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Invited Mini Review

Rewiring carbon catabolite repression for microbial cell factory

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Carbon catabolite repression (CCR) is a key regulatory system found in most microorganisms that ensures preferential utilization of energy-efficient carbon sources. CCR helps microorganisms obtain a proper balance between their metabolic capacity and the maximum sugar uptake capability. It also constrains the deregulated utilization of a preferred cognate substrate, enabling microorganisms to survive and dominate in natural environments. On the other side of the same coin lies the tenacious bottleneck in microbial production of bioproducts that employs a combination of carbon sources in varied proportion, such as lignocellulose-derived sugar mixtures. Preferential sugar uptake combined with the transcriptional and/or enzymatic exclusion of less preferred sugars turns out one of the major barriers in increasing the yield and productivity of fermentation process. Accumulation of the unused substrate also complicates the downstream processes used to extract the desired product. To overcome this difficulty and to develop tailor-made strains for specific metabolic engineering goals, quantitative and systemic understanding of the molecular interaction map behind CCR is a prerequisite. Here we comparatively review the universal and strain-specific features of CCR circuitry and discuss the recent efforts in developing synthetic cell factories devoid of CCR particularly for lignocellulose-based biorefinery. [BMB reports 2012; 45(2): 59-70]

INTRODUCTION

Microbial cell factory is gaining unprecedented momentum as the metabolic engineering has been more and more aided by "rationally" designed biological parts or pathways. Recombinant DNA technology makes synthetic microbes increasingly find their use in replacing the petrochemical processes for the production of drugs, fuels, and other value-added chemicals from renewable sources (1-3). Despite recent advances in the rising field of synthetic biology, however, the overwhelming complexity of living cells remains a formidable challenge in mapping desired traits onto a genome of a host cell. This is particularly so because even a simplest and seemingly intuitive trial of rewiring in the subcellular networks is unavoidably accompanied by the conflict of "interest" among nontrivial fitness effects. At the same time, microorganisms are amazingly good at adapting themselves to new environments, and it is often the case that the rationally developed strains would be swept away, with microbes restoring its optimal physiology for survival but not for economic viability. Here is the point where the rational construction of the production strains requires the system-level understanding of host cell physiology in light of evolution (4).

Microorganisms have limited foraging capability and so have to survive constantly fluctuating environments, where optimized uptake and assimilation of nutrients provides crucial fitness benefit. Carbon catabolite repression (CCR) is such a boon to microorganisms for their survival and dominance in ever-changing nutrient conditions. The basic principle underlying CCR is universal in all microbes, that is, the most energy efficient cognate substrate is the most preferred carbon source. This is usually achieved through the inhibition of expression of genes encoding for enzymes involved in the catabolism of carbon sources other than the preferred ones (5). Nonetheless, each group of bacteria has evolved its own way of achieving CCR. The molecular machinery behind CCR (Table 1) varies widely across the species, with CCR being enforced and operable at different levels including transcriptional (6), post-transcriptional (7), translational (8) and biochemical regulations (9) which has fascinated scientists for over half a century.

On the other hand, CCR continues to be a major hurdle to be overcome for efficient use of, particularly, the agricultural biomass, lignocellulose (10). Since lignocellulose is a highly recalcitrant substrate comprised mainly of cellulose, hemicellulose, and lignin, which can be broken down into a heterogeneous mixture of fermentable sugars, glucose, xylose and arabinose, CCR would severely affect the yield and productivity of fermentation process (Fig. 1). In fact, CCR persists even when the alternative sugar utilization phenotype is introduced as a constitutively expressing heterologous pathway (11) and the molecular mechanism behind CCR remains a contentious issue. Considering the mass of studies so far conducted in this topic, it comes as a surprise that the textbook wisdom on the major contributor to CCR even in the classical

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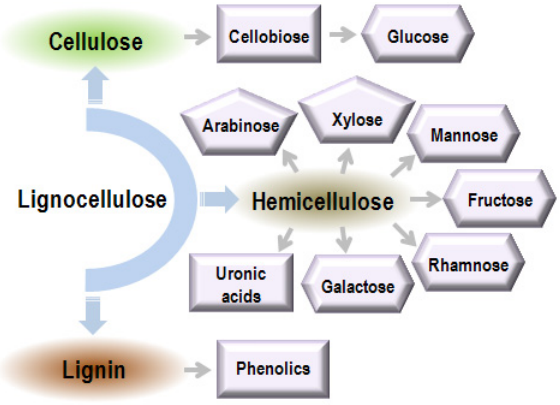

Fig. 1. Schematic representation of different kinds of sugars that could be derived from lignocellulose. Lignocelluloses are heterogeneous substrates with cellulose, hemi-cellulose and lignin as its major components, but the actual composition varies depending on the feedstock used. Further reduction of these individual components would yield a mixture of sugars (cellobiose, glucose, galactose, mannose, xylose and arabinose), phenolics and acids.

lac operon system of *Escherichia coli* is currently under debate (12). Accordingly, to achieve yield-efficient production strains, it is indispensable to scrutinize the molecular interaction network responsible for CCR in quantitative basis. In this review, we highlight the diverse molecular modes of CCR and the strategies employed to overcome the above-mentioned difficulty in industrial biocatalysts.

