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Positive regulation of *Bacillus subtilis ackA* by CodY and CcpA: establishing a potential hierarchy in carbon flow

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Summary

Conversion of pyruvate to acetate via the phosphotransacetylase-acetate kinase pathway generates ATP and is a major overflow pathway under conditions of carbon and nitrogen excess. In *Bacillus subtilis*, this pathway is positively regulated by CcpA, a global regulator of carbon metabolism genes. Transcription of the acetate kinase gene (*ackA*) proved to be activated as well by a second global regulatory protein, CodY. Expression of an *ackA–lacZ* fusion was reduced in a *codY* mutant strain. CodY was found to bind *in vitro* to two sites in the *ackA* promoter region and to stimulate *ackA* transcription in a run-off transcription assay. This is the first known case of direct positive regulation by CodY. CodY and CcpA were found to bind to neighbouring sites and their effects were additive both *in vivo* and *in vitro*. Surprisingly, positive regulation by CodY, unlike repression, responded primarily to only one type of effector molecule. That is, branched-chain amino acids (BCAAs) served as more potent co-activators of CodY-dependent *ackA* transcription than did GTP. Given the roles of CcpA and CodY in regulating genes whose products determine the metabolic fate of pyruvate, these two proteins may act together to mediate a hierarchical conversion of pyruvate to its many potential products.

Introduction

When offered a choice of multiple potential carbon sources, many bacteria respond by using one carbon source preferentially (Monod, 1942). For most bacteria, the preferred carbon source is glucose, presumably because it enters the central metabolic pathway without conversion to other compounds. A variety of mechanisms allow bacteria to regulate gene expression in such a way as to limit synthesis of enzymes for utilization of alternative carbon sources when the preferred carbon source is available (Monod, 1942; Saier, 1996; Saier and Ramseier, 1996; Crasnier-Mednansky *et al*., 1997; Kimata *et al*., 1997; Deutscher *et al*., 2002). In the Gram-positive bacterium *Bacillus subtilis*, the major regulator of gene expression in response to glucose availability is the carbon catabolite-response protein A (CcpA) (Henkin, 1996). Homologues of CcpA are found in many Gram-positive bacteria (Titgemeyer and Hillen, 2002).

CcpA is a global regulatory protein that is active in glucose-grown cells and is stimulated by the serine-phosphorylated forms of one of two alternative accessory proteins, HPr and Crh (Fujita *et al*., 1995; Voskuil and Chambliss, 1996; Galinier *et al*., 1997), to control the expression of many genes involved in carbon source utilization (Moreno *et al*., 2001; Yoshida *et al*., 2001). Phosphorylation of HPr and Crh is mediated by HprK, a kinase whose activity is stimulated by fructose 1,6-bisphosphate, an intermediate in glycolysis (Deutscher *et al*., 2002). CcpA binds to specific DNA sequences called catabolite repression elements (*cre* sites) (Deutscher *et al*., 2002). In *B. subtilis*, CcpA represses genes that encode proteins needed for utilization of alternative carbon sources and enzymes of the Krebs cycle, and positively regulates genes for glycolytic enzymes and carbon overflow pathways (Fig. 1) (Grundy *et al*., 1993a; Henkin, 1996; Saier *et al*., 1996; Turinsky *et al*., 1998; Kim *et al*., 2002).

CcpA also activates expression of the *B. subtilis ilvB* operon (Fig. 1) (Shivers and Sonenshein, 2005; Tojo *et al*., 2005), an operon involved in branched-chain amino acid (BCAA) biosynthesis (Fink, 1993). The *ilvB* operon is subject to multiple levels of regulation, including leucine-responsive transcription termination in the mRNA 5′ untranslated region (Grandoni *et al*., 1993) and regulated mRNA degradation (Mäder *et al*., 2004). The BCAA biosynthetic pathway has a second function insofar as it can serve as an overflow pathway for pyruvate that accumulates intracellularly when cells are growing in excess

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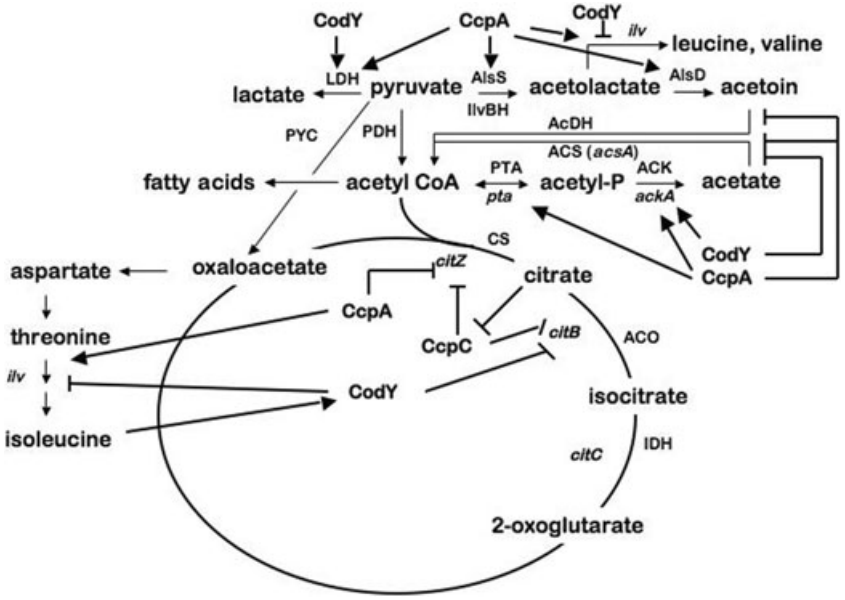

Fig. 1. Interconnections of CcpA and CodY regulons in carbon metabolism. Enzyme abbreviations as follows: AlsS and IlvBH, α-acetolactate synthetase; AlsD, α-acetolactate dehydrogenase; AcDH, acetoin dehydrogenase; ACS, acetyl CoA synthetase; PDH, pyruvate dehydrogenase; PTA, phosphotransacetylase; ACK, acetate kinase; CS, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; PYC, pyruvate carboxylase, LDH, lactate dehydrogenase. Genes coding for relevant enzymes are italicized. Bold lines with arrowheads indicate positive regulation by CcpA or CodY; bold blunted lines indicate repression of gene expression by CcpA or CcpC or CodY.

glucose (Fink, 1993), a fact that may explain positive regulation of *ilvB* by CcpA.

