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OPINION

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The importance of understanding the regulation of bacterial metabolism

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Many bacterial species have biotechnological applications, for example in the production of compounds of interest by fermentation or biotransformation, the handling of waste and the decontamination of polluted sites. The genetic manipulation of such bacteria may improve their suitability for these processes, and even allow them to synthesize or degrade new compounds. Obtaining good results usually requires that engineered microorganisms dedicate a significant amount of their resources (energy, reducing power, etc.) to the desired biocatalytic activity, but this can leave their general physiology under-optimized. When resources are compromised, a number of responses aimed at restoring proper equilibrium are triggered, likely resulting in the yield of the desired catalytic activities being reduced. This is an old problem for biotechnologists that has traditionally been tackled by optimizing the growth conditions, but it might also be confronted via a deeper understanding of the regulatory mechanisms that govern metabolite fluxes and bacterial physiology. In fact, for the optimal engineering of biocatalysts via the rational design and assembly of biological modules that work in a coordinated fashion, such knowledge is essential.

Bacteria have different global regulators that help adapt their metabolism to fluctuating conditions, such as when the supply of nutrients changes or when oxygen availability becomes limiting. But even when nutrients and oxygen are plentiful, other regulatory mechanisms may compromise biotechnological processes. For example, when different types of carbon sources are present, and in varying abundance, regulatory responses may prioritize one compound over the rest, resulting in a distribution of metabolite fluxes that may—or may not—be compatible with the microorganism's biotechnological use. Such regulation, generically known as carbon catabolite repression (CCR), has been studied for decades in a few model bacteria and has been shown not only complex but to be driven by distinct molecular mechanisms in each bacterial group (Görke & Stülke, 2008; Rojo, 2010). The preferred substrates also differ. Many bacteria prioritize glucose over other compounds (e.g. *Escherichia coli* or *Bacillus* sp.), while some prefer certain organic acids or amino acids over this sugar (e.g. *Pseudomonads*). These two strategies have been termed *classical* CCR and *reverse* CCR respectively (Park et al., 2020), and it is proposed that their complementarity helps bacteria specialize with respect to their carbon sources, thereby reducing competition for them in their natural environments (Park et al., 2020).

Optimizing a bacterium for a particular use, or engineering it to obtain a new metabolic pathway, often requires its substantial genetic modification. Powerful techniques are now available for manipulating bacterial genomes, although not all strains are equally tractable. Genome-editing techniques even allow large DNA segments to be deleted, reducing the size of the genome (Martínez-García et al., 2014; Martínez-García et al., 2015). However, it is difficult to predict the full consequences of directed genome edition on bacterial physiology. Clearly, the rational modification of any

