How nutrients shape antibiotic sensitivity of Pseudomonas aeruginosa: food for thought

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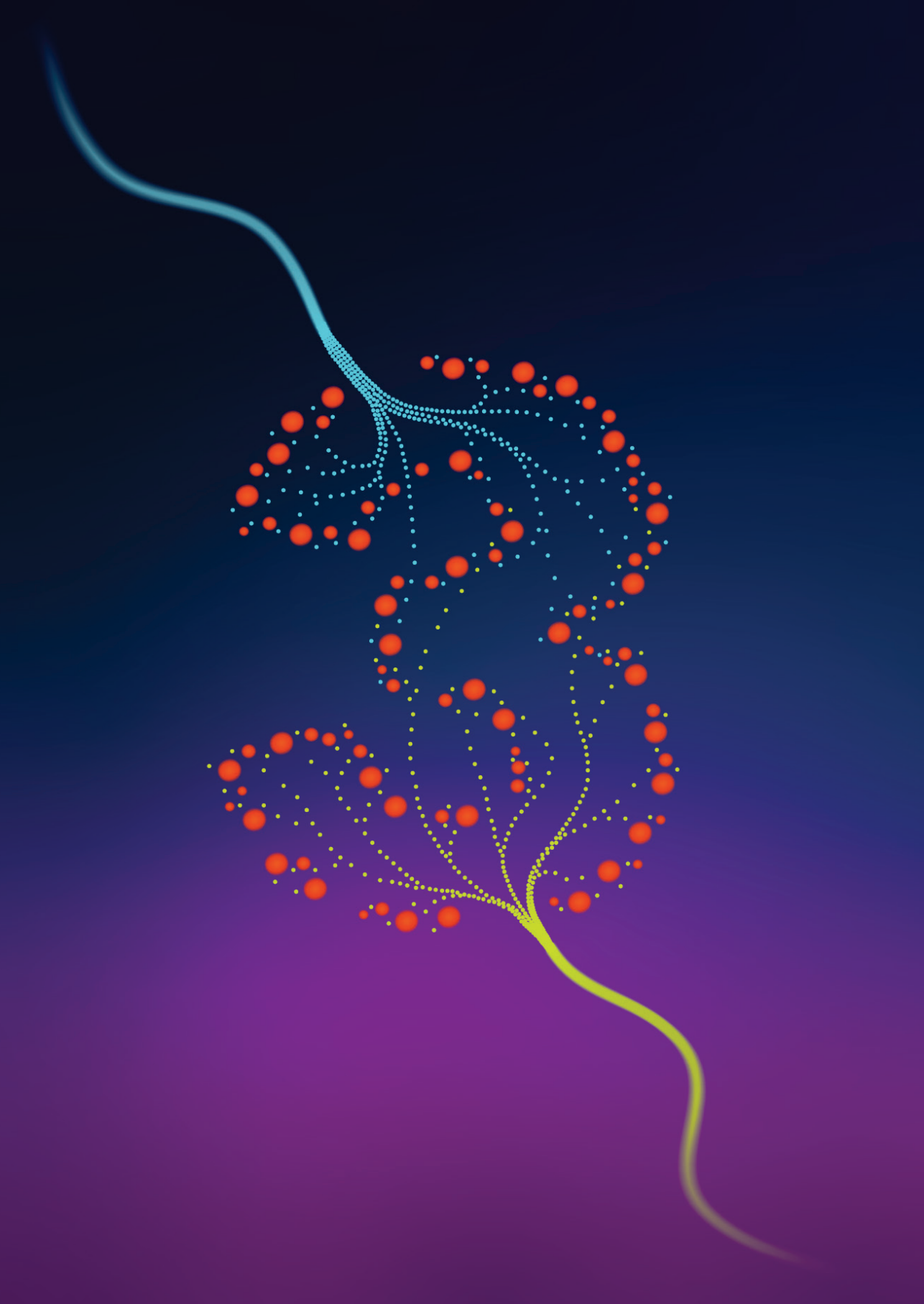

Section III

Nutrients shape antibiotic resistance evolution

How nutrients shape antibiotic sensitivity in *Pseudomonas aeruginosa*

Food for thought

Chapter 5

Unraveling antimicrobial resistance using metabolomics

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Teaser

Novel treatment strategies are needed to address the emerging threat of antimicrobial resistance (AMR) in bacterial pathogens. Metabolomics approaches may help to unravel biochemical underpinnings of AMR, to facilitate the discovery of metabolism-associated drug targets and treatment strategies.