By activating expression of the *ilvB* operon, CcpA also influences the activity of CodY, another global transcriptional regulator, whose activity is stimulated, in part, by intracellular BCAA levels (Shivers and Sonenshein, 2005). CodY is a repressor of the *ilvB* operon and of more than 100 other transcription units (Molle *et al*., 2003). The complex relationship between CcpA and CodY in regulation of the *ilvB* operon suggested to us that CodY might play a more general role in carbon utilization in *B. subtilis.*

Bacillus subtilis CodY protein (Slack *et al*., 1995; Serror and Sonenshein, 1996) represses, during rapid exponential growth, many genes that are induced in late exponential phase or early stationary phase. The products of these genes include proteins that allow cells to adapt to general nutrient limitation (Molle *et al*., 2003). Homologues of CodY are found in many low G+C Gram-positive bacteria (Ratnayake-Lecamwasam *et al*., 2001; Guédon *et al*., 2005; den Hengst *et al*., 2005; Malke *et al*., 2006). *B. subtilis* CodY is activated as a repressor by the high intracellular levels of GTP and BCAAs found in rapidly growing exponential phase cells (Ratnayake-Lecamwasam *et al*., 2001; Shivers and Sonenshein, 2004). The decrease in the intracellular GTP and BCAA levels that occurs during the transition from rapid exponential growth to stationary phase (Lopez *et al*., 1981; Soga *et al*., 2003) or upon the induction of the stringent response (Inaoka and Ochi, 2002) causes CodY to lose repressing activity, permitting transcription of its target genes (Ratnayake-Lecamwasam *et al*., 2001).

During exponential growth in a rich medium containing glucose and a mixture of amino acids, CcpA, CodY and a third protein, CcpC, cooperate to repress the genes that encode the first three enzymes of the Krebs cycle, thereby blocking a second potential overflow pathway for pyruvate (Fig. 1) (H.-J. Kim *et al*., 2002; 2003; S.-I. Kim *et al*., 2003). Other overflow pathways for pyruvate lead to the production of acetate and acetoin, both of which can be excreted into the environment (Deutscher *et al*., 2002). Acetate is produced from pyruvate via acetyl CoA by the products of the *pdhABCD* (pyruvate dehydrogenase), *pta* (phosphotransacetylase) and *ackA* (acetate kinase) genes (Deutscher *et al*., 2002). The *pta* and *ackA* genes are known to be positively regulated by CcpA (Turinsky *et al*., 1998; Moir-Blais *et al*., 2001); *ackA* has two *cre* sites upstream of the promoter region, only one of which (*cre2*) is required for CcpA binding (Grundy *et al*., 1993a; Turinsky *et al*., 1998). However, another factor must be involved as well, as sequences between the two *cre* sites are required for full activation of *ackA* transcription, but not for binding of CcpA to the *ackA* promoter region (Turinsky *et al*., 1998; Moir-Blais *et al*., 2001). Most putative CodY targets and all proved CodY targets analysed to date are repressed by CodY, but microarray data suggested that transcription of a few genes, including *ackA*, might be positively regulated by CodY (Molle *et al*., 2003) (http://www.genome.jp/dbget-bin/www_bget?exp+ex0000381). We therefore sought to determine whether CodY acts directly at the *ackA* promoter and whether CodY and CcpA control *ackA* independently (i.e. additively) or synergistically or competitively.

We show here that CodY is a direct positive regulator of *ackA* expression. This finding represents the first confirmation of positive regulation by CodY. CodY was able to bind with high affinity to a specific sequence within the

ackA promoter region and this binding was stimulated by BCAAs, as is the case for other CodY-regulated genes. Moreover, pure CodY protein stimulated *ackA* transcription *in vitro* in a BCAA-dependent manner. CodY and CcpA were shown to act additively to cause full activation of the *ackA* promoter both *in vitro* and *in vivo*.

Results

In vivo *regulation of* ackA *transcription*

Microarray analysis indicated that the *ackA* gene is underexpressed three- to fourfold in a *codY* null mutant strain compared with its wild-type parent (http://www.genome.jp/dbget-bin/www_bget?exp+ex0000381). (The same microarray analysis showed a 1.7-fold decrease in *pta* expression in a *codY* mutant, but that effect has not been explored further here.)

To verify that *ackA* is a positively regulated CodY target *in vivo*, an *ackA–lacZ* fusion was introduced into wild-type and *codY* mutant strains. Cells were grown in minimal medium supplemented with glucose, glutamine and a 16-amino-acid mixture (Atkinson *et al*., 1990) that is known to stimulate CodY activity (Shivers and Sonenshein, 2004) and samples were taken for β-galactosidase assays. The wild-type strain [~160 Miller units (MU)] (Fig. 2A) showed an approximately fourfold higher level of β-galactosidase activity throughout exponential growth phase than did the *codY* mutant (~45 MU) (Fig. 2A), suggesting that CodY is indeed a positive regulator of *ackA* expression.

To test whether regulation by CodY is independent of regulation by CcpA, the level of expression from the *ackA* promoter was compared in wild type, *codY* mutant, *ccpA* mutant and *codY ccpA* double mutant strains. Cells were grown in the same minimal medium. The *ccpA* mutant strain (~45 MU) (Fig. 2A) had a level of expression that was lower than that of the wild-type strain and quite similar to that of the *codY* mutant strain. The *ccpA codY* double mutant (~15 MU) showed ~10-fold lower expression than did the wild type (Fig. 2A) and threefold lower than did either the *codY* or *ccpA* single mutant strains, suggesting that both proteins are necessary for full expression of the *ackA* promoter.

BCAAs increase the activity of CodY at promoters at which CodY acts as a negative regulator. To see whether positive regulation by CodY also responds to BCAAs, strains were grown in minimal medium supplemented with glucose, glutamine and a 13-amino-acid mixture lacking the BCAAs. Cells were collected in mid-exponential growth phase (OD ~0.5) and then resuspended in the same medium or in the same medium supplemented with the BCAAs. Cells grown continuously in the absence of the BCAAs showed ~80 MU of activity (Fig. 2B, –ILV),

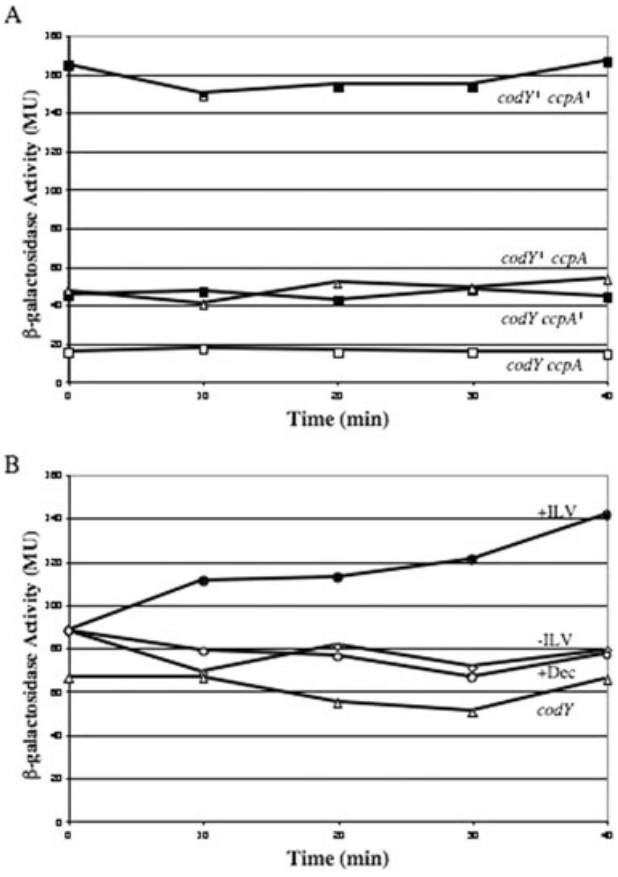

Fig. 2. Effects of *codY* and *ccpA* mutations on *ackA* expression.
A. Cells of wild-type or mutant strains were grown in minimal medium supplemented with glucose, glutamine and a mixture of 16 amino acids (see *Experimental procedures*) and samples harvested at the indicated times after resuspension in fresh medium were assayed for β-galactosidase activity [shown in Miller units (MU)].
B. Cells were grown in minimal medium supplemented with glucose, glutamine and a mixture of 13 amino acids lacking the BCAAs. At time 0, the cells were resuspended in the same medium (–ILV) or the same medium with decoyinine (+Dec) or the same medium with the BCAAs added (+ILV). These experiments were performed three times. The graphs shown are representative of the results obtained.

