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Review

Antimicrobial Tolerance and Metabolic Adaptations in Microbial Biofilms

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Active bacterial metabolism is a prerequisite for optimal activity of many classes of antibiotics. Hence, bacteria have developed strategies to reduce or modulate metabolic pathways to become tolerant. This review describes the tight relationship between metabolism and tolerance in bacterial biofilms, and how physicochemical properties of the microenvironment at the host–pathogen interface (such as oxygen and nutritional content) are key to this relationship. Understanding how metabolic adaptations lead to tolerance brings us to novel approaches to tackle antibiotic-tolerant biofilms. We describe the use of hyperbaric oxygen therapy, metabolism-stimulating metabolites, and alternative strategies to redirect bacterial metabolism towards an antibiotic-susceptible phenotype.

Antimicrobial Tolerance in Microbial Biofilms

Multidrug-resistant microorganisms pose an increasing medical risk [1] and it is estimated that yearly mortality due to infections with such organisms could increase to 10 million by 2050, while cumulative cost to the global economy output is estimated to increase to US$100 trillion (https://amr-review.org). Intrinsic antibiotic resistance is based on certain innate properties, applies to all strains of a species, and typically limits the activity of all antibiotics of a certain class, for example, the presence of a poorly permeable outer membrane in Gram-negative bacteria is responsible for glycopeptide resistance. Acquired resistance arises after spontaneous mutations in the genome of susceptible strains (e.g., mutations in *gyrA* encoding the DNA gyrase subunit A provide resistance to fluoroquinolones) or when susceptible bacteria obtain new genetic material with resistance genes (e.g., a plasmid containing a gene encoding a β-lactamase) from resistant microorganisms [2]. 'Resistance' is typically linked to an increased minimal inhibitory concentration (MIC) [3]. As well as these mechanisms of resistance, reduced susceptibility of microorganisms to antimicrobial agents can be attributed to biofilm-associated tolerance. Biofilms are aggregates of microorganisms that can (but do not necessarily have to) be attached to a surface and are embedded in a polymeric matrix [4]. Tolerance is defined as the ability of a microorganism to transiently survive exposure to high concentrations of an antimicrobial agent without an effect on the MIC [3].

Biofilm-associated sessile cells are phenotypically and physiologically very different from planktonic cells, and their reduced susceptibility to antimicrobial agents is related to both tolerance and resistance. Reduced penetration of antimicrobial agents into biofilms, occurrence of persister cells, reduced growth, and biofilm-specific protective stress responses all contribute to tolerance [5]. At the cellular level, antimicrobial access to the molecular targets can also be impaired by conventional resistance mechanisms, notably efflux pumps that expel antibiotics out of the cell, and overexpression of some of these systems has been demonstrated in biofilms [6,7]. Antimicrobial activity is known to depend on the metabolic status of the microorganisms and on the physicochemical conditions prevailing at the infection site, and specific phenotypes with low metabolic activities are frequently found in biofilms, including small colony variants (SCVs) and persister cells [8,9].

Highlights

Biofilms contain regions with low metabolic activity, and this contributes to reduced susceptibility towards antibiotics.

Biofilm heterogeneity and antibiotic tolerance *in vivo* is regulated by access to nutrients and oxygen; the microenvironment of the biofilm plays a crucial role in reduced susceptibility.

Bacteria living in a biofilm often face conditions with low levels of oxygen and nutrients and the metabolic adaptations required to survive under these conditions lead to increased tolerance.

Many of these metabolic adaptations involve the tricarboxylic acid cycle and lead to a reduced proton motive force.

The link between metabolic adaptations to the biofilm lifestyle and reduced susceptibility, opens up novel approaches to treat biofilm-related infections, for example, by using hyperbaric oxygen therapy, or by modulating microbial metabolism by adding certain carbon sources.

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In vitro Biofilm Heterogeneity

The historic focus on planktonic cells led to the assumption that physiological activities in microbial cultures are uniform. However, in highly structured microbial biofilms the environmental conditions are not homogeneous and, as a result of this spatial heterogeneity, microbial physiology and metabolism can be different in different parts of the biofilm [10]. In natural communities this spatial organization is shaped by interactions between different species and/or genotypes, and these interactions can lead to a spectrum of biofilm organization, ranging from 'intermixed' biofilms (i.e., different microbes live intermingled in the same aggregate) to biofilms in which individual aggregates predominantly consist of a single species/genotype [11–13]. Technological advances have allowed scientists to explore this heterogeneity in detail. With microsensor technology, microenvironmental conditions in biofilms (including pH and dissolved oxygen levels) can accurately be measured [14,15], advances in genomics and transcriptomics allow measurement of differences in gene expression at the genome scale [16], while advances in microanatomy, microscopy, and microfluidics have led to a detailed view of spatial, structural, and temporal heterogeneity in biofilms [17,18].

In vitro Biofilm Heterogeneity Results in Metabolic Adaptations Which Contribute to Reduced Susceptibility

The recalcitrance of *in vitro* biofilms to treatment with antibiotics at least partially depends on tolerant subpopulations generated inside the biofilm [10,19,20]. This was mostly studied in continuous-flow cell systems [21], which enable nondestructive monitoring of the biofilm developmental process in real time. In flow cells, the bacteria are growing on a glass surface with a continuous influx of oxygenated growth media. Over time, the biomass and thickness of the biofilm increase, leading to gradients of metabolites and oxygen that generate physiological heterogeneity within the *in vitro* biofilm. The outcome is a stratified biofilm with internal gradients of metabolic activity, growth rates, oxygen, and tolerance to antibiotics [22]. The competition for oxygen and metabolites was illustrated by Kragh *et al.*, showing that preformed aggregates have a fitness advantage in growth compared to planktonic bacteria [23]. It was also shown that bacteria in the outer layers of the biofilm in the flow cell have a fitness advantage compared to bacteria closer to the glass surface, because oxygen and nutrients are more readily available in these outer layers. In *Pseudomonas aeruginosa* biofilms, subpopulations with tolerance to ciprofloxacin and tobramycin are located internally, have low growth rates and low metabolic activity, and colocalize with areas in which there is a lack of oxygen due to intense oxygen consumption in the peripheral parts of the biofilm [10,19,20]. While the reduced susceptibility of biofilm to ciprofloxacin [24] and tobramycin [20] is indeed associated with low growth rates, lack of oxygen may also contribute to the tolerance of biofilm subpopulations to ciprofloxacin by preventing endogenous formation of bactericidal reactive oxygen species (ROS), which augment the killing of *P. aeruginosa* biofilms [25]. However, the lack of ROS formation in oxygen-depleted zones has not been shown to contribute to the tolerance of *P. aeruginosa* biofilm subpopulations to tobramycin. Instead, the low growth rate of the tolerant subpopulations in the *P. aeruginosa* biofilm may reflect hypoxia-induced low metabolic activity associated with decreased expression of antibiotic targets and antibiotic uptake [26]. On the other hand, colistin-tolerant *P. aeruginosa* subpopulations are typically positioned in the peripheral parts of the *in vitro* biofilm, that is, facing a continuous supply of nutrients and oxygen. These colistin-tolerant subpopulations rely on the proton motive force (PMF) for generating sufficient amounts of ATP to drive antimicrobial efflux and alteration of the antibiotic targets by lipopolysaccharide modifications [19], and the access to oxygen probably facilitates the generation of a sufficiently strong PMF. The need for oxygen to develop tolerant subpopulations towards colistin is further emphasized by the higher colistin susceptibility of *P. aeruginosa* biofilms grown under anaerobic conditions [27]. *In vitro*-grown *Staphylococcus aureus* biofilms are also heterogeneous, with subpopulations that are growing aerobically or

fermentatively, are dormant, or are dead [28]. Recently, subpopulations with hypoxia and reduced growth rates were found to affect the antibiotic tolerance of *S. aureus in vitro* biofilms [29].

