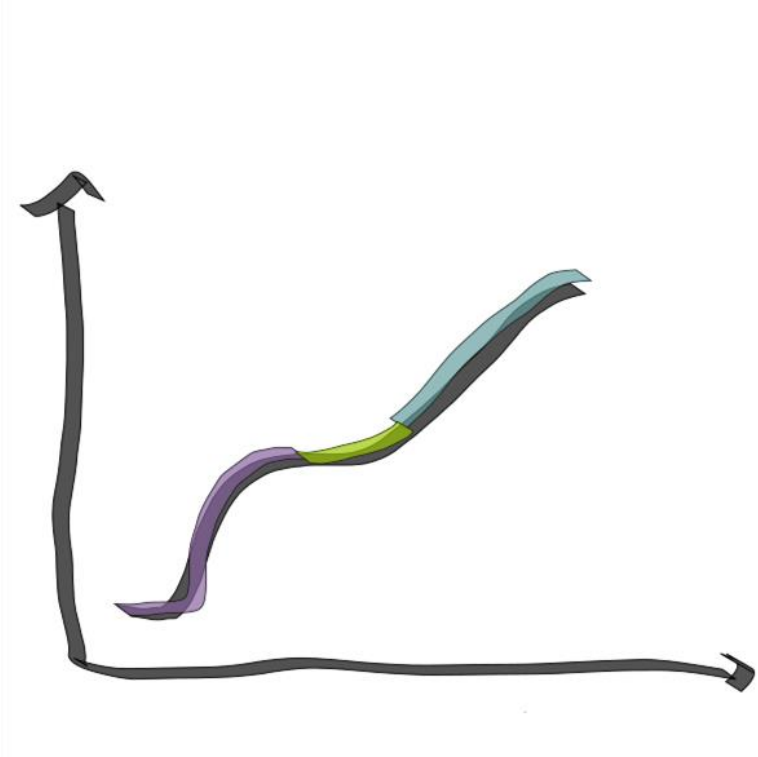
Deciphering carbon catabolite repression in bacteria



Writing Assignment

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Abstract

Organisms must constantly adapt to environmental challenges such as fluctuating resource availability and competition. Carbon catabolite repression (CCR) is a strategy adopted by many microorganisms, which selectively permits the uptake and metabolization of a more favorable carbon source until its depletion, to the detriment of other substrates, which are prohibited from entering the cell. This use of the preferred carbon source, such as glucose, is believed to aid microorganisms with rapid growth while maintaining an optimal proteomic economy. The regulatory mechanisms governing CCR vary considerably between prokaryotes. The inhibition of the uptake systems of the secondary carbon sources and their metabolizing enzymes can be implemented at the transcriptional level, post-transcriptional levels, or through direct inhibitory effects. The impact of carbon repression extends well beyond sugar uptake. CCR frequently exerts a widespread influence that overlaps with central metabolic pathways, anabolic processes, and even secondary metabolic pathways. This review aims to explore these CCR mechanisms in four bacterial groups and highlight the gaps in our current understanding.

Layman’s summary

Bacteria consume carbon sources such as sugars or various organic acids to live. When many carbon sources are present, various bacterial species choose to concentrate on metabolizing one carbon source at a time. The process of sequentially selecting carbon sources for nutritional purposes is called carbon catabolite repression (CCR). To consume a certain sugar, bacteria need to produce different kinds of proteins, consuming significant energy in the process. It is believed that CCR helps microorganisms save energy instead of using it on the production of unnecessary proteins. Carbon repression can be implemented at the molecular level in various ways. In principle, the uptake of a preferred carbon source can activate a molecular pathway which inhibits the expression of genes necessary for the uptake and processing of different carbon sources. However, many questions remain, and this review tries to summaries the current knowledge regarding this topic, point out what the missing links are and familiarize the reader with the general concepts of this topic.

1. INTRODUCTION

In 1942, famous French scientist and noble laureate Jaques Monod, quantified the growth of *Escherichia coli* when two carbon sources were provided simultaneously. Under certain combinations of carbohydrates, the culture would preferentially consume one sugar before another, as inferred by the two distinct growth curves separated by a period of lag. This phenomenon was named diauxic growth. Combining glucose as a carbon source with either mannose, fructose, mannitol did not result in diauxic growth while using lactose, sorbitol, maltose, xylose did. This phenomenon was named “the glucose effect” (Monod, 1942; Ullmann, 1996). Later, the glucose effect was renamed “carbon catabolite repression” (CCR) after anticipating that the accumulation of certain catabolites of the preferred carbon source repress the enzymes whose activities would help metabolize the less preferred sugars. It was postulated that this strategy avoids the overproduction of catabolites beyond the capacity of the anabolism (Magasanik, 1961). Later studies support the hypothesis that CCR acts as a strategy for optimal growth by allocating the resources towards anabolic processes, whereas when a non-preferred carbon source is provided, the biosynthetic resources are aimed towards increasing the carbon influx (Salvy & Hatzimanikatis, 2021; Scott & Hwa, 2022; You et al., 2013). CCR is a widespread regulatory phenomenon in most heterotrophic bacteria, and it is recognized as a strategy to enhance adaptability. CCR encompasses more than just selecting for the preferred substrate; it has implications in economically and medically relevant processes such as virulence, biofilm assembly or secondary metabolite production (Görke & Stülke, 2008; Nair & Sarma, 2021; Ruiz-Villafan et al., 2017). From a biotechnological perspective, CCR is often an unwanted effect of using microorganisms for the consumption of waste materials (Fox & Prather, 2020). These consequences of CCR highlight the importance of studying its effects beyond the fundamental incentives. However, the regulation networks underlying CCR in different species are diverse and so are the preferred carbon sources. This review aims to provide an updated summary and a general overview on our current knowledge of how CCR is implemented at the molecular level, how this process influences various cellular decisions and what are the yet lingering questions associated with it.

