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Insights into the regulatory mechanisms and application prospects of the transcription factor Cra

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ABSTRACT Cra (catabolite repressor/activator) is a global transcription factor (TF) that plays a pleiotropic role in controlling the transcription of several genes involved in carbon utilization and energy metabolism. Multiple studies have investigated the regulatory mechanism of Cra and its rational use for metabolic regulation, but due to the complexity of its regulation, there remain challenges in the efficient use of Cra. Here, the structure, mechanism of action, and regulatory function of Cra in carbon and nitrogen flow are reviewed. In addition, this paper highlights the application of Cra in metabolic engineering, including the promotion of metabolite biosynthesis, the regulation of stress tolerance and virulence, the use of a Cra-based biosensor, and its coupling with other transcription factors. Finally, the prospects of Cra-related regulatory strategies are discussed. This review provides guidance for the rational design and construction of Cra-based metabolic regulation systems.

KEYWORDS transcription factor Cra, carbon metabolism, metabolic regulation

As important components of gene expression regulatory networks, transcription factors (TFs) play a vital role in transcriptional regulation in organisms. Cra (catabolite repressor/activator) is a global TF involved in carbon flux through the central metabolic pathways of gram-negative bacteria, such as *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas putida*, and *Enterobacter aerogenes* (1–3). Cra was first identified as the transcriptional repressor of the fructose operon (named FruR) (4) but has since been recognized as a multieffect regulator that can simultaneously activate and repress the coexpression of multiple genes involved in major pathways of carbon and energy metabolism (5–7). The interaction of Cra with effectors such as fructose-1-phosphate (F1P) can allosterically regulate the affinity of the Cra protein for target DNA, which leads to weakened or even no activation or repression effects (2, 8).

In addition to target genes, Cra can regulate the expression of genes encoding other TFs (such as *crp*) (9) and subsequently affect the concentration of TF proteins to determine the degree of gene regulation, resulting in indirect regulation of more genes, ultimately leading to a broad regulatory network. This gives Cra a unique advantage in terms of its comprehensive regulatory function. Cra can be modified for different metabolic regulation purposes to exhibit various regulatory properties. For example, it can be used to strengthen the target performance of engineered strains by increasing metabolite synthesis, promoting microorganism growth, and improving environmental tolerance (10–12). It is also possible to construct dynamic regulatory systems using the sensitivity of Cra to the concentrations of certain metabolic substances. In response to changes in the intracellular environment, the flux of metabolic pathways is dynamically regulated to balance the biosynthesis of target metabolites (13, 14).

In recent years, there has been no systematic review of the regulatory mechanism of Cra and its applications in metabolic regulation processes. In this review, the structure, mechanism of action and function of Cra in carbon, and nitrogen regulation

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are highlighted. In addition, the application of Cra in metabolic regulation, including the promotion of metabolite biosynthesis and the regulation of stress tolerance and virulence, as a metabolite-responsive biosensor coupled with other TFs, is presented. Finally, the future directions of Cra-based regulatory strategies are discussed.

STRUCTURAL MECHANISM OF Cra

Structure of Cra

The Cra protein of *E. coli* consists of 334 amino acids and has a tetrameric structure in solution (1). The Cra of *P. putida* is homologous to that of *E. coli*, but the Cra protein of *P. putida* has a dimeric structure (15). Based on its similarity to other transcriptional regulators, Cra is classified into the LacI/GalR family. The Cra monomer is organized into two functional domains (Fig. 1A): (1) the N-terminal domain (NTD), which binds to specific DNA and thereby affects transcription, and (2) the C-terminal domain (CTD), which can bind effector molecules to regulate DNA affinity and activate or inhibit the expression of target genes, thus affecting their function (16–18).