In vivo Biofilms Are Not Divided into Subpopulations

The *in vivo* biofilm seems to be different from the *in vitro* biofilm [4]. Although both types of biofilm consist of aggregated microorganisms, the microenvironment is different. In infections such as chronic respiratory tract infection in cystic fibrosis (CF) patients [30], chronic wounds [31,32], soft-tissue filler-related infections [33,34], and chronic otitis media [35], bacteria occur as small nonattached biofilms (known as aggregates [36]) that are embedded in a secondary matrix consisting of pus, wound-bed sludge or CF mucus, often surrounded by inflammatory cells [20] (Figure 1). This embedment in a host-derived matrix also creates gradients as observed *in vitro*, and *in vivo* the bacteria in small aggregates all seem to be deprived of oxygen and nutrients. Indeed, based on *in vitro* studies, as little as 40 μm enables almost complete anoxic conditions [37].

In the lungs of CF patients, *P. aeruginosa* biofilms persist in the endobronchial mucus for decades despite intense antibiotic treatment [30]. Interestingly, the endobronchial mucus in chronically infected CF patients contains large zones with reduced oxygen levels, due to active oxygen consumption by polymorphonuclear leukocytes (PMNs) that are recruited to the infection site as part of the inflammatory response [38–40] (Figure 1). The metabolic activity, measured as growth rate of *P. aeruginosa*, is low and is uniformly distributed without gradients of metabolic activity in individual biofilms in the hypoxic mucus in infected CF patients [41]. However, the metabolic activity of individual biofilm aggregates varies and is inversely correlated to the number of surrounding PMNs [41], indicating that the host response may render entire biofilm aggregates tolerant to

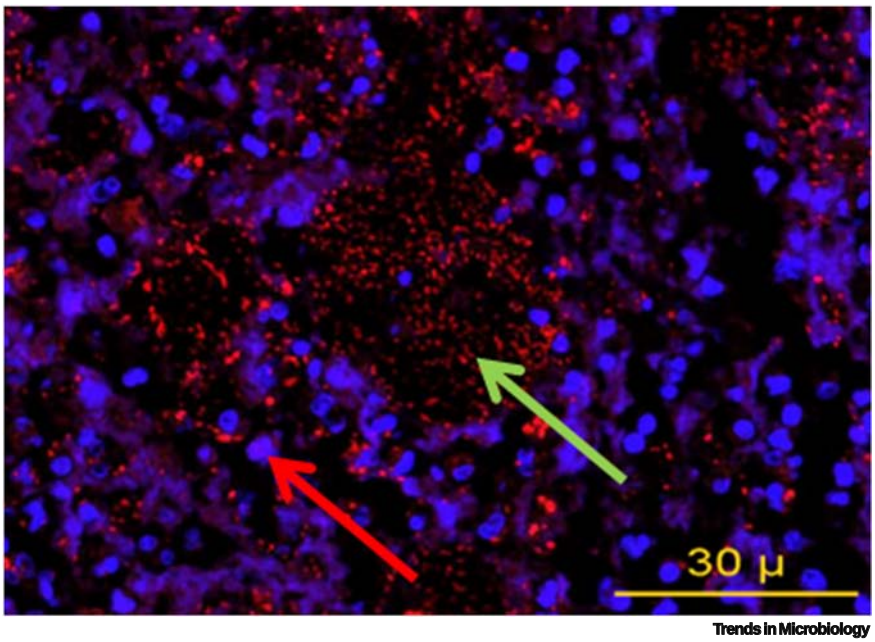

Figure 1. Endobronchial Mucus in an Explanted Lung from a Cystic Fibrosis Patient with Chronic *Pseudomonas aeruginosa* Lung Infection. Using a specific *P. aeruginosa* PNA fluorescence *in situ* hybridization (FISH) probe, the bacteria are visualized in red and are aggregated in a biofilm (green arrow). The inflammatory cells, mainly neutrophils, surrounding the biofilm are counterstained with DAPI, resulting in blue fluorescence (red arrow). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; PNA, peptide nucleic acid.

antibiotics by depleting oxygen. Treatment of CF patients with tobramycin or ciprofloxacin in combination with colistin is very effective in reducing the number of bacteria [42]. However, the need for treatment with multiple antibiotics does not seem to be due to heterogeneity within a single biofilm aggregate, but rather due to differences in growth rate between bacteria in different aggregates (determined by the layer of secondary matrix and number of PMNs). Similar investigations have not been performed for other types of chronic infections, but anoxic conditions and upregulation of bacterial genes involved in anaerobic respiration have been observed in chronic wounds [43].

Biofilm heterogeneity and antibiotic tolerance *in vivo* seem to be regulated by the access to nutrients and especially to oxygen. This means that the microenvironment surrounding the aggregates plays a crucial role, and we need to understand the driving forces of the microenvironment to improve the antibacterial treatment of chronic infections.

Metabolic Adaptations That Lead to Increased Tolerance in Biofilms and Planktonic Cells

In addition to intra- and interbiofilm metabolic differences that are due to changes in environmental factors such as oxygen and overall nutrient availability, other strategies have been discovered that contribute to bacterial tolerance. These metabolic adaptations can occur in both planktonic cultures and biofilms, and may or may not be related to environmental heterogeneity. *In vivo*, most microorganisms do not grow in conditions similar to those encountered in a shaken flask filled with microbial growth medium, and even aerobic nonfermenting biofilm-associated bacteria are often faced with microaerobic or anaerobic conditions. Surviving under these variable conditions requires high versatility which is often achieved through metabolic adaptations, and these adaptations can lead to increased tolerance. A prime example of such a versatile organism is *P. aeruginosa* and it is, for example, well known that this organism can grow anaerobically with nitrate as an alternative terminal electron acceptor (reviewed in [44]).

An overview of metabolic adaptations that influence bacterial tolerance is provided in Figure 2. Using the *P. aeruginosa* PA14 transposon mutant library in an *in vitro* screen for tobramycin-resistant mutants, it was observed that a large proportion of these resistant mutants had the transposon inserted in genes related to metabolism [45]. As uptake of tobramycin is an energy-dependent process that relies on the electron transport chain (ETC) to maintain a sufficiently high PMF, mutations in energy metabolism genes could lead to reduced tobramycin uptake and, hence, reduced susceptibility [45]. Several mutants were affected in genes encoding enzymes involved in NADH reduction and the tricarboxylic acid cycle (TCA) cycle. These results were confirmed using a completely different approach (quantitative proteomics after tobramycin or gentamicin exposure) [46]. Indeed, upon aminoglycoside exposure, a large number of proteins were differentially produced, but most belonged to the functional category 'central intermediary metabolism' and there were clear indications that the oxidative steps of the TCA cycle and the ETC were partially bypassed. Using a similar approach (transposon mutagenesis) in *S. aureus*, increased survival after exposure to lethal doses of ciprofloxacin was linked to mutations in *sucABD, sdhAB, acnA*, and *citC*, all TCA cycle genes [47]. A *Staphylococcus epidermidis* strain, in which the genes encoding citrate synthase, isocitrate dehydrogenase, and aconitase were disrupted, was found to be less susceptible to oxacillin and H_2O_2, and accumulated fourfold less ROS following oxacillin exposure [48]. In addition, decreased autolytic activity is observed in this mutant due to a positively charged cell surface to which autolysins cannot bind; the positive charge of the cell surface is likely related to the diversion of the carbon flux away from the TCA cycle towards the synthesis of the polysaccharide intercellular adhesion (PIA), the deacetylation of which leads to a positive cell surface [48]. In a large collection of clinical *S. epidermidis* isolates, 14.2% of isolates had strong TCA dysfunctions, and 57.9% of the remaining isolates showed