consistent with previous results showing that removal of the BCAAs from the growth medium partially inactivates CodY (Shivers and Sonenshein, 2004). The addition of BCAAs to the growth medium resulted within 40 min in an increase in expression to a level of ~140 MU (Fig. 2B, +ILV). A *codY* mutant strain did not respond to such changes in the growth medium (data not shown), suggesting that CodY is the protein responsible for this BCAA-induced expression.

Additionally, the removal of BCAAs from cells previously grown in medium containing the BCAAs should lead to a decrease in *ackA* expression. To test this hypothesis we grew cells in minimal medium supplemented with glucose, glutamine and 16 amino acids. When the cells reached mid-exponential growth phase (OD ~0.5), they were col-

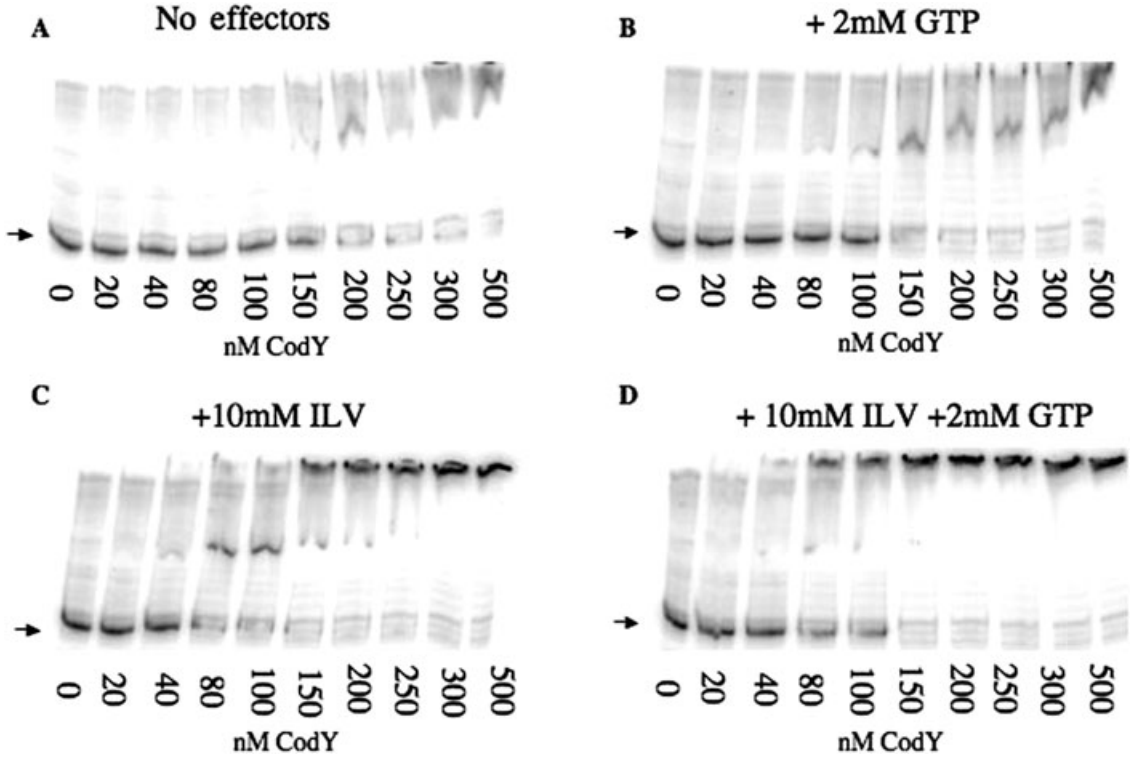

Fig. 3. *In vitro* binding of CodY to the *ackA* promoter. CodY and a 503 bp DNA fragment containing the *ackA* promoter region were incubated as described in *Experimental procedures* in the absence of effectors (A) or with 2 mM GTP (B) or with 10 mM each of isoleucine, leucine and valine (ILV) (C) or with 2 mM GTP and 10 mM ILV (D). The arrow indicates the position of free DNA. CodY concentrations are given with respect to the protein monomer. For cases in which ILV was added, the electrophoresis buffer was supplemented with 10 mM each of isoleucine, leucine and valine.

lected and resuspended in the same complete medium or in medium lacking the BCAAs. When the BCAAs were removed from the growth medium, there was an approximately twofold decrease in the level of β-galactosidase activity over the course of 40 min (data not shown).

The effect of BCAAs on the activity of CodY at the *ackA* promoter was consistent with the effect seen at promoters that are repressed by CodY (Shivers and Sonenshein, 2004). The apparent effect of GTP, however, was different. When strains carrying the *ackA–lacZ* fusion were grown in minimal medium supplemented with BCAAs and then resuspended in the same medium containing decoyinine, a GMP synthetase inhibitor that lowers the intracellular GTP pools (Lopez *et al*., 1979), the level of β-galactosidase activity was reduced, as expected, to an extent similar to that seen upon removal of BCAAs from the medium (data not shown). However, the addition of decoyinine to the growth medium lacking the BCAAs did not cause a significant change in β-galactosidase activity (Fig. 2B). The lack of an effect by decoyinine in medium lacking the BCAAs suggests that GTP contributes to the activity of CodY as a positive regulator at the *ackA* promoter, but is not by itself sufficient to activate CodY.

