

Biochemical principles enabling metabolic cooperativity and phenotypic heterogeneity at the single cell level

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Abstract

All biosynthetically active cells release metabolites, in part due to membrane leakage and cell lysis, but also in part due to overflow metabolism and ATP-dependent membrane export. At the same time, cells are adapted to sense and take up extra-cellular nutrients when available, to minimize the number of biochemical reactions that have to operate within a cell in parallel, and ultimately, to gain metabolic efficiency and biomass. Within colonies, biofilms or tissues, the co-occurrence of metabolite export and import enables the sharing of metabolites as well as metabolic specialization of single cells. In this review we discuss emerging biochemical concepts that give reasoning for why cells overproduce and release metabolites, and how these form the foundations for cooperative metabolite exchange activity between cells. We place particular emphasis on discussing the role of overflow metabolism in cells that exhibit either the Warburg or Crabtree effect. Furthermore, we discuss the profound physiological changes that cells undergo when their metabolism switches from metabolite synthesis to uptake, providing an explanation why metabolic specialization results in non-genotypic heterogeneity at the single cell level.

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Introduction

To be competitive in their environment, cells from all kingdoms of life have evolved a series of mechanisms to sense nutrients and uptake the metabolites needed for their growth and survival [1–4]. Metabolite uptake saves energy, carbon and nitrogen, and is further beneficial by reducing the number of biochemical reactions that need to run in parallel, increasing metabolic efficiency [5].

At the same time, biosynthetically active cells are known to release a complex spectrum of metabolites [6–10], and within communities, such as colonies, biofilms and tissues, these metabolites can enrich the extracellular space (Figure 1). The export of metabolites coupled with the preference for cellular import (upon reaching a critical concentration) allows for the exchange of metabolites between cells and when this occurs within microbial communities, such metabolite exchange permits the survival of otherwise unculturable cells (auxotrophs). The extent of this metabolite exchange in microbial environments is demonstrated by current estimates, that up to 90% of bacterial species are not metabolically viable outside a community environment [11–15]. Further, it is becoming increasingly clear that metabolite export and import are equally important for cells that do not completely depend on metabolite sharing for growth (prototrophs), and that a broad spectrum of metabolites are involved in these exchange events (Figure 2) [10]. Moreover, when individual cells switch from biosynthesis to uptake for a metabolite their physiology shows to be fundamentally altered [16,17]. In this review we discuss these exchange interactions, from the point of view that they do not solely emerge to confer a selective advantage in the ecological and evolutionary sense, but also as a consequence of basic biochemical properties that underlie the function of the metabolic network.

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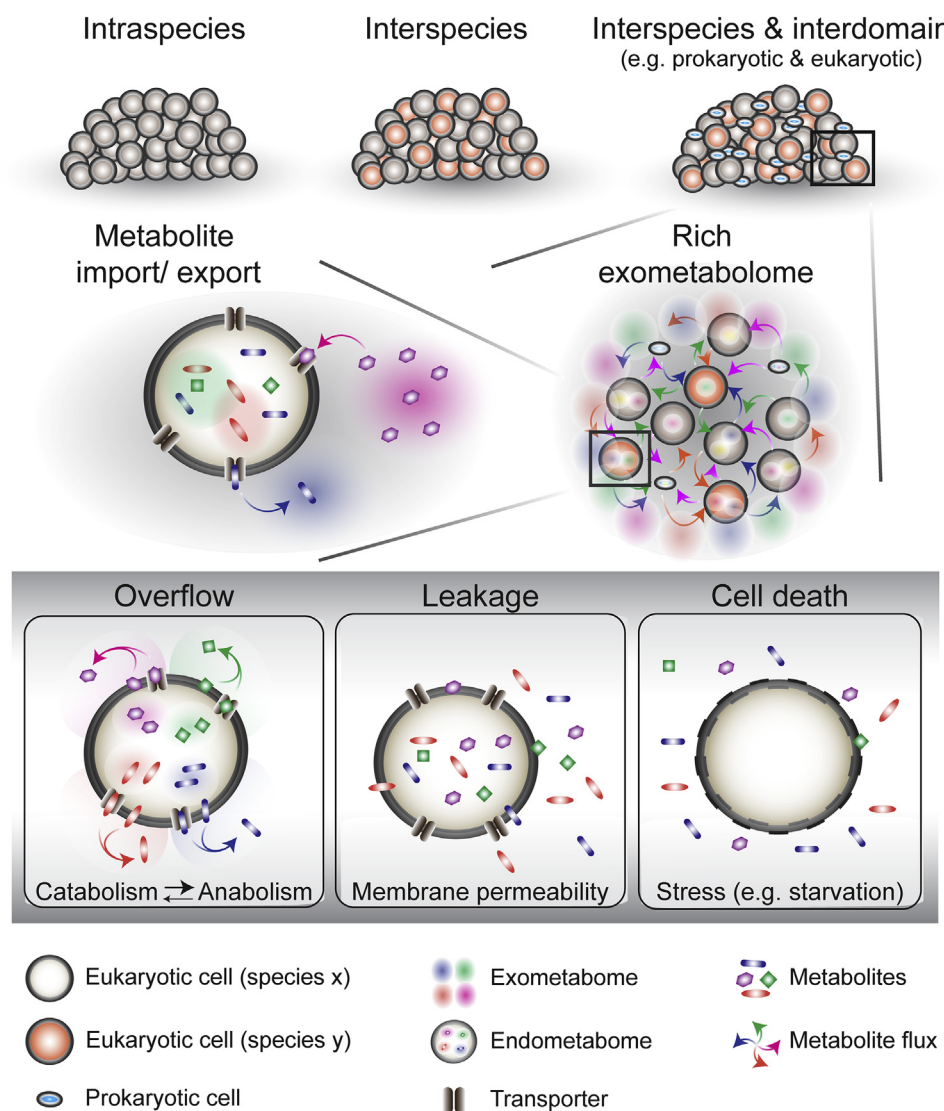
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Figure 1