2. CONTENTS

2.1. CCR IN E. COLI

Escherichia coli commonly lives in the digestive tract of mammals where it competes for resources with other enteric bacteria, but strains who inhabit the soil have also been reported (Conway & Cohen, 2015). *E. coli* can metabolize a great number of sugars, with as high as 180 predicted growth-sustaining carbon sources (Orth et al., 2011; Chang et al., 2004). Since the earliest studies investigating CCR, cAMP and later the cAMP responsive protein (Crp), were identified as antagonists of the CCR (Ullmann, 1996). In *E. coli* CCR is accomplished either via a cAMP-mediated global mechanism or operon-specific mechanisms (Görke & Stülke, 2008). Glucose is the preferred carbon source in *E. coli*. It diffuses into the periplasmic space through specific outer membrane porins (OmpF, OmpC, LamB). The posphoenolpyruvate–carbohydrate phosphotransferase system (PTS) is used to transport glucose into the cytoplasm most of the times but in certain situations transporters such as MglBac or GalP can also uptake glucose (Carreón-Rodríguez et al., 2023).

a) The molecular mechanisms E. coli CCR

Central to the *E. coli's* CCR global regulation pathway stands the PTS formed of several proteins carrying phosphotransferase reactions (**Figure 1**). In this system, the second component, EIIA (enzyme II domain A), is maintained in a phosphorylated state when non-preferred carbon sources are present (Görke & Stülke, 2008). Because each EII complex, formed by two membrane domains and two hydrophilic domains (domains A and B) is specific to a carbohydrate, *E. coli* carries multiple types of EII complexes (Deutscher et al., 2006). The phosphoryl group is transferred from phosphoenolpyruvate (PEP) to enzyme I (EI) and then to Histidine protein (HPr), which phosphorylates EIIA. EIIA is a key protein in CCR modulation. In its phosphorylated form it activates adenylate cyclase. The production of cAMP by adenylate cyclase leads to the formation of a cAMP-Crp complex, positively regulating the catabolic genes and thus mitigating the effects of CCR. When a glucose molecule is transported inside the cell through the PTS, EIIA transfers the phosphoryl group to its domain B (EIIB) and which will phosphorylate glucose, thus adopting a dephosphorylated state (Görke & Stülke, 2008; Nair & Sarma, 2021). Dephophorylated EIIA also participates in CCR by inhibiting the transporters of some non-PTS sugars, such as lactose (LacY), maltose (MalK) and melibiose (MelB). This phenomenon, independent of Crp-cAMP, is termed inducer exclusion and is considered the major CCR mechanism in Enterobacteriaceae (Deutscher et al., 2006).

An additional mechanism of CCR in *E. coli* is through Spot42, a sRNA, which is repressed by cAMP-Crp. In the presence of glucose Spot42 was shown to downregulate around 29 catabolic genes, but this number can increase as this molecule is scrutinized more in the coming years (Durica-Mitic et al., 2018).

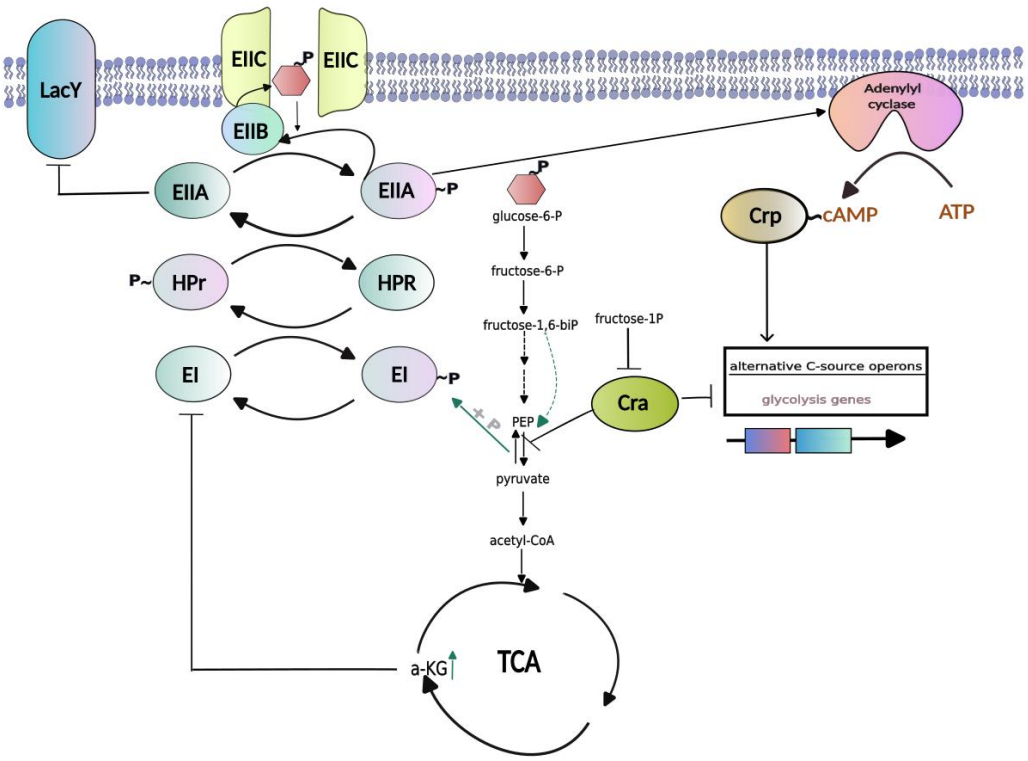

Figure 1. Carbon catabolite repression in *E. coli*. Phosphoenolpyruvate (PEP) is a phosphoryl-group donor to EI protein of the phosphoenolpyruvate–carbohydrate phosphotransferase system (PTS). A chain of phosphorylation reactions lead to the phosphorylation of EIIA. When non-preferred carbon sources are present, phosphorylated EIIA activates adenylate cyclase which converts ATP to cAMP. Crp binds cAMP forming a complex which positively regulates catabolic and glycolysis genes. When glucose or other PTS-sugars are internalized, EIIA transfers the phosphoryl group to EIIB which in turn phosphorylates the sugar, leaving EIIA dephosphorylated. In this state, EIIA acts as a repressor, directly inhibiting the transporters of less-preferred carbon sources such as lactose (here LacY) in a process called inducer exclusion. Cra is a transcription factor which mostly works by antagonizing the effects of Crp. Cra reduces the conversion of PEP to pyruvate and is inhibited upon fructose-1-phosphate accumulation. Fructose-1,6-biphosphate (FBP) allosterically positively modulates the enzyme catalyzing the conversion of PEP to pyruvate while Cra negatively inhibits this conversion. The accumulation of keto-acids such as α-ketoglutarate (α-KG) in nitrogen starvation inhibits the transfer of the phosphoryl group to EI, thus reducing the PTS-sugars transport. TCA - tricarboxylic acid cycle; the red hexagon represents a glucose molecule being phosphorylated

b) Unexplained molecular mechanisms

While most models focus on glucose-PTS system, *E. coli*'s genome contains a remarkable 21 EII complexes, associated with the transport of around 15 distinct sugars. Some of the PTS carbon sources include mannitol, mannose, fructose, GlcNAc (Tchieu et al., 2001). Certain PTS proteins from different sugar systems are interacting with each other forming networks. For example $EIIA^{Glc}$ was shown to interact with both $EIICB^{Mal}$ and $EIICB^{Glc}$. Interestingly, the phosphorylation reactions occurred equally