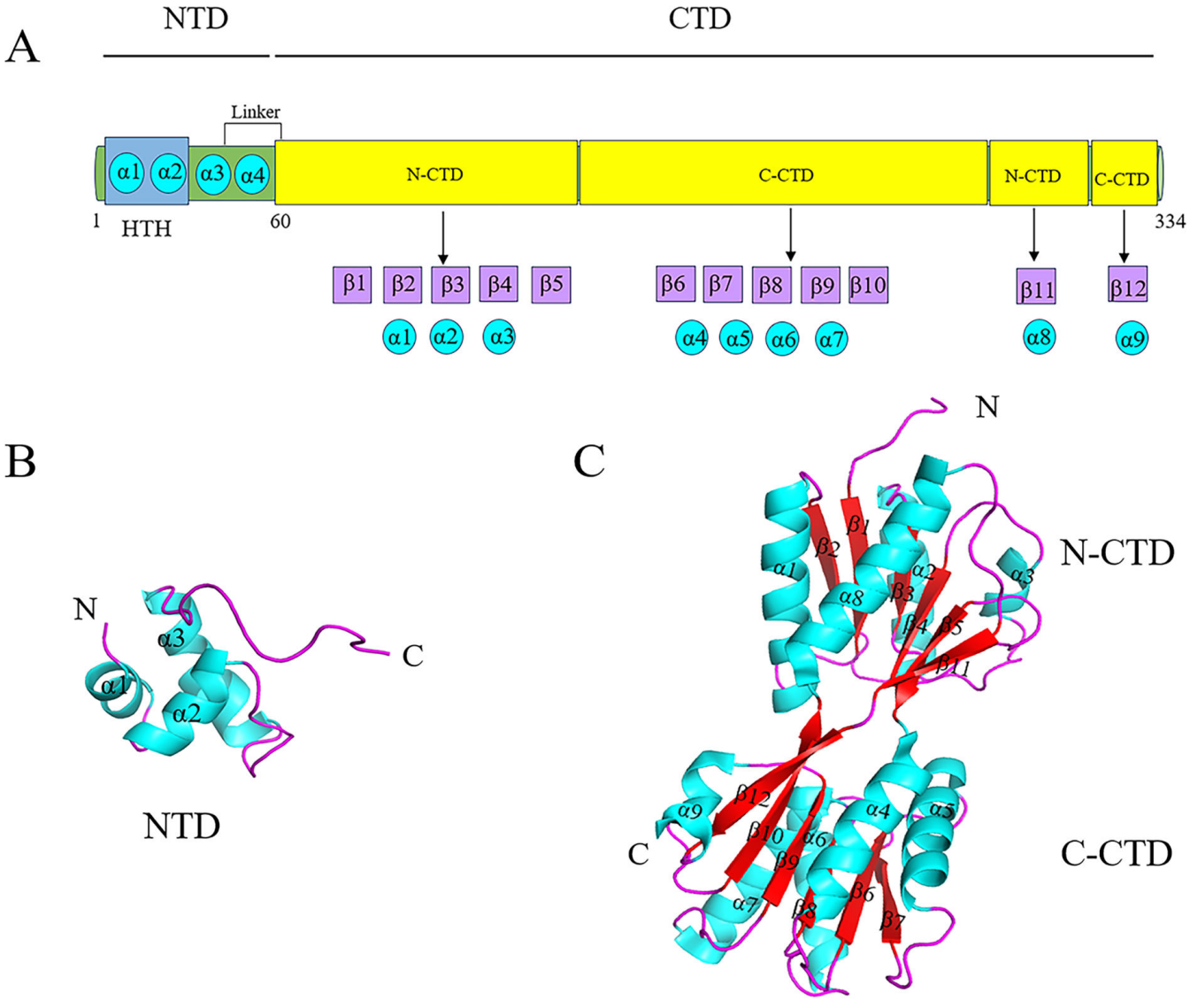

FIG 1 Schematic of the structure of Cra. (A) Structural domains of Cra. (B) Structure of NTD (PDB ID: 1UXD). (C) Structure of CTD (PDB ID: 7DOB). The α-helices are colored blue; the β-sheets are colored red, and the loops are colored purple.

The N-terminal DNA binding domain of Cra was first identified by NMR spectroscopy in *E. coli* (19). Based on the high degree of conservation in the LacI family, the structure of the Cra DNA binding domain shows good similarity to that of proteins in this family (e.g., LacI and PurR) but exhibits differences related to the specificities of the operators. The Cra NTD exhibits a typical helix-turn-helix motif (residues 3–22) stabilized by a third helix (residues 32–45) (Fig. 1A and B). The structure of the three helices is maintained by a hydrophobic core formed by several residues and stabilized by a network of hydrogen bonds (19). In contrast with the three helices, the fourth α-helix (residues 50–58) in the NTD, namely, the "hinge helix," is unfolded. Its formation is induced by repressor oligomerization and complexation with a DNA operator. It has been reported that effector binding causes the conversion of the hinge helix to a loop, which consequently causes the release of DNA from Cra (2). Furthermore, potential residues of the Cra NTD involved in DNA contact and specific operator recognition (such as Leu3, Ser13, Thr15, Thr16, Ser18, Tyr19, and Asn22) were identified. There is still no crystallographic information related to the N-terminal structural domain. The crystal structure of Cra from *E. coli* without the NTD has been reported (PDB ID:2IKS). More recently, the whole Cra structure was determined by crystallography (18). However, it has been observed in old purified *E. coli* Cra protein or during the crystallization of Cra-DNA complexes that Cra spontaneously cleaves at Asn-50 (N50), resulting in the removal of the N-terminal structural domain. In the presence of divalent metal ions, the spontaneous cleavage rate increases (18). Moreover, *in vitro* transcription experiments showed that the Cra NTD is in a functional conformation state and that its DNA binding activity is retained. However, compared with the NTD alone, the full-length Cra binds strongly to the operator DNA (18).

In studies of the *E. coli* LacI protein, the 18-amino-acid region (residues 45–62) between the N-terminal DNA binding domain and the C-terminal regulatory domain is defined as a linker (20, 21). Based on the similarity of LacI family proteins, the structure and function of the linker of Cra can be deduced by analogy with that of LacI. The linker sequence can be subdivided into an N-linker (residues 45–49), a central hinge helix (residues 50–58), and a C-linker (residues 59–62) (20). As common structural features of LacI family proteins, the hinge helix forms direct contact with DNA and with the hinge helix of the other monomer in the dimer. In addition, the N-linkers, hinge helix, and C-linkers all interact with the regulatory domain (22). The linker region is considered to potentially make long-range functional contributions. Bioinformatics and mutational analyses suggest that some sites in linker regions contribute to the specificity of DNA binding (20, 23), and some sites may be important for allostery (22, 24). Variation in the linker can lead to unique functions.

The overall fold of the C-terminal regulatory domain of Cra in *E. coli* is similar to that of other members of the LacI protein families (1). The Cra CTD can be subdivided into two similar subdomains: N-CTD (residues 60–161 and 291–321) and C-CTD (residues 162–290 and 322–332) (Fig. 1A and C). N-CTD consists of parallel chains (β1–5, β11) and is surrounded by helices (α1–3, α8). Likewise, C-CTD consists of parallel chains (β6–10, β12) and is surrounded by helices (α4–7, α9). The two subdomains are cross-connected by three discontinuous loops. The regulatory domain contains effector binding sites. A groove is formed between the two subdomains of the CTD to bind the effector molecules (18). Structural changes in the CTD can be illuminated by comparison of the apo- and ligand-bound structures, and changes in the CTD seem to determine the direction of the allosteric response for the whole protein (25).

