Hyperphosphorylation of DegU cancels CcpA-dependent catabolite repression of rocG in Bacillus subtilis

Kosei Tanaka1, Kana Iwasaki2, Takuya Morimoto3,4, Takatsugu Matsuse2, Tomohisa Hasunuma1, Shinji Takenaka1,2, Onuma Chumsakul4, Shu Ishikawa4, Naotake Ogasawara4 and Ken-ichi Yoshida1,2*

Abstract

Background: The two-component regulatory system, involving the histidine sensor kinase DegS and response regulator DegU, plays an important role to control various cell processes in the transition phase of Bacillus subtilis. The degU32 allele in strain 1A95 is characterized by the accumulation of phosphorylated form of DegU (DegU-P).

Results: Growing 1A95 cells elevated the pH of soytone-based medium more than the parental strain 168 after the onset of the transition phase. The rocG gene encodes a catabolic glutamate dehydrogenase that catalyzes one of the main ammonia-releasing reactions. Inactivation of rocG abolished 1A95-mediated increases in the pH of growth media. Thus, transcription of the rocG locus was examined, and a novel 3.7-kb transcript covering sivA, rocG, and rocA was found in 1A95 but not 168 cells. Increased intracellular fructose 1,6-bisphosphate (FBP) levels are known to activate the HPr kinase HPrK, and to induce formation of the P-Ser-HPr/CcpA complex, which binds to catabolite responsive elements (cre) and exerts CcpA-dependent catabolite repression. A putative cre found within the intergenic region between sivA and rocG, and inactivation of ccpA led to creation of the 3.7-kb transcript in 168 cells. Analyses of intermediates in central carbon metabolism revealed that intracellular FBP levels were lowered earlier in 1A95 than in 168 cells. A genome wide transcriptome analysis comparing 1A95 and 168 cells suggested similar events occurring in other catabolite repressive loci involving induction of lctE encoding lactate dehydrogenase.

Conclusions: Under physiological conditions the 3.7-kb rocG transcript may be tightly controlled by a roadblock mechanism involving P-Ser-HPr/CcpA in 168 cells, while in 1A95 cells abolished repression of the 3.7-kb transcript. Accumulation of DegU-P in 1A95 affects central carbon metabolism involving lctE enhanced by unknown mechanisms, downregulates FBP levels earlier, and inactivates HPrK to allow the 3.7-kb transcription, and thus similar events may occur in other catabolite repressive loci.

Keywords: Bacillus subtilis, Two-component regulatory system, Catabolite repression, Transcription, Metabolites

Background

Bacteria possess two-component regulatory systems comprising environmental sensor histidine kinases and cognate response regulators that are involved in adaptation to various chemical, physical, and physiological stimuli [1]. Following stimulation, autophosphorylation of specific sensor histidine kinases leads to transfer of a phosphoryl group to cognate transcriptional response regulators that control respective target genes.

In Bacillus subtilis, the DegS–DegU two-component regulatory system controls various processes during the transition from exponential to stationary growth phases, including the expression of extracellular degradative enzymes and late competence genes [2]. The phosphorylated form of DegU (DegU-P) induces a number of genes, whereas the unphosphorylated form also stimulates transcription of comK, which encodes the competence transcription factor [3,4]. In addition, DegU-P negatively regulates genes of the sigma factor SigD regulon.
which are involved in motility, chemotaxis, and auto-
lysin production [2,5,6]. In addition, the DegS–DegU
system is modulated by two regulatory genes, degQ
and degR, which encode small polypeptides of 46 and
60 amino acids, respectively [7]. In wild-type strains,
DegQ enhances phosphorylation of DegU [8], whereas
DegR protects DegU-P from dephosphorylation [9]. In
contrast in the standard laboratory strain 168, due to a
mutation in the −10 region (T-10 to C) of its cognate pro-
moter, degQ is not efficiently transcribed, and DegU-P
does not accumulate as in wild-type strains [10].

In the previous studies, mutations in DegU led to
overproduction of extracellular degradative enzymes and correlated with the loss of natural competence for DNA
uptake, the lack of flagella synthesis, filamentous morph-
ology, and higher sporulation efficiency in the presence of
glucose [11-13]. Among these mutations, the allele
degU32 in strain 1A95 has an amino acid substitution at
position 12 [14]. This mutation increases the stability of
DegU-P sevenfold, and amplifies DegU-P dependent
gene expression even with the genetic background of
strain 168 [13]. Extracellular proteomic studies of the
degU32 mutant indicated that 13 extracellular en-
zymes are overproduced and that eight proteins of
motility and cell-wall turnover were significantly downregulated, including five SigD-dependent pro-
teins [15]. The subsequent transcriptome analysis con-
firmed similar induction and repression of the known
DegU-P and DegU dependent genes, respectively [16].