CARBON CATABOLITE REPRESSION: UNIVERSALITY AND SPECIFICITY

CCR as an evolutionary outcome

CCR regulates access to different nutrients in a highly economical manner acting as a first-line cognitive screening instrument in microorganisms. Aside from controlling the uptake and the ensuing metabolism of particular carbon sources, CCR also facilitates the survival of microorganisms by influencing other adaptive behavior, such as virulence, motility, and intracellular communication.

At the same time, CCR helps microorganisms become adjusted at an optimal physiological condition that does not

Table 1. Different modes of action of carbon catabolite repression distributed across microorganisms

	Mode of action	Example
Inducer exclusion	In the presence of a preferred substrate, the inducer for secondary carbon source is excluded from the cell	*lac* operon of *E. coli*
Anti-induction	Inducer of cognate substrate acts as a repressor of secondary carbon source and hence prevent its induction	Arabinose bound AraC acts as an inducer of arabinose operon and repressor of xylose operon
Induction prevention	Selective inhibition of the first step of secondary carbon metabolic pathway leads to the prevention of further induction of the downstream genes	sRNA Spot 42 mediated regulation of galactose metabolism
Catabolite inactivation	Preferred carbon source acts as a feedback inhibitor of the key metabolic enzyme of secondary carbon source	Fructokinase enzyme is being inhibited by glucose in *Zymomonas*

Table 2. List of organisms and their most preferred and less preferred carbon sources. As indicated in the table most organisms prefer glucose to other sugars. However, reverse CCR also exists among different groups of microbes.

Organism	Preferred carbon source	Less preferred carbon source	Secondary hierarchy	Ref.
E. coli	glucose, fructose, mannose, mannitol	arabinose, xylose, rhamnose, galactose, glycerol	arabinose > xylose	(32, 90, 91)
Z. mobilis	glucose	fructose, xylose, arabinose (recombinant strains)		(79, 92, 93)
B. subtilis	glucose, fructose, mannitol, sucrose, salicin	malate, xylose, arabinose, sorbitol, maltose, glycerol	sorbitol > xylose	(34, 94)
S. cerevisiae	glucose, fructose	galactose, maltose		(42) (55, 95)
C. acetobuylicum	glucose	arabinose, xylose	arabinose > xylose	(96-98)
L. monocytogenes	glucose, fructose, cellobiose	maltose, mannose, arabitol	glucose > cellobiose	(33)
S. thermophilus	lactose, sucrose	glucose		(19, 99, 100)
B. longum	lactose, glucose, xylose	sucrose, fructose, ribose, galactose	lactose > glucose	
P. aeruginosa	organic acids (succinate, acetate, pyruvate), amino acids	glucose, fructose, mannitol, glycerol, gluconate	glucose > mannitol	(101) (102)
C. thermocellum	cellobiose	glucose		

overtax the metabolic capacities of the cell and thus preventing the cell from being overfed with the cognate carbon sources (13). In most instances, disruption of CCR is found to be detrimental to the growth of bacteria. A recent analysis shows that CCR could be a selection pressure that would help optimize the total macromolecular content of a cell, which would in turn enable a faster growth in unpredictable conditions (14).

CCR has been sometimes called as glucose effect - the inhibitory effect of glucose on the induction of catabolite enzymes required for other sugar utilization (15). This is partly because many microorganisms favor glucose as a primary carbon source as well as because the first example of CCR recognized in 1942 was glucose effect in *E. coli* (16). However, the preferred cognate substrate differs among different organisms (Table 2). For example, *E. coli* prefers monosaccharide glucose to disaccharide lactose whereas *Clostridium thermocellum* prefers cellodextrins to glucose and cellobiose (17). Considering the environmental diversity, it is quite reasonable that some microorganisms have evolved to use nutrients other than glucose as their favorites, or even not to absolutely need CCR.

For some bacteria such as *Streptococcus thermophilus*, *Bifidobacterium longum*, and *Pseudomonas aeruginosa*, glucose is only a secondary carbon source, which is referred to as reverse CCR (18-20). In *Corynebacterium glutamicum*, the co-fermentation of glucose and other carbon sources occurs albeit being stringently regulated (21, 22). Some pathogens such as *Chlamydia trachomatis* and *Mycoplasma pneumoniae*, which are highly adapted to nutrient-rich host environments, seem to lack CCR (23, 24).

Conserved signaling pathways for CCR

CCR has been most intensively studied in the Gram-negative bacterium, *E. coli* and Gram-positive bacterium, *Bacillus subtilis*. Despite the differences in detailed signaling pathways and regulatory agents (25), CCR in both the species is tightly coupled with the phosphotransferase system (PTS) that is involved in shuttling of phosphoryl group among the glycolytic intermediate phosphoenolpyruvate (PEP), the PTS proteins, and the transported sugar (Fig. 2).