General mechanism of Cra

A mechanistic study of Cra in *E. coli* revealed the direct regulation of several metabolic genes by binding the tetrameric protein to operators (26, 27). Cra has been shown to bind a 14-bp-long box with an imperfect palindromic sequence, GCTGAAACGTTTCA (26, 28). Based on this, 164 binding sites were identified using the genomic SELEX technique (26, 28). Promoter-*lacZ* fusion experiments revealed that a total of 178 promoters were

under the control of Cra. In addition, 39 binding sites and 97 regulon genes that are regulated by Cra were identified *in vivo* by using ChIP-exo and RNA-seq (6). Notably, the location of the binding site determines whether Cra mediates the activation or repression of target gene expression. Binding of Cra upstream from a promoter activates gene transcription, whereas downstream binding causes gene repression (Table 1). For example, Cra bound to the region upstream of the *icd* gene promoter and thereby activated the transcription of the *icd* gene in *E. coli* (29). Under anaerobic and aerobic conditions, inactivation of *cra* (encoding Cra) leads to increased expression of the *adhE* gene, indicating that Cra acts as a repressor of *adhE*. This finding was consistent with the DNA sequencing results showing that Cra binds downstream from the *adhE* promoter (30, 31). Transcription of the fructose operon was repressed when Cra was bound to the operator, which was located downstream of the RNA polymerase binding site (3). In some cases, the correlation has been found to not hold true. The expression of the *glk* gene was repressed by Cra binding to a site upstream of the promoter region (32). The *csgDEGF* operon was transcriptionally activated by Cra, although one of its binding sites is located downstream of the transcriptional start site (33).

The effect of Cra on transcription via binding to the promoter region of the target gene can be offset by its effectors. The binding of effector molecules to Cra in a cleft at the center of the regulatory domain leads to an allosteric response and causes DNA to be separated from Cra, which in turn activates or inhibits downstream reporter genes (2). For a long time, both F1P and fructose-1, 6-bisphosphate (FBP) were considered effectors of Cra (1). F1P is not a real intermediate in the classical diagram of central metabolism of glucose and is believed to be created in the presence of fructose as a carbon source (61). FBP is generated from F1P by fructose-1-phosphate kinase (FruK) or is derived from the metabolism of many substrates (62). In previous studies, F1P was found to be the more potent effector *in vitro*, since it interfered with Cra binding at a concentration 1,000 times lower than that required for FBP inhibition. The regulatory effect of Cra is sensitive to micromolar concentrations of F1P and millimolar concentrations of FBP *in vitro* (1, 4). However, there has been controversy over which one is the true effector of Cra. Some authors suggested that FBP is a genuine Cra effector (63). However, in later studies, *in vitro* experiments proved that there was no direct binding of FBP to Cra of *P. putida* and *E. coli*, and previous *in vitro* results could be the result of F1P contamination (2, 8, 15). Based on this, it can be considered that F1P is the only physiological effector *in vitro*, not FBP. Then, on the other hand, FBP is the key intermediate in glycolysis and plays a crucial role in flux sensing. A series of *in vivo* experiments has shown that the activity of Cra was regulated via FBP in a flux-dependent manner (14, 64, 65). In addition, the model of *E. coli* metabolic flux using F1P as the only Cra effector was insufficient to reconcile computations with experiments (66). This seems to indicate that although FBP cannot interact directly with Cra *in vitro*, it can play an indirect regulatory role *in vivo*. The paper of Chavarría and de Lorenzo raised one question, how could the perception and response of Cra on FBP take place *in vivo* (67). New findings about FruK revealed that it can function reversibly and convert FBP to F1P (68), and a linear relationship between the concentrations of F1P and FBP can be observed (8). It seems that one hypothesis can be proposed that the regulatory function of FBP on Cra can be achieved through interconversion with F1P *in vivo*. However, it is clear that the issue of the effector-mediated regulation of Cra is still open, and more studies are still necessary to interconnect all the possible factors involved.

Cra FUNCTION

Modulation of carbon flow

Cra regulators are associated with the expression of many genes that encode enzymes that constitute central pathways of carbon metabolism (69). Cra modulates the direction of carbon flow through various metabolic pathways according to the needs of cells (14). With Cra regulatory information from the public database (70), a regulatory network for Cra for core carbon metabolism was built (Fig. 2). In general, the genes encoding