Abstract

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 role of metabolic processes associated with AMR mechanisms including energy production, cell wall synthesis, cell-cell communication, and bacterial growth rate. These metabolic processes can be targeted to re-sensitizing resistant pathogens for antibiotic treatments. We discuss how state-of-the-art metabolomics approaches can be used for comprehensive analysis of microbial metabolism concerning AMR, which may facilitate the discovery of novel drug targets and treatment strategies.

5.1. Introduction

Antimicrobial resistance (AMR) in bacterial pathogens represents an urgent global health threat associated with significant morbidity and mortality[1]. To this end, there exists a need to improve our understanding of underlying molecular mechanisms of AMR 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 due to the acquisition of mobile genetic elements and mutations in chromosomal genes conferring antibiotic resistance[3]. Phenotypic mechanisms are typically linked to transiently decreased antibiotic sensitivity in either a homogeneous (e.g., tolerance) or heterogeneous fashion(e.g., heteroresistance, persistence)[4–6]. Another phenotypic mechanism which 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 may 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 (**Figure 1**). The metabolic state of bacterial cells during antibiotic treatment can either as a contributor to or as a consequence of AMR. Decreased metabolic activity contributes to AMR by reducing antibiotic uptake or secondary effects of antibiotics[9–12]. In contrast, increased metabolic activity is required to support energy-demanding AMR mechanisms such as cell-wall modifications and efflux pumps overexpression[13–18]. Understanding these underlying metabolic processes of AMR mechanisms may be used to strategically alter metabolic activity during antibiotic therapy to re-sensitize pathogens[19]. Metabolomics approaches represent a key enabling technology to help identify relationships between AMR mechanisms and microbial metabolism. Metabolomics represents the systemic study of the metabolome, all small molecules in a biological sample, providing a snapshot of the utilized biochemical processes[20,21]. The metabolome is a close link to organismal phenotype, unveiling initial responses to antibiotic pressure and the adaptations required to sustain AMR mechanisms.

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

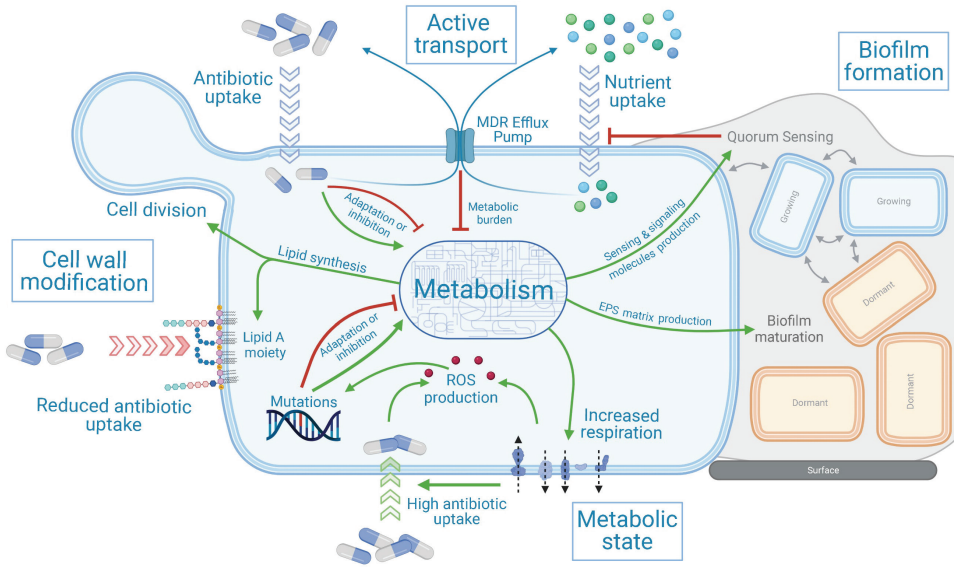

Figure 1. Schematic overview of the role of bacterial metabolism concerning antimicrobial drug action and resistance mechanisms.