In vitro *DNA binding at the* ackA *promoter*

To determine directly whether CodY is able to bind to the *ackA* promoter, we performed gel mobility shift assays using purified CodY and a 503 bp end-labelled DNA fragment containing the *ackA* promoter. The affinity of CodY for the *ackA* promoter was tested in the absence of effectors or in the presence of 2 mM GTP or 10 mM BCAAs or 2 mM GTP and 10 mM BCAAs. The addition of 2 mM GTP to the binding reaction had a small effect on the affinity of CodY for the promoter region (Fig. 3B). In contrast, the presence of 10 mM BCAAs increased the affinity of CodY several fold, giving an apparent K_D of ~80 nM monomeric CodY (apparent K_D is defined as the concentration of CodY that causes 50% of the DNA to be shifted) (Fig. 3C). The simultaneous addition of GTP and BCAAs did not increase further the affinity of CodY for the *ackA* promoter region compared with the effect of BCAAs alone (Fig. 3C and D). The presence of both GTP and BCAAs did create, however, a more slowly migrating *ackA*–CodY complex (Fig. 3D). The greater effect of the BCAAs compared with GTP in determining the affinity of CodY for the *ackA* promoter is consistent with the *in vivo* results that showed that

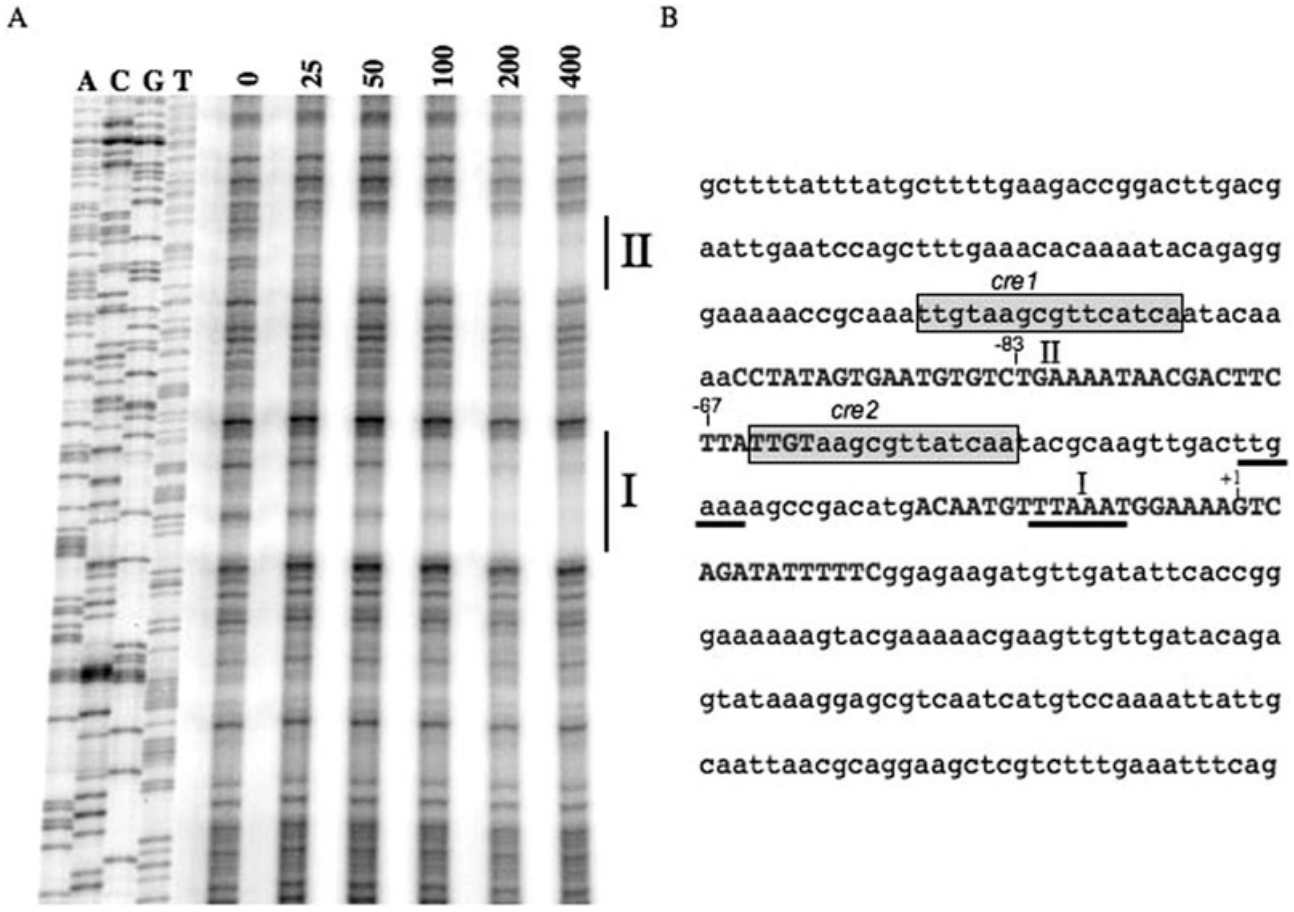

Fig. 4. DNase I protection assay for binding of CodY to the *ackA* promoter region.
A. Binding of CodY to a ^{32}P-labelled DNA fragment containing the *ackA* promoter region was carried out in the presence of 10 mM ILV and 2 mM GTP as described in *Experimental procedures* and the protein–DNA complexes were then treated with DNase I before denaturation and separation of the fragments by PAGE. The concentration of CodY varied, as indicated. Protected Regions I and II are marked by the vertical bars. A DNA sequence ladder corresponding to the template strand is shown to the left.
B. Sequence of the *ackA* promoter region. Regions I and II protected by CodY are shown in bold. The transcription start point (+1) is indicated and the −10 (TTAAAT) and −35 (ttgaaa) regions of the promoter are underlined. The *cre* sites are shown as shaded grey boxes (Grundy *et al*., 1993a; Turinsky *et al*., 1998).

reducing the GTP pool is relatively ineffective in the absence of BCAAs (Fig. 2C, +Dec).

DNase I footprinting of the ackA *promoter*

To identify the sequence within the *ackA* promoter region that is bound by CodY, we performed DNase I footprinting experiments. CodY was incubated with the 503 bp *ackA* promoter-containing DNA in the presence of 10 mM BCAAs and 2 mM GTP over a range of CodY concentrations.

Analysis of the footprints identified two protected regions (Fig. 4A). The higher affinity site (Region II) showed some protection at 25 nM CodY and was fully protected at 100 nM CodY. Region II spans the region from positions −61 to −100 with respect to the transcription start site. This binding site lies between the two *cre* sites that span the regions from positions −123 to −109 (*cre1*) and −64 to −50 (*cre2*) (Moir-Blais *et al*., 2001). The 3′ end of the CodY-protected region slightly overlaps with *cre2*, the functional *cre* site (Turinsky *et al*., 1998; Moir-Blais *et al*., 2001) (Fig. 4B). The CodY-protected Region II includes the sequence shown to be important for CcpA-independent activation of *ackA* transcription (Turinsky *et al*., 1998; Moir-Blais *et al*., 2001).

A second CodY binding site (Region I) was seen at slightly higher protein concentrations and corresponds to positions −20 to +14 with respect to the transcription start site. How CodY is able to bind within the −10 region and still act as a positive regulator is unclear, although there is precedence in *Escherichia coli* with the MerR protein (O'Halloran *et al*., 1989).

