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Molecular phenotyping approaches for the detection and monitoring of carbapenem-resistant Enterobacteriaceae by mass spectrometry

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ABSTRACT

Antimicrobial resistance is increasing in prevalence and there is a clear need for the development of rapid detection methods in clinical diagnostics. This review explores –omics studies utilising mass spectrometry to investigate the molecular phenotype associated with carbapenem resistance. Whilst the specific mechanisms of carbapenem resistance are well characterised, the resistant phenotype is poorly understood. Understanding how the acquisition of resistance affects cellular physiology and cell metabolism through molecular phenotyping is a necessary step towards detecting resistance by diagnostic means. In addition, this article examines the potential of mass spectrometry for the identification of resistance biomarkers through molecular profiling of bacteria. Developments in mass spectrometry platforms are expanding the biomarker-based diagnostic landscape. Targeted measures, such as high-resolution mass spectrometry coupled with chromatographic separation show considerable promise for the identification of molecular signatures and the development of a rapid diagnostic assay for the detection of carbapenem resistance.

1. Introduction

Carbapenems are a broad-spectrum class of β-lactam antibiotic comprised of a β-lactam ring fused to a 5-membered pyrroline ring. The unique structure and stereochemistry of carbapenems (Fig. 1) bestows a wider spectrum of antimicrobial activity compared with other β-lactams, such as penicillins and cephalosporins, and augmented stability against hydrolysis by non-carbapenemase β-lactamases [1,2]. Carbapenems are, consequently, regarded as last-resort antibiotic therapies and are used in the treatment of infections, such as those caused by extended-spectrum-β-lactamase (ESBL) producing Enterobacteriaceae.

Carbapenem-resistant Enterobacteriaceae (CRE) constitute a major global health problem, highlighted by their classification as critical priority pathogens by the World Health Organisation [3]. Enterobacteriaceae are a family of Gram-negative bacteria, which includes *Escherichia coli* and *Klebsiella pneumoniae*. Whilst some members are beneficial commensal microbiota of the gastrointestinal tract, others have far greater pathogenic potential. Moreover, Enterobacteriaceae are a major source of nosocomial infections including ventilator-associated pneumonia, hospital-acquired urinary tract infections, surgical site infections, and septicaemia [4,5]. The consequence of antimicrobial resistance (AMR) within these highly transmissible organisms, particularly to carbapenems, is of particular concern.

Whilst the natural development of drug resistance is inevitable through evolutionary means, the rate at which carbapenem resistance (CR) has emerged has been exacerbated by factors including the volume of antibiotics utilised across sectors such as medicine and agriculture, in addition to the dissemination of resistant microorganisms [6–13].

Abbreviations: AMR, antimicrobial resistance; CP, carbapenemase-producing; CRE, carbapenem-resistant Enterobacteriaceae; CR, carbapenem-resistance; CRO, carbapenem-resistant organism; DI, direct infusion; FAME, fatty acid methyl ester; FTIR, Fourier-transform infrared spectroscopy; GC, gas chromatography; HILIC, hydrophilic interaction liquid chromatography; IM, ion mobility; KPC, *Klebsiella pneumoniae* carbapenemase; LC, liquid chromatography; MALDI-TOF MS, matrix-assister laser desorption/ionisation-time of flight mass spectrometry; MIC, minimum inhibitory concentration; MOLI, metal oxide laser ionisation; MOS, metal oxide sensor; MRSA, methicillin-resistant *Staphylococcus aureus*; MS, mass spectrometry; NMR, nuclear magnetic resonance; OMV, outer membrane vesicle; PTM, post-translational modification; SESI, secondary electrospray ionisation; SIFT, selected-ion flow-tube; SPME, solid phase microextraction; TOF, time of flight.

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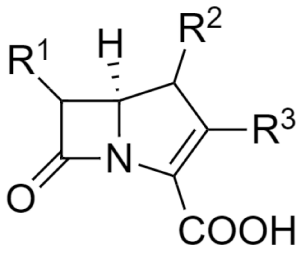

Fig. 1. Core structure for the carbapenem class of molecules.

Unsurprisingly, an upsurge in the clinical use of carbapenems, due to over-prescription combined with a lack of alternative options, has led to the subsequent increase of carbapenem-resistant organisms (CRO) [14,15]. CR in Enterobacteriaceae is mainly attributable to the acquisition of genes encoding carbapenemases, which hydrolyse and inactivate the carbapenem molecule [16]. Furthermore, cell membrane porin mutations may also confer resistance, especially in conjunction with AmpC overexpression [17,18]. The mechanisms of CR in Enterobacteriaceae are presented in Fig. 2.

Carbapenemases are typically encoded on highly mobile genetic elements, such as plasmids, which may be horizontally transferred between bacterial cells and species [19]. This signifies that the resistance itself is transmissible, a phenomenon which poses a significant threat in the healthcare environment. Rapidly identifying and isolating patients infected with carbapenemase-producing organisms (CPO) is, therefore, paramount to reducing the prevalence of nosocomial infections affiliated with carbapenem-resistant bacteria and the subsequent spread of resistance [20]. Furthermore, given the high mortality rates associated with CR infections, rapid detection will facilitate appropriate and timely treatment to improve patient outcomes [21]. Measures used in clinical practice require significant re-evaluation and improvements to meet these needs. Currently, routine clinical methodologies involve incubation periods of up to 72 h, and are based on ambiguous breakpoint criteria [22]. There is a need for rapid and accurate detection methods to improve how we manage such highly resistant infections in the healthcare environment.

