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4 Carbon catabolite control and metabolic networks mediated by the CcpA protein in *Bacillus subtilis*

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Abstract

In Bacillus subtilis and close relatives, global regulation of carbon catabolite control occurs on the binding of the complex of CcpA (catabolite control protein A) and P-Ser-HPr (seryl-phosphorylated form of a phosphocarrier protein of the phosphoenolpyruvate: sugar phosphotransferase system) to the catabolite responsive elements (cre) of the target operons, the constituent genes of which are roughly estimated to number three hundred. The complex of CcpA and P-Ser-HPr triggers the

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expression of several genes involved in the formation of acetate and acetoin, which are major extracellular products of B. subtilis grown on glucose. It also triggers the expression of an anabolic operon (ilv-leu) involved in the biosynthesis of branched-chain amino acids which subsequently leads to cell propagation. On the other hand, this complex represses many genes and operons, which include an entrance gene for the TCA cycle (citZ), several transporter genes for TCA cycle-intermediates, some respiration genes, and many catabolic and anabolic genes involved in carbon, nitrogen and phosphate metabolism as well as for certain extracellular enzymes and secondary metabolites. Thus, CcpA-mediated metabolic networks play a major role in the coordinate regulation of catabolism and anabolism to ensure the optimum cell propagation in the presence and absence of preferred carbohydrates such as glucose.

Introduction

Catabolite control, *i.e*. catabolite repression and activation, is a regulatory mechanism by which the cell coordinates the metabolism of carbon and energy sources to maximize efficiency and regulates other metabolic processes as well. Also, carbon catabolite control is specific to carbon source-mediated regulation. Carbon catabolite repression (CCR) is when genes and operons are involved not only in catabolism but also in some anabolic processes such as the synthesis of certain extracelllular enzymes and secondary metabolites that are not expressed as long as preferred sources of carbon and energy are present [1]. This can be achieved through transcription control of catabolic operons by global regulators in response to availability of preferred carbon sources such as glucose, and/or by the modulation of the intracellular availability of specific inducers of catabolic genes through assimilation of the preferred carbon source. Carbon catabolite activation (CCA) is when the expression of some operons is stimulated in the presence of preferred carbon sources.

Of numerous operons of which expression is under catabolite control, this review specifically deals with ones subjected to CCR/CCA occurring through a global transcription regulation involving catabolite control protein A (CcpA).

Elucidation of the signal transduction mechanism underlying carbon catabolite control involving CcpA

More than one-third of a century ago, a general CCR mechanism based on disappearance of the stimulatory effect of the cyclic AMP/cyclic AMP receptor protein complex was proposed for *Escherichia coli* and other enteric bacteria [2]. Since many Gram-positive bacteria including *B. subtilis* do not possess cyclic AMP, a different general CCR mechanism might operate in these organisms.

Nearly 20 years ago, the pioneering work on CCR of α-amylase synthesis in *B. subtilis* resulted in the identification of the two constituents of the major CCR/CCA mechanism: a 14-bp *cis*-acting palindromic sequence (TGTAAGCGTTAACA) subsequently called the catabolite-responsive element (*cre*) for *amyE* located in the promoter region of *amyE* [3, 4] and the CcpA protein, a member of the LacI/GalR family of transcriptional regulators [5]; mutations in either of these constituents result in relief from CCR of *amyE* expression.

Besides the *cre* for *amyE*, such *cre* sequences for the *gnt* [6, 7], *xyl* [8, 9], and *hut* [10, 11] operons have been identified in the reading frames of *gntR*, *xylA* and *hutP*, respectively, whereas those for the *acu* and *acs* operons are in their divergent promoter regions [12]. In *ccpA* mutants, the expression of the *gnt* [13, 14], *xyl* [15], *hut* [11], and *acu* [12] operons, and the *acsA* gene [12] is relieved from catabolite repression. The specific binding of CcpA to the *cre*s of *gnt* [16] and *amyE* [17, 18] with and without a corepressor protein described below has been verified by means of DNase I footprinting [16, 17] and methylation protection and interference [18], respectively. So far, a total of 45 *cre*s with a 15-bp sequence (one base addition to the 5'-end) have been identified for various genes and operons of *B. subtilis*, which are listed in Table 1.

In contrast to the situation in *E. coli*, CCR in *B. subtilis* occurs only when the repressing sugar can be converted to certain glycolytic intermediates including fructose-1,6-bisphosphate (FBP)[63-65]. Mutants unable to produce FBP do not exhibit CCR of *myo*-inositol dehydrogenase, acetoin dehydrogenase or gluconate kinase, implying the presence of a common regulatory mechanism underlying CCR in *B. subtilis*. The role of FBP is to stimulate phosphorylation of HPr at Ser-46, catalyzed by HPr kinase/phosphatase (HPrK/P), as verified later [66-69]. (HPr is a phosphocarrier protein involved in carbohydrate transport via the phosphoenolpyruvate:sugar phosphotransferase system [70]). Thus, HPr-mutants (*ptsH1*) are partially or completely relieved from CCR of several catabolic genes [15, 25, 54, 71, 72]. Most enzymes that are relieved from CCR in a *ccpA* mutant are also relieved from CCR in a *ptsH1* mutant, implying that P-Ser-HPr and CcpA might be involved in the same CCR mechanism and that they possibly interact with each other. This coincides with the fact that CcpA is a protein synthesized constitutively irrespective of the presence and absence of a preferred carbon source (14), suggesting that CcpA requires a corepressor, which might be P-Ser-HPr, to exert catabolite repression.

