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Hierarchical and simultaneous utilization of carbon substrates: mechanistic insights, physiological roles, and ecological consequences

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Bacteria grown on a mixture of carbon substrates exhibit two utilization patterns: hierarchical utilization (HU) and simultaneous utilization (SU). How and why cells adopt these different behaviors remains poorly understood despite decades of research. Recent studies address various open questions from multiple viewpoints. From a mechanistic perspective, it was found that flux sensors play a central role in the regulation of substrate utilization, accounting for the known dependences on single-substrate growth rates, substrate concentrations, and the point where the substrate enters central metabolism. From a physiological perspective, several recent studies suggested HU or SU as growth-optimizing strategies through efficient allocation of essential proteome resources. However, other studies demonstrate that a significant fraction of the proteome is dedicated to functions apparently unnecessary for growth, casting doubt on explanations based on slight efficiency gains. From an ecological perspective, recent theoretical studies suggest that HU can help increase species diversity in bacterial communities.

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Introduction

Bacteria grown on multiple carbon substrates utilize them either hierarchically or simultaneously. Despite decades of extensive experimental and theoretical attention [1–5], many questions remain regarding the regulatory mechanisms, physiological roles, and ecological consequences underlying these utilization patterns. Here we review

recent experimental and modeling studies that have provided fresh insights into this classical topic.

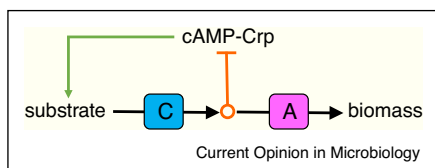
Hierarchical utilization (HU) refers to cases in which the consumption of one substrate is fully suppressed in the presence of another; simultaneous utilization (SU) to cases where multiple substrates are utilized together. In practice, however, the distinction is not clear-cut. Even if two substrates are co-utilized, the uptake of each substrate is typically reduced in the presence of the other. In *Escherichia coli*, this effect results from a generic negative feedback acting on carbon-substrate uptake [6*,7*] (Figure 1) mediated by the transcriptional regulator cAMP-Crp, which activates the expression of nearly all carbon-uptake systems. The activity of cAMP-Crp is determined by the difference between the carbon-uptake flux and the downstream biosynthesis fluxes. Hence, if carbon-uptake exceeds anabolism, further uptake is suppressed. In the presence of multiple substrates, the feedback operates on all carbon substrates, but it may not affect each equally: for instance, during growth on glucose and succinate, succinate uptake is more strongly suppressed than glucose uptake to an extent that can be quantitatively estimated based on the single-substrate growth rate [6*]. Arguably, this unequal suppression amounts to a partial substrate hierarchy. Here, however, we reserve the term HU for cases where the uptake of one substrate (almost) fully suppresses the uptake of another. cAMP-Crp-mediated feedback alone cannot account for such cases [6*].

Countless experiments, mostly on *E. coli*, have revealed several patterns in the uptake of mixed substrates. First, if two substrates give rise to HU, the preferred substrate is typically the one that supports the higher growth rate [4]. Second, if multiple substrates are present at low concentrations, as in chemostat cultures, they are co-utilized even if a hierarchy exists at higher concentrations [4,8]. Third, substrates can be classified into two categories depending on whether their degradation products merge into the ‘upper or middle’ part of the glycolysis pathway (category A), or ‘lower glycolysis’ or the TCA cycle (category B) (Figure 2). For *E. coli*, HU is typically observed only among substrates of category A [6*,9]. The challenge is to explain these general observations both from the mechanistic and the physiological perspective.

Mechanistic insights

A priori, HU could be implemented by regulators that directly repress the uptake of one substrate in the presence of another. An example is the HU of arabinose and

Figure 1



A generic negative feedback affecting the uptake of carbon substrates. A carbon substrate is taken up and catabolized through transporters and catabolic enzymes, grouped as 'C'. The precursors derived from the substrate through catabolism are utilized for biosynthesis through biosynthetic enzymes including ribosomes, grouped as 'A'. The difference between the fluxes through C and A is read out as the activity of cAMP-Crp, which is necessary for the expression of substrate uptake systems. If the flux through C is in excess, cAMP-Crp activity and thereby the substrate uptake is reduced [7].