Developments in MS platforms are rapidly expanding the biomarker-based diagnostic landscape, enabling analysis of complex biological matrices with high sensitivity. Furthermore, molecular phenotyping by MS shows immense potential for the development of robust and accurate AMR detection methods, which may be translated into routine and high-throughput clinical diagnostics. This review will explore recent developments in MS-based molecular phenotyping approaches in the fields of proteomics, lipidomics and metabolomics for the detection of CRE and identify avenues for future exploration. The techniques examined are depicted in Table 1, with an evaluation of the associated advantages and shortfalls.

2. Proteomics

2.1. The bacterial proteome

As with eukaryotes, bacterial protein networks mediate most cellular processes. Consequently, the bacterial proteome is highly dynamic and changes in response to cellular conditions. Proteins of the bacterial cell envelope, for example, have roles in sensing the outer environment and modulating regulatory pathways to maintain homeostasis [23]. Similarly, proteins involved in the stress response elicit modifications of the cell membrane to promote stability [24]. In nearly all instances, AMR is directly mediated by proteins, thus several proteins are already associated with resistance [25,26].

However, the global resistant phenotype is poorly understood. Whilst analysis of resistance-conferring proteins provides information pertinent to a particular mechanism, it offers little insight into the multitude of interlinked networks contributing to the overall resistant phenotype [27]. Exploratory (or “shotgun”) proteomics studies utilising MS facilitate a more comprehensive understanding of the networks at play and enable the identification of features not encoded by the genome or transcriptome [28,29]. Whilst genomic analysis may indicate the presence of specific genes, insight into patterns of gene expression is usually inferred, and does not provide further information on the occurrence of post-translational modifications or phenotypic changes due to environmental stresses.

Proteomics can provide both confirmation of protein presence and a direct quantitative measurement of expression levels. The proteome has greater complexity than the genome and its analysis offers a more elaborate understanding of the organism. The utilisation of proteomics for phenotypic prediction has conceivable applicability for the purposes of CRE detection, with an ability to identify novel resistance markers and post-translational modifications.

2.2. Methods of profiling

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) has revolutionised clinical protocols for

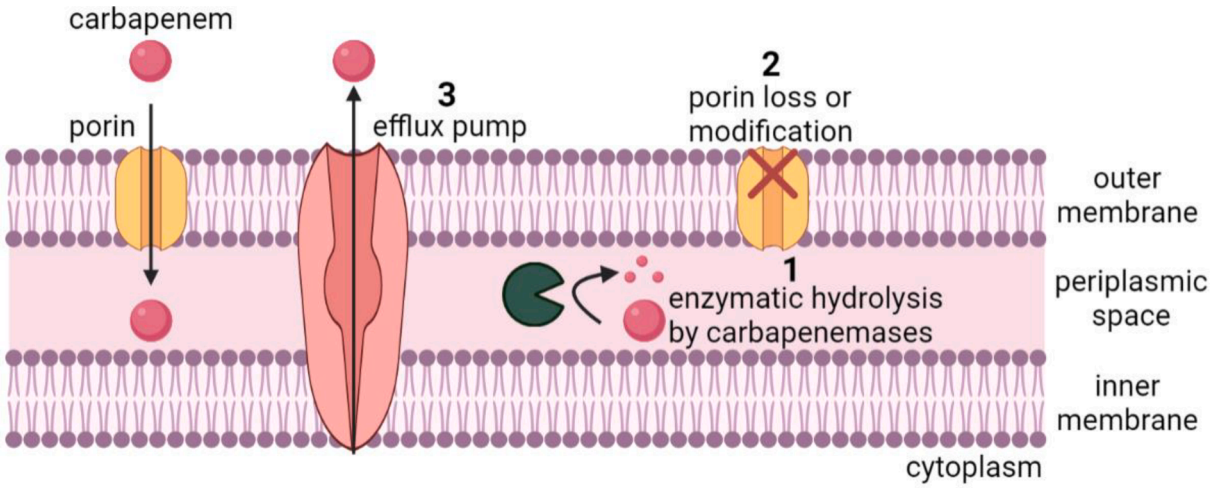

Fig. 2. The mechanisms of carbapenem resistance in Enterobacteriaceae. 1) Enzymatic hydrolysis by carbapenemase enzymes, resulting in inactivation, 2) Mutations leading to porin loss or modification, inhibiting the influx of carbapenem molecules, 3) Efflux pumps which facilitate the active transport of the carbapenem molecule out of the cell through both membranes, decreasing cellular concentrations.

Table 1
An overview of the mass spectrometry platforms discussed in this review for molecular phenotyping studies of CRE by proteomics, lipidomics and metabolomics.

Platform	-omic	Advantages	Limitations
Direct infusion-mass spectrometry (DI-MS)	Proteomics Lipidomics Metabolomics	Data acquisition is rapid as no chromatographic interface present – potential for high-throughput analysis Analysis is more simplistic and cost efficient as does not require solvents Limited issues with carryover and intra-batch drift	Lacks retention time dimension Issues with coelution, particularly in complex samples – large matrix effects Difficulty separating isomers and isobaric compounds Difficulty with spectral alignment
Liquid chromatography-mass spectrometry (LC-MS)	Proteomics Lipidomics Metabolomics	Wide variety of compounds detectable by altering chromatographic conditions Analysis of thermally labile compounds permitted	Issue of data variability between platforms and chromatographic conditions Limited compound identification libraries compared to GC–MS as retention time comparisons in databases are only relevant when chromatographic conditions match Poor coverage of metabolomics databases
Gas chromatography-mass spectrometry (GC–MS)	Lipidomics Metabolomics	Established methodologies and mass spectral libraries available Highly reproducible Gas and liquid sampling matrices possible depending on platformSecond chromatographic dimension (GCxGC) greatly improves resolution and peak identification capacity	Non-volatile compounds require derivatisation which can increase analysis time Typically longer analytical runs than LC-MS High-throughput analysis more difficult to establish than other techniques Not suitable for macromolecules
Matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-TOF MS)	Proteomics Lipidomics	Relatively low cost and minimal sample preparation Rapid analysis – potential for high-throughput analysis Already utilised in clinical laboratories for bacterial identification	Generally only induces singly charged protein ions, making ion transmission difficult Low sensitivity without pre-concentration/purification More limited to macromolecules Limited compound identification libraries
Selected-ion flow-tube-mass spectrometry (SIFT-MS)	Metabolomics	Real time online analysis Rapid analysis – potential for high throughput analysis Uses soft chemical ionisation giving less fragmentation than GC–MS (which typically uses electron ionisation)	Analysis of volatile compounds only Sampling from gaseous matrix only No retention time dimension, making identification of compounds difficult Not suitable for macromoleucles
Secondary electrospray ionisation (SESI-MS)	Metabolomics	Can be used for real time online analysis No chromatographic interface Strong potential for high throughput analysis Can detect high and low volatility compounds	No established compound identification libraries Not suitable for macromolecules No retention time dimension