To define the roles that BCAAs and GTP have in increasing the affinity of CodY for the *ackA* promoter

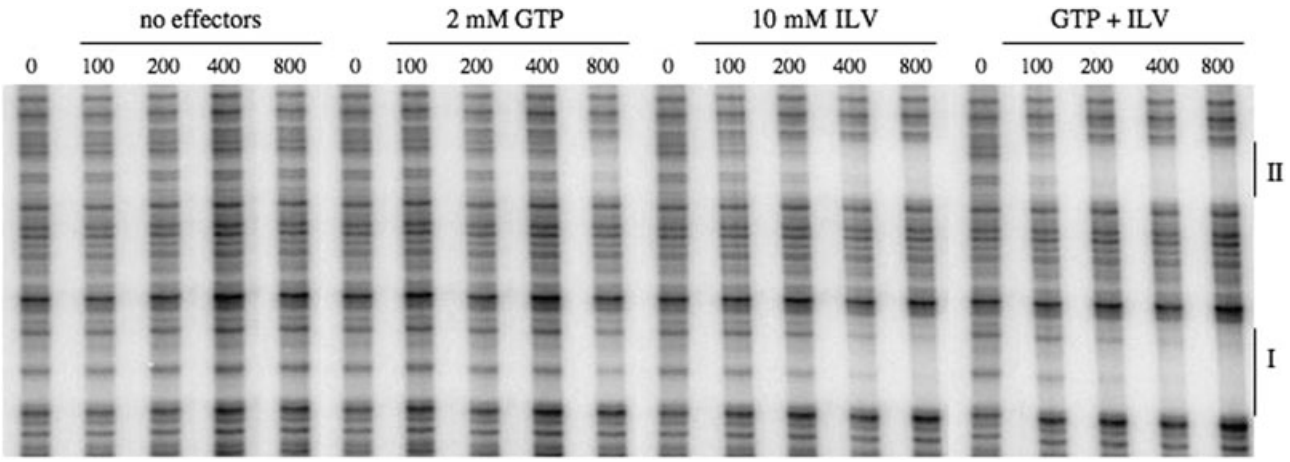

Fig. 5. DNase I protection assay for *ackA* in the presence of CodY and its effectors. Binding of CodY was carried out in the absence of effectors or in the presence of 2 mM GTP or in the presence of 10 mM ILV or in the presence of 2 mM GTP and 10 mM ILV. Concentrations of CodY varied from 0 to 800 nM. The 0 protein sample (leftmost lane) was copied and inserted between each set of reactions to facilitate comparisons. The addition of BCAAs and GTP had no effect on DNase I digestion in the absence of CodY (see Fig. 3).

region, DNase I footprinting experiments were carried out in the absence of effectors or in the presence of GTP or BCAAs or GTP and BCAAs (Fig. 5). As with the gel mobility shift assays the addition of GTP did not greatly increase the affinity of CodY for either Region I or Region II. On the other hand, the addition of BCAAs to the binding reaction caused protection of both Regions I and II at a lower CodY concentration than in the absence of effectors. When BCAAs were present, the addition of GTP did increase the affinity of CodY for the two regions, suggesting that GTP does play a role in activating CodY at the *ackA* promoter but can only do so in the context of enhancing the effect of BCAAs.

The fact that the CodY binding sites were in close proximity to and overlapped with the known CcpA binding site raised questions about how the two proteins interact at the *ackA* promoter. Binding of CcpA to the *ilvB* promoter region prevents CodY binding, thereby interfering with the ability of CodY to act as a repressor of the *ilvB* operon (Shivers and Sonenshein, 2005). From the *in vivo* results (Fig. 1) CodY and CcpA both appear to activate *ackA* expression, suggesting that both proteins should be able to bind simultaneously to the *ackA* promoter region. To test for simultaneous binding, DNase I protection assays were performed with a mixture of CcpA and CodY (in the presence of 10 mM BCAAs and 2 mM GTP). When both proteins were present (Fig. 6, lane 2) a protection pattern consistent with binding of both CcpA (Fig. 6, lane 1) and CodY (Fig. 6, lane 3) was seen. Thus, CodY and CcpA appear able to bind to the *ackA* promoter simultaneously.

In vitro *transcription from the* ackA *promoter*

The fact that CodY binds to the *ackA* promoter region led us to question whether CodY is able to stimulate transcription from this promoter. In *in vitro* transcription assays using *B. subtilis* RNA polymerase and the 503 bp DNA fragment containing the *ackA* promoter region as template, a transcript of the appropriate size (296 nt) was detected without the addition of any protein (Fig. 7). The ability of CodY to stimulate transcription in the presence of BCAAs and GTP was examined over a range of protein concentrations. At 20 nM CodY, transcript levels were increased three- to fourfold compared with transcript levels in the absence of CodY.

Previous investigations showed that CcpA is required for maximal *ackA* expression *in vivo* and that CcpA binds

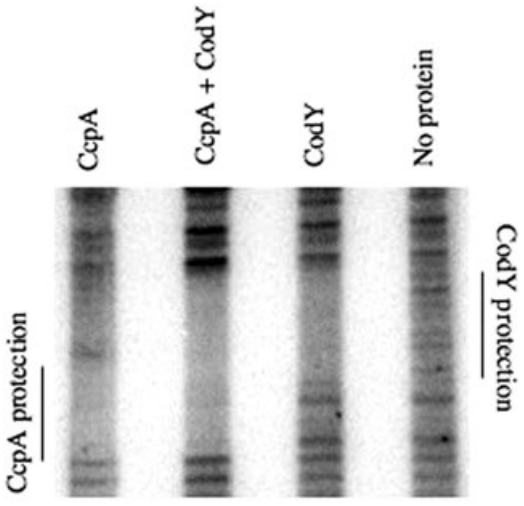

Fig. 6. DNase I protection assay for *ackA* in the presence of CodY and CcpA. Binding reactions were carried out in CcpA-binding buffer (see *Experimental procedures*) supplemented with 2 mM GTP and 10 mM ILV. The protein concentrations used were: lane 1, 400 nM CcpA; lane 2, 200 nM CcpA and 300 nM CodY; lane 3, 300 nM CodY; lane 4, no protein added.

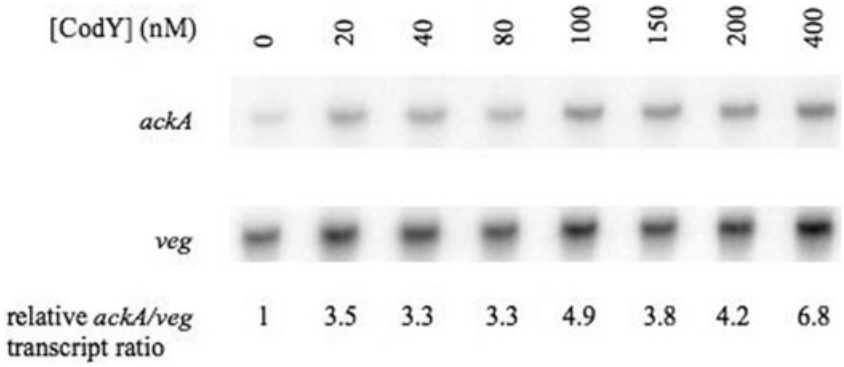

Fig. 7. *In vitro* transcription of the *ackA* promoter. *In vitro* transcription reactions were carried out using as templates a 503 bp DNA sequence containing the *ackA* promoter region that produces a 196 nt run-off RNA transcript and the 396 bp DNA sequence that contained the *veg* promoter region and produces a 136 nt run-off RNA transcript. Both DNA sequences were incubated with 0–400 nM CodY as indicated. All reactions were carried out in the presence of 10 mM BCAAs and 2 mM GTP. For each transcription reaction the ratio of *ackA* transcript to *veg* transcript was determined. The relative *ackA* transcript level was defined as the *ackA/veg* ratio for each sample containing CodY divided by the ratio obtained in the absence of CodY.

to the *ackA* promoter region (Grundy *et al*., 1993a; Turinsky *et al*., 1998; Moir-Blais *et al*., 2001). To test whether CcpA directly activates *ackA* transcription, run-off *in vitro* transcription assays were performed with purified CcpA. When either 1.5 μM CcpA or 200 nM CodY (in the presence of 10 mM BCAAs and 2 mM GTP) was added, the level of the transcript increased 1.3- to 1.6-fold (Fig. 8).