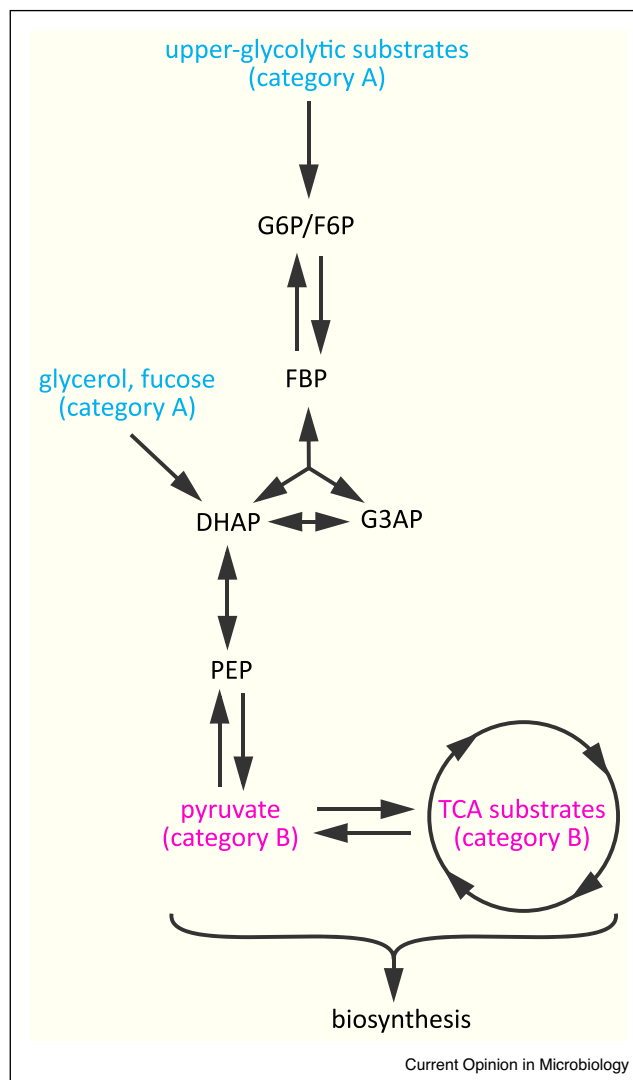
xylose in *E. coli* [10–12]. Few such direct interactions, however, have been identified. This may be because establishing a hierarchy among multiple substrates using direct interactions is impractical: the uptake system of each substrate would have to be repressed by many transcription factors — one for each preferred substrate [13]. Indeed, the preference for lactose over arabinose or xylose appears to be mediated by the generic cAMP-Crp signal rather than direct interactions [14]. Hence, the question becomes how a substrate hierarchy can be implemented using generic rather than specific molecular signals.

A recent study on HU between lactose and glycerol suggests a possible scheme [15**]. This study used engineered *E. coli* cells for which the uptake of lactose in batch culture can be tuned via an inducer. If the lactose uptake flux exceeds a particular threshold, glycerol uptake is fully suppressed (HU). Strikingly, if lactose uptake is below this threshold, glycerol uptake is 'supplemented' (i.e. SU) such that the total carbon uptake flux is maintained at the threshold value. Such observations indicate that glycerol uptake responds to the lactose uptake *flux* rather than to the substrate itself.

How can cells sense fluxes? Within metabolic pathways, particular network motifs can result in correlations between the flux through a pathway and the concentration of certain related molecules [16]. If such molecules engage in regulatory interactions, they can act as *flux sensors*, allowing the cell to respond to the corresponding fluxes [16,17].