5.2. Metabolism and antimicrobial resistance

Key cellular changes associated with AMR where 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**).

Table 1. Overview of studies researching the role of metabolism in AMR using metabolomics techniques

AMR mechanism	Main Finding	Metabolic pathways	Analytical approach	Antibiotics	Species	Ref.
Metabolic adaptation in energy production						
Energy metabolism influences antibiotic efficacy	Energy production is a better predictor for antibiotic efficacy compared to the growth rate	Nucleotides	Colorimetric*	Ampicillin[+] Carbenicillin[+] Gentamicin[±] Kanamycin[±] Streptomycin[±] Ciprofloxacin[<] Levofloxacin[<] Norfloxacin[<] Cefsulodin[>]	*E. 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[+] Gentamicin[±] Levofloxacin[<] Norfloxacin[<] Daptomicin[>] Rifampin[>]	*E. coli* *S. aureus*	30
Nutrient supplementation	Antibiotic resistant cells reduce activity in central carbon metabolism, which can be activated by nutrient supplementations	Glycolysis TCA cycle	Untargeted GC-MS	Kanamycin[±]	*E. tarda*	31,32
	Supplementation of TCA cycle stimulating nutrients increase aminoglycoside tobramycin proton motive force induced cellular intake	TCA cycle	Untargeted LC and GC-MS	Tobramycin[±] Chloramphenicol[>] Linezolid[>] Rafampin[>]	*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. 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[±]	*V. alginolyticus*	43
	Energy metabolism as a defense mechanism to reduce oxidative stress during antibiotic treatment	Glycolysis TCA cycle	(Un-) targeted LC-MS	Streptomycin[±] Isoniazid[>] Rifampicin[>]	*M. 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.	*B. glumae*	67
	Cells in the biofilm core switch to anaerobic fermentation for energy production	Lactate TCA cycle	Targeted NMR	N.A.	*S. oneidensis*	76

AMR mechanism	Main Finding	Metabolic pathways	Analytical approach	Antibiotics	Species	Ref.
Metabolic adaptation in energy production						
Metabolic adaptation in energy production		Nutrient consumption	Targeted LC-UV and LC-MS	N.A.	*P. aeruginosa*	40
	High nutrient levels promote antibiotic resistance development of resistance	Lipids Glycolysis TCA cycle	Untargeted MS	Ampicillin[+] Norfloxacin[<] Chloramphenicol[>]	*E. coli*	38
	Antibiotic treatment from different classes results in distinctive metabolic perturbations and adaptation	Amino acids Glycolysis Nucleotides TCA cycle	Untargeted MS	Kanamycin[±] Nalidixic acid[<] Norfloxacin[<] Ofloxacin[<] Chloramphenicol[>]	*E. coli*	63
	Resistant and sensitive bacteria have distinctive metabolic fingerprints	Glycolysis Lipids Nucleotides Pentose phosphate TCA cycle	Untargeted and targeted LC-MS	Methicillin[+]	*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[+] Carbenicillin[+] Ciprofloxacin[<] Ofloxacin[<] Streptomycin[±] Cefalexin[>] Doxycycline[>] Tetracycline[>]	*E. coli*	61
	Antibiotics and their corresponding mode of action can be identified based on the targeted metabolic pathways of these antibiotics	Amino acids Glycolysis Lipids Nucleotides TCA cycle	Untargeted LC-MS	Ceftazidime[>] Fosmidomycin[>] Triclosan[>]	*E. coli*	62
	Antibiotic surviving cells actively produce ATP during antibiotic treatment, dependent on the nutritional environment	Nucleotides	Colorimetric*	Ciprofloxacin[<] Streptomycin[±] Bedaquilline[>] Isoniazid[>] Rifampicin[>]	*M. 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[+] Ciprofloxacin[<] Kanamycin[±]	*S. aureus*	81
	Antibiotics induce microbiome-independent changes in the host metabolome which alter antibiotic efficacy	Amino acids Glycolysis Nucleotides Pentose phosphate	Untargeted LC-MS	Ciprofloxacin[<]	*E. coli*	41