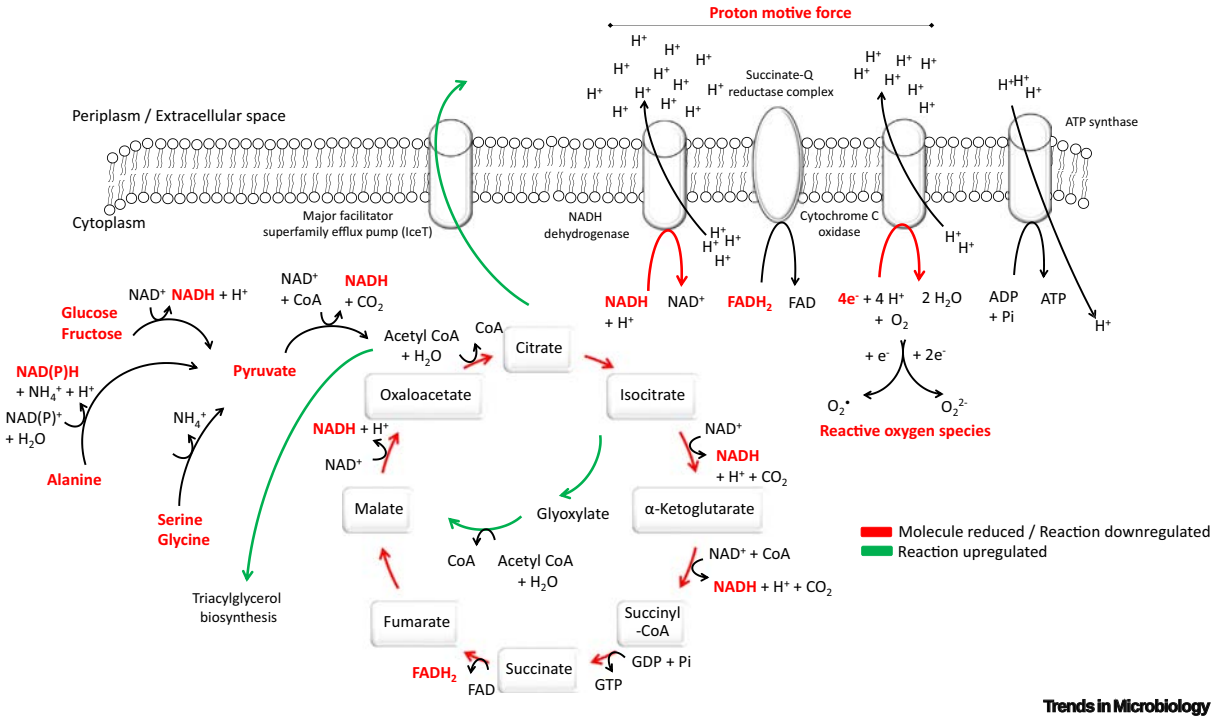

Figure 2. Bacterial Metabolic Adaptations Leading to Antibiotic Tolerance through Decreased Production of Electron Donors (NADH, $FADH_2$). Examples of metabolic adaptions in response to antibiotic exposure *in vitro* and/or in patients are given, hereby compiling adaptations observed for one and/or more bacterial species [26,45–56]. Most reported metabolic adaptations that lead to increased tolerance are mediated through decreased production of the electron donors NADH and $FADH_2$. This is possible through (i) lowering intracellular concentrations of metabolites that are processed through upper glycolysis (such as glucose, fructose) or lower glycolysis (pyruvate), (ii) lowering expression or not expressing TCA cycle enzymes (e.g., citrate synthase, isocitrate dehydrogenase) (such as through changes in gene expression, mutations, or stimulation of the glyoxylate shunt), or (iii) lowering the levels of TCA metabolites (e.g., through export using the major facilitator superfamily efflux pump, IceT, or through activation of biosynthetic pathways that utilize these metabolites – such as triacylglycerol biosynthesis). Decreased production of electron donors reduces the proton motive force (PMF, which is essential for uptake of many antibiotics) and/or may lead to reduced levels of intracellular reactive oxygen species (ROS, which can contribute to the activity of certain antibiotics). A simplified version of the glycolysis, TCA cycle, electron transport chain (ETC), and other reactions is presented. Adaptive mechanisms that involve downregulating the levels of specific molecules/metabolites or metabolic reactions are indicated in red. Adaptive mechanisms that involve inducing specific metabolic reactions are labeled in green.

adaptations leading to a reduced metabolic flux through the TCA cycle. This indicates that, despite the crucial metabolic role of the TCA cycle, adaptations to this pathway as a result of antibiotic exposure occur in wild-type clinical isolates [48]. Also, in *Burkholderia cenocepacia* biofilm cells surviving treatment with high doses of tobramycin, changes in central metabolism were observed [49]. Transcriptomic analysis of these cells revealed that genes encoding enzymes of the bottom part of the TCA cycle (i.e., conversion of isocitrate to malate) were downregulated and that isocitrate lyase- and malate synthase-encoding genes were upregulated, suggesting that increased carbon flux through the glyoxylate shunt protects against antibiotics [49]. A similar response is observed in *Mycobacterium tuberculosis* exposed to isoniazid, rifampicin, or streptomycin. These antibiotics increase cellular levels of pyruvate, succinate, and fumarate, increase the expression of isocitrate lyase, but decrease the expression of *frdA* (encoding fumarate reductase), *sdhA* (succinate dehydrogenase), *fum* (fumarase), and *mdh* (malate dehydrogenase) [50]. This points to a net change in activity of the reductive arm of the TCA cycle and increased flux through the glyoxylate shunt. Interestingly, in isocitrate lyase-deficient *M. tuberculosis*, antibiotic-induced changes in the TCA cycle are not observed, and in these mutants a 10- to 1000-fold increase in susceptibility is observed. Finally, in levofloxacin-resistant *Vibrio alginolyticus,* TCA cycle enzymes were downregulated, indicating a decreased metabolic flux coupled to a lower PMF [51].

In *M. tuberculosis*, triacylglycerol (TAG) (synthesized from glycerol and acyl-CoA by TAG synthases, including Tgs1) accumulates under stress conditions and leads to slower growth. The reason for the slower growth is that induction of *tgs1* expression redirects the carbon flux away from energy-generating pathways like the TCA cycle towards TAG biosynthesis; as acetyl-CoA is the primary substrate for both metabolic pathways, it seems that direct competition for this metabolite is the main reason for the slower growth [52]. Interestingly, both the Δ*tgs1* and a citrate synthase overexpressing strain were markedly more sensitive to antibiotics such as isoniazid, streptomycin, ciprofloxacin, and ethambutol (both *in vitro* and in a mouse model). This suggests that, under conditions of (antibiotic-induced) stress, *M. tuberculosis* can modulate its physiology, slowing down growth and metabolic activity, and by doing so, increase its antibiotic tolerance [52]. *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) uses a conceptually related strategy to slow down growth and metabolic activity under stress conditions. In this organism, the major facilitator superfamily efflux pump IceT, transports citrate out of the cell, making it effectively unavailable as a substrate for the TCA cycle. In addition, it could be demonstrated that IceT expression was linked to increased survival after exposure to ampicillin or ciprofloxacin [53]. In *Edwardsiella tarda*, kanamycin-resistant isolates have lower intracellular levels of fructose [54], glucose, and alanine [55]; as a consequence, NADH levels are lower, leading to a decreased PMF, and reduced kanamycin uptake. Similar observations were made for glycine, serine, and threonine in *Edwardsiella piscida* [56].

The studies mentioned above, either carried out with engineered mutant strains or naturally occurring resistant isolates of diverse bacterial taxa (Gram-positive, Gram-negative, and mycobacteria), paint a clear picture. Reduced susceptibility to bactericidal antibiotics with different modes of action appears to be coupled to a reduced flux through the TCA cycle, by downregulating TCA cycle genes (sometimes accompanied by activation of alternative pathways like the glyoxylate shunt) and/or by reducing the levels of metabolites feeding into the TCA cycle. In many cases these metabolic adaptations lead to a reduced PMF, limiting the influx of the antibiotic in the cell. In addition, these metabolic adaptations are frequently shown to reduce intracellular levels of ROS, which likely contributes to reduced killing [26].