within members of the same PTS network even in the presence of a non-cognate sugar. For example, $EIIA^{Man}$ -$EIICB^{Man}$ and $EIIA^{Glc}$ -$EIICB^{Glc}$ were found to interact with the same frequency in the presence of glucose, even though glucose is not the specific sugar for $EIICB^{Man}$ (Somavanshi et al., 2016). Another curiosity stems from the fact that non-PTS sugars such as glucose-6-phosphate, glycerol-3-phosphate, gluconate, lactose, xylose, arabinose can also cause CCR on other non-PTS sugars (Ammar et al., 2018; Eppler et al., 2002; Hogema et al., 1997, 1998). In the case of glucose-6-phosphate, the cause was traced to the dephosphorylation of EIIA and consequently to lower cAMP levels. The phosphorylation state of EIIA was correlated with PEP/pyruvate ratio, specifically, the lower the ratio, the more likely was for EIIA to be dephosphorylated (Hogema et al., 1998; Bettenbrock et al., 2007). For the rest of above-mentioned sugars, the mechanism of repression was shown to involve the cAMP-Crp levels (Ammar et al., 2018). This suggests that there are additional unstudied mechanisms which permit non-PTS sugars to influence cAMP levels, in some cases likely through PTS, as suggested by the dephosphorylation of EIIA.

c) The physiological role of Crp

cAMP-Crp signaling plays an important physiological function. In fact, there is an inverse linear relationship between growth rate and cAMP-Crp activity levels, known as “C-line” (You et al., 2013). The cAMP-Crp complex has been proposed as a mediator between carbon catabolism and protein biosynthesis (Kochanowski et al., 2021; Scott & Hwa, 2022). According to Kochanowski et al., 2021, the anabolic process is likely indirectly antagonized by Crp's stimulatory effects on catabolism due to the competition between catabolic and anabolic enzymes for resources necessary for their own synthesis, such as ribosomes. The relationship between catabolism and anabolism mediated by cAMP-Crp is not exclusively opposing, that is low cAMP levels can correlate with poor growth in certain situations. When the carbon uptake exceeds the anabolic capacities such as in nutrient deficiencies, the carbon flux is restricted. For example, there is a reduction in glucose uptake as a consequence of α-ketoglutarate accumulation, a product of nitrogen starvation, which directly inhibits EI phosphorylation and thus limits the glucose transport through the PTS but also keeps EIIA in a dephosphorylated state (Doucette et al., 2011; Kochanowski et al., 2021). Interestingly, a different study showed that when *E. coli* cells were grown in the presence of glucose, a second carbon source and a poor nitrogen source, the culture would preferentially consume the glucose even through glucose recorded the poorest growth rates compared to the other sugars, which is not the case when optimal nitrogen sources are present. This slow growth on glucose under nitrogen-poor conditions was indeed correlated with high α-ketoglutarate and very low cAMP levels, but perhaps this study also reveals certain “glitches” in *E. coli* metabolism (Bren et al., 2016). cAMP levels are also reduced by the accumulation of 2-oxoglutarate, another keto acid resulting from nitrogen scarcity (You et al., 2013). Moreover, it was shown that reduced cAMP led to the activation of alternative sigma factor σ^S, which activates the stress response in *E. coli.* This phenotype was associated with a thicker cell wall and an increased protection against oxidative stress (Barth et al., 2009). These studies show that information about anabolic capacities or environmental insults is integrated through Crp, which is a key coordinator of *E.coli* metabolism and physiology beyond CCR.

d) Cra is antagonizing Crp

Cra is a transcription factor in *E. coli*, governing the expression of 97 known genes in its regulatory network (Kim et al., 2018). Cra is known to activate gluconeogenesis genes and to repress glycolysis enzymes sometimes working synergistically but most often overriding the regulatory activity of Crp (e.g glycolitic enzymes) (D. Kim et al., 2018; Ramseier, 1996) While Cra and Crp seem to have mostly antagonistic effects, Cra was proposed as *crp* activator, working together with Crp itself for its transcription (Zhang et al., 2014).

e) The concept of flux sensor

While regulation of central metabolism in response to varying sugar sources and levels can in principle be sugar-specific, given the vast amount of carbohydrates *E. coli* can metabolize, a less costly strategy in terms of protein synthesis requirements would be adjusting to internal carbon influx signals, rather than to the concentration of individual carbon sources (Kochanowski et al., 2013). Kotte et al, 2010 used mathematical modeling to propose the ability of *E. coli* to discern between gluconeogenic and glycolytic substrates and adapt its central metabolism accordingly through the use of *metabolic fluxes sensors*. In this model, nutrient abundances would lead to the varying levels of a certain metabolite, the *flux-sensing metabolite*, which is sensed by molecular systems called *flux sensors*, leading to the integration of the signal at the transcription level. Later models suggested that fructose-1,6-biphoshpate (FBP) is a *flux sensing metabolite* of glycolysis rates and together with Cra, forms a metabolic *flux sensor* which adjusts the regulatory decisions in a flux-dependent manner (Kochanowski et al., 2013). Later experiments using glycerol as a substrate showed that FBP is rather a sensor of the substrates entering glycolysis upstream of FBP (Okano et al., 2020) FBP allosterically and positively modulates pyruvate kinase and PEP carboxylase while fructose-1-phosphate inhibits Cra (Bley Folly et al., 2018; Valentini et al., 2000). This suggests that FBP and fructose-1-phosphate mediate a balance between upper glycolytic flux and enzyme activities in the lower glycolysis through feed-forward allosteric modulation but also through Cra (Figure 1) (Kochanowski et al., 2013; Chubukov et al., 2014).

Recently it was shown that, when both lactose (or glucose) and glycerol are provided to *E. coli* cultures, below a certain uptake rate, the lactose/glucose influx is supplemented with glycerol to meet a minimal carbon flux threshold. In this study, FBP was proposed as a marker of the upper glycolysis flux. This small regulatory circuit of glycerol included positive upregulation of glycerol kinase through cAMP-Crp and inhibition through FBP. In other words, the flux of glycolysis is monitored through FBP and if it exceeds a certain level, gluconeogenic substrates (here glycerol) are prevented from being imported. The interesting observation about this type of regulation is that it does not matter which substrate increases the FBP pool (Okano et al., 2020) While flux sensors are abstractions of physical phenomenon, this experiment perhaps suggests that using a *flux-sensor* model could also be applied for understanding CCR.

