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Unraveling antimicrobial resistance using metabolomics

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The emergence of antimicrobial resistance (AMR) in bacterial pathogens represents a global health threat. The metabolic state of bacteria is associated with a range of genetic and phenotypic resistance mechanisms. This review provides an overview of the roles of metabolic processes that are associated with AMR mechanisms, including energy production, cell wall synthesis, cell–cell communication, and bacterial growth. These metabolic processes can be targeted with the aim of re-sensitizing resistant pathogens to antibiotic treatments. We discuss how state-of-the-art metabolomics approaches can be used for comprehensive analysis of microbial AMR-related metabolism, which may facilitate the discovery of novel drug targets and treatment strategies.

Keywords: Antibiotics; Antimicrobial resistance; Bacterial metabolism; Metabolomics

Introduction

Antimicrobial resistance (AMR) in bacterial pathogens represents an urgent global health threat that is associated with significant morbidity and mortality.[1] There is a need to improve our understanding of the molecular mechanisms that underlie AMR in order to develop innovative treatment strategies for AMR-associated bacterial infections.[2]

Bacterial pathogens can survive antibiotic exposure through a range of genetic and phenotypic AMR mechanisms. Genetic mechanisms are associated with a permanent change in antimicrobial sensitivity, for example owing to the acquisition of mobile genetic elements and mutations in chromosomal genes conferring antibiotic resistance.[3] Phenotypic mechanisms are typically linked to transient decreases in antibiotic sensitivity that occur in either a homogeneous (e.g., tolerance) or heterogeneous fashion (e.g., heteroresistance, persistence).[4–6] Another phenotypic mechanism that decreases antibiotic sensitivity is the formation of microbial biofilms, which are aggregates of bacteria protected by a polymeric matrix.[7] Importantly, the prolonged antibiotic survival of bacteria through phenotypic AMR mechanisms could act as a stepping-stone for genetic AMR development.[8] Bacterial metabolic processes have a fundamental role in cellular function and are therefore commonly associated with various AMR mechanisms (Fig. 1). The metabolic state of bacterial cells during antibiotic treatment can act either as a contributor to or as a consequence of AMR. Decreased metabolic activity contributes to AMR by reducing antibiotic uptake or the secondary effects of antibiotics.[9–12] By contrast, increased metabolic activity is required to support energy-demanding AMR mechanisms such as cell-wall modifications and overexpression of efflux pumps.[13–18] An understanding of the metabolic processes that underlie AMR mechanisms may be used to alter metabolic activity strategically during antibiotic therapy in order to re-sensitize pathogens to the antibiotic.[19]

Metabolomics is the systemic study of the metabolome, that is, of all small molecules in a biological sample, to provide a snapshot of the utilized biochemical processes.[20–21] The metabolome is closely linked to organismal phenotype, and unveils initial responses to antibiotic pressure and the adaptations required to sustain AMR mechanisms. Metabolomics approaches are key