A specific interaction between CcpA and P-Ser-HPr has indeed been demonstrated by the retarded elution of P-Ser-HPr from CcpA-carrying columns [73] and later by nuclear resonance measurements [74]. In 1995, the complex of CcpA with P-Ser-HPr was verified to recognize the *cre* of the *gnt*

Table 1. *B. subtilis* genes under control by catabolite repression/activation, for which the corresponding *cre* sequences were identified.

Gene	Rank/100[a]	Function	*cre* sequence (location)[b]	Transcription initiation base[b]	*cre* localization / function[c]	Reference
Group A (TG/CA)						
ackA	A-13	Acetate metabolism	TTGTAAGCGTTATCA(-156/-142)	(-92)	U/A	19-21
acsA	A-14	Acetyl-CoA synthetase	TTGAAAGCGTTACCA(+7/+21)	(-30)	D/R (1)	12, 22
amyE	A-5	α-Amylase	ATGTAAGCGTTAACA(-125/-111)	(-121)	P/R	3
araB	A-51	Arabinose metabolism	ATGAAAACGATTACA(+679/+693)	(-2167)	D/R (1)	23, 24
bglP	A-7	β-glucoside metabolism	ATGAAAGCGTTGACA(-253/-239)	(-212)	P/R	25, 26
cccA	A-21	Cytochrome *c*-550	TTGTAAGCGTATACA(-188/-174)	(-151)	P/R	27
citM	A-27	Mg^{2+}/citrate transporter	ATGTAAGCGGATTCA(-32/-18)	(-71)	P/R	28
dctP (*ydbH*)	A-10	C4-dicarboxylate transporter	ATGAAAACGCTATCA(-64/-50)	(-41,-42)	P/R	23, 29
dra	A-40	Deoxyribonucleoside metabolism	TTGAAACCGCATACA(+34/+48)	(-29,-31)	D/R (1)	23, 30, 31
galT	A-31	Galactose-1-P metabolism	ATGGAAGCGGATACA(+214/+228)	ND	D/R (1)	23,
glpF	A-r16, A-93	Glycerol metabolism	TTGACACCGCTTTCA(-181/-167)	ND	p/R (r)	32
glvA (*yfiA*)	A-35	6-P-α-glucoside metabolism	TTGTAAACGTTATCA(-28/-14)	(-26)	P/R	33
gntR	A-20	Gluconate metabolism	TTGAAAGCGGTACCA(+107/+121)	(-34)	D/R (2)	16, 34
gntR	A-92	Gluconate metabolism	ATGAAAGTGTTTGCA(-81/-67)!!	(-34)	P/R	35
hutP	A-43	Histidine metabolism	TTGAAACCGCTTCCA(+170/+184)	(-32)	D/R (2)	11, 36
ilvB	A-4	Branched-chain amino acid synthesis	ATGAAAGCGTATACA(-578/-564)	(-482)	U/A	37, 38
iolB	A-29	Inositol metabolism	ATGAAAACGTTGTCA(+668/+682)	(-1710)	D/R (2)	39, 40
kdgA	A-32	Hexuronate metabolism	ATGGAAGCGCTGACA(+355/+369)	(-2084)	D/R (1)	23, 41
lcfA	A-8	Acyl-CoA synthetase	ATGAAAACGTTATCA(+412/+426)	ND	D/R (1)	23, U[d]
phoP	A-3	Response regulator of PhoR/PhoP	ATGAAAGCGCTATCA(-161/-147)	(-175)P_{A6}	P/R	42
resB	A-Out	Respiration	TTGGTAACGGTTACA(+1152/+1166)	(-566)	D/R (3)r	43

Table 1. Continued

treP	A-88	Trehalose metabolism	GTGAAAACGCTTGCA(+317/+331)	(-46)	D/R (2)	23, 44
uxaC (yjmA)	A-2	Glucuronate metabolism	ATGAAAGCGTTATCA(+1177/+1191)	ND	D/R (1)	23, 45
xylA	A-37	Xylose metabolism	TTGGAAGCGCAAACA(+35/+49)	ND (not 168)	D/R (2)	9, 23
yobO	A-28	Phage-related function	ATGTAAGCGGATTCA(+1178/+1192)	ND	D/R (2)	23
yxkJ	A-86	Citrate/malate transporter	TTGCAAACGGATACA(+28/42)	ND	D/R (1)	23, 46
Group B						
acoA	B-97	Acetoin metabolism	ATGTAAGCGTTTGCT(+434/+448)	(-41)	D/R (2)	23
acoR	B-2	Activator for *acoABCL*	TTGAAAGCGCTTTAT(-67/-53)	ND	p/R	47
acuA	B-6	Acetoin metabolism	TTGAAAACGCTTTAT(-75/-61)	(-41)	P/R	12, 20
araA	B-10	Arabinose metabolism	TTGAAAGCGTTTTAT(-38/-24)	(-97)	D/R	24, 48
araE	B-28	Arabinose metabolism	ATGAAAACGCTTTAC(-38/-24)	(-102)	D/R	48, 49
ccpC (ykuM)	B-Out	Catabolite control protein C	AAGAAAGCGCATACA(-108/-94)	ND (P2)	D/R	50
citS	B-Out	Sensor kinase of CitS/CitT	TTGATAACGCTTTCG(+1298/1311)	(-28)	D/R (2)	51
citZ	B-Out	Citrate synthase	ATGTAAGCATTTTCT(-114/-100)	(-194)	D/R	52
iolA	B-23	Inositol metabolism	TTGAAAGCGTTTAAT(-106/-92)	(-191)	D/R	39, 40
levD	B-56	Levan metabolism	ATGAAAACGCTTAAC(-80/-66)	(-29)	U/R	53, 54
mmgA	B-51	Mother-cell fatty acid degradation	TTGTAAGCGCTGTCT(-37/-23)	(-50)	P/R	55
msmX	B-90	Sugar ABC transporter	AAGAAAGCGTTTACA(-35/-21)	ND	p/R	23, 46
pta	B-63	Acetate metabolism	ATGAAAGCGCTATAA(-100/-86)	(-37)	U/A	56, 57
resA	B-Out	Thiol-disulfide oxidoreductase	GTAAAAACGCTTTCT(-104/-90)	(-24)	D/R	43
rocG (yweB)	B-r67	Glutamate dehydrogenase	TTTAAAGCGCTTACA(-50/-36)	(-121)	D/R (r)	58

