infect Immun* **2009**, *77*, 4256.

[97] D. Becher, K. Hempel, S. Sievers, D. Zuhlke, J. Pane-Farre, A. Otto, S. Fuchs, D. Albrecht, J. Bernhardt, S. Engelmann, U. Volker, J. M. van Dijl, M. Hecker, *PLoS One* **2009**, *4*, e8176.

[98] K. Becker, G. Bierbaum, C. von Eiff, S. Engelmann, F. Gotz, J. Hacker, M. Hecker, G. Peters, R. Rosenstein, W. Ziebuhr, *Int J Med Microbiol* **2007**, *297*, 483.

[99] A. R. Richardson, S. J. Libby, F. C. Fang, *Science* **2008**, *319*, 1672.

[100] I. Chatterjee, S. Schmitt, C. F. Batzilla, S. Engelmann, A. Keller, M. W. Ring, R. Kautenburger, W. Ziebuhr, M. Hecker, K. T. Preissner, M. Bischoff, R. A. Proctor, H. P. Beck, H. P. Lenhof, G. A. Somerville, M. Herrmann, *Proteomics* **2009**, *9*, 1152.

[101] M. Liebeke, K. Doerries, D. Zuhlke, R. Bode, S. Fuchs, S. Engelmann, U. Voelker, M. Hecker, M. Lalk, *in preparation*.

[102] S. Donat, K. Streker, T. Schirmeister, S. Rakette, T. Stehle, M. Liebeke, M. Lalk, K. Ohlsen, *J Bacteriol* **2009**, *191*, 4056.

[103] M. Liebeke, H. Meyer, S. Donat, K. Ohlsen, M. Lalk, *Chem Biol* **2010**, *in press*.

[104] Y. Liu, J. Wen, Y. Wang, W. Xu, *Chromatographia* **2010**, *71*, 253.

[105] M. A. Wyatt, W. Wang, C. M. Roux, F. C. Beasley, D. E. Heinrichs, P. M. Dunman, N. A. Magarvey, *Science* **2010**, *329*, 294.

[106] A. U. Jackson, S. R. Werner, N. Talaty, Y. Song, K. Campbell, R. G. Cooks, J. A. Morgan, *Anal Biochem* **2008**, *375*, 272.
[107] S. Ishii, K. Tago, K. Senoo, *Appl Microbiol Biotechnol* **2010**, *86*, 1281.
[108] A. Amantonico, J. Y. Oh, J. Sobek, M. Heinemann, R. Zenobi, *Angew Chem Int Ed Engl* **2008**, *47*, 5382.
[109] A. Amantonico, P. L. Urban, R. Zenobi, *Anal Bioanal Chem*.
[110] E. J. Munoz-Elias, J. D. McKinney, *Cell Microbiol* **2006**, *8*, 10.
[111] S. A. Brown, K. L. Palmer, M. Whiteley, *Nat Rev Microbiol* **2008**, *6*, 657.
[112] W. Zhang, F. Li, L. Nie, *Microbiology* **2010**, *156*, 287.
[113] S. Fuchs, H. Mehlan, H. Kusch, A. Teumer, D. Zuhlke, M. Berth, C. Wolf, T. Dandekar, M. Hecker, S. Engelmann, J. Bernhardt, *Proteomics* **2010**, in press.
[114] W. J. Griffiths, Y. Wang, *Chem Soc Rev* **2009**, *38*, 1882.
[115] C. Hu, R. van der Heijden, M. Wang, J. van der Greef, T. Hankemeier, G. Xu, *J Chromatogr B Analyt Technol Biomed Life Sci* **2009**, *877*, 2836.
[116] O. Yanes, J. Clark, D. M. Wong, G. J. Patti, A. Sanchez-Ruiz, H. P. Benton, S. A. Trauger, C. Desponts, S. Ding, G. Siuzdak, *Nat Chem Biol* **2010**, *6*, 411.
[117] D. E. Atkinson, *Biochemistry* **1968**, *7*, 4030.
[118] K. B. Andersen, K. von Meyenburg, *J Biol Chem* **1977**, *252*, 4151.
[119] J. C. Ewald, S. Heux, N. Zamboni, *Anal Chem* **2009**, *81*, 3623.
[120] T. Kind, G. Wohlgemuth, Y. Lee do, Y. Lu, M. Palazoglu, S. Shahbaz, O. Fiehn, *Anal Chem* **2009**, *81*, 10038.
[121] M. Scholz, O. Fiehn, *Pac Symp Biocomput* **2007**, 169.
[122] J. Xia, N. Psychogios, N. Young, D. S. Wishart, *Nucleic Acids Res* **2009**, *37*, W652.
[123] A. Lommen, *Anal Chem* **2009**, *81*, 3079.
[124] M. Katajamaa, J. Miettinen, M. Oresic, *Bioinformatics* **2006**, *22*, 634.
[125] C. A. Smith, E. J. Want, G. O'Maille, R. Abagyan, G. Siuzdak, *Anal Chem* **2006**, *78*, 779.
[126] H. P. Benton, D. M. Wong, S. A. Trauger, G. Siuzdak, *Anal Chem* **2008**, *80*, 6382.
[127] E. L. Ulrich, H. Akutsu, J. F. Doreleijers, Y. Harano, Y. E. Ioannidis, J. Lin, M. Livny, S. Mading, D. Maziuk, Z. Miller, E. Nakatani, C. F. Schulte, D. E. Tolmie, R. Kent Wenger, H. Yao, J. L. Markley, *Nucleic Acids Res* **2008**, *36*, D402.
[128] J. Kopka, N. Schauer, S. Krueger, C. Birkemeyer, B. Usadel, E. Bergmuller, P. Dormann, W. Weckwerth, Y. Gibon, M. Stitt, L. Willmitzer, A. R. Fernie, D. Steinhauser, *Bioinformatics* **2005**, *21*, 1635.
[129] D. S. Wishart, C. Knox, A. C. Guo, R. Eisner, N. Young, B. Gautam, D. D. Hau, N. Psychogios, E. Dong, S. Bouatra, R. Mandal, I. Sinelnikov, J. Xia, L. Jia, J. A. Cruz, E. Lim, C. A. Sobsey, S. Shrivastava, P. Huang, P. Liu, L. Fang, J. Peng, R. Fradette, D. Cheng, D. Tzur, M. Clements, A. Lewis, A. De Souza, A. Zuniga, M. Dawe, Y. Xiong, D. Clive, R. Greiner, A. Nazyrova, R. Shaykhutdinov, L. Li, H. J. Vogel, I. Forsythe, *Nucleic Acids Res* **2009**, *37*, D603.
[130] C. A. Smith, G. O'Maille, E. J. Want, C. Qin, S. A. Trauger, T. R. Brandon, D. E. Custodio, R. Abagyan, G. Siuzdak, *Ther Drug Monit* **2005**, *27*, 747.