In fact, glycerol uptake responds to two signals: transcriptional activator cAMP-Crp and metabolite fructose-1,6-bisphosphate (FBP), which inhibits the activity of the glycerol kinase GlpK [18]. Both signals are flux sensors: the activity of cAMP-Crp correlates (negatively) with the *total* carbon-uptake flux [7*] (Figure 1) whereas the concentration of FBP correlates with the flux through upper

Figure 2



Category A and B substrates. Category A substrates, shown in cyan, are the substrates entering glycolysis either at the upper part (e.g. D-glucose, D-glucose-6-phosphate, D-gluconate, D-mannitol, D-fructose, D-sorbitol, D-mannose, lactose, melibiose, L-arabinose, and D-xylose) or in the middle part (e.g. glycerol and L-fucose). Category B substrates, shown in pink, include pyruvate and TCA substrates such as succinate, fumarate, malate, and oxaloacetate. As a rule, hierarchical utilization is observed only among substrates of category A.

glycolysis [15**,19]. A large lactose flux reduces both transcription of the glycerol uptake system (through cAMP-Crp) and GlpK activity (through FBP). These signals are amplified by another positive feedback loop, mediated by the glycerol-specific regulator GlpR, which shuts down glycerol uptake when lactose uptake exceeds the threshold flux value [15**].

Importantly, because the glycerol system responds to fluxes rather than to a specific substrate, it is also

repressed by other glycolytic substrates, such as glucose, provided their flux exceeds the threshold [15**]. Thus, a single mechanism establishes glycerol's position in the hierarchy.

Such mechanisms can explain several substrate utilization patterns described above. By sensing the total carbon flux, cells switch from HU to SU as the concentration of a preferred substrate — and hence its uptake flux — is reduced, as observed in chemostats [4,8]. Also, by sensing the upper-glycolytic flux, substrates of categories A and B can be discriminated, consistent with their differences in utilization patterns [6*]. Moreover, for co-utilized category A and B substrates, a model incorporating only the total-flux-sensor-based feedback by cAMP-Crp can successfully predict the two-substrate growth rate based on the growth rates on each substrate alone [6*].

Detailed studies on substrates beyond glycerol are needed to assess how widely flux sensors are employed. That said, other examples of flux sensors involved in the implementation of HU have been identified in the phosphoenolpyruvate:sugar phosphotransferase system (PTS). In various bacterial species, multiple carbon substrates are transported and phosphorylated via this system [3,20]. Early theoretical work already argued that the HU observed among PTS substrates could result from an inherent negative feedback of the total uptake flux of PTS substrates (i.e. the total phosphoryl flux) on the uptake of each individual PTS substrate, and that this total flux would be reflected in the phosphorylated fraction of the PTS components [21]. Indeed, this fraction correlates with the uptake fluxes of both PTS and non-PTS substrates [22,23]. Thus, these components can be regarded as flux sensors. Classical studies have demonstrated that one PTS component, EIIA^{Glc}, is involved in the HU of glucose over non-PTS sugars through inducer exclusion [3,24–26]. Recent studies demonstrate that the preference of *E. coli* for glucose over mannitol — both PTS sugars — involves the dephosphorylated form of another PTS component, HPr [27,28].

While most work has focused on minimal media containing mixtures of carbohydrates, HU and SU also occur in complex media containing mixtures of amino acids [29,30,31*,32]. Because most amino acids are degraded into category-B substrates, SU of category A and B substrates may be related to SU of category A substrates and amino acids [31*]. Future studies shall reveal the mechanism underlying utilization pattern of amino acids.

Physiological roles

While some consumption patterns can be understood from a mechanistic perspective, a different question is whether we can rationalize them from a physiological perspective.

Many attempts have been made to explain consumption patterns as the result of natural selection acting to optimize the performance of the cell. From this point of view, the task is to find a reasonable objective function, plus relevant constraints, such that the predicted optimal behavior matches the observed patterns. Often the specific growth rate is taken as a proxy for bacterial fitness and hence as the objective.