TABLE 1 Genes regulated by Cra

Gene	Function	Description	References
Carbon metabolism-related genes			
aceABK	Activator	Glyoxylate pathway	(4, 6, 34, 35)
aceEF	Repressor	Oxidation and decarboxylation of pyruvate	(28, 36)
acnA	Activator	Aconitate hydratase 1, TCA cycle, oxidative stress	(37)
acnB	Repressor	Aconitase, TCA cycle	(37)
crr	Dual	Enzyme IIA^{Glc}, PTS system, glycolysis pathway	(6, 28, 35, 38)
eda	Repressor	KHG/KDPG aldolase, entner-doudoroff pathway	(6, 28, 35, 38)
edd	Repressor	Phosphogluconate dehydratase, entner-doudoroff pathway	(6, 28, 35, 38)
eno	Repressor	Enolase, glycolysis pathway	(26)
fbaAB	Repressor	Fructose-bisphosphate aldolase, glycolysis pathway	(6, 28, 35, 38–40)
gapA	Repressor	Glyceraldehyde-3-phosphate dehydrogenase A, glycolysis pathway	(26, 28, 38)
glk	Repressor	Glucokinase, glycolysis pathway	(6, 28, 35, 38)
gpmM	Repressor	2,3-bisphosphoglycerate-independent phosphoglycerate mutase, glycolysis pathway	(6, 28)
icd	Activator	Isocitrate dehydrogenase, TCA cycle	(6, 28, 29, 38)
lpd	Repressor	Lipoamide dehydrogenase, oxidation and decarboxylation of pyruvate, formyl-THF biosynthesis	(6, 41)
pck	Activator	Phosphoenolpyruvate carboxykinase, gluconeogenesis pathway	(39)
pdhR	Repressor	Pyruvate dehydrogenase complex regulator, glycolysis pathway	(28, 42)
pfkA	Repressor	6-phosphofructokinase 1, glycolysis pathway	(6, 27, 28, 35, 38)
pgk	Repressor	Phosphoglycerate kinase, glycolysis pathway	(6, 28, 35, 38, 39)
poxB	Activator	Pyruvate oxidase, acetate producing gene	(34)
ppc	Repressor	Phosphoenolpyruvate carboxylase, TCA cycle	(28)
ppsA	Activator	Phosphoenolpyruvate synthetase, gluconeogenesis pathway	(4, 28, 34, 38, 43)
ptsHI	Dual	PTS system, glycolysis pathway	(6, 28, 35, 38)
pykF	Repressor	Pyruvate kinase 1, glycolysis pathway	(6, 35, 44)
tpiA	Repressor	Triose-phosphate isomerase, glycolysis pathway	(28)
yeaD	Repressor	Putative aldose 1-epimerase YeaD, glycolysis pathway	(6, 26, 28, 38)
zwf	Repressor	Glucose-6-phosphate dehydrogenase, pentose phosphate pathway	(28)
Transport related genes			
aroP	Activator	Amino acid transport protein	(28, 43, 45)
betT	Activator	Choline: H^+ symporter, choline transporter	(34)
fruA	Repressor	Fructose PTS system EIIBC or EIIC component, fructose metabolism	(6, 46)
fruB	Repressor	Fructose-specific PTS multiphosphoryl transfer protein FruB, 6-phosphofructokinase isozyme I, fructose metabolism	(6, 28, 35, 43)
fruK	Repressor	1-phosphofructokinase, fructose metabolism	(43)
manXYZ	Repressor	Mannose-specific PTS enzyme component	(47)
mtlA	Repressor	Mannitol-specific PTS enzyme IICBA component, PTS system, mannitol transmembrane transport	(28, 38, 39)
nirC	Repressor	Nitrite transporter NirC	(6, 35, 48, 49)
setA	Repressor	Sugar exporter, electrochemical potential driven transporters	(28, 34)
sgrS	Repressor	*ptsG* stabilizer small RNA	(28, 50)
sgrT	Repressor	PtsG glucose transporter inhibitor	(28, 51)
Energy-related genes			
cydAB	Activator	Cytochrome *bd*-I subunit, electron acceptors	(52)
cyoABCD	Repressor	Cytochrome bo_3 ubiquinol oxidase subunit, aerobic respiration, electron acceptors	(28, 53, 54)
cyoE	Repressor	Heme O synthase, aerobic respiration	(28, 55)
Biosynthesis-related genes			
adhE	Repressor	Alcohol dehydrogenase, fermentation	(30, 31, 43)
betA	Activator	Choline dehydrogenase, glycine, serine, and threonine metabolism	(34)
cysG	Repressor	Siroheme synthase, porphyrin metabolism	(6, 48)
envC	Repressor	Murein hydrolase activator EnvC, cell division	(28)

(*Continued on next page*)

TABLE 1 Genes regulated by Cra (*Continued*)

Gene	Function	Description	References
mtlD	Repressor	Mannitol-1-phosphate 5-dehydrogenase, mannose metabolism	(28, 38, 39)
prpB	Repressor	2-methylisocitrate lyase, propanoate metabolism	(28, 38)
prpD	Repressor	2-methylcitrate dehydratase, propanoate metabolism	(56)
Metabolic regulatory genes			
betB	Activator	Betaine aldehyde dehydrogenase	(34)
betI	Activator	DNA-binding transcriptional repressor BetI, betaine inhibitor	(34)
crp	Activator	DNA-binding transcriptional dual regulator CRP, cyclic AMP receptor protein	(6, 9, 28, 38)
csgDEFG	Activator	Curli production assembly, curli secretion channel, single-species biofilm formation	(33)
epd	Repressor	D-erythrose-4-phosphate dehydrogenase, vitamin B6 metabolism	(6, 28)
glcC	Repressor	DNA-binding transcriptional dual regulator GlcC, glycolate utilization	(28)
hypF	Repressor	Hydrogenase maturation protein, carbamoyl phosphate phosphatase, hydrogen metabolism, anaerobic respiration	(28, 57)
marABR	Repressor	Multiple antibiotic resistance protein MarR, drug resistance/sensitivity	(28, 35, 38, 58, 59)
mpl	Repressor	UDP-N-acetylmuramate:L-alanyl-γ-D-glutamyl-meso-diaminopimelate ligase	(28, 60)
mtlR	Repressor	Transcriptional repressor MtlR/Mannitol regulator	(28, 39)
nirBD	Repressor	Nitrite reductase (NADH) subunit, nitrogen metabolism	(6, 35, 48, 49)
pdeL	Repressor	DNA-binding transcriptional regulator/c-di-GMP phosphodiesterase PdeL	(26, 35)
yibQ	Repressor	Divergent polysaccharide deacetylase domain-containing protein YibQ	(28)

gluconeogenic enzymes and the glyoxylate shunt enzymes are activated by Cra, whereas those encoding enzymes for the glycolytic and Entner-Doudoroff (ED) pathways are repressed by Cra (4, 39, 44). Cra plays an important role in regulating the switch between glycolysis and gluconeogenesis and modulating glycolytic flux by sensing the concentration of F1P or FBP (14, 71). Given the differences in the regulatory effects and strengths of F1P and FBP, it is likely that they are sensed differently by Cra. Growth in the presence of sugars has been shown to produce a correlated increase in FBP and F1P (72). Increasing the concentration of FBP has been shown to correlate with a decrease in Cra activity (5, 14). This led to increased expression of glycolysis genes and repressed expression of gluconeogenetic pathway genes, which implied that the glycolytic flux was accelerated. A decrease in Cra activity also caused a decrease in the expression of genes involved in the TCA cycle and the corresponding repression of the TCA cycle (73). In glycerol-overproducing *E. coli*, glycerol biosynthesis decreased the concentration of FBP, which then activated Cra, resulting in the downregulation of glycolytic enzymes and the upregulation of gluconeogenesis enzymes, thereby further causing a growth burden (64). When the *cra* gene was knocked out, the glucose uptake rate increased. Similarly, the TCA cycle and glyoxylate shunt were downregulated, whereas the pentose phosphate (PP) pathway and ED pathway were upregulated, in the *cra* mutant (43).