microorganism identification over the last decade, especially as the instrumentation is already established within the clinical laboratory. Recent developments in MALDI-TOF MS analysis have demonstrated the ability to directly detect carbapenemases [30–35]. Traditionally, MALDI-TOF MS microorganism identification focuses on mass peaks <20,000 *m/z* [36]. However, it has been postulated that biomarker detection is more probable at higher mass ranges, with *Klebsiella pneumoniae* carbapenemases (KPCs) and other β-lactamases detected in Enterobacteriaceae via high range *m/z* peaks corresponding to these enzymes [30–35].

Methods that enable the direct detection of resistance markers are advantageous, facilitating accurate analysis and the development of standardised and automated workflows for diagnostic purposes. Several growth-based assays for antibiotic susceptibility testing using MALDI-TOF MS have been proposed, and include direct-on-target micro-droplet protocols, as well as the ability to sample from positive blood cultures [37–39]. The value of MALDI-TOF MS for the direct detection of CR is debated, however, with some studies demonstrating inefficacy in detecting resistance enzymes in clinical strains [40,41]. Although analysis time is modest and demonstrates high-throughput capability, this can be at the cost of sensitivity loss in complex matrices. Whilst detection appears achievable in laboratory strains, clinical strains often exhibit lesser enzyme production due to lower plasmid numbers, such that enhanced sensitivity is required for their detection [42,43]. Additionally, whilst MALDI-TOF MS methods for antibiotic susceptibility testing are compatible with direct sampling from complex biological matrices, such as blood and urine, direct detection of proteins for resistance confirmation performs poorly [36,38,39,44]. Measures to compensate for the reduced sensitivity include the use of detonated nanodiamonds to concentrate proteins [45]. However, the use of such reagents limits routine clinical applicability with increased costs and sample preparation time. Thus MALDI-TOF MS for the direct identification of proteins associated with CR is unsuitable for implementation into routine diagnostics without time-consuming and costly sample preparation.

A major drawback of direct MS techniques is ion suppression. Hart et al. sought to minimise the degree of ion suppression resulting from MALDI-TOF MS analysis of periplasmic extracts by incorporating a liquid chromatography (LC) separation step prior to digestion [43]. Ion suppression results from competitive desorption and ionisation within a complex sample, resulting in the masking of some components' signals by others of greater abundance or which have preferentially desorbed/ionised [46]. However, whilst the target proteins were detected with enhanced resolution, differentiation between enzyme subclasses was not possible and analysis time was increased by the offline coupling of LC and MALDI-TOF MS, making it suboptimal for routine clinical use.

Differential proteome analysis demonstrates great value during initial stages of biomarker discovery and phenotypic characterisation, with information obtained offering insight into the mechanisms involved in resistance and permitting identification of potential biomarker candidates. Additionally, it aids in establishing the resistant molecular phenotype, characterised by altered protein expression. Proteome profiling necessitates a separation step prior to MS analysis to enable sufficient resolution [47,48]. Previously, gel-based methods were a means of achieving such separation. Khan et al. investigated the effect of meropenem exposure on the proteome of a *K. pneumoniae* strain carrying a bla_{KPC-2} gene [49]. Protein spots observed to be upregulated after two-dimensional gel electrophoresis of whole-cell extracts were manually excised and digested prior to MALDI-TOF MS analysis. Several proteins were identified with roles in energy metabolism, detoxification

of the effects of reactive oxygen species, and mediating protein folding. Such findings are predictable given the increased energy demands associated with the stress response and the upregulation of gene expression and protein translation. Of note was the overexpression of LysM domain/BON superfamily protein, regulated as part of the general stress response and believed to play a role in the modification of peptidoglycan cross-linking when cell wall stress is detected [50]. However, whilst gel methods confer valuable information on proteins of noticeably high abundance, they are inherently biased against low abundance and hydrophobic proteins [28]. Inconclusive findings are, therefore, likely and may potentiate misrepresentation of the resistant phenotype.

The use of liquid-chromatography coupled to tandem mass-spectrometry (LC-MS/MS) confers greater sensitivity than MALDI-TOF MS for proteome profiling, with the LC component affording enhanced separation through chromatographic means. LC-MS/MS has been used to assess the dynamic changes of the CRE proteome under antibiotic stress [51,52]. Similar to the reported findings of Khan et al., carbapenem-resistance was correlated with the overexpression of proteins involved in folding, e.g. GroEL/GroES chaperonin complex proteins and heat shock proteins after carbapenem exposure [49]. Other implicated proteins included those associated with protein biosynthesis, as well as DNA/RNA modifying enzymes, which act as defence mechanisms during cellular stress. Interestingly, the presence of a fluoroquinolone antibiotic did not trigger the same expression patterns as the carbapenem. This suggests that the bacterial stress response to antibiotics is both specific and dynamic, a finding favourable for biomarker discovery. Controversially, Sidjabat et al. observed changes in the proteome in the presence of drug only, with the drug-negative proteome reflecting that of the susceptible control [52]. However, studies employing targeted methods suggest that resistant and susceptible phenotypes differ even in the absence of drug stress [53–56].