2.2. CCR IN B. SUBTILIS

a. The molecular mechanisms of CCR

In Firmicutes a similar role to that of Crp is fulfilled by the catabolic control protein A (CcpA) which represses (instead of activating like Crp) the catabolic genes containing a specific regulatory region called catabolite responsive element (*cre*). Further expanding the comparison to *E. coli*, CcpA is activated by phosphorylated HPr in a similar manner to how the formation of the cAMP-Crp complex is stimulated by phosphorylation of EIIA (**Figure 2**). However, compared to *E. coli*, B. *subtilis* possesses an extra phosphorylation site at Ser46, in addition to the conventional EI-dependent phosphorylation site at His15. This Ser46 site also undergoes phosphorylation as part of the PTS sugar transport process (Deutscher, 2008; Görke & Stülke, 2008). The phosphorylation of HPr at Ser46 is achieved by HPr-kinase/phosphorylase (Hpr-K/P) in the presence of FBP, thus when there is ongoing nutrient intake (Fujita, 2009). HPr(Ser-P) works as a cofactor for the binding of CcpA to *cre* sites. An additional protein, Crh(Ser-P), which is also phosphorylated by HPr kinase/phosphorylase, can also share this role for the regulation of certain operons. However, in a *crh* mutant, its regulatory function was supplemented by Hpr which seems to suggest a redundant role for this protein (Galinier et al., 1999).

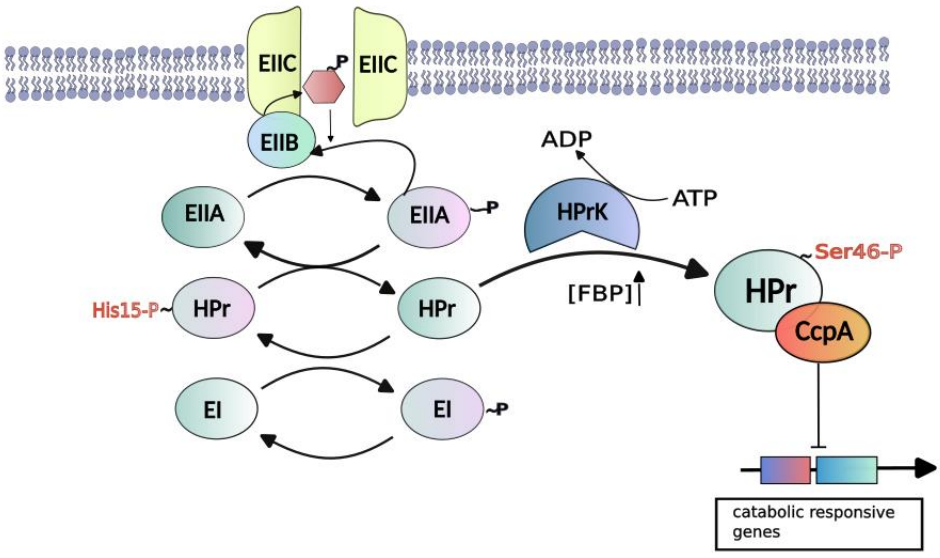

Figure 2. Glucose CCR in *B. subtilis*. The PTS complex and the cascading phosphorylation reactions can be observed on the left-hand side of the illustration, where a glucose molecule is phosphorylated as part of the uptake process. When glucose is present and FBP (fructose-1,6-biphosphate) levels are high, HPr (histidine protein) is phosphorylated at Ser46 by HprK (HPr phosphorylase). For this reaction, ATP is hydrolyzed. CcpA is a transcription factor which represses the catabolic genes containing a *cre* motif. Phosphorylated HPr acts as a cofactor of CcpA, aiding its binding to target genes.

b. CcpA-independent CCR

In *B. subtilis* glucose and malate are the preferred carbon sources but other sugars were observed induce CCR to different degrees such as salicin, mannitol, fructose (Meyer et al., 2011; Singh et al., 2008). While in most cases the repression is mediated by CcpA, there are also operon-specific responses to certain sugars. The operons needed to metabolize β-xylosides are repressed by CcpA and by the xylose repressor, XylR which is inhibited by xylose. Xylose and glucose-6-phosphate compete for binding to XylR thus glucose-6-phosphate offers less chances for the derepression of xylose-metabolizing operons. This indicates that glucose exerts extra CCR (Singh et al., 2008).

Another interesting case involves the suppression of the *pftAB* operon, which is responsible for pyruvate uptake in *B. subtilis an*d can undergo repression through both CcpA-dependent and CcpA-independent mechanisms. In this latter scenario, malate can be converted into pyruvate through the action of the malic enzyme. The accumulation of internal pyruvate is sensed, leading to a decrease in the induction level of the *pftAB* operon, responsible for pyruvate intake. Pyruvate can also be sensed externally by the LytST two-component system and consequently reduce operon induction independently of CcpA (Charbonnier et al., 2017). Besides CcpA, there are four additional transcriptional regulators, namely CcpB, CcpC, CcpN and CggR, which exert catabolite control on TCA and glycolysis genes (Fujita, 2009). However, their role in CCR, if any, has not been sufficiently investigated.

c. CcpA is a versatile global regulator

CcpA protein is a global regulator of catabolite control which can act both as repressor and activator. CcpA activity was shown to be present during different stages of growth and in the stationary phase, suggesting that it is constitutively expressed (Lulko et al., 2007). As mentioned earlier, CcpA is able to regulate genes containing *cre* sites which are mostly located in the transcription-initiation regions, but exceptions can occur, such as for the *ackA* gene (encoding acetate kinase), where *cre* was located upstream and in this situation, CcpA exhibited gene activation (Görke & Stülke, 2008). The *cre* site motif has been elucidated, however, a newer *cre* motif was found recently in Bacillus licheniformis for which, based on the nucleotide arrangement, it could be predicted whether activation or repression activity will occur (Xiao et al., 2021). Whether this new motif is present in other Firmicutes and whether it will update our knowledge of the regulon landscape for CcpA remains a topic for future research. Interestingly, the the formation of the CcpA-HPr is usually necessary for the transcriptional activity, however, when complex formation was hindered via CcpA mutagenesis, a considerable number of genes were still affected by CcpA regulation, which suggests that CcpA can also exert a regulatory function in a HPr-independent manner (Detert Oude Weme et al., 2015).

The influence of extends from carbon utilization, amino acid metabolism, nitrogen assimilation, overflow metabolism, either directly or indirectly. CcpA represses catabolic and Krebs cycle genes, while positively regulating genes of the overflow metabolism and glycolytic enzymes (H. J. Kim et al., 2002). However, unlike the Crp homologue in *E. coli*, there is no evidence of CcpA regulating iron uptake genes (Lorca et al., 2005).