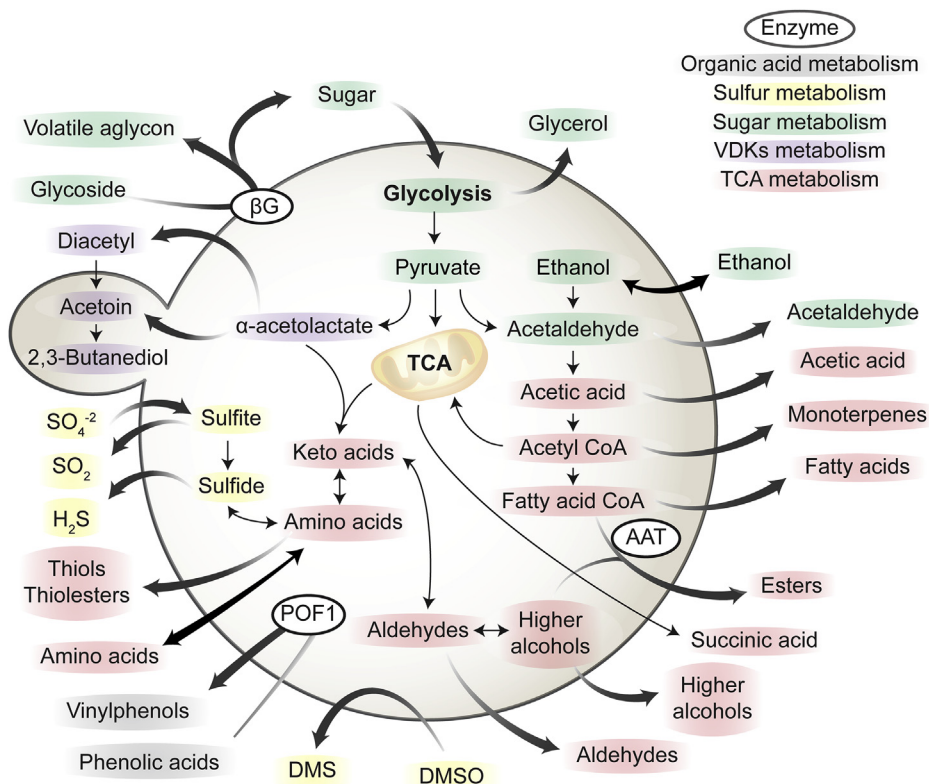
Sources of extracellular metabolites in cellular communities, tissues and biofilms. Biosynthetically active cells release a complex spectrum of metabolites, resulting in an extracellular space rich in products of metabolism. This rich exometabolome allows for the exchange of metabolites between single cells via export/ import processes, enables survival of otherwise unculturable auxotrophs, and also changes the physiology of prototrophs that reconfigure their metabolism to exploit these available nutrients. Sources of extracellular metabolites include overflow metabolism, ATP-dependent export, membrane leakage and cell death. A main driver is the topological structure of the metabolic network leading to flux-coupling, imbalances of anabolic over catabolic reactions, and non-enzymatic chemistry that requires metabolite repair through export (inset left). Membrane leakage and non-selective transport processes are other important sources of extracellular metabolites (inset), as is cell death that is of particular importance for the exometabolome in stress situations and in ageing cell communities (inset right).

Basic requirements for a metabolite exchange interaction to emerge

In order for metabolite exchange to be of biological relevance, a few basic conditions need to be fulfilled. First, cells have to export metabolites at a rate where relevant extracellular concentrations can be achieved in the given community, tissue or environment. The rate of accumulation is constrained by the environment and cell density of the community or tissue: but equally what a 'relevant' concentration is depends on the molecule's chemical nature (i.e. reactivity), cost of biosynthesis (i.e. the 'expensiveness' of a metabolite),

and the essentialness of its function. The extracellular presence of highly costly metabolites, such as thiamine (vitamin B₁), can be physiologically relevant at sub-micromolar concentrations, while much higher concentrations are involved when cells share abundant cellular metabolites, like amino acids, nucleotides or polyamines [18–20]. Related to this is the second requirement, that neighbouring cells need to sense and uptake a particular metabolite. Indeed, mechanisms that meet these conditions exist for a broad range of metabolites, released by both eukaryotic and prokaryotic cells [6,21,22].