However, ^{13}C labeling in *S. aureus* treated with high (100 × MIC) concentrations of daptomycin showed that, in surviving cells, there is actually an increased metabolic flux towards the TCA cycle. This could be related to the mode of action of daptomycin, which is permeabilization and depolarization of the bacterial cell membrane [57], whereby the increased flux through the TCA could compensate for the dissipated PMF [58]. This example illustrates that metabolic adaptations are at least partially governed by the mode of action of the antibiotic.

Impact of Metabolism on Tolerance *in vivo*

Whether the metabolic pathways that drive antibiotic susceptibility of bacterial pathogens *in vitro* are also influencing the efficacy of antibiotics *in vivo* depends in the first place on the nutritional content at the host–pathogen interface and the metabolic abilities of the pathogen. The available nutrients in the host microenvironment differ between infection sites, the intra- or extracellular location of the pathogen, and the interactions between the host, pathogen, and resident microbial communities [59–61]. It is an important consideration that both the pathogen and the infected host are constantly engaging in reciprocal interactions, where both parties are competing for the same nutrients, and that the outcome of these interactions will shape the nutritional microenvironment. An additional level of complexity is encountered at mucosal sites (e.g., oral cavity, respiratory and intestinal tracts), where diverse microbial communities colonize the host tissues and further contribute to the metabolic content at the infection site. We refer the reader to a comprehensive review of how the nutritional composition of the microenvironment is driven by the host, host–pathogen, and host–microbiome interactions [61]. Here, we describe the *in vivo*

findings that indicate that metabolic pathways playing a role in antibiotic susceptibility of pathogens are active and/or can be activated during the *in vivo* infection process.

Glucose is an essential nutrient for energy-yielding catabolism in the host, and this nutrient will often be metabolized through respiration – hereby combining glycolysis and the TCA cycle. Intracellular pathogens, such as *Salmonella enterica* and enteroinvasive *Escherichia coli*, have the ability to utilize the intracellular levels of carbon sources and/or to enhance their availability through various mechanisms [59,62]. *S.* Typhimurium has been found to depend on glucose, glycolysis, and the TCA cycle during the infection process in a mouse model, as demonstrated using metabolic deletion mutants [63,64]. Other metabolic pathways, including the glyoxylate shunt, are not essential for the *S.* Typhimurium infection process [63]. Taken together, this suggests that metabolic pathways that are involved in antibiotic susceptibility are active in pathogens *in vivo*. However, species- and tissue-specific differences in the use of central metabolic pathways exist, as was demonstrated for the urinary-tract pathogens *E. coli* and *Proteus mirabilis*. Both bacteria required an active TCA cycle for *in vivo* fitness, but specific enzymes in the TCA cycle (succinate dehydrogenase – *sdhB*) were only required for fitness during cystitis in *E. coli* and only during pyelonephritis in *P. mirabilis* [65].

At mucosal sites, microbial communities contribute to shaping the nutritional landscape at the host–pathogen interface [66,67]. External disturbance of microbial consortia, such as during antibiotic treatment, can influence levels of metabolites at the infection site [68,69]. For example, exposure to antibiotics raises the levels of microbiome-produced succinate in the mouse gut, which in turn promotes conversion of succinate to butyrate by *Clostridium difficile*, hereby providing a competitive growth advantage for the latter [70]. This type of microbiome-driven change in the nutritional microenvironment may have downstream effects on the metabolism of pathogens, and hence on tolerance. Interspecies interactions have been found to influence the susceptibility of pathogens to antibiotics [11,71], and while this involves the secretion of secondary metabolites for certain pathogens [72] the potential involvement of primary metabolism remains to be demonstrated.

An intriguing recent finding is that the exposure of mice to antibiotics induced metabolic changes that affected antibiotic efficacy, independently of effects on the gut microbiome. Using a mouse model of peritoneal *E. coli* infection treated with oral ciprofloxacin, Yang *et al*. [73] identified host-produced metabolites specifically induced by antibiotic treatment, and several of these metabolites, including adenosine monophosphate (AMP), decreased ciprofloxacin activity *in vitro*. Furthermore, a recent study described that lung epithelial cells, when grown as an *in vivo*-like three-dimensional cell-culture model, secreted aminoglycoside-potentiating metabolites (succinate, glutamate) [74]. Together, these studies may suggest a role for the metabolic microenvironment in the efficacy of antibiotics *in vivo*.

In addition to considering the host, microbiome, and their interactions in driving nutritional content of the infection site, it is important to also consider other physicochemical factors, including antimicrobial enzymes/peptides, ionic content, pH, levels of oxygen, and alternative electron acceptors. The majority of studies that reported a beneficial influence of metabolites on antibiotic efficacy involved organisms relying on aerobic or anaerobic respiration, and the tolerance of microorganisms that mostly rely on fermentation in the anaerobic gut environment may be less influenced by changes in the level of particular metabolites. Hence, biochemical factors in the host microenvironment, such as oxygen levels, are likely to influence the potentiating effect of metabolites towards antibiotics, analogous to previously discussed strategies adopted by pathogenic bacteria to limit the use of the reductive arm of the TCA cycle. Finally, it should also be mentioned that some of the metabolites that modulate the efficacy of antibiotics also influence bacterial behavior and virulence, which may in turn influence the infectious disease process [75].

Implications for Novel Approaches in Treating Biofilm-related Infections

The combination of tolerance and resistance in microbial biofilms often leads to failure of conventional antimicrobial therapy [76], and a number of alternative strategies have been explored to increase the antimicrobial efficacy of antibiotics and disinfectants towards microbial biofilms. Examples of these strategies include the use of quorum-sensing inhibitors [77] and various approaches to break up the biofilm matrix in order to facilitate access of the antimicrobial agent to the biofilm [78,79]. However, the observation that metabolic adaptations to the biofilm lifestyle contribute to tolerance, opens up novel approaches for treating biofilm-related infections.

Hyperbaric Oxygen Therapy

It is one thing to understand the microenvironment, it is another thing to change it. As oxygen is one of the limiting factors for bacterial growth, hyperbaric oxygen treatment (HBOT) could potentially revert bacterial tolerance and increase the efficacy of antibiotics. The ability to increase the susceptibility to antibiotics in biofilms by providing oxygen was initially demonstrated in *P. aeruginosa* biofilms that contained internal anoxic and peripheral oxygenated subpopulations. When combining HBOT with ciprofloxacin treatment, the oxygenated subpopulation was greatly expanded, the anoxic zone was decreased and the killing by ciprofloxacin was increased [80]. HBOT of anoxic *P. aeruginosa* biofilms grown in agarose (to mimic the *in vivo* situation with intense oxygen consumption by PMNs) generated oxygenated zones containing aerobically-respiring subpopulations with increased growth and susceptibility to ciprofloxacin, partly due to formation of bactericidal ROS [81]. Likewise, the HBOT-enhanced susceptibility to tobramycin was associated with reoxygenation of anoxic zones, inducing aerobic respiration and increased growth in *P. aeruginosa* biofilm [82]. The recent demonstration of the ability of HBOT to enhance the susceptibility of an *S. aureus* biofilm to tobramycin [83] underlines its potential to increase the susceptibility to antibiotics in biofilms of bacterial species other than *P. aeruginosa.* In fact, experimental studies have recently shown increased bacterial clearing and a better outcome by adjuvant HBOT during antibiotic treatment with tobramycin of rats with endocarditis caused by methicillin-resistant *S. aureus* and *S. aureus* [83,84]. Evidence for the clinical relevance of adjuvant HBOT during treatment with antibiotics of biofilm-related infection has recently emerged and includes improved outcome of brain abscesses and device-related infections [85,86]. However, more evidence is needed to evaluate the feasibility of applying HBOT during antibiotic treatment of patients. In particular, application of HBOT to patients with tissue damage, allowing the formation of trapped air, should be carefully considered. Trapped air is a risk factor for inflicting acute, severe tissue lesions by expansion of the trapped air during the decompression phase of HBOT; in this respect pneumothorax is the only absolute contraindication, while other conditions involving possible trapped air are relative contraindications with options to prevent expansion of trapped air [87]. The absence of oxygen dramatically reduces the occurrence of antibiotic resistance resulting from sublethal antibiotic treatment [88], and it is unclear whether the extra oxygen added during adjuvant HBOT is able to drive the development of antimicrobial resistance. The time needed for the development of antimicrobial resistance during sublethal antibiotic treatment at atmospheric oxygen levels ranges from overnight incubation to 100 generation times [88,89], which is far longer than one session of HBOT, which typically lasts for only 90 min. However, further studies are needed to determine if and when adjuvant HBOT induces antimicrobial resistance.