Cra plays a pleiotropic role in regulating the utilization of most carbon sources. Loss of Cra could impair the utilization of many carbon sources (39). With poor carbon sources such as acetate, Cra was activated to downregulate the expression of most enzymes in the glycolysis pathway, resulting in a decrease in glycolytic flux and subsequent redirection of enzymatic flux away from glycolysis toward gluconeogenesis. Subsequently, Cra activated the glyoxylate shunt pathway and TCA cycle together with the respiratory pathway and therefore enabled optimal growth (6).

Cra can also interact with phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), which is a widely distributed and highly efficient carbohydrate transport system present in many bacterial species (74, 75). The PTS can transport carbohydrates through the formation of phosphorylation cascades and influences a variety of physiological processes, such as the regulation of carbon metabolism and the coordination of carbon and nitrogen metabolism, through protein phosphorylation or interactions (76, 77). The PTS in *E. coli* consists of two cytoplasmic proteins, enzyme I (EI) and histidine-containing phosphocarrier protein (HPr), as well as the enzyme II complex (EII), consisting of EIIA, EIIB, and EIIC/D. The utilization of fructose as a carbon source

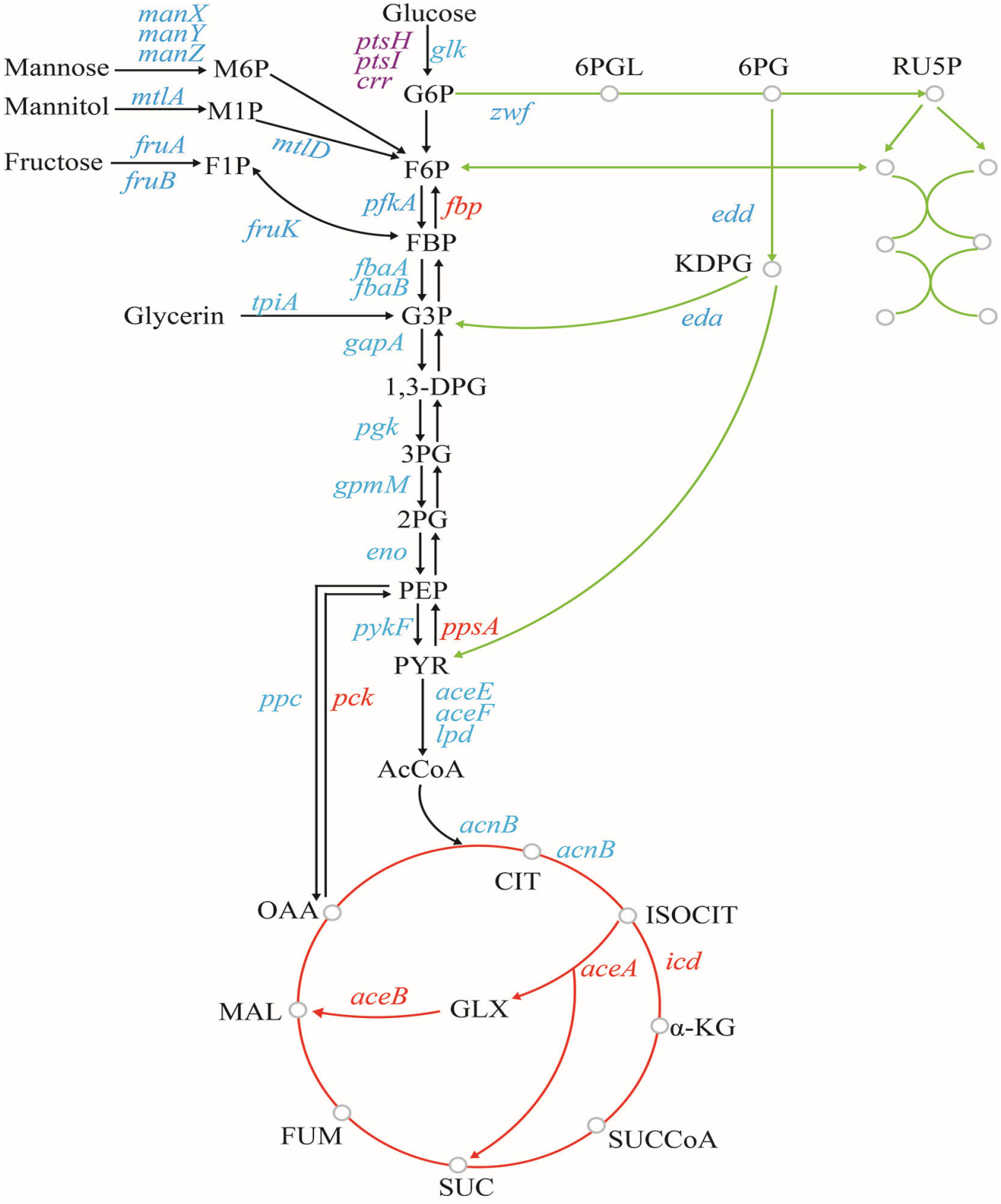

FIG 2 Metabolic regulatory network of Cra. The genes shown in red are activated by Cra, the genes shown in blue are repressed by Cra, and the genes shown in purple are both activated and repressed by Cra. Key genes for enzymes: Phosphocarrier protein HPr *ptsH*, PTS enzyme I *ptsI*, Enzyme IIAGlc *crr*, Glucokinase *glk*, Glucose-6-phosphate dehydrogenase *zwf*, Mannose-specific PTS enzyme IIAB component *manX*, Mannose-specific PTS enzyme IIC component