One salient feature of CCR circuitry shared by most of the microorganisms is the multitude of regulatory interactions interlinked with the central carbon metabolism by way of the global regulator proteins, such as CRP or CcpA. Depending on the carbon sources, 5 to 10% of all the bacterial genes are known to be subject to CCR (26). These genome-wide effects are accompanied by the substrate-specific induction system, where the catabolic genes are activated or derepressed in response to the presence of a specific carbon source. Thus the orchestrated interplay between global and substrate-specific regulation can be reduced to a simple Boolean logic of "NOT glucose AND lactose" in the classical *lac* operon system, and this combinatorial nature constitutes another salient feature of CCR. However, the detailed regulatory measures of global and substrate-specific regulation are oftentimes mediated by the competing effects, seemingly working toward opposite ends. Accordingly, critical to understanding this innate complex system is telling the relevant factors from "artifacts", and this is why the quantitative and systemic standpoint cannot be overemphasized in the rational approach to the rewiring of biological networks.

PTS helps manage the uptake of a variety of sugars and sugar alcohols across the cell membrane by phosphorylating those substrates, using PEP as the phosphate donor and energy provider (27). The PTS is composed of three distinct enzymes: Enzyme 1 (EI), histidine-containing phosphocarrier protein (HPr) and Enzyme 2 (EII). In the presence of PTS-dependent

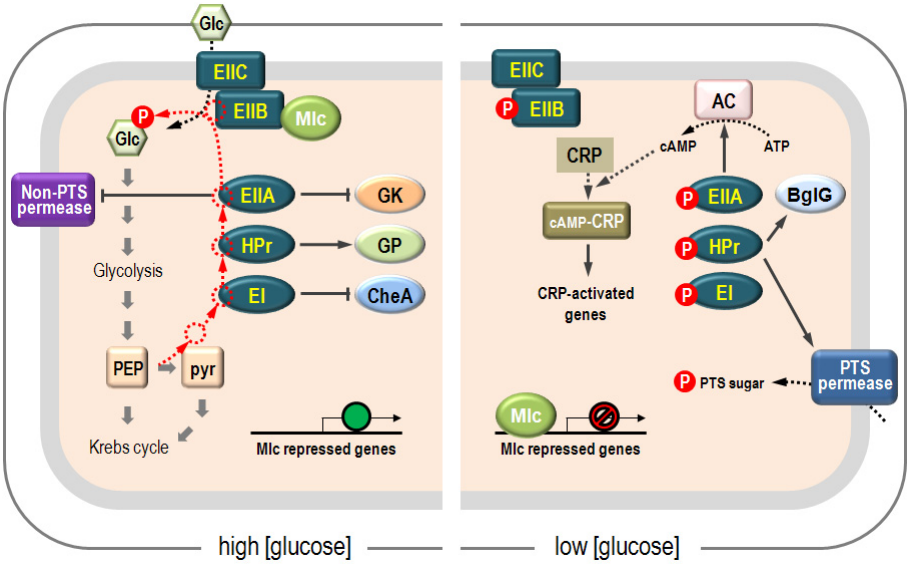

Fig. 2. Signaling pathways for carbon catabolite repression system in *E. coli*. Real lines represent enzymatic or transcriptional regulation with pointed ends for activation and blunt ends for inhibition. Dotted lines denote the transport or substrate binding. In glucose-rich media, or in case of high glycolytic flux, the phosphorylation level of PTS proteins is low, leading to the repression of catabolic genes for alternative carbon sources. When glucose is depleted from the media, the global regulator CRP is activated via the enhanced AC activity and the Mlc repressor protein is relieved from dephosphorylated EIIB to affect the expression of downstream genes. GK, glycerol kinase; GP, glycogen phosphorylase. See the main text for the other symbols.

sugars such as glucose, a phosphoryl group is transferred from PEP to the periplasmic/extracellular sugar via EI, His residue in HPr, and A, B and C domains of EII (EIIA, EIIB, and EIIC).

The phosphorylation state of PTS proteins is determined by two factors: PTS transport activity, which is dependent on the availability of PTS substrates, and the [PEP] to [pyruvate] ratio, which reflects metabolic flux through glycolysis (28). Thus, if there is abundant amount of sugars around the cell (plentiful acceptors of phosphoryl groups), or if the [PEP] to [pyruvate] ratio is low due to active glycolysis (scarce donors of phosphoryl groups), then the PTS proteins would be found most of the time dephosphorylated (Fig. 2). The latter actually accounts for the reduced phosphorylation of EIIA, and hence CCR in *E. coli*, caused by non-PTS sugars (29).

The global regulation of CCR comes with the global transcription factors such as CRP (*E. coli*) and CcpA (*B. subtilis*) whose activity is, respectively, controlled by the phosphorylation level of EIIA and HPr, which is again modulated by PTS activity. Consequently, the phosphorylation status of EIIA or HPr constitutes a master regulator that bridges CCR with PTS.