Table 1. Continued

sigL	B-44	σ54 (σH-dependent)	TGGAAAACGCTTTCA(564/578)	ND	D/R (3)	59
xynP (*ynaJ*)	B-29	β-xyloside H^+-symporter	TTGAAAGCGCTTTTA(-99/-85)	(-321)	D/R	60
yusL	B-54	Fatty acid β-oxidation	ATGAAAGCGCTTATT(1036/1050)	(-70)	D/R (1)	U[d]
yxjC	B-12	β-Hydroxybutyrate metabolism	TTGTAAACGCTTTCT(-41/-27)	ND	p/R	23, 46
Consensus sequence			WTGNAARCGNWWWCA			

[a]The ranking of *cre* sequences was performed by means of the GRSP-DNA search (http://www2.genomatica.com/grasp-dna/)[61] using 26 and 19 *cre* sequences with and without a TG/CA set (Groups A and B) as entry sequences, respectively. Another site is also available to search out such *cis*-elements on *B. subtilis* genome (http://dbtbs.hgc.jp/ motiflocationsearch.html, N. Sierro and K. Nakai, unpublished) [62]. "Out" and "r" denote "out of the 100th rank" and "reverse sequence of *cre* sequence", respectively.
[b]Numbers indicate the position of the cre sequence or the transcription start base relative to the first base of the translation start for the gene. ND in the "transcription initiation base" column denotes "not determined".
[c]"U", "P", and "D" denote the *cre* localization in the upstream, promoter and downstream regions of the transcription initiation bases, respectively. "A" and "R" indicate catabolite "activation" and "repression", respectively. Also, the lower-case "p" and "r" denote the *cre* location in the presumed promoter region and in the reverse *cre* direction as to transcription, respectively. Of the *cre* sites located downstream of the transcription initiation bases, ones associated with numbers in parentheses are located in the protein-coding regions of the target genes. When the bases of positions +1, +4, +7, +10 and +13 of the 15-base *cre* sequences correspond to the first, second and third bases of codons in the protein-coding frames of the target genes, the numbers in parenthesis, (1), (2), and (3), are assigned, respectively.
[d]"U" indicate "unpublished results by H. Matsuoka, and Y. Fujita.

operon with high affinity in footprinting experiments [16]. This finding led to the present model for the molecular mechanism underlying CCR in *B. subtilis* (Fig. 1). The high affinity binding of the complex of CcpA and P-Ser-HPr to the *amyE cre* has been actually confirmed by circular dichroism spectroscopy [74]. Moreover, the structure of the CcpA-(P-Ser-HPr)-*cre* complex is determined [75]. CcpA, HPr with Ser-46, and HPr kinase/phosphatase are well conserved among low-GC Gram-positive bacteria, suggesting that this molecular mechanism underlying CCR is operative in these bacteria.