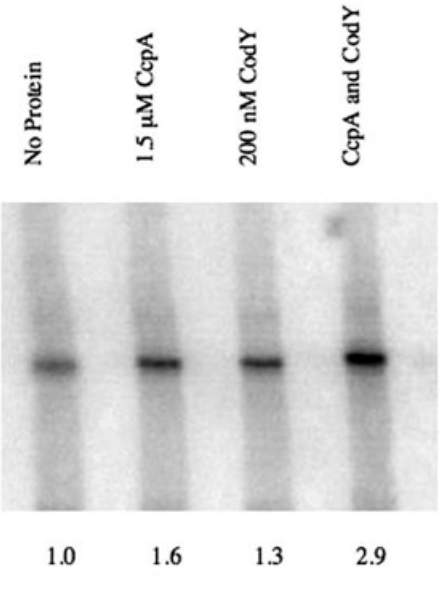

Fig. 8. *In vitro* transcription in the presence of CodY and CcpA. *In vitro* transcription reactions were carried out using the 503 bp *ackA* promoter-containing DNA sequence (see legend to Fig. 7) that was incubated with no protein or 1.5 μM CcpA or 200 nM CodY or 1.5 μM CcpA and 200 nM CodY. All reactions were carried out in the presence of 10 mM BCAAs and 2 mM GTP. This experiment was performed multiple times and the picture is of a representative gel. Relative *ackA* transcript level for this experiment was defined as the ratio of the amount of transcript obtained in samples containing CodY or CcpA to that obtained without added regulatory proteins.

(Note that the preparation of RNA polymerase used for this experiment was less active than that used for the experiment of Fig. 7). Thus, both CodY and CcpA are able independently to stimulate transcription from the *ackA* promoter. [The effect of CcpA may have been underestimated as the positive regulatory activity of CcpA *in vivo* depends on HPr and Crh proteins (Turinsky *et al*., 1998).] Consistent with *in vivo* results (Fig. 2A), when both CodY and CcpA were added to the same *in vitro* transcription reaction, the level of transcript was greater than in the presence of either protein alone (Fig. 8). Thus, simultaneous binding of CodY and CcpA to the *ackA* promoter region allows the proteins to act additively in activating transcription of the *ackA* promoter.

Discussion

Our results show that CodY acts as a positive regulator of the *ackA* gene and does so independently of and additively with CcpA. Full expression from the *ackA* promoter *in vivo* and *in vitro* requires both CodY and CcpA. Whereas GTP is a co-repressor for genes that are negatively regulated by CodY, GTP does not appear to be a major effector of CodY binding to the *ackA* promoter *in vitro* in the absence of BCAAs. Moreover, the partial deactivation of CodY *in vivo* by BCAA limitation renders the expression of *ackA* insensitive to decoyinine-induced GTP limitation. This relative insensitivity to the GTP pool suggests that the expression of *ackA* is primarily dependent on BCAA levels and only secondarily on GTP levels. Binding of CodY to every other *B. subtilis* CodY-regulated promoter tested to date has proved to be enhanced by both GTP and BCAAs. The relative inability of GTP to stimulate binding of CodY to the *ackA* promoter region in the absence of BCAAs suggests that CodY-regulated promoters may differ in their responsiveness to the GTP and

BCAA pools. The mechanisms by which the effectors stimulate binding of CodY to a given promoter are unclear, but these results suggest that BCAAs and GTP work independently and do not induce exactly the same conformational change in CodY. In fact, a significant conformational change induced by BCAAs is detectable by partial proteolysis experiments (Levdikov *et al*., 2006), but no substantial GTP-induced change in conformation can be detected by such experiments (P. Joseph, L.D. Handke and A.L. Sonenshein, unpublished). Interestingly, *Lactococcus lactis* CodY protein responds to BCAAs but not at all to GTP (Guédon *et al*., 2005; den Hengst *et al*., 2005).

While our results indicate that both CodY and CcpA are direct activators of transcription *in vitro* from the *ackA* promoter, the effect of a *ccpA* mutation *in vivo* may be the sum of both direct and indirect effects of CcpA. As CcpA stimulates BCAA synthesis (Shivers and Sonenshein, 2005; Tojo *et al*., 2005), raising intracellular BCAA pools and thereby making CodY more active, a *ccpA* mutation results in partial deactivation of CodY and reduced stimulation of *ackA* transcription.

The primary CodY binding site in the *ackA* promoter region lies at positions −100 to −61 with respect to the transcriptional start site. This location fits well with classical models of positive regulation. Moreover, multiple point mutations between positions −83 and −67 (i.e. between *cre1* and *cre2*) were shown to decrease *ackA* expression (Moir-Blais *et al*., 2001). A lower affinity CodY binding site, however, overlaps with the promoter −10 sequence and the transcription start site. It would be unusual for CodY to act as a positive regulator while bound to both sites. (Note that the centres of the two protected regions are 77 bp apart, indicating that CodY proteins would be on the same face of the DNA helix when bound to both regions.) One possibility is that CodY and RNA polymerase bind to opposite faces of the DNA helix and that CodY either stabilizes binding of RNA polymerase or alters the structure of the promoter region to facilitate melting of the DNA. It is also surprising that the promoter for *ackA* appears to have −10 (TTAAAT) and −35 (TTGAAA) regions with close-to-consensus sequences and optimal spacing and yields a transcript without the addition of any positive regulator. Thus, neither CcpA nor CodY is required for *ackA* transcription, but they act together to raise *ackA* expression to a higher level.

The cellular role of acetate kinase is twofold. The coupled activities of phosphotransacetylase and acetate kinase generate ATP by substrate-level phosphorylation. This activity can be an important source of ATP during fermentative growth. In addition, excretion of acetate is a means of removing excess end-products of glucose metabolism (e.g. pyruvate and acetyl CoA). In fact, acetate is the primary end-product of pyruvate when cells are grown with limiting phosphate (excess carbon and nitrogen) (Dauner *et al*., 2001). As we show here, expression of *ackA* is stimulated by CcpA (when cells have excess glucose) and by CodY (when cells have accumulated BCAAs and GTP).