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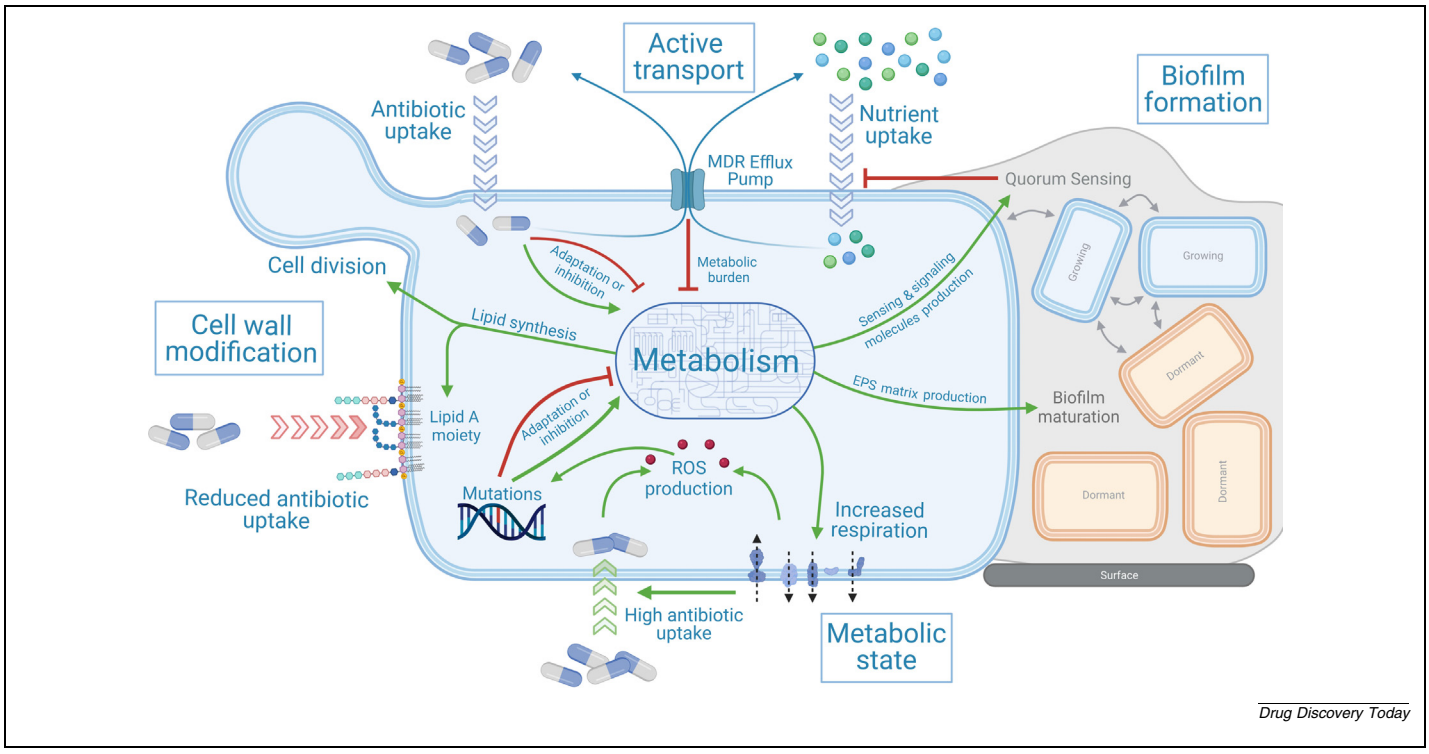

FIGURE 1
Schematic overview of the roles of bacterial metabolism in antimicrobial drug action and resistance mechanisms. Created with BioRender.com.

technologies in identifying relationships between AMR mechanisms and microbial metabolism.

In this review, we discuss the role of bacterial metabolism in AMR mechanisms and the formation of microbial biofilms for clinically relevant bacterial pathogens, as revealed using state-of-the-art metabolomics approaches. Second, we discuss how metabolomics can be applied as a key enabling technology to facilitate the discovery of innovative metabolism-associated drug targets and treatment strategies.

Metabolism and antimicrobial resistance

Key cellular changes associated with AMR in which metabolism plays an important role include: (i) cellular energy production, (ii) cell envelope modifications, and (iii) cell-to-cell interactions in biofilms. Here, we provide an overview of metabolomics studies that have identified such AMR-associated metabolic effects (Table 1).

Cellular energy production

The activity of energy-producing metabolic pathways translates into the activation of cellular functional responses or dormancy to evade the killing of microbes by antibiotics. The most efficient energy-producing metabolic pathway is aerobic cellular respiration.[22] Cellular respiration includes glycolysis and the tricarboxylic acid (TCA) cycle for the production of electron carriers, which are used in the electron transport chain (ETC) for the production of adenosine triphosphate (ATP). In case of fast energy demand or carbon source depletion, several bacterial pathogens can switch their metabolism towards less efficient anaerobic fermentative energy production.[22] Several pathogens, such as *Pseudomonas aeruginosa*, can utilize anaerobic respiration pathways, such as the nitrate respiratory chain, to maintain cellular homeostasis in oxygen-depleted environments.[23] A switch towards anaerobic energy metabolism is commonly used for evasion of host defense mechanisms but also plays an important role in increasing aminoglycoside tolerance.[24–25] For example, increasing oxygen levels using hyperbaric oxygen treatment (HBOT) to induce aerobic respiration re-sensitizes *P. aeruginosa* to aminoglycoside treatment.[26–27] Nevertheless, this approach is only of interest in specific clinical indications, such as anaerobic microenvironments in cystic fibrosis-associated lung infections.