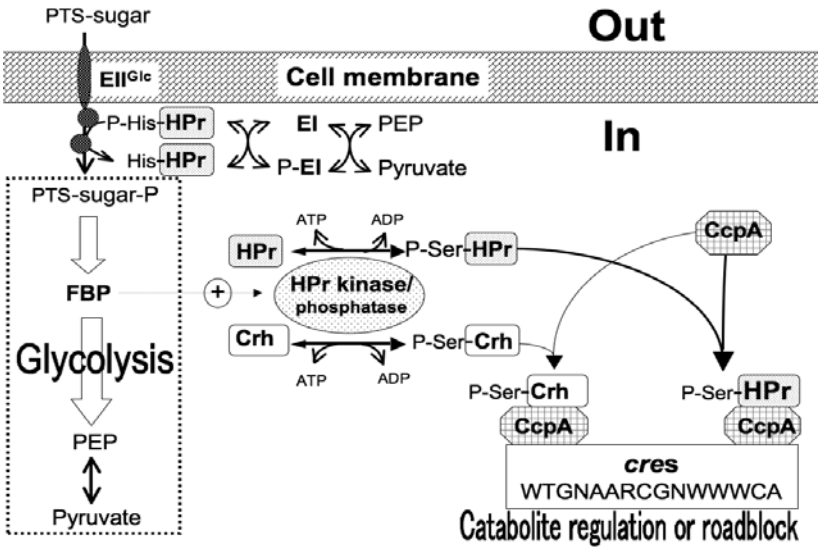

Figure 1. The molecular mechanism of carbon catabolite repression and activation in *Bacillus subtilis*. The uptake of a preferred carbohydrate (PTS-sugar), such as glucose, fructose, or mannose, leads to an increase in the FBP concentration in the cell, which triggers the ATP-dependent HPr kinase/phosphatase-catalyzed phosphorylation of HPr and Crh at Ser-46. Only the seryl-phosphorylated forms of HPr and Crh are capable of binding to CcpA. The P-Ser-HPr/CcpA and P-Ser-Crh/CcpA complexes can bind to the catabolite responsive elements, *cre*, to exert CCR or CCA, depending on the position of the *cre*. A consensus sequence for the *cre* sequences is WTGNAARCGNWWWCA [23]. If a *cre* is properly located upstream of the "-35" region of the promoter, the complex (P-Ser-HPr/P-Ser-Crh and CcpA) interacting with RNA polymerase causes CCA. The complex binds to a *cre* located in the promoter region, resulting in transcription repression, whereas that bound to *cre* located well downstream of the transcription initiation base evokes transcription roadblock. Similar mechanisms are presumably operative in most other low-GC Gram-positive bacteria, with the restriction that Crh has been found only in bacilli so far.

As shown in Fig. 1, an HPr-like protein (Crh) is included as another corepressor of CcpA, which was found during the *B. subtilis* genome sequencing project [76]. While Crh contains the conserved Ser-46, which can be phosphorylated with ATP and HPrK/P, it lacks the active site His-15 [77], so Crh is active only in CCR/CCA. Inactivation of the *crh* alone does not affect CCR or CCA, but the residual CCR observed in *pstH1* mutants disappears when the *crh* is disrupted or a *crh1* mutation (replacement of Ser-46 with Ala) is introduced [19, 22, 30, 32, 39, 42, 51, 53, 56, 58, 60, 77, 78]. The question of how P-Ser-HPr and P-Ser-Crh contribute to CCR and CCA with different efficiencies has not been clearly answered yet, although a Crh-specific function in regulation of expression during growth on substrates other than carbohydrates was recently reported [79], probably because of the drastically higher amount of HPr than that of Crh during growth on carbohydrates [80]. Also, P-Ser-Crh displays altered binding to CcpA to effect CCR/CCA [81]. Although the genomes of many Gram-positive bacteria have been sequenced, Crh has been found to be only present in bacilli, suggesting that Crh-medicated CCR/CCA with Crh as a corepressor of CcpA might be specifically operative in this genus.

Characterization of *cre* sequences and transcription regulation

Forty-five *cre* sequences that have been experimentally identified so far are listed in Table 1. Extensive base substitution analysis of a *cre* sequence for *amyE* has revealed the consensus sequence of TGWNANCGNTNWCA [4]. Genome-wide analysis of *cre* sequences has led to the proposal of similar but longer consensus sequence, WWTGNAARCGNWWWCAWW [23]; the underlined sequence corresponds to the *cre* sequences listed in Table 1. This analysis led to the following three implications. (i) Lower mismatching of *cre* sequences with the consensus sequence is required for *cre* function. (ii) Although *cre* sequences are partially palindromic, lower mismatching in the same direction as that of transcription of the target genes is more critical for the *cre* function than that in the inverse direction. (iii) However, a more palindromic nature of *cre* sequences is desirable for a better function. The left-side TG of the two palindromic consensus sequences is likely to be required for pairing with the right-side CA, resulting in proper binding of the complex. Actually, the substitution of the last C with another base rendered many *cre*s inoperative, as initially verified for the *cre*s of *amyE* [4], *gntR* [16], and *hutP* [11]. However, this pairing is likely compensated for by another pairing between the 5' and 3' parts of a *cre* sequence, as demonstrated for the *cre* sequence of *iolA*, TTGAAAGCGTTTAAT [39]; the underlined Ts, which can be paired with the underlined As, are indispensable for the *cre* function. Thus, the *cre* sequences listed in Table 1 can be classified into two groups (A and B),

i.e. ones with and without a TG-CA palindromic pair in their *cre* sequences, respectively (Table 1).

Table 1 also indicates the location of each *cre* from the translation initiation base of the gene closest to it in its target operon together with the transcription initiation base of the operon, if known. Depending on the location of the *cre* sites, the binding of the CcpA/P-Ser-HPr complex (or P-Ser-Crh) to them can regulate transcription in different manners (activation, repression and roadblock). The protein complex binding to a *cre* located upstream of the promoter results in transcription activation, i.e. CCA, as for *ackA* [19, 21], *pta* [56, 57], and *ilvB* [37]. This CCA appears to involve direct interaction of the complex with RNA polymerase, as deduced from the face-of-the-helix dependence of the *cre* sites of *ackA* [19] and *ilvB* (S. Tojo and Y. Fujita, unpublished). Interestingly, CCA of *ackA* involves not only the *cre* but also an approximately 20-bp region immediately upstream of it [21]. CCA of *ilvB* [37] and *pta* appear to also require some region upstream of their *cre*s. The *lev* operon is transcribed from the "-12, -24" promoter recognized by RNA polymerase containing σ^L, the *cre* site of which is located 43 bases upstream of the transcriptional initiation base [53, 54]. Expression of the *lev* operon is positively regulated by the transcriptional activator LevR, which binds to an activating sequence upstream of the *cre* [54]. The binding of the protein complex to the *lev cre* presumably prevents the activation by LevR interaction with the RNA polymerase through DNA looping. Moreover, a *cre* site of the *res* operon is located more than 70 bases upstream of the transcription initiation base, the binding of the protein complex to which evokes negative regulation of *res* expression [43], but the molecular mechanism underlying this negative regulation is not known.