CcpA, like Crp, balances the repartition of resources between carbon and nitrogen metabolism. Pyruvate, acetyl-CoA and oxaloacetate are metabolites necessary for branched-chain amino acids (BCAA) synthesis such as leucine, valine and isoleucine (Kaiser & Heinrichs, 2018). Another global transcription factor, CodY, acts as a sensor for nutrient availability. When BCAAs are sufficient, CodY represses genes involved in amino acid synthesis and transport (Majerczyk et al., 2010). CcpA is known to activate the *ilv-leu* operon, which houses genes encoding necessary proteins for BCAA synthesis, thus CcpA is linking information about catabolic availability to the anabolic processes ultimately leading to the indirect activation of CodY. However, in this situation, CcpA and CodY have antagonistic effects, with CcpA promoting growth and CodY favoring resource conservation. In nitrogen-rich medium, the negative activity of CodY exceeds the regulation of CcpA, thus keeping the BCAAs synthesis within appropriate bounds. In nitrogen-deficient conditions, a third transcription factor known as TnrA inhibits the positive regulation of CcpA on the *ilv-leu* operon, thus contributing to the regulation of homeostasis through the coordinated actions of these three transcription regulators (Fujita, 2009).

What are the molecular signals that inform the metabolism of a sufficient carbon intake, and would that signal correlate to CCR perhaps in a similar manner on how cAMP plays this role to a certain degree in *E. coli*? As mentioned earlier, FBP is necessary for HprK/P activity, however, when growing *B. subtilis* cells on different carbon sources, the FBP concentrations exceeded the necessary values for maximal HprK/P activity for all the carbohydrates tested (which showed high correlation levels between HprK/P and CCR), whereas their catabolite repression activity varied (Singh et al., 2008). This indicates that at least FBP alone cannot explain the variation in catabolite repression levels, therefore other molecules might be at play. GTP and ATP have been proposed as candidates for this role, but the question remains open (Chubukov et al., 2014). As for the role of FBP, like in other microorganisms, there is a high correlation between FBP and the magnitude of the glycolytic flux and therefore, FBP is rather proposed as a *flux-sensing metabolite* of glycolysis. Moreover, FBP was found to inhibit the activity of CggR which resembles the relationship between fructose-1-phosphate and Cra in *E. coli (Chubukov et al., 2013).*

2.3. CCR IN PSEUDOMONADS

Pseudomonads are a gram-negative, motile, and metabolically versatile bacterial group. They occupy diverse ecological niches in nature, with examples displaying beneficial interactions with plants (as seen in *Pseudomonas fluorescens*), plant pathogens (as demonstrated by *Pseudomonas syringae*), participation in organic waste decomposition in soils (as exemplified by *Pseudomonas putida*), or infecting animal tissues, including humans (as observed with *Pseudomonas aeruginosa*) (García-Garibay et al., 2014).

a) Reverse CCR

In contrast with other bacteria, glucose does not play a central role in catabolite repression in pseudomonas spp., nor is it one of the preferred carbon sources. Instead, lower energy substrates are

preferred such as organic acids and amino acids (Rojo, 2010). In fact, the glucose metabolism might be unique in this group. It was found that *S. putida* lacks a functional Embden-Meyerhof-Parnas (EMP) pathway and instead combines enzymatic steps from Entner-Doudoroff (EM), EMP and Pentose Phosphate Pathways (PPP) for metabolizing glucose (Nikel et al., 2015). In a recent study, *P. aeruginosa* displayed the following substrate preference in the decreasing order: amino acids (aspartate, asparagine, glutamine, glutamate), citrate, succinate, lactate, acetate, glutamate and lastly glucose (McGill et al., 2021).This inverted preference for carbon sources compared to *E. coli* and *B. subtilis* is called reverse CCR (rCCR) or inverse diauxie, while the type of CCR exhibited by *E. coli* is called classic CCR (cCCR) (Rojo, 2010).

The preference for low energy substrates might be understood in the context of natural environments where rCCR organisms such as pseudomonads live in consortia with cCCR organisms which rely on glycolysis and overflow metabolism to sustain rapid growth. Prokaryotic and eukaryotic cells use overflow metabolism, which prioritizes fermentation over high energy-yielding respiration, as respiration-related enzymes are too expensive in terms of proteome resources to support rapid growth. (Basan et al., 2015). rCCR organisms specialize in metabolizing the products released from the overflow metabolism of cCCR bacteria such organic acids, instead of competing for the same resources, which might explain the why pseudomonads adopted this strategy (Park et al., 2020).

b) The molecular mechanisms underlying CCR

In pseudomonads CCR is operating at the post-transcriptional level (**Figure 3**). In the presence of the preferred carbon source, the RNA chaperon protein Hfq recognizes the catabolite activity motif present on catabolic genes of non-preferred carbon sources and binds their mRNA, inhibiting their translation. This is only possible when the Crc (catabolic repression control) protein stabilizes the complex between Hfq and the target mRNA (Sonnleitner & Bläsi, 2014). When less preferred carbon sources are present, small RNAs (sRNA) help sequester the Hfq regulator and thus lifts the repression. In *P. aeruginosa* one such sRNA molecule is CrcZ which is induced by a two-component system unique in pseudomonads called CbrA/CbrB, with the help of the sigma factor, RpoN (Sonnleitner et al., 2009). Other pseudomonads have more than one sRNA molecule with functions which appear to be redundant such as an additional CrcZ in *P. syringae* and and CrcY in *P. putida (Franzino et al., 2022).*

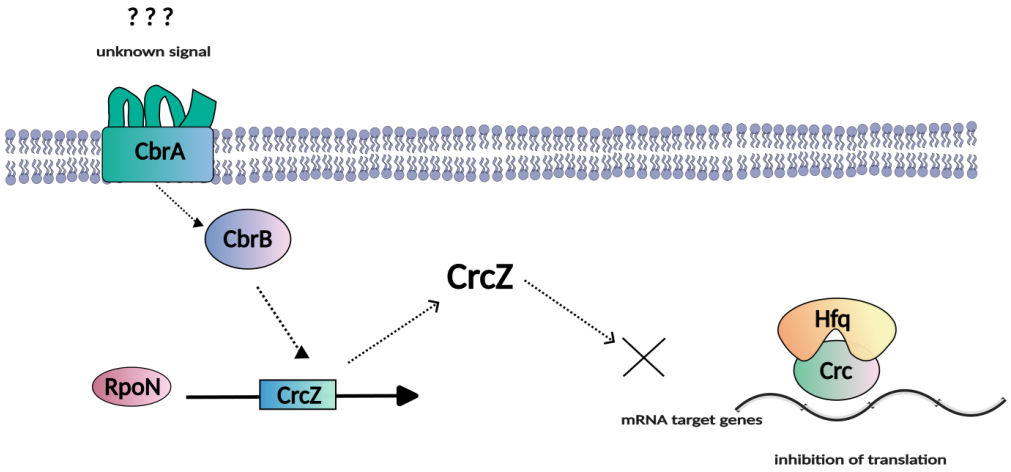

Figure 3. The CCR mechanism in pseudomonads. In the presence of the preferred carbon sources Hfq and Crc form an active protein complex and inhibit the translation of the catabolic genes. Crc stabilizes the binding of Hfq to DNA. The interactions in the absence of the preferred carbon sources can be seen with interrupted lines. Un unknown signal activates the CbrA/CbrB two component system and in the presence of RpoN, the sRNA CrcZ is expressed. CrcZ destabilizes the Hfq and Crc complex thus lifting the repression.