Stimulation of aerobic energy production as a target to enhance antibiotic sensitivity is an important potential therapeutic strategy. Specifically, the supplementation of essential carbon sources to increase aerobic respiration[19] is a promising novel approach to improve the efficacy of antibiotics, in particular aminoglycoside antibiotics. Comprehensive *in vitro* screens in bacteria using different carbon source supplements have demonstrated pathogen-dependent changes in aminoglycoside susceptibility upon nutrient supplementation.[28] Metabolomics studies have demonstrated that such carbon source supplementation changes the activity of the TCA cycle, promoting the synthesis of electron carriers that support the ETC.[29–32] Aminoglycoside efficacy can be improved by increasing the passive influx of charged molecules. Stimulation of the ETC results in a greater electric transmembrane potential, which enhances the proton-motive force (PMF)-mediated influx of positively charged aminoglycosides.[29,33] Increased antibiotic uptake resulting from nutrient-induced PMF decreased cell survival in several multidrug resistant strains.[31] Antibiotics of other classes, such as β-

TABLE 1

Overview of studies researching the role of metabolism in antimicrobial resistance (AMR) using metabolomics techniques.

AMR mechanism	Main finding	Metabolic pathways	Analytical approach	Antibiotics	Species	Reference
Metabolic adaptation in energy production						
Energy metabolism influences antibiotic efficacy	Energy production is a better predictor of antibiotic efficacy than is growth rate	Nucleotides	Colorimetric[a]	Ampicillin[b], carbenicillin[b], gentamicin[c], kanamycin[c], streptomycin[c], ciprofloxacin[d], levofloxacin[d], norfloxacin[d], cefsulodin[e]	*Escherichia coli*	37
	Bacteriostatic antibiotics inhibit the efficacy of bactericidal antibiotics due to the reduced energy demand of treated cells	Amino acids, nucleotides	Untargeted LC and GC–MS	Ampicillin[b], gentamicin[c], levofloxacin[d], norfloxacin[d], daptomicin[e], rifampin[e]	*E. coli*, *Staphylococcus aureus*	30
Nutrient supplementation	Antibiotic-resistant cells reduce activity in central carbon metabolism, which can be activated by nutrient supplementation	Glycolysis, TCA cycle	Untargeted GC–MS	Kanamycin[c]	*Edwardsiella tarda*	31–32
	Supplementation with nutrients that stimulate the TCA cycle increases the PMF-induced cellular intake of aminoglycoside tobramycin	TCA cycle	Untargeted LC and GC–MS	Tobramycin[c], chloramphenicol[e], linezolid[e], rafampin[e]	*E. coli*	25
Respiration and secondary antibiotic effects	Resistant cells demonstrate lower levels of TCA cycle intermediates, reducing ROS production	Amino acids, glycolysis, lipids, TCA cycle	Untargeted LC-MS	Chloramphenicol[e]	*E. tarda*	42
	Decreased central carbon metabolites in antibiotic-resistant cells reduce ROS production	Amino acids, glutathione, glycolysis, pentose phosphate, TCA cycle	Untargeted GC–MS	Gentamicin[c]	*Vibrio alginolyticus*	43
	Energy metabolism as a defense mechanism to reduce oxidative stress during antibiotic treatment	Glycolysis, TCA cycle	(Non–)targeted LC-MS	Streptomycin[c], isoniazid[e], rifampicin[e]	*Mycobacterium tuberculosis*	45
Biofilm heterogeneity	QS can slow down cell growth by coordinating nucleotide production and glucose utilization	Nucleotides, pentose phosphate	Targeted NMR and CE-MS	N.A.	*Burkholderia glumae*	67
	Cells in the biofilm core switch to anaerobic fermentation for energy production	Lactate, TCA cycle	Targeted NMR	N.A.	*Shewanella oneidensis*	76
Metabolic adaptation to the microenvironment						
Metabolic adaptation in antibiotic-resistant cells	Adaptative evolution in the cystic fibrosis lung of antibiotic-resistant strains results in metabolic specialization to amino acid and oxygen consumption	Nutrient consumption	Targeted LC-UV and LC-MS	N.A.	*Pseudomonas aeruginosa*	40
	High nutrient levels promote the development of antibiotic resistance	Lipids, glycolysis, TCA cycle	Untargeted MS	Ampicillin[b], norfloxacin[d], chloramphenicol[e]	*E. coli*	38
	Antibiotic treatment from different classes results in distinctive metabolic perturbations and adaptations	Amino acids, glycolysis, nucleotides, TCA cycle	Untargeted MS	Kanamycin[c], nalidixic acid[d], norfloxacin[d], ofloxacin[d], chloramphenicol[e]	*E. coli*	63
	Resistant and sensitive bacteria have distinctive metabolic fingerprints	Glycolysis, lipids, nucleotides, pentose phosphate, TCA cycle	Untargeted and targeted LC-MS	Methicillin[b]	*S. aureus*	44
Antibiotic-induced metabolic adaptation	Metabolic fingerprints identify antibiotics (secondary) mode of action for different antibiotic classes	Amino acids, nucleotides, TCA cycle	Untargeted NMR	Ampicillin[b], carbenicillin[b], ciprofloxacin[d], ofloxacin[d], streptomycin[c], cefalexin[e], doxycycline[e], tetracycline[e]	*E. coli*	61