The CcpA/P-Ser-HPr (or P-Ser-Crh) complex binds to a *cre* overlapping the promoter, interfering with the binding of the transcription machinery, as for *amyE* [3], *bglP* [26], *cccA* [27], *dctP* [29], *glpF* [32], *phoP* [42], and *acuA* [20]. The binding of the protein complex to a *cre* site located well downstream of the transcription initiation base is considered to block transcription elongation, as for most other operons listed in Table 1. This transcription roadblock was firstly demonstrated for the repression of *E. coli purB* containing an operator interacting with PurR in its reading frame [82]. PurR as well as CcpA belong to the LacI/GalR family of bacterial regulatory proteins, which are supposed to potentially possess the ability to cause this transcription roadblock. CCR of the *gnt* operon carrying a *cre* in the *gntR*-coding region is partially promoter-independent, and the amounts of the transcripts containing regions downstream of the *cre* considerably decrease on the addition of glucose [7], implying that transcriptional roadblock might be involved in this catabolite repression. This idea is supported by the finding [22] that a mutation of *mfd* encoding a transcription-repair coupling factor, Mfd [83],

relieves CCR of *hut* and *gnt* expression at the *cis*-acting *cre* sequences located downstream of their transcriptional start site, but does not affect CCR at the promoter-proximal *cre* sites such as in *amyE* [3] and *bglP* [26], suggesting that the Mfd protein displaces RNA polymerase stalled at downstream *cre* sites to which the CcpA/P-Ser-HPr (or -Crh) is bound. Nonetheless, CCR of *acsA* expression is not affected by an *mfd* mutation in spite of the location of *acsA* *cre* 44 bp downstream of the *acsA* transcriptional initiation sites, but CCR is relieved by it if the *cre* is placed 161 bp downstream of the initiation site. So, transcription roadblock occurring near the transcription initiation base might not require the Mfd protein. Mfd was also found to be involved in catabolite repression of *dra–nupC–pdp* expression [30]. Furthermore, CCR of *sigL* expression is likely exerted by transcription roadblock through CcpA binding to a *cre* in the *sigL*-coding region [59]. Contrary to the these findings, CCR of *xyl* expression through CcpA-binding to a *cre* in the *xylA*-coding frame has been reported to be unlikely to be regulated by the roadblock mechanism [84].

Among 44 *cre*s experimentally identified, 17 are located in the protein-coding regions of the target genes, so it would be interesting to know where these l7 *cre* sequences are localized in the three possible protein-coding frames. As shown in Table 1, the bases of positions +1, +4, +7, +10 and +13 of WTGNAARCGNWWWCA correspond to the first or second bases of codons in all cases except for a *cre* in *sigL*. The bases at these positions are W, N, or R, allowing more flexibility of the base species, whereas the first or second bases of codons in the protein-coding frames of the target genes require the less flexibility. The other bases of the *cre* consensus sequence are conserved, frequently corresponding to the third bases of codons in the protein-coding frames, where more base degeneracy is allowed. This fact implies the elegant harmony between the establishment of a *cre* sequence and the evolution of a functional protein encoded by a catabolite-repressive gene.

Metabolic networks mediated by CcpA

Determination of the complete genome of *B. subtilis* [76] has allowed the detection of many genes which are likely subject to CcpA-mediated CCR and CCA by means of transcriptome and proteome analyses [85-89] as well as an electronical search for the *cre* sequence in the genome sequence [23, 87]. The transcriptome and proteome analyses revealed that out of the nearly1,000 of the *B. subtilis* 4,107 protein genes whose expression in cells growing in a nutrient sporulation medium is actually detected on DNA microarrays or 2D gels, roughly 10% are repressed or activated more than 3-fold upon the addition of glucose to the medium [86], which implies that a total of several hundreds of genes might be regulated by glucose. More than a few of the candidate glucose-regulated genes searched for in the above transcriptome and

proteome analyses were experimentally proven to be under CcpA-mediated CCR or CCA through identification of their *cre* sequences. The total forty-four *cre* sequences including those described above have been experimentally identified so far (Table 1). To search for as many candidate *cre*s in the genome as possible, the *cre* sequences listed in Table 1 are classified into two groups (A and B), i.e. ones with and without a TG-CA palindromic pair in their *cre* sequence, respectively (Table 1). The *cre* sequences belonging to groups A and B have been separately subjected to the web-application of GRASP-DNA (http://www2.genomatica.com/grasp-dna/) [61], each search providing the rank numbers of the respective *cre*s out of the 100 *cre* sequences searched for. This ranking is indicated in Table 1, which shows that 5 *cre*s are out of rank. Out of the more than candidate 150 *cre*s resulting from the above two GRASP-DNA searches, at least 100 are supposed to function, if examined, because of the high sequence-dependency of this *cis*-element (*cre*), as described above. Therefore, this microorganism is assumed to carry nearly 150 *cre*s to regulate roughly 300 genes if two genes are under the control of each *cre* on average. The results of the transcriptome and proteome analyses described above implied various connections of central control of carbon metabolism with various metabolic networks. However, the following sections deal only with networks connecting the control of carbon metabolism with other metabolic regulation that has been experimentally verified. Also, it is notable that a CcpA-defective mutant of *B. subtilis* grows at a slower rate in a minimal medium with glucose and ammonium as carbon and nitrogen sources than the wild-type cells [90], which reveals an intimate connection between catabolism and anabolism as described below.