Eradication of Tolerant Cells by Modulating Microbial Metabolism

The realization that shifts in microbial metabolism contribute to tolerance led to two novel approaches to tackle (biofilm-related) infections: (i) stimulation of metabolic pathways that enhance killing by antibiotics, and (ii) inhibition of alternative metabolic strategies.

Table 1 presents an overview of metabolites that have been evaluated for their effect on bacterial tolerance. This table highlights that the supplementation of antibiotics with specific carbon sources, such as carbohydrates and amino acids, and their downstream processing in specific metabolic pathways (such as upper glycolysis, TCA cycle) can lead to a decrease in bacterial tolerance. In contrast, activation of other metabolic pathways (such as the glyoxylate shunt and pentose-phosphate pathway) through supplementation of targeted metabolites (such as glyoxylate or arabinose) is mostly ineffective at potentiating antibiotic activity. However, it also becomes clear from this overview that the effect is species- and antibiotic-dependent. Specific examples are discussed below. Already in 2011 it was shown that persister cells could be eradicated from *E. coli* and *S. aureus* biofilms by combining aminoglycoside antibiotics with specific carbon sources, including fructose, mannitol, glucose, and pyruvate [90]. The mechanism behind the potentiation was the induction of the PMF, which facilitates aminoglycoside uptake. Interestingly, this potentiation did not require activation of the pentose-phosphate pathway, Entner–Doudoroff pathway, or TCA cycle, but relied entirely on NADH dehydrogenase and pyruvate dehydrogenase activity. Mannitol also potentiated tobramycin activity in *P. aeruginosa* biofilms, and the potentiating effect was based on generating a stronger PMF [91]. Mannitol is already used in CF patients to improve mucociliary clearance [92], and formulations containing aminoglycosides plus mannitol could potentially be promising to treat chronic respiratory-tract infections in CF. In *Edwardsiella tarda*, fructose was found to activate the TCA cycle, leading to increased NADH production, an increased PMF, and increased uptake of kanamycin [54]. Similarly, the addition of alanine and/or glucose potentiated the activity of kanamycin [55,93]. After uptake, these metabolites are converted to acetyl-CoA, which stimulates the TCA cycle, increases the intracellular NADH concentration and the PMF, which ultimately leads to increased kanamycin uptake and cell death. Also, other metabolites of the TCA cycle, in particular 2-oxoglutarate, lead to the same effect, and it was shown that activation of oxoglutarate dehydrogenase is particular important in this context [55]. The aminoglycoside-potentiating activity of alanine and/or glucose was confirmed in *Vibrio parahaemolyticus*, *Klebsiella pneumoniae, P. aeruginosa*, and *S. aureus* [55]. In another *Enterobacter* species, *E. piscida*, a similar observation was made for the amino acids glycine, serine, and threonine [56]. In a seminal study, Meylan and colleagues [94] demonstrated that components of the lower part of the TCA cycle (fumarate, succinate) and lower glycolysis (pyruvate) sensitized *P. aeruginosa* cells to tobramycin, while upper TCA metabolites such as citrate had little effect. In contrast, glyoxylate was shown to protect against tobramycin. Through a series of elegant experiments, it was shown that fumarate enhances activity of the ETC by a transcriptional metabolic program (involving increased expression of glycolysis, TCA, and ETC genes), again leading to increased respiration, increased PMF, and increased tobramycin uptake. Finally, it is worth mentioning that two studies have already shown that changing the nutritional content *in vivo* through supplementation of specific antibiotic-potentiating metabolites, such as mannitol and fructose, improves the efficacy of different antibiotics in mouse models of infection [54,90]. These findings support the idea that stimulation of select metabolic pathways *in vivo* may have the potential to improve the efficacy of antibiotics in the host. As a result, researchers are currently exploring the possibility of supplementing antibiotics with metabolites as a novel drug formulation [95,96].

The approach of inhibiting alternative metabolic strategies has not been explored extensively. In *B. cenocepacia* biofilms, inhibiting the glyoxylate shunt with itaconate or 3-nitropropionate (inhibitors of isocitrate lyase) increased killing by tobramycin 10-fold [49]. However, the concentrations required were high (millimolar range), and it remains to be seen whether this approach has any clinical applicability. A different approach was used by Slachmuylders *et al*. [97]. Baicalin hydrate had previously been shown to potentiate the activity of tobramycin against *Burkholderia cepacia* complex bacteria [77], but its potentiating activity was found to be independent of its

Table 1. Effect of Metabolites on Bacterial Tolerance to Antibiotics

Metabolite	Antibiotic[a]	Effect on tolerance	Species	Refs
Upper glycolysis				
Glucose	Km, Gm	Decrease	*Escherichia coli*	[90]
	Km, Gm	No effect	*Staphylococcus aureus*	[90]
	Km, Gm	Decrease	*Edwardsiella tarda*	[55]
	Am, Cf, Bf	No effect	*E. tarda*	[55]
	Km	Decrease	*Edwardsiella piscicida*	[56]
	Tb	Decrease	*Pseudomonas aeruginosa*	[94]
Glucose 6-P	Cip	Increase	*E. coli*	[73]
Fructose	Km, Gm	Decrease	*E. coli, E. tarda, S. aureus, Klebsiella pneumoniae, Vibrio parahemolyticus*	[54,90]
	Am, Of	No effect	*S. aureus*	[90]
	Tb	Decrease	*P. aeruginosa*	[94]
Mannitol	Km, Gm	Decrease	*E. coli*	[90]
	Am, Of	No effect	*E. coli*	[90]
	Gm	No effect	*S. aureus*	[90]
	Tb	Decrease	*P. aeruginosa*	[91]
Maltose	Km	Decrease	*E. tarda*	[55]
Lower glycolysis				
Pyruvate	Km, Gm	Decrease	*E. coli*	[54,90]
	Tb	Decrease	*P. aeruginosa*	[94]
	Gm	No effect	*S. aureus*	[90]
Phosphoenolpyruvate	Cip	Increase	*E. coli*	[73]
Glycerol	Gm	No effect	*E. coli*	[90]
Glycolate	Gm	No effect	*E. coli*	[90]
Galactarate	Gm	No effect	*E. coli*	[90]
Acetate	Tb	Decrease	*P. aeruginosa*	[94]
(Acetyl-/Malonyl-)coenzyme A	Cip	Increase	*E. coli*	[73]
1,3-Bisphosphoglyceric acid	Cip	No effect	*E. coli*	[73]
2-Phosphoglycerate	Cip	No effect	*E. coli*	[73]
Dihydroxyacetone phosphate	Cip	No effect	*E. coli*	[73]
TCA cycle				
Oxaloacetic acid	Km	Decrease	*E. coli*	[54]
Citrate	Km	Decrease	*E. coli*	[54]
		No effect	*P. aeruginosa*	[94]
	Cip	No effect	*E. coli*	[73]
Fumarate	Tb	Decrease	*P. aeruginosa*	[94]
Succinate	Tb	Decrease	*P. aeruginosa*	[94]
α-ketoglutarate	Km	Decrease	*E. tarda*	[55]
	Tb	Decrease	*P. aeruginosa*	[94]