Complementary approaches of bottom-up and top-down proteomics have been employed for the targeted detection of carbapenemases. Wang et al. developed a protocol for the detection of peptides belonging to KPC enzymes [54]. Potential target peptides were identified using sequence alignments of the KPC subclasses and *in silico* digestion. The subsequent detection of specific peptides in known KPC-producers refined these to three peptides for targeted LC-MS/MS. The diagnostic performance of the peptides resulted in an overall accuracy of 100 % with a turnaround time <90 min. Lovison et al. later adapted and validated the method and reported a sensitivity of 96.07 % and specificity of 100 % [56]. Similar protocols have been published for the detection of NDM and OXA-48-like CP isolates [53,55]. The OXA-48-like method demonstrated low specificity when performed on OXA-48 β-lactamases without carbapenemase activity, struggling to differentiate between family members with different levels of resistance.

Top-down LC-MS/MS methods for the detection of intact KPC and OXA-48-like proteins have been recently proposed [57,58]. Compared with peptide-based methods, intact protein detection confers several advantages. Firstly, less sample preparation is required due to the omittance of a digestion step, reducing overall analysis time. In addition, detection of intact proteins offers a greater degree of accuracy than peptide analysis, enabling the distinction of enzymes within each subclass. This confers particular value for the detection of OXA-48-like carbepenemases, permitting their differentiation from OXA-48 β-lactamases without carbapenemase activity, a feat unachievable using peptide analysis alone [55].

The full capacity of high-resolution LC-MS/MS for protein identification is exemplified by the work of Foudraine et al. They detected KPC, OXA-48-like, NDM and VIM carbapenemases within a single assay, demonstrating the potential for multiplexing and detecting multiple resistance markers simultaneously [59]. Expanding analysis to include mechanisms aside from those that are enzyme-mediated is also plausible with the methodology applicable to the detection of any protein observed to be implicated in the resistant phenotype. The identification of prospective biomarkers across the proteome in discovery profiling experiments will prove paramount to characterising the resistant phenotype and developing a rapid diagnostic assay for the detection of resistance.

3. Lipidomics

3.1. The bacterial lipidome

Like proteins, lipids are major constituents of the bacterial membrane, and their subcellular organisation is key to facilitating numerous cellular processes, including cell signalling [60,61]. Structurally, lipids are an exceptionally diverse group of molecules, with differences in moieties conferring distinct functions [62,63]. Lipids are comprised largely of fatty acids, and structural differences in these molecules aid in their characterisation. Similarly, bacteria are known to display species-specific characteristics concerning their lipidome, enabling their differentiation [64]. Such diversity presents lipids as prospective candidates for biomarkers in a range of biological phenomena. Furthermore, lipid homeostasis is a highly dynamic process, and it is well documented that bacteria regulate their membrane composition as a response to fluctuations in environmental and cellular conditions [65–68]. Alterations in the physicochemical properties and composition of lipids regulate membrane bilayer properties, such as fluidity which subsequently affect protein position, binding and function [62,69]. Thus, a comprehensive understanding of proteins and lipid-protein membrane interactions cannot be thoroughly achieved without adequate investigation into the lipidome.

Lipidomics provides a complement to proteomics due to its ability to circumvent certain complexities associated with the identification and characterisation of microbes by protein analysis [70]. However, there is a paucity of literature relating to bacterial lipidomics for the purposes of investigating AMR in Gram-negative pathogens. The exception to this is in the case of polymyxin resistance, for which there is extensive research [70–74]. However, resistance to polymyxins is generally conferred by the modification of the lipid A portion of lipopolysaccharide, and thus relates directly to lipids. Nevertheless, owing to the highly diverse and dynamic nature of bacterial membrane lipids, it may be speculated that differences in the lipidome of resistant and susceptible bacteria may also be observed in the context of other resistance mechanisms. Thus, methods currently employed for bacterial lipidome profiling, including those for the detection of polymyxin resistance, demonstrate potential application for the detection of CRE and will be reviewed.

3.2. Methods of profiling

Early methods of lipid profiling generally employed gas chromatography (GC) for fatty acid analysis [75–78]. Whilst the structural diversity of these molecules was not observed to be species-specific, distinct differences in the relative abundance of each fatty acid were noted, such that each species exhibited unique fatty acid profiles based on quantitative differences [77–79]. However, due to the requirement of extraction and derivatisation of fatty acids prior to analysis, these earlier techniques have lost popularity in favour of methods which require less laborious and time-consuming sample preparation. In addition, fatty acids have been shown to vary significantly when experimental parameters including growth medium and growth phase were modified, emphasising the need for standardisation [77,79]. However, whilst this may have prevented the introduction of these methods into routine clinical use, such observations offer valuable insight into the dynamic and responsive nature of bacterial fatty acid profiles and aid in establishing fatty acids as prospective biomarkers. Furthermore, a relationship between fatty acid composition and antibiotic resistance has been demonstrated in penicillin- and tetracycline-resistant *E. coli,* polymyxin-resistant *Serratia marcescens* and *K. pneumoniae,* and methicillin-resistant *Staphylococcus aureus* (MRSA) [76,80–82]. The fatty acid profiles

associated with the resistant bacteria were observed irrespective of drug stress, suggesting that the resistant phenotype is intrinsic in nature. The influence of CR on the bacterial lipidome, however, does not appear to have been extensively investigated. Since the mechanism of action of carbapenems directly involves disruption of cell wall biosynthesis through the inhibition of peptidoglycan crosslinking, it is relevant to analyse the lipidome of CRO. These studies, which demonstrate specific lipidomes associated with β-lactam resistance and Enterobacterales, provide strong support for further lipidomic investigation of CR.