DIVERSE MODES OF ACTION OF CCR

CCR mediated by inhibition of transcriptional activation

In *E. coli*, when glucose supply is sufficient, dephosphorylated EIIA prevents the uptake of less attractive carbon sources by a mechanism called inducer exclusion. For example, uptake of lactose is necessary to form allolactose, the inducer of *lac* operon, which enables *lac* operon to be expressed by inhibiting the *lac* repressor. Dephosphorylated EIIA inhibits the formation of this inducer by binding and inactivating LacY, the lactose transporter (30). Dephosphorylated EIIB also mediates CCR by inhibiting Mlc, a transcriptional repressor for the genes of glucose metabolism (31). On the other hand, when the glucose supply is depleted, it leads to the increase in the phosphorylation level of PTS. Highly phosphorylated EIIA activates the adenylate cyclase, which converts adenosine-5′-triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP then binds to the global regulator, CRP (cAMP Receptor Protein), and cAMP-CRP complex activates the promoters of many catabolic operons, such as *lac*, by recruiting RNA polymerase (6, 32).

CCR mediated by transcriptional repression

B. subtilis also utilizes the PTS system to transport sugars but pursues a different strategy of CCR partly because *B. subtilis* and low GC content Gram-positive bacteria neither synthesize cytosolic cAMP nor possess CRP-like proteins (33). Instead, they use a transcription repressor in order to achieve CCR. In the presence of high intracellular level of ATP (and a low level of inorganic phosphate) and intermediates of glycolysis (such as glucose-6-phosphate and fructose-6-phosphate), HPr of the PTS component is phosphorylated at the serine residue by HPr kinase/phosphatase. Ser-phosphorylated HPr (p-Ser-HPr) is kinetically stable and binds to CcpA, a catabolite control protein A. The p-Ser-HPr-CcpA complex interacts with the regulatory sequence named catabolite responsive elements, *cre*, present in the promoter region of CCR responsive genes. p-Ser-HPr-CcpA complex can act either as a transcriptional repressor or activator depending on the orientation of *cre* element with respect to the promoter (34).

Signal transduction through duplicate PTS domain

CcpA-independent catabolite repression system of *B. subtilis* is mediated by the mechanism of induction prevention, by which transcription factors or RNA-binding anti-termination proteins of the operons for less preferred PTS substrates is inhibited (35). The transcription factors controlled by induction prevention often contain duplicated PTS-regulatory domains (PRDs), which can be phosphorylated by the components of PTS and provide information on the glucose availability. For instance, LicT is an anti-terminator, which promotes the expression of *bglPH* operon for the metabolism of β-glucoside, a less preferred carbon source than glucose. LicT has two PRD domains, which are phosphorylated by the components of PTS (PRD1 by β-glucoside specific EII, and PRD2 by p-His-HPr, respectively). The activity of LicT depends on the phosphorylation status of its PRD domain, which, in turn, is determined by the availability and composition of the surrounding carbon sources (36, 37).

Role of sRNAs in CCR

cAMP-CRP complex not only mediates CCR of protein-coding genes, but also of non-coding small regulatory RNAs (sRNA) such as Spot 42, CyaR, and SgrS (7). For example, CRP represses the *spf* gene encoding the base-pairing sRNA, Spot 42 that is abundant in the presence of glucose. Spot 42 directly suppresses the expression of the galactose catabolic (*gal*) operon by base paring with *galK* mRNA (38). While sRNA Spot 42 mediates CCR in *E. coli*, sRNA, CrcZ helps in the reversal of CCR in *Pseudomonas spp.* (39).

CCR mediated by biochemical regulation

While CCR observed in *E. coli* and *B. subtilis* is mediated by PTS system that is dedicated for glucose uptake, microorganisms such as *Saccharomyces cerevisiae* and *Zymomonas mobilis* employ a single transporter for the uptake of glucose and other secondary carbon source. Hence, CCR observed in these organisms are not enforced merely through the transcription regulation of other catabolic genes. *S. cerevisiae* has a strong preference for glucose as its carbon source (40). Glucose transporter repression has been known to play an important role in CCR in *S. cerevisiae* (41). This pathway is responsible for the suppression of genes involved in the metabolism of alternative carbon sources (e.g. galactose, sucrose and maltose), respiration, and gluconeogenesis when glucose is available (42). The major components of this pathway are the transcription repressor, Mig1, and the protein kinase, Snf1;

Snf1 inhibits Mig1 by phosphorylating it. When high level of glucose is supplied, glucose might be transported into the cell by Hxt1, one of the 18 glucose transporters of *S. cerevisiae*, which has a lower affinity but a higher transport capacity for glucose. Intracellular glucose is then converted to glucose 6-phosphate primarily by Hxk2, which would be further metabolized to yield energy. Since Snf1 is inactivated at a high energy level condition, Mig1 can keep inhibiting genes for the secondary carbon sources when glucose is available (43).