Histidine can activate the CbrA/CbrB but the complex was also shown to be activated in a His-independent manner by an unknown cue, consequently it not clear what are the signals which activate CbrA/CbrB two component system (Monteagudo-Cascales et al., 2022). In *P. putida*, CbrA levels were shown to have an inverse linear relationship with available carbon levels when grown on succinate, oxaloacetate, or LB media. The same authors propose that Crc is indirectly regulating CbrA levels based on the existence of a putative Hfq consensus binding site on a previously unknown ORF, *cbrX*, situated upstream of *cbrA*, which together share dependent transcription. Furthermore, the observation of high repression levels by Hfq/Crc and low CbrA expression in carbon-rich conditions supports this hypothesis (Monteagudo-Cascales et al., 2019; Moreno et al., 2012). Recently, a new protein named CrcA was discovered in *P. aeruginosa*, which helps sequester Crc in the absence of the preferred carbon source (Sonnleitner et al., 2023). Nonetheless, due to the limited understanding of the specific cues that trigger Crc or CbrA activation, the molecular networks responsible for coordinating CCR in these species remain insufficiently elucidated.

2.4. CCR IN STREPTOMYCES

Streptomycetes are soil-dwelling gram-positive bacteria known for their capacity to produce anti-infective secondary metabolites. They are characterized by large chromosomes, a complex multicellular life cycle and a high number of protein-encoding genes with predicted with regulatory function, which reflect their ability to adapt to the competitive soil environment (Borodina et al., 2005; Hodgson, 2000). Streptomycetes secrete hydrolytic enzymes used to digest a wide range of polysaccharides available in the soil such as lignin, cellulose and chitin and break them down into oligo- and monosaccharides which can be internalized for further usage. These immobile bacteria form entangled mycelial structures, which enable the hydrolytic enzymes to concentrate locally, thus enhancing the digestive efficiency. When the available nutrients neighboring the mycelium are finally exhausted, the mycelium undergoes a local process of autocatalytic cell death, known as programmed cell death (PCD), which subsequently fuels the formation of aerial hyphae. These structures permit the dispersion of spores, some of which will germinate, forming a new mycelium (Barka et al., 2016; Hodgson, 2000). As such, nutrient scarcity is finely tuned with the onset of development, and thus the capacity to respond to different nutrients and coordinate external signals is vital for these species.

a) The role of glucose

Glucose is one of the most abundant monosaccharides found in soil which, in this environment, primary originates from the decomposition of cellulose (Gunina & Kuzyakov, 2015). In *Streptomyces,* glucose is not transported via the PTS system, but instead internalized via sugar permease GlcP, a major facilitator superfamily (MFS) transporter (Van Wezel et al., 2005). Like in many other species, glucose elicits CCR on alternative carbon sources in streptomycetes. Early studies in *S. coelicolor* showed that glucose-related CCR cannot solely be explained by an inducer exclusion mechanism in this species, but there is an additional system governing catabolite repression relating to the activity of glucose kinase (Glk). The significance of Glk became evident when it was observed that mutants of *S. coelicolor*, which no longer exhibited sensitivity to glucose repression, consistently displayed mutations in the *glkA gene* (Hodgson, 1982; Hodgson, 2000). It is believed that Glk plays a dual in role in these species, having both glucose-metabolizing activity by phosphorylating glucose and a role in glucose-mediated regulation of alternative carbon-source catabolic genes. This assumption is based on the fact that in *S. coelicolor glkA* deletion mutants complemented with *Zymomonas mobilis glkA* gene regain their catalytic function, but glucose repression is still absent (Angell et al., 1994; Romero-Rodríguez et al., 2017).

It is likely that Glk could be part of a regulatory system triggered by glucose but to this date, the molecular processes that lead to Glk-mediated catabolite repression have not been elucidated. As a first step of this proposed repression system, Glk binds the GlcP transporter at the membrane level and likely dissociates when glucose is internalized but the downstream process remains a mystery (Van Wezel et al., 2007). Glk bears some resemblance to ROK transcription factors, but it lacks the DNA-binding domain, thus it is unable to exert a regulatory function directly (Romero-Rodríguez et al., 2017). Instead, it has been proposed that Glk likely works by interaction with other regulators, interaction which might be triggered by post-translational modifications (Van Wezel et al., 2007). In line with this, recently it was shown Glk can be reversibly crotonylated in *S. roseosporus*. High Glk

crotonylation levels were associated with decreased kinase activity while Glk and global high crotonylation levels were associated with enhanced CCR and high glucose consumption (Sun et al., 2020). How crotonylated-Glk might interact with the presumable partners and how can the glucose influx be maintained in the presence of high crotonylated-Glk are questions which need to be investigated. Additional studies showed that glucose-mediated transcriptomic and proteomic changes in primary metabolic processes such as glycolysis or pentose phosphate pathway, cannot be explained by primarily by Glk-mediated responses (Romero-Rodríguez et al., 2017). This contrasts with *E. coli* or *B. subtilis*, where the global effects of certain carbon sources are mainly mediated via a global system (via Crp-cAMP and CcpA respectively).

Nevertheless, a Glk-independent regulator has been characterized. In *Streptomyces avermitilis*, Rok7B7, a ROK-family regulator, responds to the presence of xylose and glucose. When glucose is present, it actively inhibits the xylose transporter operon, *xylFGH*. In contrast, when xylose binds to Rok7B7 in the absence of glucose, Rok7B7 is released from the *xylFGH* promoter, subsequently leading to the repression of the *glcP1* gene, which codes for glucose permease, although it is not known if this latter effect is direct or indirect. In addition, Rok7B7 was found to both supress and enhance the production of certain antibiotics and regulated a couple of genes involved in primary metabolism (Lu et al., 2020; Ruiz-Villafán et al., 2022). In *S. colicolor, rok7B7* deletion mutants displayed reduction in agarase production, which stands as a marker for CCR, and an increase in *glcP1* and *glkA* expression. Moreover, the mutants showed delayed development and an altered pattern of antibiotic production (Świątek et al., 2013). All this data indicates a global regulatory role for Rok7B7 which integrates the carbon source availability information to life cycle progression.

b) CCR, development and antibiotic production

As mentioned earlier, nutrient availability and morphological development are correlated events in streptomycetes. When the vegetative mycelium undergoes programmed cell death (PCD) is also the moment antibiotic production is initiated. This is likely a defensive measure, protecting the nutrients released during PCD against competitors. Key for triggering development are *bld* genes, with *bldB* mutants incapable of producing antibiotics and aerial hyphae while also displaying insensitivity to glucose repression (van Bergeijk et al., 2020). Moreover, deletion of *glkA* restored antibiotic production in a bldA-null background. (Van Wezel & McDowall, 2011). These observations tap into the role of CCR and Glk in furthering the life cycle of these species.