AMR mechanism	Main Finding	Metabolic pathways	Analytical approach	Antibiotics	Species	Ref.
Metabolic adaptation in energy production						
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 envelope 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$^{\Delta}$	*K. pneumoniae*	55
	Lipid A reconstruction results to increase pentose phosphate activity and reduced TCA cycle activity in colistin resistant cells	Lipids Pentose phosphate TCA cycle	Untargeted LC-MS	Colistin$^{\Delta}$	*A. 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$^{\Delta}$	*M. tuberculosis*	57
	Combination therapy with colistin and doripenem antibiotics affect 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$^{\Delta}$ Doripenem^{+}	*A. baumannii*	64
	The addition of phosphoethanolamine to lipid A for colistin resistance has a high fitness cost	Lipids	Targeted MALDI-MS	Colistin$^{\Delta}$	*E. coli*	59
MDR overexpression	The overexpression of MDR efflux pumps initiates metabolic rewiring to anaerobic respiration	Oxygen and nitrates	Colorimetric* Oximeter	N.A.	*P. aeruginosa*	17

*Fluorescent staining of targeted metabolites

$^{+}$β-Lactam antibiotics

$^{<}$Fluoroquinolone antibiotics

$^{\pm}$Aminoglycoside antibiotics

$^{\Delta}$Polymixin antibiotics

$^{>}$Other antibiotic classes

Cellular energy production

The activity of energy producing metabolic pathways translate the activation of cellular functional responses or dormancy to evade antibiotic killing. The most energy efficient 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 may switch towards less efficient anaerobic fermentative energy production[22]. Several pathogens such as *Pseudomonas aeruginosa* can utilize anaerobic metabolic respiration 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 to increase 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]. However, this approach is only of interest in specific clinical indications, e.g. anaerobic microenvironments in cystic fibrosis-associated lung infections.

Stimulation of aerobic energy production as a therapeutic target to enhance antibiotic sensitivity is an important potential therapeutic strategy. Specifically, supplementation of essential carbon sources to increase aerobic respiration[19], is a promising novel approach to improve antibiotic efficacy, in particular for aminoglycoside antibiotics. Comprehensive *in vitro* screens in bacteria using different carbon source supplements have demonstrated pathogen-dependent changes in aminoglycoside susceptibility with nutrient supplementation[28]. Metabolomics studies demonstrated such carbon source supplementation changes the TCA cycle activity for the synthesis of electron carriers to support the ETC[29–32]. Aminoglycoside efficacy can be increased by increasing the passive influx of charged molecules. Stimulating the ETC results in a higher electric transmembrane potential which enhances the proton-motive force (PMF) mediated influx of the positively charged aminoglycosides[29,33]. Increasing the antibiotic uptake by nutrient-induced PMF demonstrated decreased cell survival in several multi-drug resistant strains.[31]Other antibiotic classes, like β-lactams and fluoroquinolones, also partly depend on cellular respiration for their antimicrobial effects by inducing a redox disbalance as a secondary antibiotic effect. Fluoroquinolones exert better bactericidal effects in metabolic active cells by 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 bactericidal killing in cells with accelerated respiratory activity[30]. In accordance, metabolic activity has experimentally shown to better forecast antibiotic effect than growth rate[37].