Rees et al. used GC–MS to assess the fatty acid methyl ester (FAME) profile of CP *K. pneumoniae*, implementing a single-step method to simultaneously lyse cells and transesterify the lipid portion [83]. Interestingly, differential profiles of FAMEs linked with fatty acid, lipid and amino acid biosynthesis were found between CC258, a dominant CP lineage, and non-CC258 isolates. However, no significant differences were observed between KPC-producing and non-KPC-producing isolates. These results suggest that resistance phenotypes involve a complex interplay of multiple factors giving rise to an altered cellular metabolism, and consequently phenotype, rather than simply modifications in the expression of those proteins known to directly mediate resistance.

Alternative MS platforms should also be considered for the characterisation of bacterial lipids. MALDI-TOF MS coupled with CeO_2 in metal-oxide laser ionisation (MOLI MS) has recently been used to characterise bacterial species and strains based on fatty acid profiles [82,84]. Cerium displays catalytic properties and acts as a biocatalyst to convert lipids into fatty acids *in situ*. The use of CeO_2 as the MALDI matrix reduces background noise due to the absence of matrix ion peaks and the cleavage of fatty acyls produces negative ions similar to those generated by classic derivatisation methods, but without the need for hazardous chemicals or lengthy procedures [84]. Results indicated accuracy superior to protein profiling by MALDI-TOF MS, with the successful characterisation of closely related strains that had been previously misidentified by protein analysis. Furthermore, the method successfully discriminated between MRSA and methicillin-susceptible *S. aureus* strains, supporting the concept that differences between the lipidomes of resistant and susceptible strains exist [82].

The ubiquitous nature of phospholipids can complicate analysis of biological samples, with molecules shared between host and pathogen eliciting difficulties in discerning origin [70]. The use of membrane glycolipids for the rapid identification of bacteria with MALDI-TOF MS has been proposed [72,74]. Several complex glycolipids, such as lipopolysaccharide, are found exclusively in bacterial membranes, establishing them as potential biomarkers. Leung et al. demonstrated that mass spectral analysis of bacterial glycolipids provides a chemical fingerprint for both the identification of pathogens, as well as for colistin resistance [74]. Furthermore, detection of the resistant phenotype remained possible when polymicrobial mixtures and blood cultures were examined. Drawbacks of the protocol relate to the time-consuming and hazardous extraction method [85]. Further method development by Liang et al. introduced a sodium acetate lysis buffer, as proposed initially by Zhou et al., reducing sample turnaround time to <1 h and eliminating the need for hazardous chemicals [72,86]. As observed with blood, the ability to sample directly from polymicrobial urine was demonstrated, furthering the method's clinical applicability. Future lipidomics studies encompassing CR should consider glycolipids, amongst other molecules, to improve the chances of identifying biomarkers which are detectable even in complex biological matrices and facilitate applicability in the clinical setting.

Owing to the outer membrane origin of outer membrane vesicles (OMVs), these structures are highly representative of cell membrane composition and state [87,88]. Furthermore, as OMVs are not enclosed within the cellular membrane, they are easily isolated and remain in the cell-free supernatant after centrifugation of cultures [88,89]. This offers significant practicality in terms of sample preparation and may help to minimise suppression effects by other cellular components. Jasim et al. proposed the analysis of OMV lipids using LC-MS/MS to better understand pathogenicity and resistance in *K. pneumoniae* and demonstrated outer membrane remodelling in polymyxin-resistant strains through intrinsic differences observed in OMV lipidomes [89]. Whilst the observed changes in lipid composition directly relate to polymyxin resistance mechanisms, the concept of resistance-mediated membrane remodelling is supported by these findings. Thus, it may be speculated that the increased levels of lipids involved in maintaining membrane integrity and bacterial vitality, including sphingolipids, fatty acids and glycerophospholipids, may also be observed in other resistance mechanisms including CR.

It is also important to consider non-membrane-based lipids, and protocols which permit the profiling of both free and membrane lipids. Such analysis provides insight into the metabolite component of the lipidome in addition to membrane state, meaning that unique phenotypic differences are more likely to be observed. Following this approach, Allwood et al. demonstrated distinct grouping of the lipid profiles of ciprofloxacin-resistant and –susceptible *E. coli* and highlighted the potential of LC-MS lipidomics for the investigation of AMR [90]. The use of LC-MS permits the analysis of whole lipids rather than merely the fatty acid constituents, allowing for greater confidence in molecular characterisation and in ascertaining cellular origins [91]. Furthermore, lipids may be separated according to either fatty acid chain composition or polar head group, depending on whether reverse or normal phase is selected, allowing for greater molecular discrimination. Rashid et al. assessed membrane lipid composition in *Enterococcus faecalis* strains with resistance to daptomycin, a cationic lipopeptide antibiotic, and observed correlation between resistance and quantitative measures of lipids [92]. The lipid profile was initially obtained through untargeted direct infusion-MS with characterisation optimised via targeted analysis using several LC-MS/MS methods. However, whilst singular analysis time may be modest, employing numerous analytical methods for the quantification of multiple components is time-consuming and does not translate to practical clinical application. Nonetheless, studies such as these lay the foundation for future work, with biomarker discovery not feasible without the availability of comprehensive profiles and initial exploratory analysis. Studies by Hines et al., however, circumvented these issues by employing a hydrophilic interaction LC-ion mobility-MS (HILIC-IM-MS) method to separate lipids based on both head group polarity and gas phase structure, facilitating more powerful discrimination [93–95]. Results validated those of Rashid et al., and revealed alterations in the lipid profiles of daptomycin-resistant MRSA and *Corynebacterium striatum* [92,93]. Furthermore, the abundance of long-chain fatty acyl phosphatidylglycerols was observed to be negatively correlated with β-lactam susceptibility in MRSA [95]. Importantly, these studies demonstrate the development of a sensitive, high-throughput and multi-analyte MS method that may detect perturbations in molecular profiles for the purposes of assessing AMR.