TABLE 1 (*CONTINUED*)

	Antibiotics and their corresponding mode of action can be identified on the basis of the metabolic pathways targeted by these antibiotics	Amino acids, glycolysis, lipids, nucleotides, TCA cycle	Untargeted LC-MS	Ceftazidime[e], fosmidomycin[e], triclosan[e]	*E. coli*	62
	Antibiotic-surviving cells actively produce ATP during antibiotic treatment, dependent on the nutritional environment	Nucleotides	Colorimetric[a]	Ciprofloxacin[d], streptomycin[c], bedaquilline[e], isoniazid[e], rifampicin[e]	*Mycobacterium smegmatis*	100
	Methicillin-resistant and -sensitive strains demonstrate different metabolic responses to treatment with other antibiotics	Amino acids, nucleotides, TCA cycle	Targeted LC-MS	Ampicillin[c], ciprofloxacin[d], kanamycin[c]	*S. aureus*	81
	Antibiotics induce microbiome-independent changes in the host metabolome that alter antibiotic efficacy	Amino acids, glycolysis, nucleotides, pentose phosphate	Untargeted LC-MS	Ciprofloxacin[d]	*E. coli*	41
Biofilm formation	There is a heterogeneous distribution of quorum-sensing molecules over the biofilm population	Quorum sensing	Targeted MALDI-SIMS	N.A.	*P. aeruginosa*, *S. aureus*	72
	The production of antimicrobials and signaling molecules is influenced by the nutritional environment	Quorum sensing	Targeted MALDI-SIMS	N.A.	*P. aeruginosa*, *S. aureus*	79
Cell wall modifications						
Cell wall disruption and synthesis	The loss of envelope and membrane biogenesis processes results in complete lipid reconstruction, including changes in lipid A moiety, resulting in the energy metabolic switch to glycolysis	Amino acids, lipids, pentose phosphate, TCA cycle	Untargeted (LC-)MS	Colistin[f]	*Klebsiella pneumoniae*	55
	Lipid A reconstruction results in increase pentose phosphate activity and reduced TCA cycle activity in colistin-resistant cells	Lipids, pentose phosphate, TCA cycle	Untargeted LC-MS	Colistin[f]	*Acinetobacter baumannii*	56
	Colistin treatment induces metabolic flux towards cell wall repair, forcing the energy production flux to glucose utilization and shuttled TCA cycle	Glycolysis, lipids, TCA cycle	Untargeted GC–MS	Colistin[f]	*M. tuberculosis*	57
	Combination therapy with colistin and doripenem antibiotics affects metabolic pathways in cell wall synthesis and energy production differently in a time-dependent manner	Amino acids, glutathione, lipids, nucleotides, pentose phosphate	Untargeted LC-MS	Colistin[f], doripenem[c]	*A. baumannii*	64
	The addition of phosphoethanolamine to lipid A for colistin resistance has a high fitness cost	Lipids	Targeted MALDI-MS	Colistin[f]	*E. coli*	59
MDR overexpression	Overexpression of MDR efflux pumps initiates metabolic rewiring to anaerobic respiration	Oxygen and nitrates	Colorimetric[a] oximeter	N.A.	*P. aeruginosa*	17

Abbreviations.
CE-MS, capillary electrophoresis–mass spectrometry.
GC–MS, gas chromatography with mass spectrometry.
LC, Liquid chromatography.
LC-UV, liquid chromatography with UV detection.
MALDI-SIMS, matrix assisted laser desorption ionization - secondary ion mass spectrometry.
NMR, nuclear magnetic resonance.
PMF, proton motive force.
ROS, reactive oxygen species.
TCA cycle, tricarboxylic acid cycle.
[a] Fluorescent staining of targeted metabolite.
[b] β-Lactam antibiotic.
[c] Aminoglycoside antibiotic.
[d] Fluoroquinolone antibiotic.
[e] Other antibiotic classes.
[f] Polymixin antibiotic.