Carbon catabolite repression (CCR) of catabolism of secondary carbon and nitrogen sources, and anabolism of secondary products

CCR is when genes and operons involved not only in catabolism but also some anabolic processes such as the synthesis of certain extracelllular enzymes and secondary metabolites like antibiotics are not expressed as long as preferred sources of carbon and energy are present. As described above, hundreds of catabolic and anabolic genes and operons are supposed to be subject to CCR. Out of the genes and operons involved in carbon and nitrogen metabolism, only those known to be direct targets of the complex of CcpA and P-Ser-HPr are mentioned (Table 1 and Fig. 2).

The operons involved in the catabolism of secondary carbon sources are as follows, the carbon sources being given in parentheses; *gntRKPZ* (gluconate)[16, 34, 35], *xylAB* (xylose)[9], *iolABCDEFGHIJ* (*myo*-inositol)[39, 40], *trePAR* (trehalose)[23, 44, 94], *galKT* (galactose)[23], *glpFK* (glycerol)[32], *glvARC* (6-P-α-glucoside)[33], *bglPH* (β-glucoside) [25, 26], *yjlBCD-uxaC-yjmBCD-uxuA*-

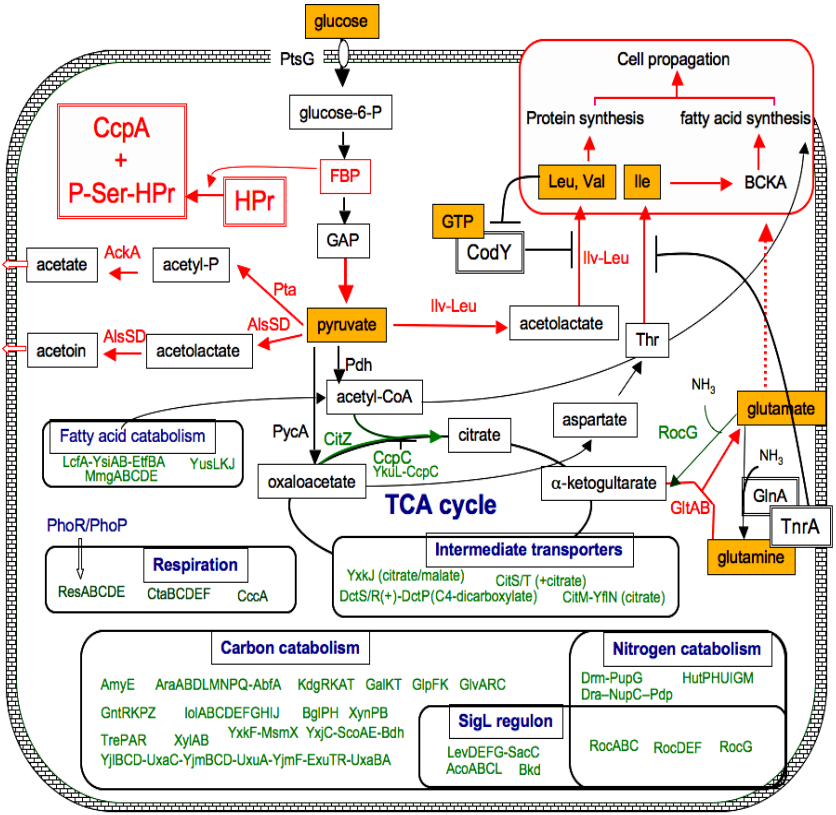

Figure 2. CcpA-mediated metabolic networks in *Bacillus subtilis*. The genes and operons subject to catabolite activation mediated by the complex of CcpA and P-Ser-HPr are indicated in red. This complex activates the *ackA* [19] and *pta* [56] genes directly, and the *alsSD* operon [91, 92] indirectly, which are involved in the formation of acetate and acetoin, which are major extracellular products of *B. subtilis* grown on glucose, respectively. It also triggers the expression of the *ilv-leu* operon involved in the biosynthesis of branched-chain amino acids directly [37, 38]. Besides, the complex indirectly triggers the expression of the *gltAB* operon encoding glutamate synthase [93]. On the other hand, the complex of CcpA and P-Ser-HPr repressed numerous genes and operons, indicated in green, which include many genes invoved in carbon, nitrogen and phosphate metabolism, *citZ* coding for the entrance enzyme of the TCA cycle, several genes encoding TCA cycle intermediate transporters, and the genes involved in respiration. Thus, the CcpA-mediated metablic networks play a major role in the coordinated regulation of catabolism and anabolism to ensure the optimum cell propagation under given growth conditions. The details are given in the text.

yjmF-exuTR-uxaBA (hexuronate) [23, 45], *xynPB* (β-xyloside)[60], *yxjC-scoAE-bdh (*β-hydroxybutyrate)[23, 46], *araABDLMNPQ-abfA* (arabinose) [24, 48, 49], and *kdgRKAT* (hexuronate)[23, 41]. The *yxkF-msmX* operon likely involved in the transport of unknown sugars has been proven to be under CcpA-mediated catabolite repression [23, 46]. Besides, the *amyE* gene encoding the extracellular α–amylase hydrolyzing starch [3, 5], and the *levDEFG-sacC* operon encoding a fructose-specific phosphotransferase system and extracellular levanase hydrolyzing fructose polymers and sucrose [54, 95] are also known to be subject to CcpA-mediated catabolite repression.