The sensory system which ties the nutrient availability to life cycle progression in *S. coelicolor r*evolves around *N*-acetylglucosamine (GlcNAc) and DasR, one of the best studied regulators in *S. coelicolor*. GlcNAc enters the composition of chitin and murein and it is a preferred carbon and nitrogen source in streptomycetes. GlcNAc is obtained by releasing chitinases which are used to hydrolyze chitin (Urem et al., 2016). When nutrients are abundant, the GlcNAc presence is interpreted as a result of chitinase activity, indicates available resources and consequently fosters growth, whereas when nutrients are scarce, GlcNAc is a marker of PCD and triggers development and antibiotic production (Świątek et al., 2012; van Bergeijk et al., 2020). In the latter case, GlcNAc is taken up via PTS, which is in accordance with *E. coli* and *B. subtilis* where GlcNAc is internalized via a EII

GlcNAc-specific complex belonging to PTS (Bertram et al., 2011; Nothaft et al., 2010; Plumbridge & Kolb, 1991).

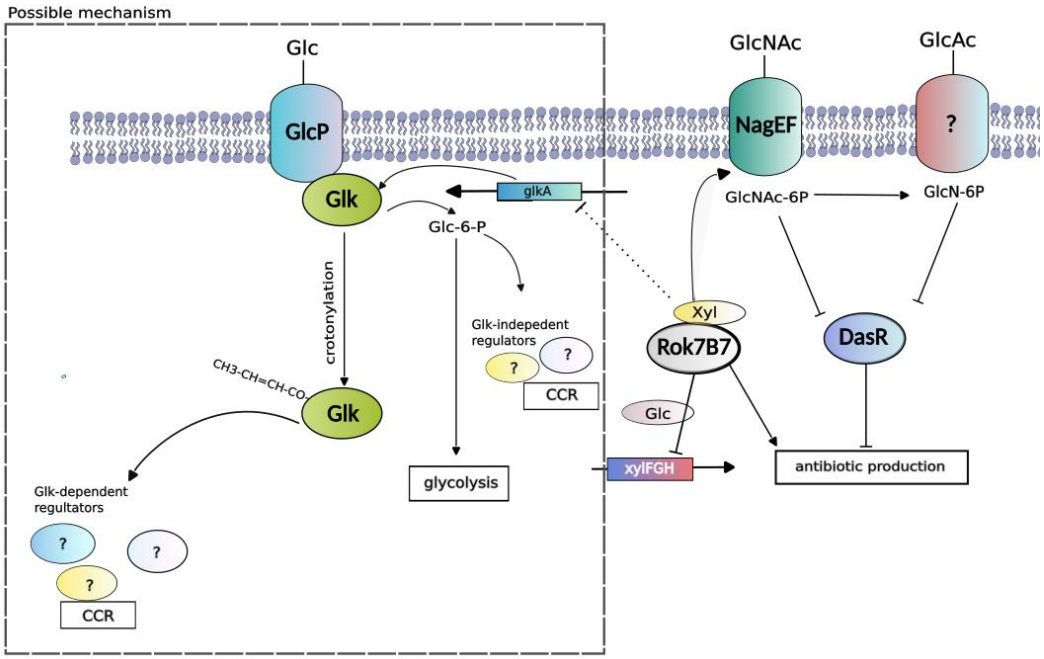

Figure 4. Possible CCR mechanism displaying the interconnectedness with other regulatory networks. The model combines data from different *Streptomyces* species. Based on this model, there are two mechanisms of CCR, Glk-dependent and Glk-independent. Likely, Glk-dependent signal is carried with the help of other regulators. Rok7B7 is known to bind glucose in a Glk-independent manner. When Rok7B7 binds glucose, it represses the *xylFGH* operon, thus exhibiting catabolite repression on xylose uptake. When glucose becomes scarce, Rok7B7 is free from the *xylFGH* operon. In Rok7B7's absence, the genes for Glk and GlcP are unregulated, therefore it must act as a repressor, directly or indirectly (dotted lines). Rok7B7 also induces or repress antibiotics in a life-cycle dependent manner. The illustration is indicative of the late life cycle stages. Rok7b7 is indirectly inhibiting DasR through stimulating the transport of GlcNAc.

The molecular mechanisms which tie CCR to secondary metabolite production are not elucidated but it is believed this process is connected to the regulatory networks of DasR, Rok7B7 and AtrA. DasR is a GntR-family repressor which plays a central role in development and inhibition of antibiotic production in *S. coelicolor.* During internalization, GlcNAc is phosphorylated to GlcNAc-6P. GlcNAc-6P can then be deacetylated to GlcAc-6P and then deaminated to fructose-6-P, thus linking the carbon and amino-sugar metabolisms. GlcAc can also be taken up independently by unknown transporter. GlcAc-6P and GlcNAc-6P in turn inhibit DasR, relieving the repression on antibiotic production. AtrA is indirectly antagonizing the activity of DasR by stimulating the uptake of GlcNAc transport and additionally exhibiting positive regulation on actinorhodin production. Likewise, RokB7 stimulates the GlcNAc transport, thus sharing a similar role (**Figure 4**) (Van Wezel & McDowall, 2011; Urem et al., 2016).

The CCR model in streptomyces spp. shows that this process encompasses more than just the preferential use of carbon-sources but is also fundamental for assessing the environment, making appropriate physiological decisions and coordinates the secondary metabolism.

3. DISCUSSIONS AND CONCLUSIONS

Microorganisms need to adapt to varying resources in their environment and make appropriate decisions. Prioritizing the consumption of certain carbon sources over others is such a strategy. Various studies using quantitative models in *E. coli* argue that CCR favors fitness and growth and optimizes proteome resources allocation (Towbin et al., 2017; Wang et al., 2019). These models describe how *E. coli* is an optimal bioenergetic machine, but it is not clear why these microorganisms evolved to perfect this strategy, as there could be multiple solutions to a problem. *Mycobacterium tuberculosis*, for instance adopts a co-utilization of carbon sources strategy, but this can be justified by its relatively uncompetitive and resources-scarce environment (De Carvalho et al., 2010). It is probably through the lenses of microbial ecology that the constraints on the metabolic choices can be understood. For example, fast-growing bacteria are not necessarily the most competitive in their environment. Instead, there is a tradeoff between rapid growth and adjusting quickly to a new carbon-source (shorter lag-phase during diauxic growth). The tradeoff comes from upregulating genes necessary for the uptake of secondary carbon sources or anticipating nutrient scarcity before the actual primary nutrient depletion had occurred. This preparation is done at the expense of growth. This tradeoff was shown to exist in *E. coli, B. subtilis and Saccharomyces cerevisiae* (J. Wang et al., 2015 ; Basan et al., 2020). Moreover, in a co-culture model it was shown that adopting this tradeoff can induce co-existence of two species instead of mutual exclusion (Bloxham et al., 2022).