Many studies assume that the amount of enzyme a cell can produce or contain is constrained due to some limiting resource, be it nutrients, ribosomal capacity, cytosolic or membrane space [13,33,34**]. This results in a resource allocation problem: given a choice of two substrates, what fraction of the resource should be invested in the uptake of either substrate? Under a wide range of model assumptions, if the return on investment (ROI), that is, the carbon-uptake flux per unit of resource invested, differs among substrates, HU is optimal [13,33]: the cell only consumes the substrate providing the largest ROI. If this ROI also dictates the specific growth rate on substrates, this naturally explains that the observed rank order of substrates typically follows their growth rate. However, this argument does not readily explain SU. One possibility is that SU results from nonlinear cost/benefit functions [13], but no specific mechanisms have been identified.

Recent mathematical work provides more general insight by studying Elementary Growth Modes (EGMs): minimal sets of enzymes that can support balanced growth under given conditions [35*]. In this framework, SU can be optimal in two ways. First, if no specific constraints are imposed, optimal growth is characterized by a *single* EGM. SU can then be optimal only if each substrate plays a distinct role in biomass production. Second, if additional constraints *are* imposed, optimal growth may involve multiple EGMs consuming different substrates, also resulting in SU. However, it is difficult for this theory to explain transitions from HU to SU observed when substrate concentrations are reduced. Also, detailed information, such as rate constants of the metabolic enzymes, is needed to generate quantitative predictions.

A recent study on substrate consumption patterns reveals the importance of the topology of the metabolic network [34**]. The production of biomass requires multiple precursors that are derived from various metabolite pools. Some of these pools are more efficiently supplied by glycolytic substrates, others by gluconeogenic substrates. Importantly, SU of category A and B substrates can hence be rationalized as the most efficient way to supply all pools. However, it is difficult to judge whether the modest improvements in ROI thus obtained account for the observed 20–50% differences between single-substrate and mixed-substrate growth rates [6*,34**]. To validate growth-optimization theories, quantitative predictions on

growth rate differences between HU and SU, would be valuable.

While many studies consider growth rate as the objective function supplemented with a resource allocation constraint, several facts known for *E. coli* are hard to square with this framework. First, glucose is preferentially utilized over lactose, and yet in several laboratory strains the growth rate on lactose is indistinguishable from that on glucose and possibly slightly higher [15^{••},36]. Second, the regulation of several uptake/catabolic systems seems suboptimal. For example, GlpK mutations that abolish allosteric inhibition by FBP increase the growth rate on glycerol by over 20% [37,38] and such mutants co-utilize glucose and glycerol without a measurable reduction in growth rate (Okano, unpublished). Third, mass spectrometry shows that the transporter systems of quite a few substrates, such as DppA, RbsB, and MglB, are expressed regardless of the presence of these substrate, each occupying ~0.5% of the proteome, comparable to the glycerol uptake system expressed on glycerol (1.15% for GlpK and 0.25% for GlpD [39,40]). If the consumption patterns are to be explained by ROI gained through such modest fractions of the proteome, it is hard to see why other uptake systems are expressed when they are clearly not required. Indeed, growth increased with deletion of these useless transporters [41]. Generally, a large fraction of proteomes is allocated towards proteins that are unnecessary for growth (but presumably required for other functions, such as motility and chemotaxis) [39,42,43]. Moreover, even the abundances of proteins essential for metabolism and growth are not necessarily set optimal [44]. Deletion of transcriptional regulator Cra, which represses the expression of glycolytic enzymes, increases the growth rate on fructose and mannose by at least 10% [45[•]]. Additionally, recent work combining proteomic and metabolomic analysis of *E. coli* suggests that the abundances of most metabolic enzymes far exceed what it takes to maintain desired metabolic fluxes [46].

Together, such facts suggest that objectives beyond growth rate and constraints other than resource allocation should be considered. Examples of other potential constraints include an upper bound on the Gibbs free energy dissipation [47[•]] and toxicity of metabolic intermediates [48–51]. As an alternative objective function one might also consider the average growth rate in a fluctuating environment [45[•],52]. Adaptation of growth after an environmental switch usually involves a lag time. A recent study demonstrates that this lag time increases with the preshift growth rate [45[•]]. Thus, if substrate availability fluctuates, a larger growth rate on one substrate might result in a smaller growth rate on average.