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Fig 2 (Continued)

manY, Mannose-specific PTS enzyme IID component *manZ*, Mannitol-specific PTS enzyme IICBA component *mtlA*, Mannitol-1-phosphate 5-dehydrogenase *mtlD*, Fructose PTS system EIIBC or EIIC component *fruA*, fructose PTS system EIIA component *fruB*, 1-phosphofructokinase *fruK*, 6-phosphofructokinase 1 *pfkA*, Fructose-1,6-bisphosphatase *fbp*, Fructose-bisphosphate aldolase class II *fbaA*, Fructose-bisphosphate aldolase class I *fbaB*, Triose-phosphate isomerase *tpiA*, Glyceraldehyde-3-phosphate dehydrogenase A *gapA*, Phosphoglycerate kinase *pgk*, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase *gpmM*, Enolase *eno*, Pyruvate kinase 1 *pykF*, Phosphoenolpyruvate synthetase *ppsA*, Pyruvate dehydrogenase E1 component aceE, Pyruvate dehydrogenase E2 component *aceF*, Lipoamide dehydrogenase *lpd*, Phosphoenolpyruvate carboxylase *ppc*, Phosphoenolpyruvate carboxykinase *pck*, Aconitase *acnB*, Isocitrate lyase *aceA*, Malate synthase *aceB*, Isocitrate dehydrogenase *icd*, Phosphogluconate dehydratase *edd*, KHG/KDPG aldolase *eda*. Metabolic intermediates: Glucose 6-phosphate G6P, Fructose 6-phosphate F6P, 1,6-fructose diphosphate FBP, glyceraldehyde-3 phosphate G3P, 1,3-disphosphoglycerate 1,3-DPG, 3-phosphoglycerate 3 PG, 2-phosphoglycerate 2 PG, Phosphoenolpyruvate PEP, Pyruvate PYR, Acetyl-CoA AcCoA, Citrate CIT, Isocitrate ISOCIT, α-Ketoglutaric acid α-KG, Succinyl coenzyme A SUCCoA, Succinate SUC, Fumarate FUM, Malate MAL, Oxaloacetate OAA, 6-phosphogluconolactonase 6 PGL, 6-phosphogluconate 6 PG, Ribulose 5-phosphate RU5P, and 2-keto-3-deoxy-6-phosphogluconic acid KDPG.

involves the function of the PTS and a fructose operon (*fruBKA*). The fructose PTS has an HPr-like protein (FPr) (78). The phosphoryl group of PEP is sequentially transferred to EI and FPr and then to fructose via fructose-specific $EIIA^{Fru}$ and $EIIBC^{Fru}$. The phosphorylated fructose is converted to F1P, which inhibits Cra activity (7). Cra also has a weak repressive effect on the downstream *ptsH* (encoding HPr) promoter (79, 80). The *fruBKA* operon, encoding components of the fructose PTS, contains genes encoding a diphosphoryl transfer protein (*fruB*), a fructose 1-phosphate kinase (*fruK*), and the fructose-specific enzyme EIIB'BC (*fruA*) and is repressed by Cra (73, 81). Cra regulates the expression of the fructose PTS in a manner that is dependent on the endogenous concentrations of F1P in *P. putida* (82). It is known that glucose is preferentially transported by glucose PTS and consumed when glucose coexists with other sugars. The *cra* knockout mutant coconsumed glucose and fructose, with fructose being consumed faster, although glycolysis activity increased. The total carbon source consumption rate was greater than that of the wild type (81). Furthermore, when a general PTS component is defective (i.e., *ptsH* mutant), the presence of a PTS sugar can exert selective pressure to induce immediate Cra mutations. The mutations in *cra* resulted in constitutive expression of the *fruBKA* operon, and thus, the FPr domain could replace the activity of HPr in the PTS (83).

Nitrogen regulation

Nitrite reduction is of paramount importance for nitrogen assimilation and anaerobic metabolism. The *nir* operon, which encodes a NADH-dependent nitrite reductase expressed during anaerobic growth, is a critical enzyme involved in nitrogen metabolism (84). Transcription from the *nir* promoter was repressed by Cra, and the *nir* operon expression was increased by the disruption of *cra* (48).

In addition to the sugar PTS dedicated to carbohydrate uptake, many gram-negative bacteria have a nitrogen PTS, which has been shown to regulate diverse processes implicated in nitrogen and carbon metabolism (85). There is cross-talk between the fructose-related and nitrogen-related branches of the PTS. The high-energy phosphoryl group (~*P*) can be transferred from the sugar PTS to the nitrogen PTS (86). Cra and F1P can not only control the expression of the fructose PTS and check the activity of the nitrogen PTS but also regulate the cross-talk between the two branches of the PTS. The transfer of ~*P* was greatest when the loss of Cra led to high expression of the *fruBKA* operon and was lowest when the cells were unable to produce sufficient Cra protein in P. putida (82).

APPLICATION OF Cra IN METABOLIC ENGINEERING

As a global TF, Cra can simultaneously activate or inhibit the coexpression of multiple genes in a specific pathway, thereby enhancing the target performance of engineered microbial strains, such as increasing target metabolite synthesis, improving environmental tolerance, and improving stress tolerance (87).