Various amino acids and nucleotides are utilized as carbon and nitrogen sources. The *hutPHUIGM* operon involved in histidine utilization is a direct target of CcpA [10, 11]. The *dra-nupC-pdp* [30] and *drm-pupG* [96] operons involved in deoxyribonucleoside metabolism are subject to CcpA-mediated catabolite repression; the former has been proven to be a direct target of CcpA. Moreover, the *sigL* gene encoding a σ^{54}-type factor of *B. subtilis* σ^{L} partly involved in nitrogen metabolism [95] is a direct target of CcpA [59]. The σ^{L} regulon contains the levanase operon, *levDEFG-sacC*, involved in fructose and levan metabolism [54, 95], three *rocABC*, *DEF*, and *G* operons associated with arginine catabolism [97-99], an *acoABCL* operon encoding the acetoin dehydrogenase complex [47, 100], and the seven-cistronic *bkd* operons [101] encoding enzymes involved in leucine and valine degradation. Out of these, *acoABCL*, *levDEFG-sacC*, and *rocG* and has been experimentally proven to carry the respective *cre* sequences [23, 53, 54, 58].

Enhancement of carbon flow, and shutdown of the TCA cycle and respiration

CcpA is indirectly involved in triggering of the synthesis of the enzymes encoded by the *gapA* operon, catalyzing the central part of glycolysis, including the steps of interconversion of triose phosphates from dihydroxyacetone-phosphate to phosphoenolpyruvate [85, 102, 103].

As shown in Table 1, all the *cre*s except the three for *ackA* [19], *pta* [56], and *ilv-leu* [37, 38] have been shown to be involved in negative regulation of catabolic and anabolic genes. The *pta* and *ackA* genes encode phosphotransferase and acetate kinase, respectively, which catalyze the conversion of acetyl-CoA to acetate via an acetyl~P intermediate. Acetate is one of the major by-products during the growth of *B. subtilis* cells in a rich medium containing rapidly metabolizable carbohydrates such as glucose. The *ilv-leu* operon is one of the major anabolic operons involved in the biosynthesis of branched-chain amino acids (isoleucine, valine and leucine)(BCAA). In addition, the *alsSD* operon [91], which is involved in acetoin biosynthesis, is known to be under CcpA-dependent positive regulation

[91, 92]. However, no *cre*-like sequence was found in the promoter region of the *alsSD* operon in our *cre* search involving a web-based GRASP-DNA application [61], implying that an unknown factor might be involved in this positive regulation [92]. The fate of pyruvate is a major concern for the cell [104]. So, the CcpA-dependent positive regulation of the *ackA* and *pta* genes, and the *alsSD* and *ilv-leu* operons appeared to play a certain similar role in reduction of the intracellular concentration of pyruvate accumulated during growth in a rich medium containing rapidly metabolizable carbon sources by means of enhancement of the excretion pathways for acetate (*ackA* and *pta*) and acetoin (*alsSD*), and BCAA biosynthesis from pyruvate (*ilv-leu*).

On the other hand, CcpA represses the expression of *citZ* encoding citrate synthetase to condense acetyl-CoA with oxaloacetate directly and decreases it indirectly through CcpA-induction of CcpC able to repress *citZ* expression [50,52]. This negative regulation of entrance to the TCA cycle allows cells to avoid the production of excess ATP as long as they can obtain enough ATP from glycolysis. Accordingly, the transport of the intermediates of the TCA cycle is also shut down. The *citM-yflN* operon involved in citrate transport is a direct target of CcpA-mediated catabolite repression [51], which is positively regulated by a two-component regulatory system, CitS/CitT, whose synthesis is also directly repressed by CcpA [28]. The *yxkJ* gene likely encoding a citrate/malate transporter is subject to CcpA-mediated repression [23, 44, 46]. Transport systems for C4-dicarboxylates, such as malate, fumarate and succinate, are encoded by *dctP*, whose expression is positively regulated by a two-component regulatory system encoded by *dctS*/*dctR* and is subject to CcpA-mediated catabolite repression [29].

The *B. subtilis* respiration system is severely repressed by glucose. The *resABCDE* operon indispensable for respiration encodes a three-protein complex involved in cytochrome *c* biogenesis [105] as well as the ResE sensor kinase and the ResD response regulator that control electron transfer and other functions in response to oxygen availability [106, 107]. This operon is subject to CCR, and is likely a direct target of CcpA [43]. Besides, the *cccA* gene encoding small cytochrome c_{550} has been proven to be glucose-repressed through the direct interaction of the CcpA/P-Ser-HPr complex [27].

The transcription of the *resABCDE* operon requires the PhoP/PhoR two-component system [108]. The PhoP~P response regulator directly binds to the *cis*-element of the *res* promoter and is essential for transcriptional activation of the *resABCDE* operon as well as being involved in repression of the internal *resDE* promoter during phosphate-limited growth. CcpA plays a significant role in the transcriptional regulation of the *phoPR* promoter, which is achieved through its direct binding to the *cre* sequence present in a *phoPR* promoter A6 [42].