The fate of pyruvate is a major concern for the cell (Sauer and Eikmanns, 2005). Pyruvate is a direct precursor of alanine, valine and leucine, and, through conversion to oxaloacetate, of aspartate, asparagine, isoleucine, threonine, lysine, diaminopimelate and methionine. Synthesis of oxaloacetate is also required to prime the Krebs cycle. Other possible fates of pyruvate in *B. subtilis* are conversion to lactate or acetyl CoA. Lactate production by lactate dehydrogenase is important for regeneration of NAD^+ from the NADH that accumulates during glycolysis.

Acetyl CoA also has multiple possible fates, including ligation to oxaloacetate to form citrate for metabolism through the Krebs cycle, utilization as a precursor for fatty acid and amino acid biosynthesis, and conversion to acetate under conditions of glucose excess. Thus, the involvement of both CodY and CcpA in regulating biosynthesis of isoleucine, leucine and valine (Shivers and Sonenshein, 2004; 2005; Tojo *et al*., 2005), the activity of the tricarboxylic acid (TCA) branch of the Krebs cycle (H.-J. Kim *et al*., 2002; 2003; S.-I. Kim *et al*., 2003), and acetate excretion suggests that these two proteins play a significant role in controlling cellular carbon flow (Fig. 1). [Microarray analysis suggests that CodY and CcpA are both positive regulators of *lctE*, the lactate dehydrogenase gene (Moreno *et al*., 2001; http://www.genome.jp/dbget-bin/www_bget?exp+ex0000381)].

CcpA represses the expression of Krebs cycle genes both directly (*citZC*) and indirectly (*citB*) when glucose is in excess (Kim *et al*., 2002; H.-J. Kim *et al*., 2003). Activated CodY also contributes to repression of the TCA branch of the Krebs cycle (H.-J. Kim *et al*., 2003). Perhaps to compensate for the failure to direct acetyl CoA to the Krebs cycle under such conditions, CcpA positively regulates the expression of the *ilvB* operon. Proteins encoded by the *ilvB* operon catalyse the condensation of pyruvate with either itself or α-ketobutyrate to initiate the synthesis of branched-chain keto acids, the precursors of the branched-chain fatty acids found in membranes, and BCAAs, which can serve as carbon and nitrogen storage molecules. Stimulation of the *ilvB* operon by CcpA, however, is self-limiting, as the consequent rise in BCAA pools leads to repression of the same operon by CodY. This interplay of CcpA, BCAAs and CodY would determine a level of *ilvB* expression that is constant as long as glucose is in excess. If this level of expression is insufficient to deplete accumulated pyruvate, other pathways would need to be activated.

Another carbon overflow pathway leads to production and excretion of acetoin. The *alsSD* operon, which encodes an α-acetolactate synthase (similar to IlvBH) and α-acetolactate decarboxylase, is required for the produc-

Table 1. *Bacillus subtilis* strains used.

Strain	Genotype	Source or reference
HKB5	*ccpA::*Tn*917::ble*	Kim *et al.* (2002)
ACKLAC-E	*lys-3 trpC2* SPβc2 Δ2::Tn*917*::pSK10Δ6::Φ[*(ackA–lacZ) cat*] *erm*	Grundy *et al.* (1993a)
SMY	Prototroph	P. Schaeffer
HKB7[a]	SPβc2 Δ2::Tn*917*::pSK10Δ6::Φ[*(ackA–lacZ) cat*] *erm*	SMY X ACKLAC-E DNA
RPS40	*codY::spc*	Shivers and Sonenshein (2005)
RPS51	*met*[+]	Shivers and Sonenshein (2005)
RPS86	SPβc2 Δ2::Tn*917*::pSK10Δ6::Φ[*(ackA–lacZ) cat*] erm	RPS51 X HKB7 DNA
RPS87	SPβc2 Δ2::Tn*917*::pSK10Δ6::Φ[*(ackA–lacZ) cat*] erm *codY::spc*	RPS40 X HKB7 DNA
RPS88	SPβc2 Δ2::Tn*917*::pSK10Δ6::Φ[*(ackA–lacZ) cat*] erm *ccpA::ble*	RPS86 X HKB5 DNA
RPS89	SPβc2 Δ2::Tn*917*::pSK10Δ6::Φ[*(ackA–lacZ) cat*] erm *codY::spc ccpA::ble*	RPS87 X HKB5 DNA

a. This strain was created by H.-J. Kim.

tion of acetoin from pyruvate and is positively regulated by AlsR (Renna *et al.*, 1993). Acetate is thought to be the co-activator for AlsR. CcpA is a direct activator of the transcription of the *alsSD* operon (Turinsky *et al.*, 2000) and a putative *cre* site has been identified upstream of the *alsS* gene (Deutscher *et al.*, 2002). Thus, expression of this pathway is linked to synthesis of both BCAAs and acetate. Positive regulation of *ackA* by both CodY and CcpA may be needed to generate enough acetate to activate AlsR (Renna *et al.*, 1993; Turinsky *et al.*, 2000).

When glucose becomes limiting, acetate and acetoin can be re-imported (Grundy *et al.*, 1993b; 1994) and converted to acetyl CoA by acetyl CoA synthetase and acetoin dehydrogenase respectively. Interestingly, the *acsA* gene, encoding acetyl CoA synthetase, and *acuABC*, an operon involved in post-translational modification of this enzyme (Gardner *et al.*, 2006), are among those that appear to be repressed by both CodY and CcpA (Grundy *et al.*, 1994; Zalieckas *et al.*, 1998; Moreno *et al.*, 2001; Molle *et al.*, 2003) (http://www.genome.jp/dbget-bin/www_bget?exp+ex0000381).

The purpose of having CodY and CcpA control both *ilvB* and *ackA* may be to create a hierarchy for carbon flow. If neither CcpA nor CodY is active, as would be true under poor growth conditions, pyruvate will enter the Krebs cycle to generate reducing power and ATP. If CcpA is active but CodY is relatively inactive (as would occur in minimal-glucose-glutamine medium, for instance), the Krebs cycle genes will be repressed through the actions of CcpA and CcpC and pyruvate will be directed towards BCAA biosynthesis. If CcpA and CodY are both active (as in rich medium), the Krebs cycle genes and the *ilvB* operon will be repressed, while the *ackA* and *alsSD* operon will be maximally expressed, directing pyruvate toward production of acetate and acetoin.

Experimental procedures

Bacterial strains, growth and β-galactosidase activities

Bacterial strains used in this study are described in Table 1. To clone the *ackA* promoter region, a 503 bp DNA sequence was amplified from strain RPS40 chromosomal DNA using primers ORPS92 (GCTTTTATTTATGCTTTTGAAGACCG GACTTGACG) and ORPS93 (CAGCAGCATTTTAACAGC TACCGCATGATCTGG) and ligated to TOPO TA cloning vector 2.1 (Invitrogen). Transformants of *E. coli* strain JM107, carrying plasmid pAckA, were selected on L-medium plates (Miller, 1972) containing ampicillin (50 μg ml^{-1}).