Role of CCR machinery beyond sugar metabolism

CCR has been reported to modulate virulence of some pathogens (44). It makes sense because, in general, the host cell cytosol is not simply a nutrient-rich culture medium but, rather, is a complex environment that requires specific physiological adaptation by the pathogen. In *Listeria monocytogenes*, virulence genes that are essential for its intracellular life cycle is under the control of a single transcriptional activator, PrfA, which belongs to the cAMP receptor protein/fumarate and nitrate reduction regulator (CRP/FNR) family of transcriptional activators (45). PrfA activity seems to be modulated by the phosphorylation state of the major PTS transporter for glucose, mannose, and cellobiose. The dephosphorylated state of EIIA component, which is predominant during active uptake of the PTS dependent substrates, correlates with low PrfA activity and vice versa (46). Second, in *E. coli*, dephosphorylated EI, at a high glucose level, inhibits the chemotaxis protein, CheA, changing the motility pattern of the cell in such a way that it is able to approach the source of food (47). Not only that, but the cAMP-independent catabolite repression control protein Crc of *P. aeruginosa* is necessary for the formation of biofilm (48).

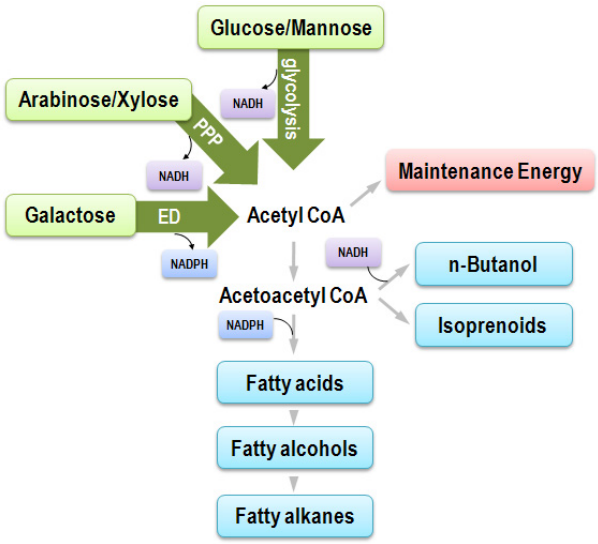

Fig. 3. Enhancing the metabolic capacity of industrial biocatalyst by disruption of CCR. Elimination of CCR would enable a cell to operate all metabolic pathways simultaneously resulting in the surplus supply of all intermediates needed for bioproduct formation. For example, NAD(P)H and acetyl-coA are the two important precursors needed for the production of advanced biofuels (like fatty acids, n-butanol). Simultaneous utilization of many substrates would therefore ensure the continuous supply of these intermediates for advanced biofuel production. ED, Entner-Doudoroff pathway; PPP, Pentose Phosphate Pathway.

CATABOLITE DEREPRESSION: MOLECULAR CHALLENGE TO LIGNOCELLULOSIC BIOREFINERY

All of the above-discussed examples might reconfirm that CCR is important for the survival of microorganisms and thus CCR remains as a positive selection force in microorganisms in evolution. However, CCR would reduce their ability to perform as an efficient host in lignocellulosic fuel production. Hence, it becomes necessary to develop strategies to overcome CCR without disturbing the evolutionary fitness of the microbes. The highly heterogeneous substrate, lignocellulose is particularly rich in sugars such as cellobiose (dimer of glucose), glucose, xylose, arabinose, galactose and mannose. Glucose (cellobiose) and xylose are the major constituents of hardwood whereas arabinose occupies a significant portion of softwood (49). Biofuels and bio-based chemicals derived from lignocelluloses are promising alternatives that would pacify the threats posed on fossil fuels (50, 51). Development of a strain with a greater flexibility to utilize completely all of the sugars derived from agricultural biomass is one of the major challenges to cellulosic fuel production (50).

Deciphering and eliminating CCR in microbial cell factories have several advantages. First, the yield and productivity of the bioproducts are significantly enhanced as all of the substrates could be utilized completely (10). CCR is the major cause of auto-regulation in the utilization of a cognate substrate. Deregulated uptake of a preferred substrate, exceeding the original metabolic capability of the cell, would lead to the accumulation of metabolic intermediates that in turn would lead to the disturbance of homeostasis. Hence, CCR helps in maintaining the balance by reducing the uptake of a cognate substrate which is disadvantageous in cellulosic fuel production process as it would limit the total amount of carbon source consumed ultimately affecting the productivity (13). Catabolite derepressed strain would thus help enhance the sugar uptake rate. Simultaneous utilization of two substrates metabolized through independent pathways might help in the surplus supply of intermediates needed for bioproduct formation (Fig. 3). Further, one substrate could be used to derive energy for cell growth while the other substrate could be used for the biofuel production, a strategy widely employed in xylitol production (52). Finally, accumulation of unused carbon substrate would provide an additional challenge in the downstream processing to obtain the desired product which would not be the case in catabolite derepressed strains (50, 53).

As mentioned above, the hidden molecular mysteries behind CCR is far more complex than previously assumed.

Despite the lack of complete knowledge on CCR, several recombinant and native strains devoid of diauxie and CCR were isolated and have been proven to efficiently co-metabolize glucose or cellobiose with xylose and/or arabinose, major sugars of lignocellulose. Few other strains were shown to co-metabolize a mixture of carbon sources.