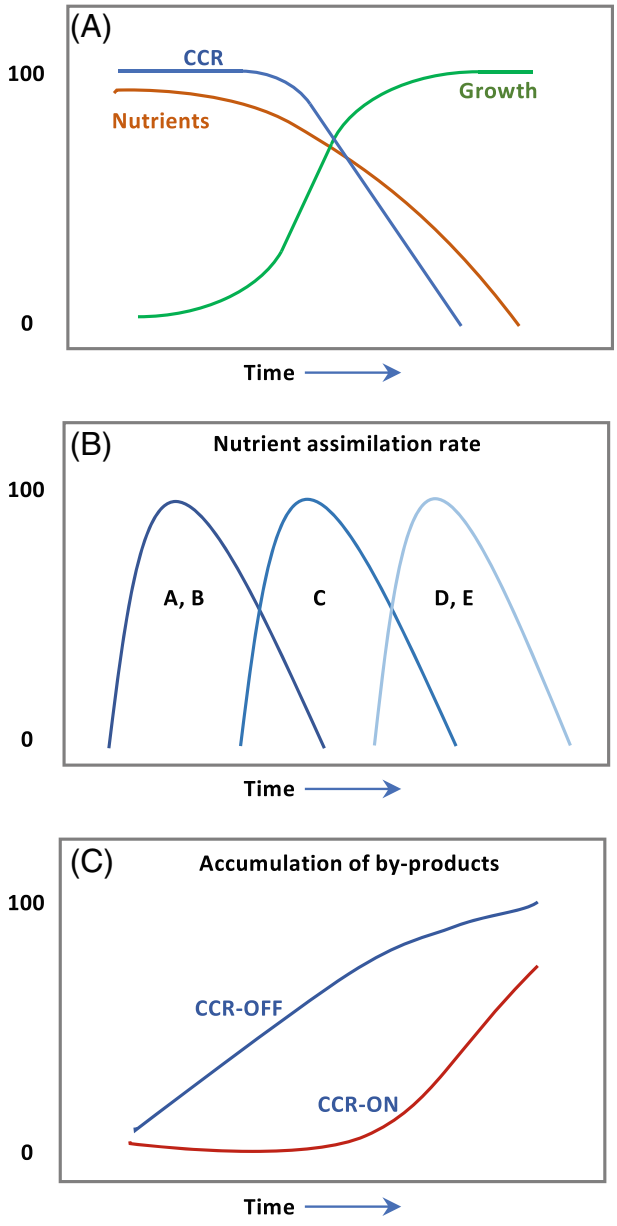

FIGURE 1 Schematic representation of the CCR phenomenon and its influence on the accumulation of products during a biotransformation process. (A) Effect of nutrients on CCR strength and on bacterial growth in a batch culture. (B) Sequential and hierarchical assimilation of different nutrients ('A' and 'B', preferred ones; 'C', intermediate preference; 'D' and 'E', non-preferred), represented as rate. (C) Effect of CCR on the accumulation of a desired by-product during a biotransformation reaction. Please note that representations are schematic, and values might change substantially according to conditions in different settings. CCR, carbon catabolite repression

strain for a specific purpose requires prior in-depth knowledge of its metabolism, and of the regulatory networks that coordinate and optimize the expression of its genome. However, we are far from having such knowledge at our disposal despite the impressive progress made in recent years. Indeed, with the number of known regulatory elements and interconnections increasing every year, plus the realization of the importance of regulatory small RNAs (Aoyama et al., 2022; Bobrovskyy & Vanderpool, 2013), the puzzle is becoming ever more complex.

The control of metabolite fluxes is influenced by many factors, complicating their study (Chubukov et al., 2014). Certainly, the use of [^{13}C]-labelled substrates to analyse these fluxes, plus the development of strain-specific genome-scale metabolic models that help in interpreting transcriptomic and metabolomic datasets, have greatly increased our understanding of which pathways metabolites flow through under given growth conditions, and of how these fluxes are controlled (Hyduke et al., 2013; Schwechheimer et al., 2018). However, unless growth occurs in a chemostat in which steady-state conditions can be maintained, the concentrations of nutrients must fall as they are consumed while those of by-products, and waste products, must increase. This necessarily influences the metabolite fluxes at work, as recently illustrated in the model bacterium *Pseudomonas putida* KT2440 when batch-cultured in a complete medium. The configuration of its metabolism changed substantially over the exponential growth phase as cells sequentially exhausted the different nutrients present and started to use those less preferred [Figure 1(A,B)]. In particular, the configuration of the tricarboxylic acid cycle changed over growth, providing no energy in the early phase (*P. putida* can use glucose as a source of energy only, or as an energy and carbon source), then switching to a reductive mode at mid-growth, and to an oxidative configuration in late exponential phase (Molina et al., 2019b). Furthermore, the inactivation of the main CCR regulatory network led to a metabolic imbalance in which the uptake and assimilation of substrates did not match cellular needs, leading to the overflow of some pathways and the leakage of pyruvate and acetate—something not seen in the wild-type strain (Molina et al., 2019a). The CCR-deficient strain also assimilated many of the available nutrients significantly more quickly than did the wild-type strain, but rather than increasing the growth rate, it actually reduced it, revealing the importance of CCR in coordinating metabolism and optimizing growth.

An important question is whether the addition of new genes and pathways to a bacterium might alter its metabolite fluxes and overall performance, and whether or not it is desirable to keep the new genes beyond the influence of the cell's regulatory networks. In connection with this, it is interesting to know whether native genes that provide bacteria with metabolic versatility, but which are present only in certain strains of a given species (and are therefore components of the so-called 'accessory genome'), fall under the influence of these regulatory networks. Several *Pseudomonas* strains with accessory genes that allow them to assimilate hydrocarbons and aromatic compounds have been

studied. In most cases, these genes were found to be under the strict influence of global regulatory networks, including CCR (Hernández-Arranz et al., 2013; Madhushani et al., 2015; Moreno et al., 2010). It would therefore seem to be advantageous for cells to keep newly acquired genes under the control of their global regulatory networks. But this might not always be desirable for genes involved in biotechnological applications. In fact, there are several examples in which global regulatory networks, and CCR in particular, have hampered such applications (Vinuselvi et al., 2012). In these cases, uncoupling the expression of genes of interest from global regulatory networks would therefore seem advisable [Figure 1(C)].