There is a dearth of knowledge regarding the bacterial lipidome and CR, and more generally, β-lactam resistance as a whole. Further research is warranted to determine the correlation between lipid composition and CR, especially given the role of carbapenems in the disruption of cell wall biosynthesis. Greater insight into this relationship will facilitate elucidation of the resistant phenotype and will promote the identification of prospective molecular biomarkers that may be used in the rapid identification of AMR. Since a close relationship exists between the lipidome and proteome, especially in the context of the cell membrane, the implementation of complementary -omics disciplines in investigations of resistance may provide a more comprehensive and unique insight into the mechanisms of resistance. Furthermore, the analysis of extracellular components, such as OMVs, should be considered, owing to their easily isolatable nature and close relationship with cellular state.

4. Metabolomics

4.1. The bacterial metabolome

It is well recognised that alterations in bacterial metabolism may modulate antimicrobial susceptibility through phenotypic resistance, thus a relationship between resistance and metabolism has already been demonstrated [96]. Previously, the acquisition of resistance was assumed to be a burden on bacterial metabolism, associated with loss of function and increased fitness cost [96,97]. However, it has since been postulated that the acquisition of resistance can generate modifications in the bacterial physiology that do not convey loss of function, but rather compensate for metabolic burden [96,98–100].

Both intracellular and extracellular metabolites convey valuable information about cellular state. Whilst the metabolic fingerprint of a microorganism is characterised by intracellular metabolites, the entire complement of extracellular molecules secreted and taken up by bacteria defines the metabolic footprint [101]. The environment exerts influence on the uptake and secretion of bacterial metabolites through regulation of the genome and proteome (Fig. 3) [102–104]. Consequently, the metabolic footprint is highly reflective of the cellular state [105,106]. Furthermore, since metabolites are the end-products of upstream biochemical processes, they offer insight into intracellular metabolism [107]. Thus, extracellular metabolites may prove equally as useful in determining the molecular basis of certain phenomena and identifying the biological networks involved in cellular processes. Similarly, genomic modifications may be indirectly observed through alterations in metabolism [108]. Characterising the metabolome associated with a particular phenotype is paramount to identifying metabolites with biomarker potential and to better understanding the interconnected molecular networks which give rise to specific phenotypes, such as CR.

4.2. Methods of profiling

Metabolomics is largely concerned with biomarker discovery and involves the study of the small molecules involved in metabolism, or metabolites, as well as their associated chemical interactions within a biological system. Recent studies have sought to assess the metabolic profiles associated with AMR [109–111]. Lin et al. utilised a GC–MS method to analyse the metabolic profile of multi-drug resistant *E. coli* [109]. Results demonstrated differential expression of metabolites between susceptible and resistant strains with many implicated in amino acid biosynthesis. Interestingly, differing metabolic profiles within the resistant strains were also observed, with the more resistant strain exhibiting a greater degree of differential expression. This suggests that the metabolic profile of resistant bacteria may be specific to the resistance mechanism and that metabolic profiling demonstrates applicability for the assessment of drug susceptibility. Owing to the use of GC, the extraction procedure utilised was inherently complex; however, results indicate potential for the development of more refined targeted methods for the detection of resistance. Additionally, whilst the microorganisms assessed in this study demonstrated resistance to many classes of antibiotics, the strains selected were all susceptible to carbapenems. Given the current crisis and the consequence of CR within multidrug resistant organisms, further analysis is warranted that includes analysis of CR bacteria.

The ability to sample directly from biological fluids holds significant clinical applicability. There is minimal literature surrounding metabolomics analysis of CR in the context of biological samples. To the best of our knowledge, the only such study to date investigated the metabolome of CR *K. pneumoniae* infected patients using a GCxGC-TOF-MS method for plasma analysis [110]. Differential expression of 58 metabolites was observed in patients with and without CR *K. pneumoniae*. Interestingly, the metabolites exhibiting the greatest differential change were related to bacterial metabolism rather than host. Results suggest that key components of bacterial metabolism, which may also be implicated in resistance, can be detected within biological matrices with sufficient