Bacillus subtilis cultures for β-galactosidase assays were grown overnight at 37°C in TSS minimal medium supplemented with 0.5% glucose, 0.2% glutamine, 0.02% $MgSO_4$, $FeCl_3$-Na_2-Citrate (40 μg ml^{-1}) and a 16-amino-acid mixture (Atkinson *et al.*, 1990). Cells were diluted in the same medium, grown at 37°C until the OD_{600} reached ~0.5, pelleted, and resuspended in minimal medium supplemented with mixtures of 16 or 13 amino acids. The 13-amino-acid mixture was the same as the 16-amino-acid mixture but without isoleucine, leucine and valine. When indicated, decoyinine was added to a final concentration of 500 μg ml^{-1} from a stock solution at 100 mg ml^{-1} in 1 M KOH. For control cultures an equivalent volume of 1 M KOH was added. At various times during growth samples of 1 ml were removed and β-galactosidase assays were performed as previously described (Ratnayake-Lecamwasam *et al.*, 2001). All *lacZ* fusion strains carried the ACKLAC-E fusion of Grundy *et al.* (1993a) integrated at the SPβ phage locus.

Purification of CodY, CcpA and RNA polymerase

Purification of CodY and CcpA was carried out as previously described (Shivers and Sonenshein, 2004; 2005). To purify *B. subtilis* RNA polymerase, *B. subtilis* strain MG5636 (Qi and Hulett, 1998), containing a 10-histidine codon extension at the 3′ end of the *rpoC* gene, was grown for 16 h in L-broth (Miller, 1972) containing chloramphenicol (2.5 μg ml^{-1}) and then diluted 1000-fold in DS medium (Fouet and Sonenshein, 1990) containing chloramphenicol (2.5 μg ml^{-1}). The culture was grown at 37°C to an OD_{600} ~1.0. Cells were collected by centrifugation at 5000 r.p.m. at 4°C and resuspended in Novagen binding buffer (20 mM Tris HCl, 500 mM NaCl, 5 mM imidazole, 5% glycerol, 1 mM PMSF, pH 8.0). After resuspension cells were passed through the French press (12 000 psi) and then sonicated using a Branson Sonifier Cell Disrupter 200 for six cycles of 30 s each with 15 s rests between sonication cycles. After sonication the cell debris was pelleted at 19 000 r.p.m. for 50 min at 4°C. The super-

natant fluid was loaded on a Ni^{+} His-binding column (Novagen). The column was washed with binding buffer containing increasing concentrations of imidazole and then eluted with binding buffer containing 100–400 mM imidazole. Fractions containing pure RNA polymerase (as judged by SDS-PAGE and staining with Coomassie blue) were pooled and concentrated using an Ultrafree-4 centrifugal filter and then dialysed against storage buffer (10 mM Tris HCl pH 8.0, 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 50% glycerol) and stored at –20°C.

Gel mobility shift assays

A DNA probe containing the *ackA* promoter region was generated by PCR amplification of plasmid pAckA as template DNA using primer pairs ORPS92 and ORPS93. Primer ORPS92 was radioactively labelled by incubation with T4 polynucleotide kinase and [γ-^{32}P]-ATP as described previously (Kim *et al*., 2002).

Labelled DNA (~0.2 fmol) was mixed with increasing amounts of CodY protein in reactions of 10 μl as described previously (Molle *et al*., 2003; Shivers and Sonenshein, 2004; 2005). Where indicated, BCAAs (10 mM each of isoelucine, valine and leucine) or GTP (2 mM) or both were added. After incubation for 30 min at room temperature, samples were loaded onto a 12% non-denaturing polyacrylamide gel prepared in Tris-Glycine buffer and pre-warmed at 110 V in 35 mM HEPES/43 mM imidazole electrophoresis buffer (pH 7.4). Subsequent electrophoresis was at 150 V for 180 min. For some reactions containing isoleucine, leucine and valine, the HEPES/imidazole electrophoresis buffer was supplemented with 10 mM of each amino acid.

In vitro *transcription reactions*

The 503 bp *ackA* promoter-containing DNA includes the first 296 bp of the *ackA* transcription unit. This DNA sequence was amplified by PCR using primers ORPS92 and ORPS93 and plasmid pAckA as template. A 396 bp DNA sequence containing the *veg* promoter served as a template for a 138 nt RNA product. The promoter-containing DNAs were incubated with CodY or CcpA, as indicated, in a reaction mix containing 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0), 0.1 mg ml^{-1} bovine serum albumin, 50 mM KCl, 1 mM dithiothreitol, 150 μM each of ATP, GTP and CTP, 20 μM unlabelled UTP, 0.1 units of RNaseOUT (Invitrogen), 0.5 μCi [α-^{32}P]-UTP (600 Ci $mmole^{-1}$; NEN) and 5% glycerol in a 10 μl reaction volume. BCAAs and GTP were added to the reactions as indicated. The mixtures were incubated for 30 min at 37°C to allow for binding of the proteins. *B. subtilis* RNA polymerase was then added to a final concentration of 75 nM and the reaction was further incubated for 15 min at 37°C. To stop the reaction, 4 μl of sequencing gel loading dye was added and the reaction tube was incubated at 80°C for 2 min; 8.5 μl of the reaction was loaded on a pre-warmed 6% sequencing gel. Electrophoresis was at 1200 V for 150 min.

DNase I footprinting

The DNA sequence used for DNase I footprinting was amplified by PCR using primers ORPS92 and ORPS93 and plasmid pAckA as template. Primer ORPS93 was radioactively labelled with T4 polynucleotide kinase and [γ-^{32}P]-ATP as described previously (Kim *et al*., 2002). The labelled PCR product was fractionated on a 12% polyacrylamide gel and eluted using the Qiagen Qiaex II Gel Extraction Kit.

Conditions for CodY binding were the same as for gel mobility shift assays, but in a 20 μl volume. DNase I footprinting reactions in Fig. 6 were performed in CcpA-binding buffer [10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 50 mM KCl; 1 mM dithiothreitol; 0.05% Nonidet P-40 (Igepal); 10% glycerol; 50 μg of bovine serum albumin per ml; 10 μg of calf thymus DNA per ml]. After incubation for 30 min at room temperature, 6 mM $MgCl_2$, 6 mM $CaCl_2$ and RQ1 DNase (Promega; 0.11 U) were added to each reaction tube and incubation continued for 1 min at room temperature. Each reaction was stopped by addition of 0.9 μl of 0.5 M EDTA and transferred to a dry ice-ethanol bath. Samples were extracted with phenol/chloroform, and the DNA was ethanol precipitated and resuspended in 4 μl of sequencing gel loading buffer (Sambrook *et al*., 1989). The samples were incubated at 80°C and analysed on a 6% urea-polyacrylamide DNA sequencing gel. Dideoxy sequencing reactions performed with a Sequenase Kit (United States Biochemical), primer ORPS93, and plasmid pAckA were run with each experiment. The incorporated radioactive label was [α-^{35}S]-dATP.

Analysis of mobility shift, footprinting and in vitro *transcription reactions*

Gels were dried under vacuum and exposed to a phosphorimager screen before analysis with a Molecular Dynamics 860 Imager. Quantification of gel images made use of ImageQuant version 1.2 Macintosh software.

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