Ecological consequences

Nutrient utilization strategies adopted by bacterial species may also significantly influence the emergent

properties of microbial communities. For example, a model inspired by the stable marriage problem of Game Theory predicts that multi-stability emerges in microbial populations composed of species adopting HU [53].

In some natural habits, such as rivers or intestines, bacteria may encounter growth conditions that resemble chemostat cultures. In chemostat cultures, nutrients are supplied at a fixed low rate and the concentration of a limiting nutrient changes until the cellular growth rate matches the dilution rate. Recent theoretical studies have assessed the effects of substrate utilization strategies on the biodiversity of microbial communities under chemostat-like conditions.

Classical competition theory predicts that the number of coexisting species cannot exceed the number of limiting nutrients — the ‘competitive exclusion principle’ [54,55]. In reality, however, many bacterial species have been found to coexist on a few limiting nutrients [56,57]. This puzzle, known as ‘the paradox of the plankton’ [58], has been studied through consumer-resource models [59–61,62[•],56,63^{••},64]. Recent studies introduced trade-offs in allocating cellular resources into such consumer-resource models under chemostat-like conditions [62[•],63^{••},64]. Posfai *et al.* [62[•]] showed that, on p substitutable substrates, an arbitrary number of bacterial species can coexist irrespective of their strategies of substrate utilization, provided substrate supply fluxes are located within the p -dimensional space defined by strategies of ‘keystone’ species that preferentially consume different substrates. In the context of substrate utilization strategies, if a population includes p species each adopting a different HU strategy, then those species would act as keystone species that maximize the p -dimensional space and thereby allow any species to coexist in the population at any supply fluxes. Under the assumption of optimal allocation of essential resources, species adopting a distinct HU strategy can invade the preexisting bacterial communities [65], thereby supporting coexistence of more species with different utilization strategies. More recently, Pacciani-Mori *et al.* [63^{••},64] showed that if each species can dynamically adjust their utilization strategy to optimize its own growth rate, then the keystone species of Ref. [62[•]] are not even necessary because the system can ‘self-organize’ to find the right conditions for coexistence.

Currently, experimental validation of these intriguing predictions is lacking. A critical requirement for the model of Posfai *et al.* to support broad coexistence is that each coexisting species has an *identical* constraint on total resource. This constraint is somewhat relaxed in the model of Pacciani-Mori *et al.*, which provides an explicit mapping [63^{••},64] of the model components to a model of proteome allocation established for *E. coli* [66]. However, currently it is unknown whether such resource constraints and variability exist across microbes.

Conclusion

While substrate utilization patterns have been studied extensively for over half a century, recent developments reviewed above demonstrate that this subject is far from settled.

The notion of a flux sensor provides a new mechanistic perspective. Flux-sensor-based regulation can serve as a general strategy to implement HU, readily rationalizing the observed hierarchical order of utilization according to single-substrate growth rates, as well as the switch to SU at low substrate concentrations, and the lack of HU between category A and B substrates. While the involvement of flux sensors in the regulation of several uptake systems has been demonstrated, more work is needed to survey the generality of these mechanisms.

The proposed physiological functions of HU and SU have mainly been based on growth-rate optimization achieved through the efficient allocation of essential resources. Recent developments have widened the scope of such arguments considerably. However, several experimental observations, including those from proteomic and metabolomic studies, show that the specific growth rate is often not optimal and gene expression often not streamlined, casting doubt on the notion that the adoption of HU or SU is based on small gains in ROI. Possibly, the identification of additional constraints and/or the application of different (multi-)objective functions can help explain the discrepancies. The dependence of HU and SU on fluxes suggests that such constraints and/or objective functions are condition dependent. Also, further studies will benefit greatly from incorporating elements from the environmental and ecological context of bacterial cells.

Conflict of interest statement

Nothing declared.

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