The question thus arises as to what might be the best strategy for separating a metabolic pathway of interest from CCR control. One solution might be to reduce as far as possible the interaction between pathways that generate biomass (growth) and those that produce compounds of interest (Pandit et al., 2017). This might be useful in some settings, but not in all, since many pathways are strongly interconnected. An alternative is to use a carefully selected mixture of carbon sources that optimizes both growth and biocatalyst performance (Liu et al., 2020), but again, no size fits all. A third possibility is to eliminate the influence of CCR on the biosynthetic pathway of interest by deactivating the complete CCR network, for example by deleting some key genes associated with it. Some examples are known in which inactivating the genes responsible for CCR improved the simultaneous utilization of several compounds that would otherwise be used sequentially (Elmore et al., 2020; Johnson et al., 2017). However, once again, this solution was found not to be helpful in other situations (Lu et al., 2021), probably because eliminating the global regulators responsible for CCR causes imbalanced metabolism. A further possibility might be to conserve the general CCR network but detach the genes of interest from its influence. This implies engineering these genes to eliminate the elements recognized by global CCR regulators, and indeed this strategy has proved helpful in optimizing a *P. putida* strain engineered to generate medium-chain-length α,ω-diols (building blocks for polymer production) (Lu et al., 2021). The drawback here is that such an approach requires detailed prior knowledge of the molecular mechanisms that drive CCR in the strain of interest, including the identification of the targets recognized by the global regulators of the genes to be manipulated. In addition, the regulatory mechanisms responsible for CCR differ substantially among bacterial groups. The molecular details are relatively well known for model bacteria such as *E. coli*, *B. subtilis* or *Pseudomonads* (Görke & Stülke, 2008; Moreno et al., 2015; Pei et al., 2019; Rojo, 2010; Sonnleitner et al., 2018), but certainly not for other bacteria of biotechnological importance (Ruiz-Villafán et al., 2021).

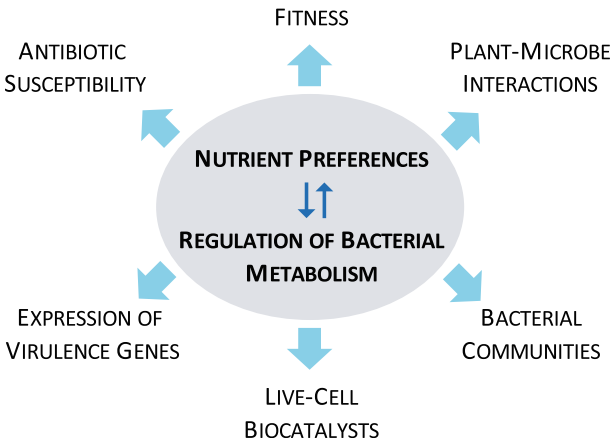

FIGURE 2 Some important traits influenced by the regulation of bacterial metabolism

Moreover, even if the problems related to CCR could be solved, other regulatory elements might also interfere transcriptionally or post-transcriptionally affecting the expression of the genes of interest. Particular metabolites might even allosterically modulate the activity of key enzymes. In other words, eliminating one regulatory element might not avoid the effects of others.

A final problem is that the signals that trigger CCR are not well understood. Cells probably sense the concentrations of key metabolites that act as flux sensors, although other elements might be monitored as well. A recent report indicated that the hierarchy of utilization of carbon sources in *E. coli* was ordered by the total carbon-uptake flux rather than by the precise compounds being used (Okano et al., 2020; Okano et al., 2021). Below a given threshold of carbon-uptake flux, CCR faded away and the compounds present were utilized simultaneously. In addition, certain metabolites such as fructose-1,6-bisphosphate (an intermediate of the glycolytic pathway), α-ketoglutarate (a component of the tricarboxylic acids cycle) and others may also act as flux sensors regulating the activity of particular enzymes (Chubukov et al., 2014; Okano et al., 2020). The final picture is that of finely tuned, highly intertwined and regulated metabolic pathways that are complicated to study, but much deserving of attention.

In summary, learning to control bacterial metabolism, or at least making good predictions regarding its behaviour, will require considerable effort. It would be useful to investigate this from complementary perspectives, for example examining how the expression of genes making up complete metabolic pathways is regulated, how global regulators change transcription programs, how the activity of particular enzymes is regulated by key signalling metabolites, which metabolites flow through which pathways under given conditions, how these flows change when conditions change, how pathways are interconnected, and so

on. Obtaining this knowledge requires bringing together traditional molecular biology techniques, analysis of the metabolite fluxes under different conditions, the use of *in silico* genome-scale metabolic models of increasing complexity that will allow predictions to be experimentally verified, and so on. The more complete information we obtain, the better the chance of succeeding when engineering bacterial biocatalysts.

A detailed knowledge of how cells regulate and coordinate their metabolism is important in other fields too (Figure 2). For example, the nutrients used by pathogenic bacteria can influence the expression of virulence genes (Eisenreich et al., 2010), the adaptation of the pathogen to a specific host (La Rosa et al., 2018), or its susceptibility to different antibiotics, for example those that enter the cell using cell envelope proteins involved in the transport of nutrients (Martínez & Rojo, 2011). Metabolic preferences can also have a huge impact on the assembly and behaviour of bacterial communities, the members of which have to share—or compete for—nutrients present in limited amounts (Bajic & Sánchez, 2020; Estrela et al., 2021). CCR also affects the expression of genes involved in plant–microbe interactions (Franzino et al., 2022) with its many implications. Clearly, reaching an in-depth understanding of the regulation of metabolism will require much effort over the coming years, but will bring many benefits as well.

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