(continued on next page)

Table 1. (continued)

Metabolite	Antibiotic[a]	Effect on tolerance	Species	Refs
Glyoxylate shunt				
Glyoxylate	Tb	Increase	*P. aeruginosa*	[94]
	Cip	Increase	*E. coli*	[73]
Entner–Douderoff pathway				
Gluconate	Gm	No effect	*E. coli*	[90]
	Tb	Decrease	*P. aeruginosa*	[94]
Pentose-phosphate pathway				
Arabinose	Gm	No effect	*E. coli*	[90]
Ribose	Gm	No effect	*E. coli*	[90]
	Tb	Decrease	*P. aeruginosa*	[94]
Ribulose 5-P	Cip	No effect	*E. coli*	[73]
Sedoheptulose 7-phosphate	Cip	No effect	*E. coli*	[73]
Fatty acid metabolism				
Propionate	Tb	Decrease	*P. aeruginosa*	[94]
Amino acids				
Alanine	Km, Gm	Decrease	*E. tarda*	[55,93]
	Am, Cf, Bf	No effect	*E. tarda*	[55]
	Cip	No effect	*E. coli*	[73]
Glutamate	Km	Decrease	*E. coli*	[54]
	Cip	Increase	*E. coli*	[73]
Isoleucine	Km	Decrease	*E. tarda*	[55]
Threonine	Km	Decrease	*E. tarda, E. piscicida*	[55,56]
Glycine	Km	Decrease	*E. piscicida*	[56]
Serine	Km	Decrease	*E. piscicida*	[56]
Phenylalanine	Km	Decrease	*E. piscicida*	[56]
Others				
AMP, NADP, oxidized/reduced glutathione, NAD, GDP	Cip	Increase	*E. coli*	[73]
Thymine, cytidine (monophosphate), cAMP, guanosine (monophosphate), guanine, riboflavin, methionine, aicar, UDP, UDP-D-glucuronate, adenine, hypoxanthine, inosine, adenosine	Cip	No effect	*E. coli*	[73]

[a]Am, ampicillin; Km, kanamycin; Gm, gentamicin; Cip, ciprofloxacin; Of, ofloxacin; Cf, ceftazidime; Bf, belofloxacin; Tb, tobramycin.

quorum-sensing inhibitory activity. A transcriptomic analysis showed that baicalin hydrate downregulates glyoxylate shunt genes and upregulates genes involved in the TCA cycle, oxidative phosphorylation, and glucarate metabolism; and adding glucarate mimicked the effect observed with baicalin hydrate. While the mechanism(s) behind this transcriptional reprogramming by baicalin hydrate are yet unknown, this study shows that, in addition, compounds that do not directly interfere with carbohydrate metabolism can be used to potentiate antibiotics by modulating metabolism [97]. Results obtained with an *M. tuberculosis* strain, in which malate synthase

was knocked down, showed that interfering with the glyoxylate shunt increased killing by rifampicin [98], suggesting that strategies that target this biochemical pathway could be widely applicable to potentiate the activity of antibiotics.

While much of the potentiating activity observed when adding specific metabolites is due to increased antibiotic uptake linked to an increased PMF, in many studies the involvement of ROS was also demonstrated. Indeed, ROS play an important role in the antibiotic-mediated killing of bacteria [26,99], and as ROS are an inevitable by-product of aerobic metabolism, their levels are expected to be increased in cells in which the metabolic rate is high and oxygen is sufficiently available. Several studies have shown that interfering with defense mechanisms against ROS, either by inactivating genes coding for catalases or superoxide dismutases, by inhibiting the activity of these enzymes, or by reducing intracellular levels of antioxidants, increases antibiotic killing (e.g., [49,93]). However, also for this approach, the clinical applicability remains to be investigated.

Concluding Remarks

The microenvironment and metabolic adaptations can render antibiotic treatment of microbial biofilms ineffective. Antimicrobial tolerance induced by metabolic adaptations typically downregulates the PMF and the ETC, and may result from changing availability of nutrients in the microenvironment imposed by the host response and/or other microbes. In this respect, the possibility to revert tolerance to susceptibility by restocking lacking metabolites and electron acceptors could be of major clinical potential. The unleashing of this potential for advancing antibiotic treatment of infectious biofilms, however, awaits further research to determine the optimal timing and safety of supplemental carbon sources and oxygen (see Outstanding Questions).

Outstanding Questions

To what extent does biofilm heterogeneity contribute to reduced susceptibility? Is there a relevant difference between heterogeneity observed *in vitro* vs. *in vivo*?

How important is reduced metabolic activity in the overall antimicrobial tolerance in biofilms? To what extent is this species- and/or tissue-specific?

Which bacterial metabolic pathways are active during infection, and which ones can be activated by adding specific metabolites?

Can bacterial metabolism be stimulated during the infection process without increasing virulence and without adverse effects on host tissues?

Can the metabolism of fermenting microorganisms be modulated to increase tolerance?

Can strategies be designed to selectively target metabolism of pathogens, without affecting commensal microorganisms and host metabolism?

Why does the potentiating effect of certain metabolites differ between bacterial species with a similar metabolism?

Which metabolite concentrations need to be reached *in vivo* to exert antibiotic-potentiating effects? Can these concentrations be achieved at the site of infection?

References

1. Theuretzbacher, U. (2017) Global antimicrobial resistance in Gram-negative pathogens and clinical need. *Curr. Opin. Microbiol.* 39, 106–112
2. Fernandez, L. and Hancock, R.E. (2012) Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin. Microbiol. Rev.* 25, 661–681
3. Brauner, A. *et al.* (2016) Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nat. Rev. Microbiol.* 14, 320–330
4. Bjarnsholt, T. *et al.* (2013) The *in vivo* biofilm. *Trends Microbiol.* 21, 466–474
5. Van Acker, H. *et al.* (2014) Molecular mechanisms of antimicrobial tolerance and resistance in bacterial and fungal biofilms. *Trends Microbiol.* 22, 326–333
6. Van Acker, H. and Coenye, T. (2016) The role of efflux and physiological adaptation in biofilm tolerance and resistance. *J. Biol. Chem.* 291, 12565–12572
7. Zaborskyte, G. *et al.* (2017) Real-time monitoring of nfxb mutant occurrence and dynamics in *Pseudomonas aeruginosa* biofilm exposed to subinhibitory concentrations of ciprofloxacin. *Antimicrob. Agents Chemother.* 61, e02292-16
8. Conlon, B.P. *et al.* (2015) Persister cells in biofilm associated infections. *Adv. Exp. Med. Biol.* 831, 1–9
9. Garcia, L.G. *et al.* (2013) Antibiotic activity against small-colony variants of *Staphylococcus aureus*: review of *in vitro*, animal and clinical data. *J. Antimicrob. Chemother.* 68, 1455–1464
10. Stewart, P.S. and Franklin, M.J. (2008) Physiological heterogeneity in biofilms. *Nat. Rev. Microbiol.* 6, 199–210
11. Burmolle, M. *et al.* (2014) Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* 22, 84–91
12. Liu, W. *et al.* (2016) Interspecific bacterial interactions are reflected in multispecies biofilm spatial organization. *Front. Microbiol.* 7, 1366
13. Nadell, C.D. *et al.* (2016) Spatial structure, cooperation and competition in biofilms. *Nat. Rev. Microbiol.* 14, 589–600
14. Beyenal, H. and Babauta, J. (2014) Microsensors and microscale gradients in biofilms. *Adv. Biochem. Engineer. Biotechnol.* 146, 235–256
15. Sonderholm, M. *et al.* (2017) The consequences of being in an infectious biofilm: microenvironmental conditions governing antibiotic tolerance. *Int. J. Mol. Sci.* 18, E2688
16. Williamson, K.S. *et al.* (2012) Heterogeneity in *Pseudomonas aeruginosa* biofilms includes expression of ribosome hibernation factors in the antibiotic-tolerant subpopulation and hypoxia-induced stress response in the metabolically active population. *J. Bacteriol.* 194, 2062–2073
17. Moormeier, D.E. *et al.* (2013) Use of microfluidic technology to analyze gene expression during *Staphylococcus aureus* biofilm formation reveals distinct physiological niches. *Appl. Environ. Microbiol.* 79, 3413–3424
18. Serra, D.O. *et al.* (2013) Microanatomy at cellular resolution and spatial order of physiological differentiation in a bacterial biofilm. *mBio* 4, e00103–e00113
19. Pamp, S.J. *et al.* (2008) Tolerance to the antimicrobial peptide colistin in *Pseudomonas aeruginosa* biofilms is linked to metabolically active cells, and depends on the *pmr* and *mexAB-oprM* genes. *Mol. Microbiol.* 68, 223–240
20. Sonderholm, M. *et al.* (2017) *Pseudomonas aeruginosa* aggregate formation in an alginate bead model system exhibits *in vivo*-like characteristics. *Appl. Environ. Microbiol.* 83, e00113-17
21. Moller, S. *et al.* (1998) In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. *Appl. Environ. Microbiol.* 64, 721–732
22. Jensen, P.O. *et al.* (2017) Microenvironmental characteristics and physiology of biofilms in chronic infections of CF patients are strongly affected by the host immune response. *APMIS* 125, 276–288
23. Kragh, K.N. *et al.* (2016) Role of multicellular aggregates in biofilm formation. *mBio* 7, e00237
24. Evans, D.J. *et al.* (1991) Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. *J. Antimicrob. Chemother.* 27, 177–184
25. Jensen, P.O. *et al.* (2014) Formation of hydroxyl radicals contributes to the bactericidal activity of ciprofloxacin against *Pseudomonas aeruginosa* biofilms. *Pathog. Dis.* 70, 440–443