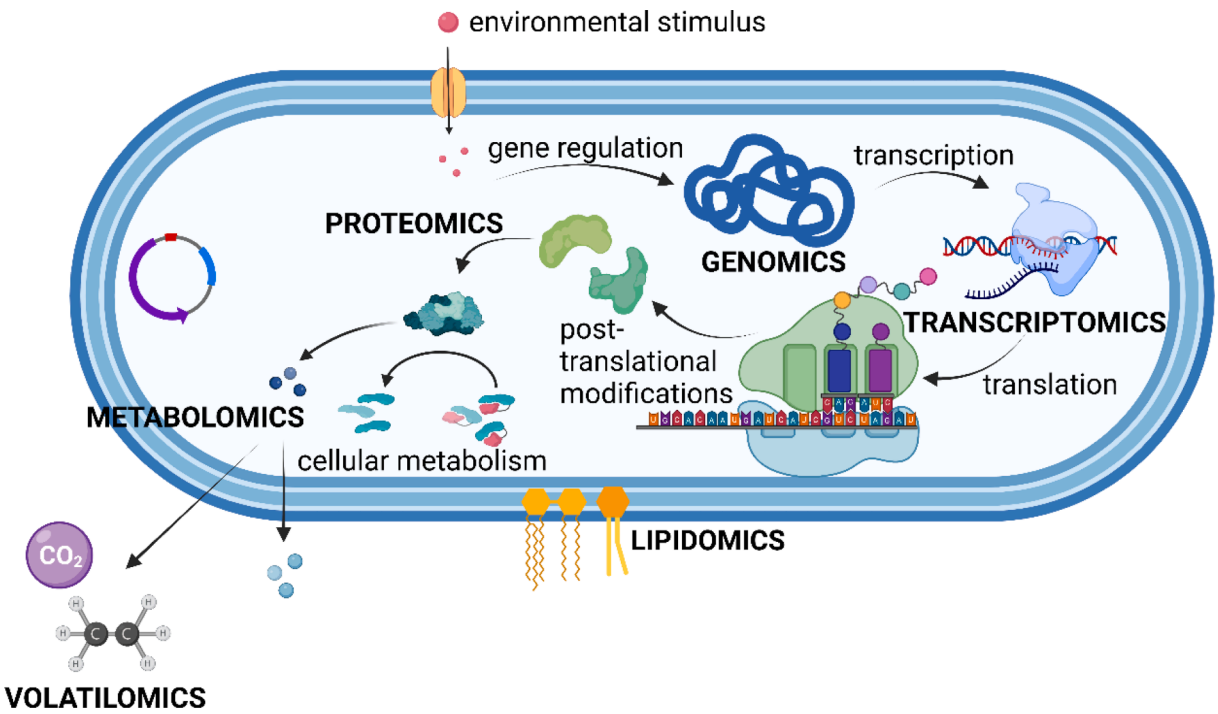

Fig. 3. Bacterial cellular processes and their relation to the omics fields based on the central dogma model. The environment exerts influence on gene expression, resulting in alterations in transcription and, thereby, the abundance of mRNA transcripts. Transcripts are translated into polypeptides, which undergo post-translational modifications to form functional proteins. Proteins mediate cellular biochemical processes, which alter the uptake and secretion of metabolites. Some of these secreted compounds are by-products, whilst others act as building blocks for macromolecules, such as lipids, that are required by the cell.

sensitivity despite the complex nature of these samples. However, the study contained several limitations. It was not clear whether the CR negative group was also infected with *K. pneumoniae* or an alternative microorganism, and thus whether the detected metabolites were in fact resistance-related or arose from species-level differences in metabolism. The challenge associated with translational studies is highlighted here, with false positives easily obtained when proper consideration has not been given to alternative factors. For this reason, it is desirable to start analysis within a controlled laboratory environment prior to performing analyses on biological samples. Furthermore, there were inconsistencies in the method for susceptibility characterisation. The carbapenem minimum inhibitory concentration (MIC) was determined to be $\leq$4 μg/mL in just as many CR-positive as CR-negative strains (n = 15), with just 5 positive strains displaying a MIC >4 μg/mL compared to 3 negative strains. In addition, VIM-1 carbapenemase production was observed in several strains in the CR-negative group. Lastly, the use of GCxGC-TOF-MS is highly complex and requires expertise for both sample preparation and data analysis. Such instrumentation is, therefore, unfit for use in routine clinical practice. However, its use as a highly sensitive analytical method within initial exploratory studies supports future work employing alternative methods once target molecules have been identified.

One growing area of interest surrounds the microbial volatile metabolome. It has been demonstrated that bacteria exhibit characteristic volatile metabolic footprints, and there is a body of literature centring on the analysis of volatile organic compounds (VOCs) for pathogen identification in infections [112–115]. Several MS methods have been proposed for the analysis of antibiotic susceptibility and resistance using VOC analysis, including selected ion flow tube (SIFT)-MS and secondary electrospray ionisation (SESI)-MS [116–118]. However, whilst these methods allow online sampling, it is more challenging to identify compounds as the dimensionality of retention time is missing. More recently, a metal oxide sensor (MOS) was proposed for the detection of VOC signatures associated with AMR [119]. The MOS demonstrated superior sensitivity for several VOCs compared to MS, at the cost of losing sensitivity to prominent bacterial VOCs, such as sulfides. Statistical analysis permitted the construction of a successful model for the identification of resistance after just 2 h of incubation using MOS compared to the 4 h required by MS. However, as MOS detection is based on changes in electrical resistance, it is not possible to identify compounds unless the sensor is trained prior to analysis or performed simultaneously with a method that can, e.g. MS [116]. Consequently, whilst the MOS can detect signals where the MS cannot, the compounds giving rise to these cannot be directly identified. Using unidentified signals as biomarkers presents several problems, with identification based on pattern recognition rather than direct confirmation. Only a handful of research groups have investigated AMR VOC profiles using GC–MS methods [114,119,120]. All groups employed different methods of headspace sampling, including the use of solid phase micro extraction (SPME) fibres for the sampling of both cell supernatant and cell culture headspace, or via active pumping of collected headspace onto adsorbent material. Smart et al. reported differences between the VOC profiles of susceptible and resistant bacteria, and Rees et al. demonstrated that volatile metabolic fingerprints could be used to discriminate between CR and susceptible strains [114,120]. However, the protocol of Rees et al. involved a 12 h incubation step and GCxGC–MS, excluding it from application in the clinical setting as a rapid and simple detection method [120]. Further investigation into the VOC profiles and metabolic pathways found to be associated with resistance using GC–MS is warranted to identify potential compounds of interest. Direct detection and validation of these compounds will be key to developing a method with high confidence. In addition, the use of stable isotope tracers may aid in uncovering the endogenous origin of VOCs and other metabolites through temporal metabolite tracing [121].