Figure 2



The brewing process as a well-studied example of overproduction and release by a eukaryote for a complex spectrum of metabolites, under conditions that do not require cooperation between cells. A multitude of metabolites are released by yeast cells, even under typical growth conditions where there is no ecological pressure to cooperate in metabolism (adapted from [27]). The brewing process can result in the release of carbohydrates, amino acids, and aromatic flavour compounds such as ethyl acetate, isoamyl acetate and ethyl phenyl acetate. The biological reasons behind overflows are still debated, with causes appearing to be multifactorial and applying across species' borders. While metabolites like acetate or ethanol are common overflow metabolites, the production of flavour related components is not a generally observed phenomenon (i.e. it is species specific) and could have evolved before domestication [102], potentially to attract fruit flies in order to promote their dispersal, outbreeding and survival under adverse conditions [103]. Abbreviations: β G: β -glycosidase; DMS: dimethyl sulfide; DMSO: dimethyl sulfoxide; AAT: alcohol acetyltransferase; POF1: phenolic acid decarboxylase; SO_4^{2-} : sulphate; SO_2 : sulphur dioxide; H_2S : hydrogen sulfide; tricarboxylic acid cycle; VDK: vicinal diketone; CoA: coenzyme A.

The impact of metabolite uptake on cell physiology depends on the ability of cells to re-configure their metabolism to prioritise import over self-synthesis. Without the ability to reconfigure their metabolism, metabolite import would not provide any physiological advantage. Furthermore, due to flux coupling within the metabolic network [23], these re-configurations have shown to have a system-wide impact, affecting regulation at the transcriptome, proteome and metabolome level; As a consequence, the physiology of the cell, its stress resistance and growth, as well as its response to gene deletions, can be altered to various degrees (Figure 3) [17].

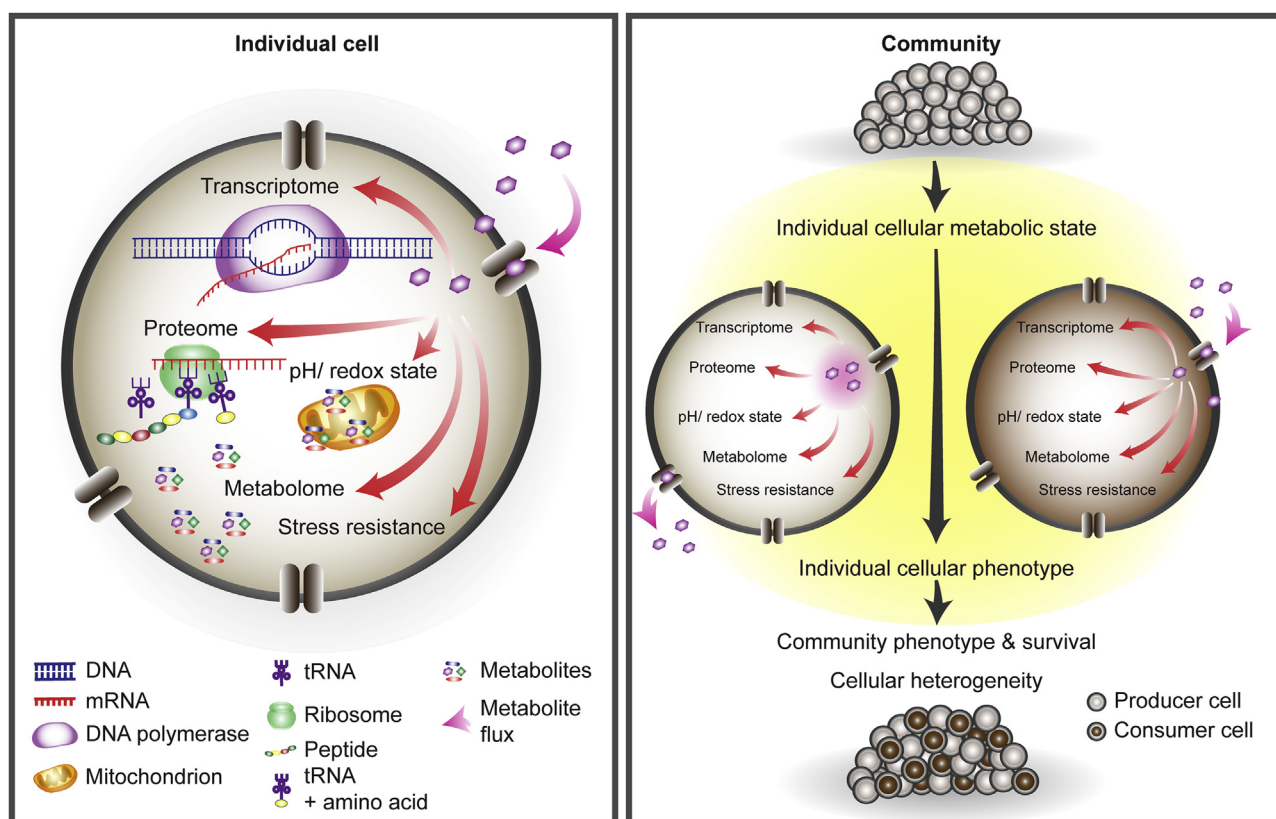
The mechanisms inducing metabolic re-configuration have been best studied for amino acids and nucleotides. The response to extracellular nutrient presence is, at least in part, explained by concerted feedback inhibition of the biosynthetic pathways involved. This is demonstrated by feedback-resistant mutants, which

have been identified for several amino acid producing pathways, where cells are deficient in sensing or uptake processes and continue to produce a particular metabolite even though it remains present in the extracellular environment [24].