RECOMBINANT CELL FACTORIES DEVOID OF CCR

Industrial solventogens such as *E. coli*, *Z. mobilis*, *S. cerevisiae*, *C. acetobutylicum* and *B. subtilis* were proposed to be an efficient host for lignocellulosic fuel production (50, 54). *E. coli*, *C. acetobutylicum* and *B. subtilis* are capable of efficient utilization of the major hexose and pentose sugars of lignocellulose whereas *Z. mobilis* and *S. cerevisiae* are capable of utilizing only the hexose sugar (glucose) (8, 53-56). Several genetic and evolutionary engineering approaches helped achieve efficient pentose utilization in *Z. mobilis* and *S. cerevisiae* (57). Substrate ranges of the above mentioned recombinant strains were also expanded to utilize the disaccharide cellobiose (58-60). Cellobiose is a major breakdown product of cellulose and recombinant strains capable of utilizing cellobiose would help reduce the need for additional saccharifying enzymes used in the hydrolysis of lignocellulose. Even though recombinant strains with a wide substrate range were achieved, CCR remains to be a major bottleneck. The heterologous pathways introduced in recombinant strains for pentose and disaccharide utilization remain pointless because of CCR. Several strategies were employed to overcome CCR some of which are discussed below.

Escherichia coli

E. coli is one of the most promising candidates for biofuel production. While native solventogens like *S. cerevisiae* and *Z. mobilis* are dedicated ethanol producers, *E. coli* had been extensively engineered to produce a wide range of solvents like free fatty acids and short chain alcohols (61). With its wide substrate range and extensive genetic tools for easy manipulation, *E. coli*, remains to be one of the unassailable cell factories. Its natural ability to utilize a wide range of carbon sources implies that its CCR system would be more complex to be deciphered. For example, xylose metabolism was completely inhibited when glucose concentration exceeds 40% of the total sugars (49). Further, there is a strong debate on which of the cAMP-CRP complex or the inducer exclusion by glucose PTS is the major determinant of CCR in *E. coli* (6, 12, 62). Despite these complexities and disputes, several catabolite derepressed strains of *E. coli* have been constructed.

In *E. coli*, glucose is transported into the cells by means of the PTS system. Complete deletion of PTS would ultimately impair growth on glucose. PTS deleted strains (△*ptsGHI*) of *E. coli* are still capable of growth on glucose through the activation of galactose permease, a transporter that can transport glucose non-specifically. Such a PTS^{-} $Glucose^{+}$ strain of *E. coli* was capable of co-metabolizing glucose together with arabinose; however, glucose still exhibited a partial impact on xylose metabolism. With this strain, the rate of sugar utilization was accelerated by a factor of 16% (63). Similarly, an *E. coli* strain carrying an inactive *ptsG*, which encodes glucose tranporter in PTS system, was capable of simultaneous utilization of glucose, arabinose and xylose (53). *ptsG* deleted strains were capable of utilization of 75% of xylose as against the wild type strains that could utilize only 18-20% of xylose in the presence of glucose (64). In addition to the simultaneous utilization of xylose and glucose, *ptsG* deleted strains (△*ptsG*) were capable of co-metabolizing sugars and fatty acids (65, 66).

The △*pts*G strain exhibits a partial relief from CCR favoring simultaneous utilization of glucose and xylose but the co-metabolism is achieved by impairing glucose metabolism, which is disadvantageous. In order to achieve an efficient co-metabolism of xylose and glucose without an impairment of glucose metabolism, the native *crp* gene was replaced with a *crp* mutant (*crp**) that is active irrespective of the cAMP level inside the cell. The extent of glucose impairment was lesser in crp* strains than in △*ptsG* strains. In addition, xylose utilization was improved in the presence of arabinose in *crp** strains but not in △*ptsG* strains (52, 67).

Both the △*ptsG* and *crp** strains result in some degree of growth impairment on glucose. Ultimate output of mere elimination of CCR by impairing glucose uptake would be to increase the portion of xylose in the total sugars consumed. However, elimination of CCR in microbial cell factories would mean an increase in the total sugar uptake by favoring pentose assimilation in addition to glucose metabolism. Hence, a co-culture strategy was employed to simultaneously utilize glucose and xylose. Two substrate-selective strains (one for glucose and another for xylose), when co-cultured exhibited an improved rate of sugar utilization (68). The gene *mgs* encoding methylglyoxal synthase, responsible for the activation of the shunt pathway of glycolysis in glucose excess condition, has also been deleted with a view to eliminating CCR. The resulting strain was capable of co-fermenting xylose and glucose and shows accelerated sugar metabolism in a mixture of glucose, xylose, arabinose, mannose and galactose (69). In another approach, glucose was made to be metabolized in the form of its dimer, cellobiose, by exploiting the native cryptic genes of *E. coli*. Cellobiose metabolizing strains were capable of co-metabolizing cellobiose with xylose, galactose or mannose (70).