lactams and fluoroquinolones, also partly depend on cellular respiration for their antimicrobial effects as they induce a redox disbalance as a secondary antibiotic effect. Fluoroquinolones exert better bactericidal effects in metabolic active cells through the production of reactive oxygen species (ROS) during oxidative phosphorylation.[11–12,34] β-Lactams induce systemic ROS-associated cellular toxicity by creating an energy-demanding futile cycle of peptidoglycan synthesis and degradation by obstructing cell wall synthesis.[35–36] The close kinship between energy production and antibiotic lethality is further demonstrated by the increased killing of bacteria that show accelerated respiratory activity.[30] In accordance, experiments have shown that metabolic activity, rather than growth rate, is better able to forecast antibiotic effect.[37]

The use of nutrient supplementation to increase antibiotic uptake and to induce secondary antibiotic effects relies on the metabolic specialization of the targeted bacterial cell. Clinically relevant strains potentially lose the ability to utilize certain pathways during the acquisition of mutations that confer antibiotic resistance and as a result of adaptation to specific microenvironments at infection sites.[38–41] Antibiotic-resistant strains demonstrate distinctive metabolic footprints.[42–44] The observed decline in energy metabolism reduces ROS production, thereby further enhancing AMR.[45] Subsequently, limiting the frequency of mutations in core metabolic genes directly results in the development of antibiotic resistance.[46] However, metabolism-affecting mutations, such as PMF-limiting mutations, can only be sustained in nutrient-rich environments because they are associated with a high fitness burden.[47] To further unravel the metabolic effects and adaptations that are associated with antibiotic efficacy, mathematical flux analysis of central metabolic pathways could be used to scrutinize the effect of nutritional supplements on metabolic processes during antibiotic treatment.[31,48] Although these approaches targeting cellular energy metabolism are of interest, there remains a significant knowledge gap concerning the broad spectrum of bacterial species and clinically occurring strains.

Modifications of the cell envelope

Cell wall permeability is essential for effective antibiotic treatment because most antibiotics rely on passive transport across the outer membrane.[49] The cell wall of Gram-negative pathogens in particular can be challenging for antibiotics to penetrate, in part because the outer layer of negatively charged lipopolysaccharides (LPS) prevents passive transport of large and hydrophobic antibiotics through the cell wall. The uptake of antibiotics by Gram-negative pathogens is mainly dependent on transport through membrane porins. Here, porin permeability is greatest for positively charged small-molecule antibiotics, possibly because of the role of the PMF discussed above.[50]

The LPS layer in gram-negative pathogens is an important target for drugs that can disrupt cell envelope integrity, leading to AMR. Polymyxin antibiotics, which are currently used as last-resort antibiotics, bind lipid A in the LPS layer of Gram-negative bacteria to initiate lethal disruption of both the outer and the cytoplasmic membrane, and thus increase intracellular levels of combination therapeutics.[51–52] Modifications to lipid A structure resulting from changes in the biosynthetic pathway of LPS can lead to resistance to polymyxins. Polymyxin resistance mechanisms that reduce the frequency of lipid A binding sites in the LPS layer involve active membrane modifications, which may be intrinsic, acquired chromosomally encoded, or plasmid-mediated adaptations.[53–55] These cell modifications are supported by a wide range of fatty acid biosynthetic pathways.[13] Nevertheless, the rewiring of fatty acid synthesis comes with a high energy demand, which is demonstrated by increased efficiency in the killing of metabolically inactive cells.[56] Metabolomics studies of polymyxin-resistant strains have demonstrated that modifications in lipid biosynthesis result in metabolic rewiring in energy metabolism.[57–58] Metabolic flux analysis in another strain supported this finding, as the upper carbon flux in the glycolysis pathways was elevated while the TCA cycle was shunted.[59] This suggests that there is a switch to glucose-fermenting metabolism for energy production in polymyxin-resistant cells, evidence that is supported by the pH-mediated detection of lactic acid production in polymyxin-resistant Enterobacteriaceae.[60] Although fermentative metabolism can sustain cell homeostasis, the high metabolic burden of fatty acid synthesis during the acquisition of resistance results in a fitness cost.[14–15,61] To this end, enhancing our understanding of the biosynthetic routes for LPS and their fitness costs during polymyxin resistance can potentially improve the development of drug candidates for targeting the cell envelope.