The role of the metabolic network topology in the establishment of metabolite exchange interactions

Why are cells producing more metabolites than needed by themselves? At first, there are important ecological-evolutionary models to consider in respect to the evolution of metabolite exchange, cheating and cooperativity [3,25,26]. However, in this review we concentrate only on several metabolic network properties that result in metabolite release, irrespective of there being an ecological benefit to the cell. Indeed, many ground-breaking findings, considering the principles of metabolite overproduction and release, are not described within the ecological context but emerge from research

Figure 3



Physiological consequences of metabolite uptake versus synthesis at an individual cell and community level. The metabolic network is tightly interlinked with the cell's response to its environment, i.e. the change from metabolite synthesis to uptake has a huge impact on a cell's physiology. Metabolic reconfigurations are reflected on the transcriptome as well as the proteome, altering biochemical reactions and the metabolome, the cellular redox state and, with it, the cell's ability to tolerate stress. The metabolic state of a cell hence strongly determines its survival chances when environmental conditions change. If cells specialize in metabolism, these differences are a source of cellular heterogeneity.

into biotechnology. In the fermentation process for example, metabolite export can both be desired or undesired. We illustrate this situation for the brewing process, a well-studied example, in which high quantities of metabolites including ethanol, amino acids, and flavour compounds are released from yeast, defining the very nature of the product, the fermented beverage (Figure 2, [27]).

One overriding reason for the overproduction of certain metabolites can be found in the topological structure of the metabolic network itself. The metabolic network is constrained by the underlying chemistry, in particular the stoichiometry of chemical reactions, thermodynamics, and the availability of catalysts (enzymes) and cofactors [5,28–30]. Many reactions within the metabolic network have been increasingly speculated to originate back to a time of non-enzymatic chemistry, wherein the evolution of early metabolic pathways was constrained by the reaction spectrum achievable, with metabolism's first catalysts (most likely metal ions and simple amino acids) — and not by the exact need of the (now modern) cell [31–33]. Furthermore, as metabolic

reactions can also interfere with one another, due to common moieties and structural similarity between metabolites, the metabolic network is also constrained in its expansion [5]. The result of these processes is a remarkable conservation of the metabolic network topological structure among the kingdoms of life, which is in stark contrast with the great variability of individual metabolic requirements of different organisms within different environmental niches. Therefore despite metabolism being highly regulated, it still needs to overcome this deterministic stoichiometry. As a result, in order for metabolic flux to permit all metabolites being produced at a rate that fulfils the minimum demand, some need to be overproduced. This is a consequence of the high interconnectivity within the metabolic network that prevents fluxes through biosynthetic pathways occurring independently to one another. For example, in yeast, >25% metabolites are involved in >3 reactions, leading to system-wide flux coupling, whereby it can be common for an increase in the production of one metabolite leading to flux changes for several other metabolites across the metabolic network [23,34,35]. And with thousands of metabolites

being connected by hundreds of enzyme-catalysed reactions, a frequent solution for achieving a growth-optimal flux distribution is to export, rather than recycle, a subset of the metabolites that have been overproduced. Prominent examples for this have been shown in *Escherichia coli* that, remarkably, lacks a degradation pathway for many expensive amino acids, leading to metabolite export being the sole option to avoid accumulation beyond their optimal concentration range [36–38]. Moreover, an imbalance in amino groups can cause overflow metabolism whereby some amino acids can be used as sinks connected to transamination reactions [39]. Another example is the preferential export of uracil for balancing pyrimidine biosynthesis. Here, when there is excess biosynthetic flux in the pyrimidine pathway of *E. coli*, feedback inhibition occurs in a downstream pathway step for uridine monophosphate (UMP) kinase by uridine triphosphate (UTP), in effect triggering the export of uracil [40].

Tradeoffs between proteins synthesis and metabolic cost as a cause of overflow metabolism

When both oxygen and nutrients are abundant, cells often switch from respiration to fermentation, a process which can be related to the ‘Warburg effect’ in cancer cells or the ‘Crabtree effect’ in yeast. Under these conditions, there is a high degree of overflow metabolism, leading to the extracellular accumulation of energetically expensive carbohydrates (Figure 1). Despite their high ATP demand, rapidly growing cells export lactate, ethanol or acetate, instead of oxidizing pyruvate in the Krebs cycle (also referred to as the tricarboxylic acid (TCA) cycle), which would generate more ATP through oxidative phosphorylation. Several explanations have been proposed for why overflow metabolism occurs in this context. One of the most recently discussed causes is proteome resource allocation [7,41,42]. Under nutrient rich conditions, respiratory enzymes are substituted by smaller glycolytic enzymes allowing extra space, for example, for additional substrate transporters and proteome allocation for other processes such as translation [43]. Glycolysis consequently becomes the pathway of choice when intermediates need to be turned over quickly, despite enzymes in both pathways having similar k_{cat} s, meaning both modes of metabolism operate at the same speed, and despite oxidative phosphorylation generating more mmol ATP per gram dry weight per hour. Therefore after correcting for protein production, the small molecular weights of glycolytic enzymes mean that glycolysis is more catalytically efficient per unit protein (k_{cat}/MW) than oxidative phosphorylation, for generating ATP [7,42,44–46].