As observed with other -omics disciplines, the use of LC over GC can be advantageous. In the current context, there is broader metabolome coverage with less complex sample preparation since derivatisation is not usually required. However, integration of data from both platforms remains the most credible means of achieving near-global coverage. Extraction methods for intra- and extracellular metabolites of *E. coli* have been adapted for use with both LC-MS and GC–MS [90,122]. LC-MS for metabolomic analysis has not been extensively utilised in examination of resistant phenotypes, with methods such as Fourier transform infrared spectroscopy (FTIR) or nuclear magnetic resonance (NMR) being more commonly employed to date [90,123,124]. Direct infusion-MS followed by targeted LC-MS/MS has been used for the assessment of drug susceptibility based on metabolic profile in *Mycobacterium tuberculosis*, with strains being grouped as susceptible, multidrug resistant or extensively drug resistant [111]. This study introduces the concept of modelling antibiotic susceptibility rather than simply detecting a marker of resistance. Whilst results could not confirm complete association between grouped resistant phenotype and metabolic profile, clear differences could be seen when just two strains were compared, suggesting that molecular phenotype may be more specific to the mechanism of resistance than initially thought.

5. Future perspectives

Measures routinely utilised in clinical practice for the detection of CR involve subjective antibiotic susceptibility testing methods that fail to exploit underlying molecular mechanisms. In addition, current procedures are time-intensive with detrimental consequences for the identification of infections caused by resistant pathogens both in terms of patient outcomes and in mitigating the spread of resistance. Establishing the molecular phenotype associated with CR establishes a precedent for the development of highly specific methods that permit its detection. Functional analysis of molecules linked to the resistant phenotype within each of the -omics disciplines may be utilised to investigate system-wide alterations and to explore the mechanisms surrounding resistance. This greater breadth of knowledge will provide a more complete picture of the mechanisms involved in resistant phenotypes and will facilitate the development of detection methods that are more direct, reliable, and rapid than those currently in place. It is likely that machine learning models will play a central role in future predictions of resistance. As biology-based disciplines become more reliant on bioinformatics and machine learning approaches, the use of predictive models based on MS data for a panel of analytes seems the most plausible diagnostic assay for detecting resistance. The ability to distinguish CR *K. pneumoniae* from its susceptible counterparts by machine learning methods using whole cell MALDI-TOF MS data has already been demonstrated [125]. Consideration should be given towards incorporating both detection of resistance and susceptibility into such models, as whilst detection of resistance is of great importance, prediction of susceptibility is pertinent to implementing an effective treatment regimen.

It is inherently challenging to achieve controlled conditions within the clinical setting. Patients are often treated with several antibiotics prior to and throughout hospitalisation [110]. In addition, many are prescribed other medications for comorbidities. The identification of prospective biomarkers for the detection of CR, therefore, hinges on several factors. It is imperative than any prospective biomarkers are either present in all conditions (including basal), or, if they are induced by a specific drug stress, they are not influenced negatively by the presence of factors such as other antibiotics/medications. Whilst initial work in this area is likely to focus on laboratory strains cultured in growth media, future studies should assess clinical strains and biological samples in order to develop methodologies that may be translated into clinical practice. Given the prevalence of CRO within the clinical setting, and the critical priority assigned to this issue by the World Health Organisation, there is a dire need to develop methodologies for the rapid detection of CR that are suitable and applicable to the clinical laboratory [3]. Whilst previous studies have aided in expanding our current

understanding of resistance, a bench-to-bedside approach should be considered in future work whereby results from the laboratory are translatable to the clinical setting for the direct benefit of patients. However, it is important that sufficient time is spent within the exploratory stage, such that translated analysis solutions provide meaningful results.

Lastly, whilst studies into the molecular phenotype and metabolism of CR pathogens may centre around developing methods for the detection of resistance, it is highly probable that insight gained from such studies will inadvertently facilitate developments in associated fields. There is an urgent need to develop novel antimicrobials that may overcome resistant bacteria, especially those identified as being resistant to last-resort therapies, such as carbapenems. Expanding the knowledge surrounding the mechanisms of CR at the molecular level using MS is likely to reveal information pertinent to the development of novel therapeutics and treatment strategies, such as the identification of potential drug targets.

6. Conclusion

Multi-omics approaches are facilitating integrated insights into biological phenomena that cannot be entirely explained by a singular -omics field alone. This 'whole-cell' approach demonstrates the interconnectedness between the various -omics disciplines. The implementation of complementary omics approaches using high resolution MS to assess whole and sub- proteomes/lipidomes/metabolomes coupled with bioinformatics is more likely to provide insight into mechanisms of resistance than analysis of single components. Whilst the specific mechanisms of CR are well characterised, the molecular phenotype associated with resistance is poorly understood. Understanding just how the acquisition of CR affects cellular physiology and cell metabolism through molecular phenotyping is a necessary step towards detecting resistance by diagnostic means. Ideally, this will facilitate the implementation of effective methods to tackle the current AMR crisis by means of detection, surveillance, and the development of efficacious antimicrobials. Targeted measures using high-resolution mass spectrometry show considerable promise for the identification of molecular signatures and the development of a rapid diagnostic assay for the detection of CR. However, whether future clinical methods will utilise such platforms is unclear. Whilst these instruments show considerable promise for biomarker discovery, their translational applicability for the clinical setting is debated. Nonetheless, use of these platforms in this current age of discovery is likely to play an important role in advancing the field towards clinical translation.

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CRediT authorship contribution statement

Breanna Dixon: Writing – original draft. **Waqar M Ahmed:** Supervision, Writing – review & editing. **Tim Felton:** Supervision, Writing – review & editing. **Stephen J Fowler:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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