26. Van Acker, H. and Coenye, T. (2017) The role of reactive oxygen species in antibiotic-mediated killing of bacteria. *Trends Microbiol.* 25, 456–466
27. Kolpen, M. *et al.* (2016) Increased bactericidal activity of colistin on *Pseudomonas aeruginosa* biofilms in anaerobic conditions. *Pathog. Dis.* 74, ftv086
28. Rani, S.A. *et al.* (2007) Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J. Bacteriol.* 189, 4223–4233
29. Pabst, B. *et al.* (2016) Gel-entrapped *Staphylococcus aureus* bacteria as models of biofilm infection exhibit growth in dense aggregates, oxygen limitation, antibiotic tolerance, and heterogeneous gene expression. *Antimicrob. Agents Chemother.* 60, 6294–6301
30. Bjarnsholt, T. *et al.* (2009) *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatr. Pulmonol.* 44, 547–558
31. Bjarnsholt, T. *et al.* (2008) Why chronic wounds will not heal: a novel hypothesis. *Wound Repair Regen.* 16, 2–10
32. Kirketerp-Moller, K. *et al.* (2008) Distribution, organization, and ecology of bacteria in chronic wounds. *J. Clin. Microbiol.* 46, 2717–2722
33. Christensen, L. *et al.* (2013) Bacterial infection as a likely cause of adverse reactions to polyacrylamide hydrogel fillers in cosmetic surgery. *Clin. Infect. Dis.* 56, 1438–1444
34. Bjarnsholt, T. *et al.* (2009) Detection of bacteria by fluorescence in situ hybridization in culture-negative soft tissue filler lesions. *Dermatol. Surg.* 35, 1620–1624
35. Jensen, R.G. *et al.* (2017) Recurrent otorrhea in chronic suppurative otitis media: is biofilm the missing link? *Eur. Arch. Otorhinolaryngol.* 274, 2741–2747
36. Alhede, M. *et al.* (2011) Phenotypes of non-attached *Pseudomonas aeruginosa* aggregates resemble surface attached biofilm. *PLoS One* 6, e27943
37. Folsom, J.P. *et al.* (2010) Physiology of *Pseudomonas aeruginosa* in biofilms as revealed by transcriptome analysis. *BMC Microbiol.* 10, 294
38. Kolpen, M. *et al.* (2014) Nitric oxide production by polymorphonuclear leucocytes in infected cystic fibrosis sputum consumes oxygen. *Clin. Exp. Immunol.* 177, 310–319
39. Kolpen, M. *et al.* (2010) Polymorphonuclear leucocytes consume oxygen in sputum from chronic *Pseudomonas aeruginosa* pneumonia in cystic fibrosis. *Thorax* 65, 57–62
40. Worlitzsch, D. *et al.* (2002) Effects of reduced mucus oxygen concentration in airway *Pseudomonas infections* of cystic fibrosis patients. *J. Clin. Invest.* 109, 317–325
41. Kragh, K.N. *et al.* (2014) Polymorphonuclear leukocytes restrict growth of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients. *Infect. Immun.* 82, 4477–4486
42. Hansen, C.R. *et al.* (2008) Early aggressive eradication therapy for intermittent *Pseudomonas aeruginosa* airway colonization in cystic fibrosis patients: 15 years experience. *J. Cyst. Fibrosis* 7, 523–530
43. James, G.A. *et al.* (2016) Microsensor and transcriptomic signatures of oxygen depletion in biofilms associated with chronic wounds. *Wound Repair Regen.* 24, 373–383
44. Schobert, M. and Jahn, D. (2010) Anaerobic physiology of *Pseudomonas aeruginosa* in the cystic fibrosis lung. *Int. J. Med. Microbiol.* 300, 549–556
45. Schurek, K.N. *et al.* (2008) Novel genetic determinants of low-level aminoglycoside resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 52, 4213–4219
46. Mikkelsen, H. *et al.* (2010) Proteomic analysis of the adaptive responses of *Pseudomonas aeruginosa* to aminoglycoside antibiotics. *FEMS Microbiol. Lett.* Published online August 2010. https://doi.org/10.1111/j.1574-6968.2009.01729.x
47. Wang, Y. *et al.* (2018) Inactivation of TCA cycle enhances *Staphylococcus aureus* persister cell formation in stationary phase. *Sci. Rep.* 8, 10849
48. Thomas, V.C. *et al.* (2013) A dysfunctional tricarboxylic acid cycle enhances fitness of *Staphylococcus epidermidis* during beta-lactam stress. *mBio* 4, e00437-13
49. Van Acker, H. *et al.* (2013) Biofilm-grown *Burkholderia cepacia* complex cells survive antibiotic treatment by avoiding production of reactive oxygen species. *PLoS One* 8, e58943
50. Nandakumar, M. *et al.* (2014) Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. *Nat. Commun.* 5, 4306
51. Cheng, Z.X. *et al.* (2018) The depressed central carbon and energy metabolisms is associated to the acquisition of levofloxacin resistance in *Vibrio alginolyticus*. *J. Proteom.* 181, 83–91
52. Baek, S.H. *et al.* (2011) Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PLoS Biol.* 9, e1001065
53. Frawley, E.R. *et al.* (2013) Iron and citrate export by a major facilitator superfamily pump regulates metabolism and stress resistance in *Salmonella* Typhimurium. *Proc. Natl Acad. Sci. U. S. A.* 110, 12054–12059
54. Su, Y.B. *et al.* (2015) Fructose restores susceptibility of multidrug-resistant *Edwardsiella tarda* to kanamycin. *J. Proteome Res.* 14, 1612–1620
55. Peng, B. *et al.* (2015) Exogenous alanine and/or glucose plus kanamycin kills antibiotic-resistant bacteria. *Cell Metab.* 21, 249–262
56. Ye, J.Z. *et al.* (2018) Identification and efficacy of glycine, serine and threonine metabolism in potentiating kanamycin-mediated killing of *Edwardsiella piscicida*. *J. Proteom.* 183, 34–44
57. Taylor, S.D. and Palmer, M. (2016) The action mechanism of daptomycin. *Bioorg. Medicin. Chem.* 24, 6253–6268
58. Lechner, S. *et al.* (2014) Metabolic and transcriptional activities of *Staphylococcus aureus* challenged with high-doses of daptomycin. *Int. J. Med. Microbiol.* 304, 931–940
59. Abu Kwaik, Y. and Bumann, D. (2015) Host delivery of favorite meals for intracellular pathogens. *PLoS Pathog.* 11, e1004866
60. Fuchs, T.M. *et al.* (2012) Metabolic adaptation of human pathogenic and related nonpathogenic bacteria to extra- and intracellular habitats. *FEMS Microbiol. Rev.* 36, 435–462
61. Passalacqua, K.D. *et al.* (2016) Bacterial metabolism shapes the host–pathogen interface. *Microbiol. Spectr.* Published online June 2016. https://doi.org/10.1128/microbiolspec.VMBF-0027-2015
62. Gotz, A. and Goebel, W. (2010) Glucose and glucose 6-phosphate as carbon sources in extra- and intracellular growth of enteroinvasive *Escherichia coli* and *Salmonella enterica*. *Microbiology* 156, 1176–1187
63. Bowden, S.D. *et al.* (2009) Glucose and glycolysis are required for the successful infection of macrophages and mice by *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* 77, 3117–3126
64. Tchawa Yimga, M. *et al.* (2006) Role of gluconeogenesis and the tricarboxylic acid cycle in the virulence of *Salmonella enterica* serovar Typhimurium in BALB/c mice. *Infect. Immun.* 74, 1130–1140
65. Alteri, C.J. *et al.* (2015) Preferential use of central metabolism *in vivo* reveals a nutritional basis for polymicrobial infection. *PLoS Pathog.* 11, e1004601
66. Nicholson, J.K. *et al.* (2012) Host-gut microbiota metabolic interactions. *Science* 336, 1262–1267
67. Takahashi, N. (2015) Oral microbiome metabolism: from 'who are they?' to 'what are they doing?'. *J. Dental Res.* 94, 1628–1637
68. Zarrinpar, A. *et al.* (2018) Antibiotic-induced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism. *Nat. Commun.* 9, 2872
69. Perez-Cobas, A.E. *et al.* (2013) Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. *Gut* 62, 1591–1601
70. Ferreyra, J.A. *et al.* (2014) Gut microbiota-produced succinate promotes *C. difficile* infection after antibiotic treatment or motility disturbance. *Cell Host Microbe* 16, 770–777
71. Tavernier, S. *et al.* (2017) Community composition determines activity of antibiotics against multispecies biofilms. *Antimicrob. Agents Chemother.* 61, e00302-17
72. Limoli, D.H. *et al.* (2017) *Pseudomonas aeruginosa* alginate overproduction promotes coexistence with *Staphylococcus aureus* in a model of cystic fibrosis respiratory infection. *mBio* 8, e00186-17
73. Yang, J.H. *et al.* (2017) Antibiotic-induced changes to the host metabolic environment inhibit drug efficacy and alter immune function. *Cell Host Microbe* 22, 757–765 e3
74. Crabbé, A. *et al.* (2019) Host metabolites stimulate the bacterial proton motive force to enhance the activity of aminoglycoside antibiotics. *PLoS Pathog.* 15, e1007697