Is ATP always the metabolically limiting factor?

In view of the textbook-promoted picture of ATP production as a limiting factor in central carbon metabolism, some additional restrictions need to be considered. This perception most likely originated from, and applies to,

the study of energy metabolism in the skeletal muscle, which was the focus of many key metabolic studies in the 1960s and 1970s. When exercising, ATP available in skeletal muscle cells is consumed rapidly. The ATP pool then needs to be replenished readily, first with metabolites with a higher phosphotransfer potential, such as phosphocreatine [47]. Once phosphocreatine becomes limiting as well (within minutes), other metabolic sources of ATP have to be activated. This is achieved in the following order a) glycolysis (which can increase up to a thousand-fold in flux), b) the Krebs cycle and oxidative phosphorylation, which occur during mid-level exercise, and c) β -oxidation of fatty acids, during long-term exercise and in the adult heart [48].

In the context of the Warburg effect, one has to consider however that there is a fundamental difference between ATP consumption in the active muscle and when a cell needs ATP for biomass growth. The latter process couples ATP consumption by definition to anabolism, which requires access not only to ATP, but also to biosynthetic precursors. Full glucose oxidation over the Krebs cycle produces more ATP, but has a negative carbon balance: In each complete round of the Krebs cycle, two carbon molecules are converted into CO_2 , and are lost to the cell in the absence of carbon fixation mechanisms. The carbon balance is equally negative once carbon equivalents are converted into fatty acids, as β -oxidation depends on the Krebs cycle to generate ATP from acetyl-CoA, with two carbon units entering the Krebs cycle as acetyl-CoA, which are then released as CO_2 . For this reason, mammals can not recreate a sufficient glucose pool from carbon equivalents once they have been oxidized in the Krebs cycle or converted into fatty acids. Instead, glucose oxidation over glycolysis has a much more favourable carbon balance permitting the cell greater metabolic efficiency; During fermentative metabolism lactate is excreted from cells, however, it is not ‘lost’, as lactate (or ethanol, acetate or glycerol in the case of microbes) can be re-imported into cells, serving as a substrate for gluconeogenesis. In mammals, this process is known as the Cori cycle [49], an archetypical example of metabolic cooperativity between tissues. The Cori cycle appears to also be implicated in metabolic decision making. The lactate circulated can indeed not only be used as a source for gluconeogenesis, but can also serve as a substrate for the Krebs cycle to replenish ATP when required. Indeed, recent results indicate that in mice, the majority of Krebs cycle activity is fed through lactate [50]. This situation appears to also be associated with the ongoing exchange of lactate between cancer cells, a metabolic feature typical to many glycolytic tumour cells [51].

Another facet of this problem is described in the Membrane Real Estate Hypothesis [52]. This addresses the problem that oxidative phosphorylation has its own metabolic demands, with it depending on a supply of

oxygen to function as well as concomitantly, needing to export CO₂ produced in the Krebs cycle. When cells grow bigger, they have a less favourable surface-to-volume ratio, limiting gas exchange for oxidative phosphorylation [52]. For similar reasons, a restriction in oxidative phosphorylation emerges also in solid tumours that are characterized by hypoxia [53].

Redox balancing and overflow metabolism

The choice between fermentative and oxidative metabolism also affects redox homeostasis, with the electron transport chain and glycolysis respectively having different effects on the release of reducing or oxidizing molecules, during ATP generation [54]. The maintenance of redox balance is indeed one reason why microbes use overflow metabolism when there is excess glucose available, as secreted metabolites can act as electron sinks for NADH, to regenerate NAD⁺ for glycolysis to occur [55]. In *E. coli*, the overflow of acetate and other fermentation metabolites [56], and in *Saccharomyces cerevisiae*, glycerol and ethanol production [57], for instance, can be manipulated by interfering with the NADH/NAD⁺ balance.

Indeed, redox homeostasis is reflected mainly by the redox cofactors, nicotinamide adenine dinucleotide (phosphate) (NAD(P)⁺ and NAD(P)H) and flavin adenine dinucleotide (FAD and FADH₂) [58]. Although the redox potential of a cell is affected by both oxygen and glucose utilisation, when glucose is in excess, the cellular response is similar regardless of aerobic or anaerobic conditions - metabolic intermediates accumulate. This is due to the rate of glucose consumption being greater than the capacity for reduced redox cofactors to be oxidised. In glycolysis, glucose is oxidised to two molecules of pyruvate, additionally two molecules each of ATP and NADH are formed. To acquire enough ATP through this low ATP yield pathway, cells consume high amounts of glucose and subsequently generate high levels of reducing equivalents (each NADH corresponds to two reducing equivalents). As NAD⁺ is a substrate for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in glycolysis, NADH needs to be reoxidised to maintain glycolytic flux [59]. One way this is achieved is by transferring the reducing equivalents to partially oxidised metabolic intermediates, such as lactate and ethanol, which are then released by the cell [60]. Overflow product formation is therefore considered a rapid way in which cells restore a high NAD⁺/NADH ratio, resulting in the excretion of multiple catabolites such as lactate, acetate, succinate, alcohols and CO₂.