The overexpression of multidrug resistance (MDR) efflux pumps in the cell envelope is another mechanism that restricts the intracellular concentrations of antibiotics, leading to AMR and a fitness cost. MDR efflux pump-associated AMR occurs for a range of broad antibiotic classes across pathogenic species.[62] Metabolic rewiring is an important mechanism in enabling pathogens to overcome the metabolic burden that accompanies MDR efflux pump overexpression.[16–18] For instance, the switch towards the nitrate respiratory chain and anaerobic fermentative metabolism compensates for the use of oxygen as an alkaline agent, which enables the acquisition of mutations that promote the activity of MDR efflux pumps in the absence of selective pressure.[17] The reliance on metabolic adaptation to maintain cellular homeostasis during AMR could potentially be utilized therapeutically. For instance, metabolic adaptation upon antibiotic exposure[63–65] can be used to design combination treatments that involve antibiotic agents. Antibiotics disrupt the homeostasis of bacterial cells by affecting different key metabolic pathways, and these differences in targets largely explain the synergistic effect of colistin and doripenem in combination therapy.[66] In conclusion, the metabolic changes that result from efflux pump upregulation are of interest as therapeutic targets.

Cell–cell interactions in biofilms

The formation of microbial biofilms is an important mechanism in decreasing antibiotic sensitivity through the production of extracellular polymeric substances (EPS). Nevertheless, the production of EPS is a metabolically expensive activity, which requires efficient cellular communication and metabolic adaptation.[67] Bacteria utilize quorum sensing (QS) systems to coordinate cell–cell interactions in all biofilm stages. QS occurs through the production of various hormone-like small molecules that are excreted into the biofilm microenvironment, and it is

essential in biofilm formation and maintenance because it synchronizes the metabolism of macromolecules necessary to establish the protective layer of EPS.[68–69] QS-associated metabolic processing may thus represent an important target for the treatment of biofilm-associated infections.

A promising approach to improving the treatment of biofilms exploits the role of QS molecules in biofilm physiology. Treatments that disrupt microbial communication can interfere with biofilm integrity over multiple biofilm stages,[70] enabling therapy at different stages of infection. The link between metabolic activity in biofilms and QS is can also be utilized to disrupt biofilm integrity. The use of QS-controlled circuits for dynamic control of cellular fluxes[71] demonstrates that cell–cell communication is a key regulator of bacterial metabolism, which indirectly affects antibiotic susceptibility. Therefore, the use of QS systems as a treatment target[72–73] creates an opportunity to obtain universal control over metabolism-associated antibiotic potentiation and biofilm physiology. However, the high variety of QS systems and the differences between species require the further identification and characterization of QS molecules that partake in biofilm biology. Spatially oriented mass spectrometry techniques can identify the QS molecules and characterize the population dynamics in biofilms.[74]

Chronic bacterial infections are commonly associated with well-developed mature biofilms and are associated with reduced antibiotic efficacy. In particular, well-developed mature biofilms are associated with steep nutrient gradients that are induced by the biofilm structure.[75] The biofilm maturation process is often dependent on the ability of pathogens to switch metabolically to alternative nutrient sources.[76–77] Real-time analysis of the metabolites from central carbon metabolism demonstrated metabolic adaptations to anaerobic fermentation pathways over time and biofilm depth.[78] Redirecting metabolism in *P. aeruginosa* biofilms by TCA cycle carbon source supplementation resulted in increased aminoglycoside eradication,[29,33] which highlights the potential of nutrient supplementation to reduce metabolically induced tolerance in biofilms.[7]

Metabolomics technologies and approaches

Metabolomics approaches enable organism-wide metabolite identification and the quantification of biochemical networks. Metabolomics approaches can be broadly differentiated into untargeted metabolite profiling and targeted methods. Untargeted methods aim for broad metabolite coverage, but may not allow full identification of molecular structures. Targeted metabolomics approaches aim for quantitative analysis of a set of metabolites, with enhanced structural resolution of identified metabolites.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the most commonly used detectors in the metabolomics field, allowing the determination of mass-to-charge (m/z) ratio on the basis of the manipulation of ionized analytes by an electric or magnetic field, respectively. MS systems generally have superior selectivity and sensitivity, and can detect a larger range of analytes. In MS, the charge-dependent detection requires the ionization of the metabolites in the ion source before entering the system. Complementary to MS, NMR detectors provide quantitative and structural information in a non-destructive manner.

Even though metabolomics technologies have advanced extensively over the years, analysis of the full metabolome of an organism in a single analytical method is still not possible because of the great diversity in the physicochemical characteristics and the broad range of concentrations of the metabolites. Here, we discuss the practical considerations that affect the utility of different metabolomics approaches, in particular in the context of microbial metabolism and AMR (Table 1).