75. Eisenreich, W. *et al.* (2010) Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nat. Rev. Microbiol.* 8, 401–412
76. Hoiby, N. *et al.* (2015) ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. *Clin. Microbiol. Infect.* 21, S1–25
77. Brackman, G. *et al.* (2011) Quorum sensing inhibitors increase the susceptibility of bacterial biofilms to antibiotics *in vitro* and *in vivo*. *Antimicrob. Agents Chemother.* 55, 2655–2661
78. Teirlinck, E. *et al.* (2018) Laser-induced vapour nanobubbles improve drug diffusion and efficiency in bacterial biofilms. *Nat. Commun.* 9, 4518
79. Belfield, K. *et al.* (2017) Evaluation of combinations of putative anti-biofilm agents and antibiotics to eradicate biofilms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 72, 2531–2538
80. Kolpen, M. *et al.* (2016) Reinforcement of the bactericidal effect of ciprofloxacin on *Pseudomonas aeruginosa* biofilm by hyperbaric oxygen treatment. *Int. J. Antimicrob. Agents* 47, 163–167
81. Kolpen, M. *et al.* (2017) Hyperbaric oxygen sensitizes anoxic *Pseudomonas aeruginosa* biofilm to ciprofloxacin. *Antimicrob. Agents Chemother.* 61, e01024-17
82. Moller, S.A. *et al.* (2019) Hyperbaric oxygen treatment increases killing of aggregating *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *J. Cyst. Fibrosis* Published online 30 January 2019. https://doi.org/10.1016/j.jcf.2019.01.005
83. Lerche, C.J. *et al.* (2017) Hyperbaric oxygen therapy augments tobramycin efficacy in experimental *Staphylococcus aureus* endocarditis. *Int. J. Antimicrob. Agents* 50, 406–412
84. Ozkan, M.T. *et al.* (2016) Is hyperbaric oxygen or ozone effective in experimental endocarditis? *J. Surg. Res.* 202, 66–70
85. Bartek Jr., J. *et al.* (2016) Hyperbaric oxygen therapy in spontaneous brain abscess patients: a population-based comparative cohort study. *Acta Neurochirurg.* 158, 1259–1267
86. Bartek Jr., J. *et al.* (2018) Hyperbaric oxygen therapy as adjuvant treatment for hardware-related infections in neuromodulation. *Stereotact. Function. Neurosurg.* 96, 100–107
87. Heyboer 3rd, M. *et al.* (2017) Hyperbaric oxygen therapy: side effects defined and quantified. *Adv. Wound Care* 6, 210–224
88. Kohanski, M.A. *et al.* (2010) Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Mol. Cell* 37, 311–320
89. Jorgensen, K.M. *et al.* (2013) Sublethal ciprofloxacin treatment leads to rapid development of high-level ciprofloxacin resistance during long-term experimental evolution of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 57, 4215–4221
90. Allison, K.R. *et al.* (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* 473, 216–220
91. Barraud, N. *et al.* (2013) Mannitol enhances antibiotic sensitivity of persister bacteria in *Pseudomonas aeruginosa* biofilms. *PLoS One* 8, e84220
92. Nevitt, S.J. *et al.* (2018) Inhaled mannitol for cystic fibrosis. *Cochrane Datab. Syst. Rev.* 2, CD008649
93. Ye, J.Z. *et al.* (2018) Alanine enhances aminoglycosides-induced ROS production as revealed by proteomic analysis. *Front. Microbiol.* 9, 29
94. Meylan, S. *et al.* (2017) Carbon sources tune antibiotic susceptibility in *Pseudomonas aeruginosa* via tricarboxylic acid cycle control. *Cell Chem. Biol.* 24, 195–206
95. Collins, J.J. *et al.* (2017) Proton-motive force stimulation to potentiate aminoglycoside antibiotics against persistent bacteria. U.S. Patent 100807667
96. Collins, J.J. *et al.* (2015) Intermediate metabolism products to potentiate aminoglycoside antibiotics in bacterial infections. U.S. Patent application 14/914
97. Slachmuylders, L. *et al.* (2018) Elucidation of the mechanism behind the potentiating activity of baicalin against *Burkholderia cenocepacia* biofilms. *PLoS One* 13, e0190533
98. Singh, K.S. *et al.* (2017) Down-regulation of malate synthase in *Mycobacterium tuberculosis* H37Ra leads to reduced stress tolerance, persistence and survival in macrophages. *Tuberculosis* 106, 73–81
99. Kohanski, M.A. *et al.* (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130, 797–810