Membrane leakage and promiscuous transport activity

We consider it an unanswered question for which among the above listed arguments is the main cause of overflow

metabolism in cells showing Warburg/ Crabtree effects. It is however intuitive that multiple metabolic constraints apply in parallel. Depending on the species and metabolic state, the different constraints may contribute to a different extent. In other words, its intuitive there is more than one single cause of the Warburg or Crabtree effect. Nonetheless, this example shows that in typical overflow, metabolites are removed from cells through active and energy consuming transport processes, revealing that metabolite export must confer an advantage in typical metabolic situations, otherwise cells would employ alternative mechanisms of metabolite removal, such as catabolism and degradation. In parallel however, another important source of extracellular metabolites is leakage of metabolites through the cell membrane and non-selective transport [61].

Membranes can only have obtained their present (closed) structures upon the evolution of modern transport systems, explaining why our cells could not have evolved when membranes were completely sealed already in early evolution [62]. It is therefore most likely that membranes evolved to be highly selective barriers over time, with changes in their lipid composition and a myriad transport systems originating in order to fulfil the specific function of regulating the intracellular pool of metabolites [61]. Membrane permeability however, although intrinsic, increases with age, size of the cell, adverse environmental conditions and/ or properties of the leaking metabolites themselves [63]. As a consequence, any variable that causes a change in membrane permeability also has a direct impact on metabolite leakage. Some of these variables being temperature, pH, osmolarity and nutrient availability, as well as the cell cycle, growth rate and changes in lipid metabolism [64,65].

Many membrane transporters and channels also demonstrate varying degrees of specificity: Exporting metabolite A can often not be achieved without also exporting at least some of a structurally similar metabolite B, wherein the given transport system, such as efflux pumps and transmembrane channels, lack the required discriminatory power to distinguish between the two metabolites [66]. The basic problem causative to this is a finite structural diversity that exists within the small-molecule metabolome, with the vast majority of cellular metabolites possessing homologues with highly overlapping structural features [5]. Recently, it was proposed that these conditions could explain the existence of low affinity transporters when nutrients are plentiful, as they allow sufficient import while preventing leakage of expensive metabolites [67].

Overflow metabolism caused by metabolite repair

A related cause of overflow metabolism is so called metabolite repair. Not only transporters, but also

enzymes are promiscuous, and together with non-enzymatic reactions, a large number of metabolites lacking biological function are formed within the cell [68]. Some of these metabolites are toxic for cells; in many cases for the simple reason that they possess structural similarity to the metabolites they derive from, and hence act as competitive inhibitors for the associated metabolic enzymes [5]. By exporting metabolites formed by non-enzymatic or promiscuous reactivity for which no specific export system exists [42], promiscuous clearance subsequently prevents deleterious effects on metabolism.

Cell death

Under some conditions, programmed or spontaneous cell death can also be a highly relevant source of extracellular metabolites (Figure 1), and can indeed be an evolutionary adaptation. In microbes, the recovery of metabolites released from cell death, when nutrients become limiting, helps remaining living cells to obtain resources against competitors, exploiting rapid cell growth followed by programmed cell death. This type of ‘harvesting’ strategy at the community level has also shown to confer population survival up to several years, whereby cell death and growth is balanced by nutrient input originating only from dead cells [69]. Interestingly at the point of starvation prior to cell death, some cells are also known to release expensive secondary metabolites with antibiotic properties as a survival strategy. Such secondary metabolites, when taken up by neighbouring competitors, lead to cell death and the release of nutrients that can be taken up by producer cells to exit starvation [70].

During apoptosis and other forms of programmed lysis, the degradation of cellular proteins, nucleic acids, lipids and polysaccharides by endogenous enzymes is known to occur; these breakdown catabolites such as amino acids and sugars are subsequently exported from the cell, at the expense of a decline in cell biomass, cell density, and an increasingly leaky cell wall before death occurs [71]. In bacteria, cell death has also shown to be involved in biofilm formation, providing cells with nutrients, enzymes and polymers required for the biofilm matrix, or with signals that trigger specific developmental and evolutionary processes such as sporulation or horizontal gene transfer [72–75]. Moreover, programmed cell death in yeast and bacteria has shown to support community metabolism and the feeding of younger cells when nutrients become limiting [72,76,77].

How the exploitation of extracellular metabolites affects cell physiology

Successful survival in competitive environments, where resources are limited, relies on nutrient sensors and transporters to efficiently bind and uptake metabolites required by the cell [78,79]. These are active within

communities and cellular tissues, where the source of matrix metabolites are the co-growing cells. Cells nurtured from the so-called ‘pool of shared goods’ can feedback-inhibit their respective biosynthetic pathways and hence become metabolically different to a cell producing the respective metabolite. As the metabolic network is tightly interlinked with the cell’s response to its environment, the change from metabolite synthesis to uptake has a broad impact on gene expression and cell physiology (Figure 3) [80]. Phenotypically, this situation is relevant as metabolism is highly interconnected with the stress response machinery, demonstrated by the fact that a metabolically reconfigured cell will respond differently to a stress perturbation and have a different chance of survival, relative to the same cell that had not undergone any metabolic changes.