Promotion of metabolite biosynthesis

Due to the complexity of the metabolic network, the desired cell phenotype may not be obtained through gene manipulation. As an important part of gene expression regulation, modification of the global TF Cra can induce changes in carbon flux in multiple metabolic pathways. Thus, Cra-related manipulation has been proven to be useful for the construction of bacterial strains suitable for the synthesis of target components (88). In previous studies, a common approach was to knock out the *cra* gene, but the effects on the biosynthesis of target products were different. Deletion of the *cra* gene in *Enterobacter aerogenes* significantly increased the rate of fructose consumption and eliminated carbon catabolite repression (CCR) in fructose utilization, thus increasing the production of 2, 3-butanediol (3). The effect of *cra* on L-tryptophan biosynthesis showed that *cra* knockout significantly enhanced metabolic flow toward glycolysis, the PP pathway, and the TCA cycle, increasing the levels of key precursors and substrates for L- tryptophan biosynthesis in *E. coli* (89). Conversely, also in *E. coli*, knockout of *cra* led to a significant decrease in L-phenylalanine (L-PHE) production (90). This may be due to the imbalance of central carbon metabolism caused by the disruption of Cra. By integrating random mutagenesis libraries of Cra into the chromosome, it was possible to screen for strains with higher L-Phe production. The highest L-PHE concentration obtained with the best mutant strain (Cra^{E173K}) was 70.50 ± 1.02 g/L, which was 23.34% greater than that of the control strain. Metabolomic and transcriptomic analysis revealed that the mutation enhanced metabolic flow through the gluconeogenic pathway, alternative Krebs cycle, whole glyoxylate shunt, and PP pathway; improved the utilization of carbon sources; enhanced the supply of precursors (PEP and erythrose 4-phosphate); and thus switched carbon fluxes to the L-PHE biosynthetic pathway (90).

By screening for mutations in the DNA binding and effector binding sites of Cra, the regulatory effects of Cra on multiple genes can be altered, ultimately resulting in the promotion of target product synthesis. Zhu et al. constructed a *cra* mutant library using error-prone PCR in *E. coli*. By testing combinations of various mutations in Cra, they identified Cra mutants where the PEP carboxylation and glyoxylate pathways were activated. The best Cra mutant showed a stronger binding affinity for FBP and a more significant effect on the synthesis of succinate (91). On this basis, Wei et al. further demonstrated the relationship between the affinity of Cra for FBP and succinate production. Computer-assisted analysis was used to identify the sites that contributed to the binding affinity. The mutant strain with a combination of three sites (D101R/D148R/G274R) showed the highest binding affinity of Cra for FBP and DNA and the highest succinate production, proving that there was a positive correlation between succinate biosynthesis and the affinity of Cra for FBP (92).

In addition to focusing on the modification of Cra, studies on the interaction between Cra and promoters have also shown that this modification is effective for product biosynthesis. According to a previous study, the regulation of Cra contributes to the overproduction of glycerol in *E. coli* (64). The pBAD promoter, which was engineered by inserting a Cra-binding site, was repressed by Cra. There were interactions among the pBAD promoter, FBP, and Cra. A decrease in the FBP concentration led to an increase in Cra activity, which in turn inhibited the pBAD promoter, reduced the activity of key enzymes involved in glycerol synthesis, and ultimately restored the concentration of FBP. This feedback regulation enabled high FBP levels and, at the same time, high glycerol production rates, which presumably prevented *E. coli* from switching glycolysis to gluconeogenesis (64).

Regulating the stress tolerance of microorganisms

The normal growth of microorganisms is challenged by a variety of environmental stresses, such as osmotic stress, redox stress, organic solvent stress, and high concentrations of bioproducts (93). These stresses are important in microbial fermentation and can

affect the metabolic activity and productivity of cells (94, 95). Therefore, enhancing stress tolerance is considered an effective measure for improving cell growth and increasing product yield. However, stress tolerance is a complex mechanism controlled through multilayered regulatory networks (96). The use of TFs promotes the modulation of overall cellular processes and can be a viable strategy for improving the tolerance of microorganisms (97). Cra reportedly affects numerous stress response genes (28). This finding suggested that the manipulation of Cra can affect the tolerance of bacteria to different environmental conditions.

In *Yersinia pseudotuberculosis*, Cra negatively regulates acid survival. The expression of *cra* was repressed in response to acidic pH, and deletion of the *cra* gene increased acid survival 10-fold (98). Knockout of *cra* can increase the sensitivity of *E. coli* to environmental stresses such as alcohols and redox stress (99). Cra is also related to osmotic pressure regulation. Decreased expression of the *bet* operon, which plays a role in protecting *E. coli* against high osmotic pressure, has been reported in *E. coli* K-12 Δ*cra* mutants (34). Gayán et al. reported that mutations in Cra (L56R) were associated with increased resistance to high hydrostatic stress in *E. coli* (10). Combinations of mutations in *cra*, *aceA,* and *rpoD* could enable *E. coli* strains to comfortably survive pressures of 600–800 MPa (10).

The formation of biofilms is a response to environmental pressure (100). Bacterial cells wrapped by biofilms exhibit stress tolerance to physical and chemical stimuli (101). Biofilms are multicellular structures encased in matrices that generally consist of exopolysaccharides (EPSs), proteins, extracellular DNA, and other factors (102). EPSs have been shown to play an important role in the formation and colonization of biofilms (103, 104). Cra was demonstrated to influence biofilm formation. In the endophytic bacterium *Pantoea alhagi*, Cra stimulated EPS production and biofilm formation by directly activating the expression of the *eps* operon, *ydiV*, and *yedQ*, and the activating effects were much more significant in a gluconeogenic environment (105). In *S. enterica*, Cra coordinates the carbon flux from glycolysis to gluconeogenesis and promotes biofilm formation via the end products of gluconeogenesis (11). In *E. coli*, the biosynthesis of curli, which is involved in biofilm formation, was strongly inhibited by the repression of the transcription of the *csgDEFG* operon in the *cra* mutant (33, 106).