Major link between carbon and nitrogen regulations

B. subtilis assimilates ammonium through the concerted actions of glutamine and glutamate synthetases. The expression of the *gltAB* operon encoding the latter enzyme depends on the accumulation of glycolytic intermediates, which cannot occur in the *ccpA* mutant [93], although no candidate *cre* was found in the GRASP-DNA search [61]. The lack of *gltAB* induction is the bottleneck that prevents growth of a *ccpA* mutant on glucose/ammonium media. On the other hand, the *rocG* gene, encoding catabolic glutamate dehydrogenase, was found to be subject to direct CcpA-dependent glucose repression [58]. The glutamate pool would be low in *ccpA* mutants due to the loss of CCR of *rocG*, which contributes to the slow growth rate in glucose/glutamate medium.

CcpA-mediated CCA of BCAA biosynthesis

BCAAs are the most abundant amino acids in proteins and form the hydrophobic cores of the proteins. Moreover, these amino acids are precursors for the biosynthesis of *iso*- and *anteiso*-branched fatty acids, which represent the major fatty acid species of the membrane lipids in *Bacillus* species [109]. The initial step of isoleucine or valine synthesis is the condensation of 2-oxobutanoate derived from threonine and pyruvate or two pyruvates, leading to the formation of branched-chain keto-acids [110]. Leucine is synthesized from one of the branched-chain keto acids, i.e. α-ketoisovalerate. The *B. subtilis ilv-leu* operon comprises seven genes (*ilvB, H, C, leuA, B, C,* and *D*) necessary for the biosynthesis of BCAAs [111]. Besides a plausible role of the CcpA-dependent positive regulation of *ilv-leu* in glycolysis to continuously proceed for draining of accumulated pyruvate, it is notable that this positive regulation links carbon metabolism to amino acid anabolism. Recent global gene expression studies on amino acid availability [112] and CodY regulation [113], as well as ones on metabolic links of *ilv-leu* expression to glucose and nitrogen metabolism [37, 38, 90, 114], revealed that the *ilv-leu* operon is under direct negative transcriptional control through two major global regulators of nitrogen metabolism (CodY and TnrA).

The CodY protein is a GTP-binding repressor of several genes that are normally quiescent when cells are growing in a nutrient-rich medium [115]. A high concentration of GTP activates the CodY repressor, which serves as a gauge of the general energetic capacity of the cells. CodY is also induced through direct interaction with BCAAs to bind to the promoter regions of its target genes including the *ilv-leu* operon for their repression [116]. Thus, the *in vivo* BCAA concentrations serve as a gauge of the nutrient conditions through the activation of CodY. TnrA is known to both activate and repress nitrogen-regulated genes during nitrogen-limited growth [117]. When nitrogen sources

are in excess, the concentrations of intracellular glutamine and other metabolites are thought to become high enough to cause feedback inhibition of glutamine synthetase (GlnA). The feedback-inhibited GlnA captures TnrA to form a protein–protein complex and thereby abolishes the DNA-binding ability of TnrA. By contrast, during nitrogen-limited growth, TnrA is released from the GlnA–TnrA complex and binds to its specific sites on DNA for the regulation of transcription. Thus, TnrA only exerts its regulatory function in cells grown under nitrogen-limited conditions [117].

It is notable that CcpA-dependent positive regulation of *ilv-leu* is associated with the respective counter negative regulation mediated by CodY and TnrA under both nitrogen-rich and -limited conditions. To achieve the full growth potential of rapidly metabolizable carbohydrates such as glucose, CcpA attempts to enhance the expression of *ilv-leu* to make the cell synthesize more BCAAs for rapid cell growth. However, when enough BCAAs are supplied from a nitrogen-rich medium, negative regulation exerted by CodY interacting with these amino acids overwhelms the CcpA-dependent positive regulation to prevent their excess synthesis for the maintenance of their appropriate concentrations *in vivo*. The repression of *ilv-leu* expression through CodY is overwhelming regardless of whether or not the CcpA-dependent positive regulation occurrs. On the other hand, when cells are grown in a nitrogen-limited medium containing glutamate as the sole nitrogen source, TnrA decreases the CcpA-dependent positive regulation to adjust the amounts of BCAAs in response to a poor nitrogen supply. Branched-chain amino acids are the most abundant amino acids in proteins, and precursors for the biosynthesis of *iso*- and *anteiso*-branched-chain fatty acids. The intracellular BCAA concentrations appear to serve as a gauge of the nutrient conditions, that is, a pacemaker of the synthesis of proteins and membranes probably representing cell growth. Thus, global regulators of cellular metabolism (CcpA, CodY, and TnrA), each of which controls the expression of a certain set of numerous catabolic and anabolic genes, participate in transcription regulation of the *ilv-leu* operon, unlike in the regulation of other biosynthetic pathways, in which only their own feedback systems are generally involved. Therefore, BCAA biosynthesis is elaborately regulated according to the cellular energetic and nutritional conditions through the intracellular concentrations of the signal compounds of individual global regulators, such as GTP, BCAAs, glutamine and fructose-bisphosphate. Thus, the elegant regulation of *ilv-leu* most likely plays a central role in linking catabolism to anabolism in the overall metabolism of *B. subtilis*.

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