Saccharomyces cerevisiae

S. cerevisiae has long been used in the industrial-scale production of alcoholic beverages due to its efficient ethanol-producing capability. Despite its superiority as an industrial host for cellulosic fuel production, *S. cerevisiae* lacks the ability to utilize wide range of substrates. Several strategies have been employed to impose heterologous xylose-, arabinose-, and/or cellobiose-utilizing pathways into *S. cerevisiae*. Although heterologous, these pathways also suffered from CCR. Unlike in *E. coli*, CCR encountered in the heterologous pathways may

not be restricted to the transcriptional level.

In the first step, co-utilization of two completely heterologous pathways (arabinose and xylose) was achieved. Arabinose pathway was introduced into the recombinant xylose fermenting strains of *S. cerevisiae*. Even though xylose was preferred over arabinose, the strain was capable of co-consuming arabinose and xylose indicating that the effect of CCR on completely heterologous pathway is lesser than that on the native pathway (71).

The main reason for CCR observed in the heterologous xylose pathway in *S. cerevisiae* might be due to the lack of dedicated xylose transporter. *S. cerevisiae* has low and high affinity hexose transport systems, which accounts for 18 different transporters (Hxt1-17 and Gal2) that could transport a wide range of substrate including xylose and glucose. Hexose transporters are differentially expressed depending on the concentration of glucose. The specificity of these transporters to facilitate xylose uptake varies as a function of glucose concentration. Of the 18 transporters, Hxt4p, Hxt5p, Hxt7p and Gal2P exhibit a higher specificity towards xylose. *S. cerevisiae* expresses the xylose specific transporters, Hxt4p and Hxt7p, only at the low concentration of glucose. Hence, in the initial stages of fermentation glucose concentration will be relatively high and hence the expression of xylose specific hexose transporters would be inhibited. With time, glucose concentration would reduce leading to the expression of Hxt4p and Hst7p transporters and hence favor xylose uptake. Strong competition between glucose and xylose for a single transporter would also limit xylose uptake leading to CCR (72).

A committed pentose transporter might help circumvent this problem. Hence, glucose/xylose facilitator from *Arabidopsis* was expressed in xylose fermenting *S. cerevisiae*. The heterologous transporter conferred an improved growth on xylose and glucose-xylose co-metabolism. However, glucose still remains to be more preferred carbon source indicating the need for more specific xylose transporter (73). Many monosaccharide transport proteins were screened in order to search for one with a higher affinity towards xylose rather than glucose but with little success (74).

Cellobiose metabolizing strains of *S. cerevisiae* was developed in an attempt to resolve the issue of CCR between glucose and xylose. In the first phase, β-glucosidase (the enzyme that cleaves cellobiose to glucose) was expressed on the cell surface. As mentioned above, glucose concentration is the major determinant of xylose consumption. Hence, providing glucose in the form of cellobiose would limit the glucose concentration outside the cell and would help improve xylose utilization. Thus, expression of β-glucosidase on the cell surface favored efficient co-metabolism of cellobiose and xylose in *S. cerevisiae* (58). Surface display of β-glucosidase would still pose a threat to xylose utilization when considering a large-scale fermentation, as glucose produced is extracellular. In case of large-scale fermentation, glucose obtained from cellobiose would be sufficiently high in concentration, and thus CCR is triggered to block xylose utilization. Hence, a new strain was developed in which β-glucosidase was expressed along with a cellodextrin transporter. With this approach, cellobiose would be cleaved to glucose inside the cell and hence, would pose no threat to xylose utilization even on a large scale. This new strain was capable of co-metabolizing cellobiose together with xylose or galactose (56, 75).

Clostridium acetobutylicum

C. acetobutylicum is one of the most famous industrial hosts capable of producing acetone, ethanol and butanol at a relatively higher titer. It is also capable of utilizing both pentose and hexose sugars present in lignocellulose. Hence, these strains are not exempted from CCR. Similar to *E. coli*, glucose metabolism in *C. acetobutylicum* is mediated by the PTS system. Disruption of the PTS in *C. acetobutylicum* leads to an improved co-utilization of arabinose and xylose without greatly impairing the glucose metabolism. Over-expression of xylose metabolic pathway together with the PTS knockout enhanced the co-metabolism of the three sugars (55). CCR system observed in *C. acetobutylicum* was relatively simple than that observed in *E. coli*.

Zymomonas mobilis

Z. mobilis is one of the efficient ethanol producers, known for its higher productivity with a remarkably higher glucose uptake rate. *Z. mobilis* utilizes Entner-Doudoroff pathway to ferment glucose to ethanol and hence yields only a single mole of ATP per mole of glucose that imposed robust sugar consumption characteristic. Lower maintenance energy is one of the most important traits of an industrial microbe, as it would reduce the non-productive consumption of the substrate. By far, *Z. mobilis* is the only know industrial microbe with lower maintenance energy. Even though deregulated glucose uptake rate is an advantageous feature, the narrow substrate range limits its use in cellulosic biofuel production.