Untargeted metabolomics

Untargeted non-selective screening enables the broad characterization of (changes in) metabolism. The analysis of multiple metabolite classes, with high contrast in polarity, requires long separation methods to maintain accurate metabolite identification. The use of high-resolution mass spectrometers (HRMS) enables high-throughput metabolite profiling, without the need to combine multiple or time-consuming analytical platforms. HRMS refers to mass analyzers, such as time-of-flight, Orbitrap, and Fourier-transform ion cyclotron resonance systems, that offer high mass accuracy, dissociating metabolites with a resolution as small as 0.001 atomic mass units. This high metabolite resolving power facilitates the confident identification of metabolites by HRMS, allowing both the study of metabolic changes without a time-consuming separation step and confident comparison of the acquired m/z features to those of previously identified metabolites stored in mass spectral libraries.[79] This enables relatively fast metabolic fingerprinting, which can be used to screen for metabolic adaptation during AMR development in a larger set of conditions with higher throughput.

The high throughput provides the possibility of researching metabolic evolution and immediate metabolic response during treatment with a variety of antibiotics of different classes within a single study.[38,65] Single studies that rely on metabolic data to compare different bacterial species, environments, or antibiotic classes do not fully rely on data acquired in other studies, reducing the influence of variability caused by differences in experimental design. The high mass accuracy achieved with HRMS can also be used to determine the molecular composition of completely unknown metabolites, and can be applied for instance for the discovery of novel metabolites. All of the metabolites in central carbon metabolism are covered in most metabolite databases, whereas many secondary metabolites such as QS molecules are yet to be discovered.[80] However, the robust identification of chemical structures requires the addition of low-resolution fragmenting mass analyzers or the multidimensional information provided by NMR detectors.

Metabolite identification methods using HRMS in combination with spatial oriented ionization techniques have demonstrated the influence of the nutritional environment and biofilm formation in the production of signaling molecules.[74,81] Thus, the advances in HRMS technologies provide the opportunity to screen confidently for metabolic adaptations or unidentified metabolites that are involved in AMR, providing a better understanding of AMR mechanisms or facilitating the development of metabolism-targeted treatment strategies.[82]

Targeted metabolomics

Targeted metabolomics approaches require prior knowledge of metabolite targets to enable their efficient extraction and isolation from the sampled cells, providing sensitive and selective quantitative analysis. By contrast, untargeted methods come with the bottleneck of limited detection range and quantitative accuracy due to detector saturation by thousands of signal-producing analytes. The confident identification and high sensitivity of targeted metabolomics enable the characterization of exact changes in the concentrations of metabolites. However, this requires the use of expensive or complex standardization procedures and time-consuming analytical validation. The specialization of targeted methods limits the metabolic targets, which results in studies that focus on specific metabolic pathways.[40,45,69,78,83] The absolute quantitative data obtained in targeted metabolomics are superior for biological interpretation. For example, in one study, the quantitative analysis of nutrient uptake and metabolism with both NMR and MS was combined with RNA sequencing data to determine QS-controlled metabolic repression.[69] This study was not able to analyze the broad spectrum of potential carbon or nitrogen sources in the nutrient-rich culture medium, impeding the full characterization of the metabolic phenotype, but this can be addressed by using either a combination of analytical methods or elaborate targeted methods that use chemical derivatization.[84–85] Targeted metabolomics platforms enable the interpretation of metabolite utilization data, in particular data from metabolic flux studies using isotope labeling, during or after the development of AMR. The isotope labels in core nutrients can be followed over time until a metabolic steady state is achieved, providing information about enzyme function and metabolite transport through various metabolic pathways.[86] The high precision of targeted methods is of utmost importance as changes in the measured metabolite levels influence the metabolic network model. The metabolic networks, in combination with transcript and protein changes, are key to understanding cellular regulatory systems. For example, extensive fluxomics research demonstrated specialized metabolism, with changes in the physiologically relevant carbon sources, during infection in the cystic fibrosis lung.[24] Similar metabolic flux adaptation was observed during AMR development, which was successfully targeted using nutrient supplementation.[31] The targeted analytical methods used in metabolic flux studies benefit from the high sensitivity and metabolite coverage provided by MS detectors used in combination with separation methods, but can also use NMR studies to determine nutrient exchange and utilization at the intercellular level. The non-destructive nature of NMR detectors enables the real-time quantification of metabolic fluxes in living samples, enabling studies on nutrient interchange between biofilm subpopulations.[78]