It needs to be taken into account that nutrient supplementation to increase antibiotic uptake and induce secondary antibiotic effects rely on the metabolic specialization of the targeted bacterial cell. Clinically relevant strains potentially lose the ability to utilize certain pathways during the acquisition of antibiotic resistant conferring mutations and 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 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 due to the high fitness burden[47]. To further unravel such metabolic effects and adaptations associated with antibiotic efficacy, the use of mathematical flux analysis of central metabolic pathways could help 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.

Cell envelope modifications

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

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

The overexpression of multidrug resistance (MDR) efflux pumps in the cell envelope is another mechanism to regulate 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 enabling mechanism to overcome metabolic burden accompanied by 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 MDR efflux pump promoting mutations in the absence

of selective pressure.[17] The reliance on metabolic adaptation to maintain cellular homeostasis during AMR mechanisms could potentially be utilized therapeutically. For instance, metabolic adaptation upon antibiotic exposure[63–65] can be used to design combination treatments with antibiotic agents. Antibiotics affect different key metabolic pathways, disrupting cell homeostasis, expected to be one of the driving forces between the synergistic effect of the combination therapy of colistin and doripenem[66]. In conclusion, targeting metabolic changes due to efflux pump upregulation are of interest to target therapeutically.

Cell-cell interactions in biofilms

The formation of microbial biofilms forms an important mechanism to decrease antibiotic sensitivity, through the production of extracellular polymeric substances (EPS). Production of EPS is however 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 excreted in the biofilm microenvironment and is essential in biofilm formation and maintenance by synchronizing metabolism for the production of macromolecules to establish the protective layer of extracellular polymeric substances (EPS) layer[68,69]. Targeting QS-associated metabolic processing may thus represent an important target for biofilm-associated infections.

A promising approach to improve the treatment of biofilms exploits the role of QS molecules in biofilm physiology. Disruptive microbial communication treatments can interfere with biofilm integrity over multiple biofilm stages[70], enabling treatment approaches for 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-to-cell communication is a key regulator of bacterial metabolism, which indirectly affects antibiotic susceptibility. Therefore, QS systems create an opportunity to be used as a treatment target[72,73] to get a universal control over metabolic-associated antibiotic potentiation and biofilm physiology. However, the high variety of QS systems and differences between species require further identification and characterization of QS molecules which partake in biofilm biology. Spatial-oriented mass spectrometry techniques can

identify utilized QS molecules and characterize 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 induced by the biofilm structure[75]. The biofilm maturation process is often dependent on the ability of pathogens to metabolically switch to alternative nutrient sources[76,77]. Real-time analysis of the metabolites from the 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 has resulted in increased aminoglycoside eradication[29,33], which highlights the potential of nutrient supplementation to reduce metabolic induced tolerance in biofilms[7].

5.3. Metabolomics technologies and approaches

Metabolomics approaches enable organism-wide metabolite identification and 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 for a set of metabolites, with enhanced possibilities for structural resolution of identified metabolites.

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

Even though metabolomics technologies advanced extensively over the years, the analysis of the full organismal metabolome in a single analytical method is still not possible due to the high diversity in physicochemical characteristics and broad range of concentrations of the metabolites. Here, we

discuss practical considerations considering 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 for combining multiple or time-consuming analytical platforms. HRMS refers to mass analyzers, such as time-of-flight, Orbitrap, and Fourier-transform ion cyclotron resonance, with high mass accuracy, dissociating metabolites up to 0.001 atomic mass units. This high metabolite resolving power of HRMS facilitates the confident identification of metabolites to study metabolic changes without a time-consuming separation step and confident comparison of acquired m/z features to the masses 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 provided the possibility to research metabolic evolution and immediate metabolic response during antibiotic treatment with a variety of antibiotic classes within single studies[38,65]. Comparison studies between bacterial species, environments, or antibiotic classes relying on metabolic data measured within a single do not fully rely on data acquired in other studies, reducing the variability caused by differences in experimental design.