Several recombinant strains capable of utilizing the pentose sugars have been engineered previously. Similar to *S. cerevisiae*, the xylose-utilizing strains of *Z. mobilis* also suffered from CCR presumably due to the reduced kinetics of the indigenous glucose transporter towards xylose (76-78). However, CCR observed in *Z. mobilis* is different from that observed with *S. cerevisiae* as the rate of inhibition of xylose uptake is a function of glucose concentration in the latter but not in the former. Similarly, glucose in its free-form inside the cell (rather than the extracellular glucose) was the major reason for CCR. Several lines of evidence indicate that the slow growth rate of *Z. mobilis* in xylose medium might be responsible for this CCR. It has been established that the energy content of xylose-grown cells were less than that in glucose grown cells (79). Further, xylitol, sugar alcoholic byproduct of xylose metabolism, is a potent inhibitor of cell growth. Xylitol also reduces the activity of xylose isomerase, the first enzyme of xylose metabolism. The energy content of xylose-grown cells might be reduced because of xylitol formation, which would ultimately affect the growth rate. The re-

duced growth rate might have been reflected as a CCR (57).

Bacillus subtilis

As against other industrial microbes, *B. subtilis* has natural protein secretion machinery, an advantageous feature in lignocellulosic fuel production. Several saccharifying enzymes are needed to hydrolyze lignocelluloses to soluble sugars before being fermented by the microbes. Engineering heterologous secretable cellulases is a major challenge with the recombinant microbes as most of them lack native protein secretion machinery. Native protein secretion pathway is a peculiar feature of *B. subtilis* making it superior to other microbes in lignocellulosic fuel production (54). Similar to *E. coli*, *B. subtilis* can utilize a wide range carbon sources like glucose, xylose, cellobiose, xylosides arabinose and mannose. Hence, mechanisms of CCR are more complex in *B. subtilis*. For instance, XylR represses xylose operon of *B. subtilis* in the absence of xylose. Binding of xylose to XylR helps in the inactivation of XylR leading to the transcription of xylose operon. However, glucose and glucose-6-P binds to XylR with a higher affinity than xylose. Unlike xylose, repressive effect of XylR is not relieved with the binding of glucose leading to the continued repression of xylose operon in the presence of glucose (80, 81). AraR regulates arabinose metabolism in a similar manner as XylR (81). Successful diversion of glucose flux from glycolysis to the oxidative branch of the pentose phosphate pathway (PPP) in *B. subtilis* favored the co-metabolism of glucose and other carbon sources like arabinose and xylose that is extensively utilized via non-oxidative branch of PPP (82).

Use of native organisms devoid of CCR for biofuel production

While many engineering attempts were made to develop recombinant strains devoid of CCR, several bacteria with broad substrate range and still devoid of CCR are isolated in nature. *Sulfolobus acidocaldarius*, a hyperthermophilic archeaon, is capable of metabolizing glucose, xylose, arabinose and galactose simultaneously. Another advantage of exploiting this organism in lignocellulosic biofuel production would be because it is a hyperthermophile capable of growth at higher temperature and at lower pH, an ideal condition used in the pre-treatment of lignocellulosic substrates (83). Co-utilization of glucose and xylose is a common phenomenon observed in *Lactobacillus spp*. Lactic acid bacteria are gaining increased importance in the fermentation of lignocellulose to lactic acid. These bacteria do not generally exhibit preference over any sugar and can utilize a mixture of carbohydrates simultaneously (84, 85). Understanding the ability of these strains to grow on a mixed substrate without preference would help in unwinding the molecular mysteries of CCR.

SUMMARY AND OUTLOOK

CCR is an evolved trait that has optimized the microbial carbon utilization in a fluctuating nutritional environment. CCR is not just restricted to mono- and disaccharide utilization but also employed by native cellulolytic organisms in order to control the titer of the different cellulase systems based on the availability of carbon sources (86, 87). A key engineering goal for the lignocellulose-based biorefinery lies in the rational rewiring of molecular networks underlying CCR to achieve yet another optimization for producing value-added chemicals. Understanding and deciphering the regulatory logics of CCR would also pave way for the development of synthetic microbes with minimal genome. This brings about an intervention against the robustness of the evolved biological network, requiring quantitative and system-wide understanding of CCR.

Since Monod's seminal contribution (16), the basic principles of CCR have been well established around the model organisms. In addition, various strategies to work around CCR by inactivating PTS components have shown promising impact on the development of production strains (52, 53, 63-67), some of which showed product-specific improvement. Assembly of modular synthetic parts needed for specific sugar metabolism is a recently emerging engineering solution to the never ending battle against CCR (88, 89). Nevertheless, due to the deep entanglement with the host cell physiology and the global regulatory effects, many fundamental questions remain unanswered. For instance, even in the well-known model organism *E. coli*, there is still a missing component that fills the gap between the protein EIIA and adenylate cyclase activity. In fact, these "missing parts" become a norm rather than an exception if we go beyond *E. coli* or *B. subtilis*. It is the genome-wide systems biology approaches to transcriptome, proteome, and metabolome level that will be of fundamental importance to yield valuable insights into and, thus, rewiring strategies applicable to CCR (26).

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