Sample preparation

Metabolomic data should represent the metabolic state of the microbial population at the moment of sample collection. Metabolic quenching is a critical initial step in the sample preparation process that is necessary to provide an unbiased snapshot of metabolism, given that many metabolites have a rapid turnover rate.[87] In particular, the role of energy metabolism in AMR mechanisms demands efficient quenching techniques as energy- and electron-carrying molecules are chemically labile metabolites that have extremely high turnover rates. Quenching methods need to be chosen on the basis of the cell wall composition of the strain of interest in order to prevent the leakage of intracellular metabolites.[87–88]

Further sample preparation steps depend on the chosen metabolite extraction procedure and the sample clean-up method. The extraction of intracellular metabolites can be achieved by the chemical or mechanical lysis of the cell wall.[87,89–90] Chemical lysis reduces metabolite degradation or the leakage of macromolecules, but requires that a protocol is chosen to suit the analytes of interest. Here, the polarity of the lysis solvent influences the extraction efficiency of different metabolite classes. For example, a study on the influence of colistin treatment on membrane profiles and energy metabolites used two different extraction methods.[57] Combining chemical lysis with mechanical cell disruption is another method to increase the metabolite coverage of the analytical method. Changes in the sample extraction method and the targeted bacterial species can impact the extraction efficiency differently for each metabolite.[90] The final step of the sample preparation procedure is sample clean-up, which is especially important for MS-based methods. The ionization step in MS can be interfered by common components such as salts, sugars, lipids, and proteins.[91] Ionization-suppressing elements can be removed by non-selective protein precipitation or with the use of optimized techniques such as liquid–liquid extraction and solid-phase extraction.[91–92] The sample preparation decisions are dependent on the analytical approach: untargeted metabolomics approaches aim for high metabolite coverage that is achieved by non-selective sample preparation, whereas targeted metabolomics uses sample preparation methods that result in high recovery values for the analytes of interest.[93] Importantly, the development and use of standardized protocols for sample preparation are beneficial for the comparison of metabolomics data between studies because of the dynamic nature of metabolism.

Future perspectives on metabolomics technologies

The metabolome is closely related to biological phenotype, and consequently, metabolomics is expected to be an essential tool in unraveling the phenotypical AMR mechanisms and metabolic adaptations that occur during genetic AMR. The integration of metabolomics with microfluidic systems enables further elucidation of the complex communication systems that are involved in AMR.[94–95] Metabolomic analysis of co-cultivated strains and their environment can be used to study small molecule virulence factors, such as those involved in QS, in both commensal and competitive interactions, as well as their effect on metabolic diversity during host colonization. Here, the advances in resolution and sensitivity of MS analysis will enable both the identification of QS molecules and the elucidation of the metabolic footprint.

The next fundamental step in unraveling phenotypic heterogeneity and its role in AMR mechanisms is the characterization of metabolic profiles from single cells within a heterogeneous population.[96] Slow growth and dormancy are considered essential in antibiotic-tolerant or -persistent subpopulations.[5,97–98]

Nevertheless, the metabolic activity in bacterial subpopulations and its role in AMR is still debated.[7,97,99–100] NMR imaging and spatial ionization techniques have demonstrated different metabolite profiles within bacterial populations, but currently lack the resolution necessary to scrutinize the contribution of single bacterial cells. A prevalent single-cell technique attains its resolution by sampling one cell in an ionization probe before MS analysis, and provides metabolic coverage that is mainly dependent on MS resolution or the integration of innovative separation techniques such as ion mobility.[101] The application of these techniques to resolve bacterial heterogeneity during antibiotic treatment requires multiple improvements that will make it possible to handle low bacterial intracellular volumes and stochastic distribution.

Conclusions

Metabolic changes resulting from evolution and phenotypic adaptation at the infection site are associated with a broad range of AMR mechanisms. Metabolomics technologies can help to unravel and characterize these AMR-associated metabolic effects. To date, metabolomics studies have focused on a limited number of bacterial species and antibiotics. The systematic application of metabolomics studies, in conjunction with complementary next-generation sequencing approaches and experimental evolution models in clinically relevant conditions, will allow further unraveling of the role of microbial metabolism in AMR. The improved understanding may support the discovery of novel metabolism-targeted treatment strategies that can be used in combination with established antibiotic agents.

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