The carbon source preference can be understood as hierarchy of repression potency, with the most preferred carbon-source exhibiting the most repression over the secondary sources. This phenomenon is most studied in *E. coli*, where PTS sources occupy the upmost positions in the preference spectrum. However, glucose repression over fructose was shown to be incomplete in these bacteria, showing that the most preferred source does not necessarily have to be completely dominant *(Wang et al., 2019)*. Non-PTS carbon sources can also exhibit inhibition over the uptake of other substrates but to this day the molecular mechanisms remain unknown, although it has been shown repeatedly that this inhibition involves controlling cAMP-Crp levels (Ammar et al., 2018; Hogema et al., 1998). However, if cAMP-Crp levels constitute a global untargeted response, how can one carbon source skip its own repression? It was shown that Crp-cAMP displays differential activation of catabolic/ sugar-specific promoters which suggests the hierarchy of dominant sugars is encoded in the affinity of Crp for its promoters and therefore these promoters compete for their activation (Aidelberg et al., 2014). *B. subtilis* and *P. aeruginosa* also displayed the existence of a sugar preference hierarchy, but less is known about how it is established at the molecular level (Singh et al., 2008; McGill et al., 2021). Finally, the hieracy

between PTS-sugars has not been resolved as of yet but it was shown in *E. coli* that glucose repression over mannitol is a consequence of HPr negatively interacting with the mannitol regulator operon during the transport of glucose (Choe et al., 2017).

Most microorganisms adopt CCR, although the specific molecular mechanisms can vary significantly. Out of all the organisms analyzed in this thesis, Firmicutes and Enterobacteriaceae, specifically *B. subtilis* and *E. coli,* exhibit the highest degree of similarity in their CCR strategy, particularly in how the PTS plays a central role in influencing a global regulator which controls the expression of catabolic genes. The PTS system couples sugar internalization and phosphorylation with glycolysis through PEP, which provides the phosphoryl group for the subsequent phosphorylation cascades. In *E. coli*, the PEP to pyruvate ratio was shown to correlate with the phosphorylation state of EIIA, thus information about the carbon influx might be integrated with later regulatory decisions (Hogema et al., 1998). This could constitute one of the advantages of coupling PTS to the CCR mechanisms. However, it is not exactly known why the PTS transporters were chosen for implementing the CCR mechanisms in so many evolutionary-distant species.

In pseudomonads, the CCR is implemented at the post-transcriptional level through directly inhibiting mRNA translation (Rojo, 2010). In a quantitative study in *E. coli*, this type of regulation, especially in the case of sRNAs, was associated with quicker phenotypic responses compared to what can be achieved by transcription factors (Mehta et al., 2008). One theory suggests that Pseudomonas spp.'s strategy is to uptake the available substrates as fast as possible and thus, CCR evolved more as a means to overcome the accumulation of toxic intermediates and maintain a metabolic balance (Y. Liu et al., 2017). In contrast, *E. coli* can overtake the competition either by outgrowing it or by being able to consume less *popular* sugars (Conway & Cohen, 2015). As such, in *E. coli* CCR appears as a strategy which aids the uptake of the most efficient substrate which can sustain fast growth. In other words, it could be that pseudomonads have faster responses to environmental/nutrient changes, helped by a CCR strategy based on post-transcriptional modifications as opposed to rapid biomass building. This view perhaps explains why pseudomonads do not tend to maximize for quick growth and were shown to display short diauxic lags (Bloxham et al., 2022).

In *Streptomyces* the CCR mechanisms are less understood compared to the other bacterial groups analyzed in this thesis. While the picture of CCR in these species is still under investigation, the current knowledge seems to indicate that there is no global response to the presence of glucose that is predominately implemented by a single actor in the same way CcpA or Crp carry the repression in *E. coli/ B. subtilis*. Rather, there could be multiple regulators which enact the repression on the secondary carbon sources. If this is the case, why would this division of labor be preferred over a more all-in-one global regulator approach? One possible explanation could come from the fact that these bacteria have a complex lifestyle and consequently more information about the environment has to be integrated before certain regulatory choices can be made. For example, the nutrient availability status has to be correctly assessed before initiating the process of PCD and furthering the life cycle. In theory, a hierarchical preference to carbon sources could aid these bacteria make a correct assessment about their habitat. If more favorable carbon sources completely repress the least preferred ones, then the intake of the least preferred source is a signal for the absence of favorable substrates and would indicate a suitable time to trigger development. For example, xylose could be such an unfavorable carbon-source

in *S. colicolor*. D-xylose enters the composition of lignocellulose which is abundant in soil (Domingues et al., 2021). In such a case, Rok7B7 could be activated by the presence of xylose and aid the process of antibiotic production and development.

CCR is understood as the process known for suppressing the uptake and metabolism of secondary carbon sources. However, this process is intertwined with various other aspects of cellular life to the extent that it becomes challenging to discern where the boundaries of CCR lie. CCR decisions are closely linked with anabolic choices, as exemplified in the *E. coli* and *B. subtilis* sections. Crp and CcpA play important roles in regulating anabolic processes, albeit indirectly in the case of Crp and directly through gene regulation for CcpA (Doucette et al., 2011; Fujita, 2009). Furthermore, information concerning anabolic capabilities can serve as signals to directly inhibit CCR, as observed in *E. coli* where the accumulation of ketoacids hinders sugar uptake through the PTS (Doucette et al., 2011). Additionally, global CCR systems have been found to modulate biofilm formation, mobility, and virulence in various bacteria (C. Liu et al., 2020; Seidl et al., 2008; Stella et al., 2008).

Since Monod's discovery of diauxic growth, substantial efforts have been invested in unraveling the intricate molecular networks governing the CCR process. In well-studied model organisms like *B. subtilis* and *E. coli*, details require further research, while in other groups, the molecular aspects are only beginning to be understood. For instance, even today, there is no precise quantitative answer to the question of what factors render one carbon source preferable than another. Perhaps exploring the dynamics of the environment and interactions between organisms could help shed light on the actual constraints under which microorganisms have evolved and perfected. Fortunately, systems biology approaches can provide valuable answers to these questions.

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