A helpful model for studying the biological impact of cells re-configuring their metabolism from biosynthesis to uptake, are auxotrophic marker alleles, which confer nutrient dependency of otherwise prototrophic species. Auxotrophic markers have been exploited for the specific reason of being essential metabolic deficiencies that can be complemented with extracellular metabolites, as genetic selection markers in laboratory experiments. In *S. cerevisiae*, it has been shown that the transcriptional response induced through four commonly exploited auxotrophic markers, interfering with histidine, leucine, uracil and methionine metabolism, affects the expression of up to $\frac{2}{3}$ rds of cellular transcripts. Differentially expressed transcripts also show to overlap with one third of the broad range of transcriptional changes reported across a series of independent gene expression datasets, revealing that a majority of cellular responses to gene loss are confounded by metabolism [17]. The rationale for this observation is the tight interconnectivity within metabolism, leading to network-scale reconfiguration when cells switch from metabolite uptake to synthesis. Indeed, the transcriptional changes induced by auxotrophy correlate strongly with the metabolic flux distribution. As a consequence, the metabolic background is not confounding gene expression experiments in a linear manner but will lead to different impacts on cell physiology when the same perturbations are applied.

These metabolic differences affect the survival chances of cells in a variety of stress situations. For instance, yeast cells which differ in either uptaking or synthesizing methionine have altered survival when cells are exposed to oxidative stress, induced by the thiol oxidizing reagent diamide. This phenotype depends on NADP⁺ to NADPH reduction in the pentose phosphate pathway. When cells do not need to synthesize methionine, NADP(H) availability increases for the anti-oxidative machinery [81]. Another example is the dissimilar response of uracil producing and uracil consuming cells to the oxidant hydrogen peroxide (H₂O₂), even when

these cells grow together under the same conditions within the same colony. Here, the mitochondrial network in uracil producers undergo a significantly higher degree of mitochondrial fission compared to uracil consumers, and at the same time, cells mount a higher resistance to oxidant treatment, suggesting their altered uracil metabolism confers a benefit to their survival [16]. A third case is the role of the polyamine exporter *TPO1*, that determines timing of the oxidant-induced cell cycle through the export of antioxidant polyamines, that as a consequence, become available to co-growing cells during stress conditions [82].

Not only does the exchange of metabolites have a direct metabolic role but also those metabolites with a signalling function contribute to a cell's stress response. When it comes to interspecies' landscapes, these metabolite interactions can have sizeable physiological consequences. Gut bacteria, for instance, have shown to secrete N-acyl amides that activate G-protein coupled receptors of the host's intestinal cells [83], similarly, the physiology and fitness of the fruit fly, *Drosophila melanogaster*, has shown to be influenced by the metabolites produced from their gut microbiome [84].

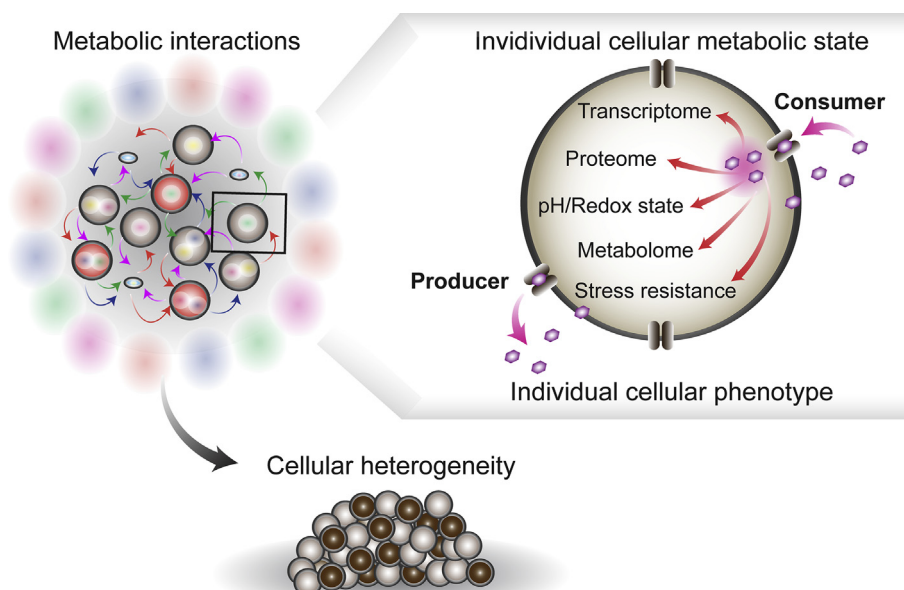
Phenotypic heterogeneity emerges as a consequence of metabolic specialisation

The intrinsic ability of cells to take up extracellular metabolites, and the physiological changes that emerge from this process, understandably can alter the

phenotype of the cell to a broad extent. As cells sense nutrients independently, this situation can be associated with phenotypic differences that arise between cells in a population (Figure 4). Phenotypic heterogeneity is known to enable bet-hedging strategies, providing a population of isogenic cells with the flexibility to adapt to a constantly changing environment [85]. It has also been associated with infection [86,87], formation of persister cells [62,88], resistance to environmental stresses [16] and triggering specific developmental processes [89,90]. In yeast, metabolic divergences between cells adapted to the uptake of a specific metabolite (auxotrophs) and those which have a broad metabolic functionality (generalists), allow the community to find strategies for both the efficient adaptation to new environments as well as to grow at a competitive rate when conditions stay constant [91].