Modulation of the bacterial virulence

The level of virulence is usually correlated with the ability of the pathogen to multiply within the host (107). Pathogens use TFs to express their virulence genes in subtle and precise ways and may exhibit different extents of virulence (108). Cra regulates virulence in pathogenic bacteria such as *Listeria monocytogenes*, *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and enterohemorrhagic *E. coli* (EHEC) (11, 109–111). Cra was shown to be a facilitator of *S. flexneri* pathogenesis, and *S. typhimurium* lacking Cra exhibited reduced virulence (110, 112). Sugar metabolism is crucial for virulence (113). Carbohydrates are not only known as energy substances but also exploited as structural materials, such as for bacterial peptidoglycan, lipopolysaccharide, and biofilm production. Due to the important role of Cra in carbon metabolism, *Salmonella* lacking Cra may not be able to proliferate due to imbalanced carbon flow and may also undergo structural changes in peptidoglycan and lipopolysaccharide composition (112). The EHEC pathogen links the expression of virulence factors to metabolic sensing through Cra by improving the expression of virulence-associated genes in response to the gluconeogenic environment (114). Cra utilizes changes in the concentration of carbon metabolism intermediates to positively regulate the virulence of EHEC, and a lack of Cra impairs bacterial virulence during host infection (108). EHEC infection is characterized by the formation of intestinal attaching and effacing (A/E) lesions due to the activity of the type III secretion system (T3SS) (115). Robertson et al. reported that the phosphorylation of Cra (Y47 residue) diminishes DNA binding to regulate the expression of *ler,* which encodes the major activator of EHEC virulence gene expression; subsequently, the

production and secretion of T3SS proteins are negatively affected, resulting in decreased A/E lesion formation (111).

Metabolite response biosensors

In microbial strains, the concentration of intracellular metabolites typically varies throughout the biological production process. To promote the synthesis of target metabolites, key enzymes in each pathway need to be expressed at appropriate times and in appropriate amounts to precisely adjust dynamic metabolism and prevent the accumulation of metabolic intermediates to toxic levels (116). Biosensors, which are devices used to determine the presence or concentration of biological analytes, play an important role in the real-time monitoring of metabolite concentrations during fermentation (117). Biosensors based on TFs can realize fast detection and *in-situ* screening of desired properties by converting the intracellular concentration of target metabolites into detectable signals and have been widely applied in the monitoring and regulation of microbial metabolic products (118).

As a crucial pathway of cellular metabolism, glycolysis plays a fundamental role in energy production and metabolic balance. High glycolytic flux may cause overflow metabolism, whereas low glycolytic flux may lead to low growth efficiency and decreased product titers (119). The FBP concentration followed a linear trend with the glycolytic flux. Cra is considered a sensor of glycolytic flux due to its sensitivity to the metabolic intermediate FBP (14, 65). Changing the glycolytic flux would affect FBP concentration and thus Cra activity. Based on the relationships among Cra, FBP, and the promoters regulated by Cra, a series of bifunctional glycolysis flux biosensors with various dynamic ranges and thresholds was constructed, which exhibited positive and negative responses. The biosensors could be used to characterize the intracellular FBP concentration in different gene-deficient strains and dynamically regulate glycolytic flux. Finally, the biosensors were used to efficiently improve the biosynthesis of mevalonate and N-acetylglucosamine (13). In addition, to reduce the burden of overexpression and structural alterations of Cra on the metabolism of host cells, Zhu et al. focused on the de novo design of Cra-regulated promoters to adjust the function of glycolytic flux biosensors. By replacing native promoters with a series of artificial promoters of varying strengths or phage-derived promoters and deploying the DNA-binding sites of Cra into promoters at different positions and distances, a series of biosensors with a wide dynamic range and low basic leakage was constructed. The biosensors were successfully applied to the dynamic regulation of pyruvate and lycopene biosynthesis (12).

Coupling Cra with other transcription factors

Due to the complexity of cellular metabolism, sometimes using a TF alone to regulate metabolic pathways cannot meet regulatory needs. TFs can interact with each other to form a complex network. Through the network, the transcription level of target genes can be jointly regulated by TFs (120). KdpE is the response regulator of the two-component system, which was extensively studied due to its important role in potassium transport (121). As a transcriptional regulator, KdpE has been shown to regulate many essential genes involved in key metabolic processes, stress tolerance, and virulence (121–123). Cra could interact with KdpE, and the two regulators shared several common virulence targets in EHEC (108, 114). Njoroge et al. confirmed that the expression of target genes was regulated through direct interaction of Cra and/or KdpE with their promoter regions (114). The interaction between Cra and KdpE had an additive effect on the expression of the target gene. This regulation led to the activation of EHEC virulence, including the formation of signature A/E lesions (108, 114). The TF FNR, a regulator of fumarate and nitrate reduction, repressed the expression of genes involved in aerobic respiration and activated the expression of genes allowing the reduction of alternative electron acceptors (124, 125). The *cydAB* operon, encoding cytochrome oxidase *d*, is known to be controlled by both Cra and FNR. However, the transcriptional

effects exerted by Cra were dependent on FNR, and the interactions between FNR and Cra were crucial to the regulation of the *cydAB* operon (52).

CONCLUSIONS AND PERSPECTIVES

With further research, it has become clear that TF Cra plays a crucial role in bacterial cell growth and central carbon metabolism. By analyzing the structure and function of Cra, the structural characteristics, mechanism of action, and regulatory network complexity of Cra have gradually been revealed. Because of its important physiological functions and multiple regulatory effects, Cra has been widely used in the study of metabolic regulation. However, there are many aspects worth further study. First, maintaining the balance between metabolic flux and biosynthetic pathways is a key issue in metabolic engineering. Therefore, the dynamic regulation of metabolic flux is very beneficial for optimizing the cell phenotype and maintaining cell homeostasis. Based on the rational design of protein engineering methods, the development of novel Cra biosensors with a wider response range is conducive to exploring more accurate adjustment times and nodes and providing new ideas for dynamic regulation. Second, the design of artificial Cra offers opportunities to overcome limitations associated with natural Cra, such as limited versatility or weak binding to DNA. The artificial design and construction of high-performance Cra can be achieved through rational protein design such as domain substitution or engineering regulatory domains. Finally, the interactions between Cra and other TFs form a complex network of actions that should be further studied.

In summary, the rapid development of biotechnology will facilitate more detailed and comprehensive study and application of Cra.

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