The high mass accuracy achieved with HRMS can also be used to assign the molecular composition of completely unknown metabolites, which was for instance applied for the discovery of novel metabolites. All metabolites covered in central carbon metabolism are covered in most metabolite databases, while many secondary metabolites like 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 from NMR detectors. This metabolite identification method combined with spatial oriented ionization techniques 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 confidently screen for metabolic adaptations or unidentified metabolites concerning AMR, which can be used to better understand AMR mechanisms or develop metabolism-targeted treatment strategies[82].

Targeted metabolomics

Targeted metabolomics approaches require prior knowledge of metabolite targets to enable efficient extraction and isolation from the sampled cells, providing sensitive and selective quantitative analytical methods. This is in contrast with untargeted methods which 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 enables the characterization of exact changes in metabolites concentrations. 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 focusing on specific metabolic pathways[40,45,69,78,83]. Nevertheless, the absolute quantitative data obtained in targeted metabolomics are superior for biological interpretation. For example, the quantitative analysis of nutrient uptake and metabolism with both NMR and MS was combined with earlier obtained RNA sequencing data to determine QS-controlled metabolic repression[69]. This study was not able to analyze broad spectrum of potential carbon or nitrogen sources in the nutrient-rich culture medium, impeding the full characterization of the metabolic phenotype, which can be addressed by using a combination of analytical methods or elaborate targeted methods using chemical derivatization[84,85]. Targeted metabolomics platforms enable the interpretation of metabolite utilization during or after AMR development, in particular for metabolic flux studies using isotope labeling. 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 measured metabolite levels influence the metabolic network model. The metabolic networks in combination with transcript and protein changes are key for the understanding of cellular

regulatory systems. For example, extensive fluxomics research demonstrated different metabolic specialization on physiological 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 of MS detectors hyphenated to separation methods but can be transferred to NMR to determine nutrient exchange and utilization on an intercellular level. The non-destructive nature of NMR detectors enables the real-time quantification of metabolic fluxes in living samples, used for studies on nutrient interchange between biofilm sub-populations[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 to provide an unbiased snapshot of metabolism, given that many metabolites have a rapid turnover rate[87]. Especially the role of energy metabolism in AMR mechanisms demands efficient quenching techniques as energy and electron carrying molecules are chemically labile metabolites with extremely high turnover rates. Quenching methods need to be chosen based on the cell-wall composition of the strain of interest to prevent the leakage of intracellular metabolites[87,88].

Composing the further sample preparation steps consists of the choice for the 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 the metabolite degradation or leakage of macromolecules but needs to be chosen based on 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 uses 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 targeted bacterial species can impact the extraction efficiency differently per metabolite[90]. The final step of the sample preparation procedure is sample clean-up, in

particular important for MS-based methods. The ionization step in MS can be interfered by common components such as salts, sugars, lipids, and proteins[91]. The ionization suppressing elements can be removed by non-selective protein precipitation or optimized techniques such as liquid-liquid extraction and solid-phase extraction[91,92]. The sample preparation decisions are dependent on the analytical approach since untargeted metabolomics approaches aim for high metabolite coverage achieved with non-selective sample preparation, and targeted metabolomics aims for sample preparation methods resulting in high recovery values for the analytes of interest[93]. Importantly, the development and use of standardized protocols in sample preparation techniques are beneficial for the comparison of metabolomics data between studies because of the dynamic nature of metabolism.

Future perspectives on metabolomics technologies

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

The next fundamental step in unraveling phenotypic heterogeneity and their 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]. However, the metabolic activity in these cells and its role in AMR is still debated[7,97,99,100]. NMR imaging and spatial ionization techniques demonstrated different metabolite profiles within bacterial populations, but currently lack the resolution 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, where metabolic coverage is mainly dependent on MS resolution or the integration of innovative separation techniques such as ion

mobility[101]. Applying these techniques in resolving bacterial heterogeneity during antibiotic treatment requires multiple improvements to handle low bacterial intracellular volumes and the stochastic distribution.

5.4. Conclusion

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

5.5. References

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