Single cells with non-genetic phenotypic differences in metabolism can partially be explained by stochastic noise in gene expression [92,93]. This "noise" is believed to propagate to the cell cycle [94], growth rate [95], epigenetic modifications and differences in transcriptional activity [96]. Alternatively, metabolite exchange interactions that allow the specialisation of single cells in metabolism, are a biochemical source of heterogeneity. For instance, cell-to-cell differences in gene expression attributed to noise have shown to decline when amino acids are supplemented, giving reason to speculate that gene expression variability at the single cell level is, at

Figure 4



Metabolite exchange interactions between co-growing cells within communities. Metabolic interactions, relying on the exchange of metabolites between single cells via export/ import processes, are critical within microbial and other multicellular communities. This process of switching between self-synthesis (producer) and uptake (consumer) is inherent to intercellular metabolite exchange interactions and affects fundamental cellular physiological features alongside the transcriptome, proteome, and metabolome, on a genome wide scale. This leads to wide ranging impacts on how cells respond to environmental cues, reflecting different individual cellular phenotypes that contribute to cellular heterogeneity within a microbial community.

least in part, caused by metabolism [94]. Indeed, a metabolic cause of cell heterogeneity has been supported by multiple observations: at the single cell level, measuring lactulose abundance in an *E. coli* population showed that the levels of this enzyme is variable across the population causing growth fluctuations, and heterogeneity [95]. Another example is provided in self-establishing communities (SeMeCos), that allow the tracking of individual metabolotypes in the community context [8]. In SeMeCos, a progressive increase in metabolic co-dependencies as a colony forms is achieved via the stochastic segregation of mini-chromosomes that contain essential metabolic enzymes, to complement genomic auxotrophies. This way, cells overcome their inability to cooperate as observed upon direct mixing of auxotrophs [97]. A key lesson to be learned from SeMeCos is that there are several metabolotypes that do not make successful cooperators, while other combinations of the same auxotrophic alleles are compatible with effective cooperation [8]. One potential explanation for the latter is that all metabolite export, sensing and import is semi-selective. This means that although yeast cells release a broad spectrum of metabolites [6,10], as several belong to the same chemical category, such as aromatic or branched chain amino acids, they are therefore coordinately regulated, synthesized and transported [80,98]. Co-synthesis and co-transport hence puts constraints on the ability of cells to exchange connected metabolites independently from one another.

Metabolotypes that are successful cooperators in SeMeCos, diverge strongly in their stress tolerance in a metabolism-dependent manner [16]. In contrast, inefficient cooperators do not diverge in stress tolerance, even though they possess the same auxotrophic alleles and co-grow inside the same colony [16]. This indicates that active metabolite exchange is responsible for the phenotypic diversity of the cooperating single cells. A role of metabolic cooperativity in the establishment of cellular heterogeneity may also be of therapeutic relevance, as it indicates a therapeutic window to address cellular heterogeneity without genetic intervention. While noise in gene expression is difficult to target pharmacologically, metabolic exchange interactions are accessible by targeting the extracellular space, and could therefore be altered by using intelligently designed metabolic inhibitors.

Finally, metabolic cooperativity can also arise as a consequence of spatial heterogeneity, whereby cells diverge in stress tolerance due to spatial and, concomitantly, temporal differences in access to nutrients. For bacteria and yeast, where cells can grow into colonies on agar, the cells that locate closer to the bottom of the colony have a more abundant supply of nutrients than cells located proximal to the top. As the colony develops, subpopulations emerge with mixed

metabolic specializations, caused by varying access of colony cells to available nutrients. Cells with different spatial localisation will subsequently undertake different uptake and self-synthesis activity. The metabolic niche will therefore determine the phenotype of the cells, which then also leads to divergence in stress tolerance between individual cells within the same colony [99,100]. A related situation has recently been described in bacterial biofilms, in which metabolic exchange activity results in collective growth oscillations that spread over spatial distances, allowing populations to have increased resilience to chemical attack, as well as to increase in size and viability [101].

Conclusions

Metabolite exchange interactions are an indispensable feature of cellular physiology in both prokaryotic and eukaryotic cells, and can affect the physiology of both auxotrophic and prototrophic cells (Figure 4). The underlying principles of metabolite export and import emerge both from evolutionary adaptations to metabolite exchange, but also from the consequences of fundamental functional constraints operating within the metabolic network. Overflow metabolism, concerning cells exhibiting the Warburg and Crabtree effect, as well as metabolite diffusion, through leaky membranes or non-selective transport, all lead to the enrichment of the cellular environment for a diverse array of metabolites. In parallel, cells have evolved the ability to sense a wide spectrum of metabolites and typically tend to prefer import over biosynthesis. This situation enables individual cells to exploit the exometabolome in order to specialize in metabolism, that is, to streamline the number of active metabolic reactions necessary for growth, while also optimising their survival chances. As the reprogramming of metabolism has wide ranging physiological implications, the affected cells with different metabolotypes can subsequently diverge extensively on the phenotypic level. Elucidating the biological impact of metabolism-induced non-genetic phenotypic heterogeneity, wherein cells dynamically reconfigure metabolism based on nutrient availability, will shed light on this key but barely understood feature of single-cell physiology.

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