Bacterial carbon catabolite repression (CCR) is a global
regulatory mechanism that occurs in the presence of a
preferred carbon source and represses genes involved in
metabolism of other minor carbon sources, coordinating
metabolic responses to efficient carbon and energy
sources. In B. subtilis, CCR occurs primarily at the tran-
scription level and involves phosphorylation of CcpA
and HPr at the Ser-46 residue (P-Ser-HPr) [17]. Preferred
carbon sources such as glucose are readily converted into
glycolytic intermediates, including fructose-1,6-bispho-
sphate (FBP), and the accumulation of intracellular FBP
stimulates HPr kinase/phosphatase, which phosphorylates
HPr to P-Ser-HPr. CcpA is a member of the LacI/GalR
family of transcriptional regulators, which are synthesized
constitutively irrespective of the presence or absence
of repressing sugars. The CcpA/P-Ser-HPr complex is
formed only in response to P-Ser-HPr, and exerts sitespecific DNA binding activity with 14-bp cis-acting palindromic sequences that are known as catabolite
responsive elements (cre). Because DNA binding of
CcpA/P-Ser-HPr interferes with both initiation and
elongation of target gene transcription under CCR,
CcpA/P-Ser-HPr functions as a classical repressor and
a transcriptional roadblock. In contrast, HPr originally
functions as the energy coupling protein of the
phosphotransferase system (PTS), which mediates
carbohydrate transport following phosphorylation of
the His-15 residue.

B. subtilis expresses the two glutamate dehydrogenase
genes gudB and rocG. In the standard laboratory strain 168 and its derivatives, gudB is a cryptic gene with a
short repeat within the coding region, whereas in the wild-type B. subtilis strains this gene encodes an intact
enzyme [18]. Potentially, the ancestral gudB gene be-
came inactive during domestication under laboratory
conditions. In contrast, RocG functions as an active
catabolic glutamate dehydrogenase and is induced in the
presence of arginine, ornithine, or proline following
transcriptional activation of the SigL-dependent pro-
moter and enhancement by the transcription factor
RocR [19]. RocG is also regulated by CcpA-dependent
CCR, involving a cre situated just downstream of the
rocG promoter [20]. Moreover, RocG is involved in
ammonia-releasing reactions during the production of
the Japanese food natto, which is produced by fermenta-
tion of soybeans using strains of B. subtilis natto that are
equivalent to wild-type B. subtilis strains [18].

As described above, 1A95 cells carry the degU32 mu-
tation on the genetic background of the laboratory strain
168. In the present study, we noticed that the pH of
soytone-based medium significantly increased during
growth of 1A95 cells after the onset of the transition
phase. Thus, we speculated that this change in pH re-
ffects ammonia release following glutamate degradation
by RocG. Subsequent investigations of transcription in
the rocG locus revealed a novel transcript in 1A95 cells.
Further experiments indicated that hyperphosphoryla-
tion of DegU may proactively abolish CcpA-dependent
CCR, modulating the regulatory network involving intra-
cellular metabolites and phosphorylation of HPr.

Results
The degU32 mutation led to elevated pH of growth
medium following activation of rocG encoding glutamate
dehydrogenase
During growth of B. subtilis strains in soytone–glucose
medium, pH levels decreased to around 5.5 prior to
transition from the logarithmic to the stationary phase,
and then increased during the stationary phase (Figure 1).
However, pH levels gradually increased to around 6.5
and no higher than 7.0 during growth of the laboratory
standard strain 168, whereas the 1A95 (degU32) strain
significantly elevated pH to almost 8.0.

rocG encoding glutamate dehydrogenase mediates
ammonia-releasing reactions during secondary natto
fermentation [18]. Thus, we suspected that ammonia
may contribute to increases in the pH of growth
media, and observed ammonia accumulation in the
culture medium of 1A95 cells (Figure 1). Inactivation
of rocG in the 168 strain only had a slight effect on pH and ammonia accumulation (Figure 1A and C, strain TM013). However, inactivation of rocG in 1A95 cells prevented increases in pH and accumulation of ammonium, which remained at similar levels to those observed in 168 cells (Figure 1B and C, strain TM014). These data indicate that degU32 contributes to increases in pH, potentially by enhancing RocG-mediated release of ammonia.

Cells expressing degU32 produced a novel 3.7-kb transcript containing rocG

Total RNAs were extracted from cells during the transition from logarithmic to stationary phases, just prior to pH increases (Figure 1B). In subsequent northern blotting analyses (Figure 2), three mRNA species containing rocG were detected in 1A95 cells under low-stringency conditions, with sizes of 5.0, 3.7, and 1.5 kb (Figure 2A). Control of rocG by RocR reportedly activates the SigL-dependent promoters for rocABC, rocDEF, and rocG in the presence of arginine metabolites such as citrulline and ornithine [19-21]. Both 5.0- and 1.5-kb transcripts were detected in 168 cells, but were not present in TM016 cells (degLI32 rocR::kan). These observations indicate probable dependence of these two transcripts on rocR but not on degLI32, although further experiments were not performed on these transcripts because they were not detected under high-stringency conditions (see below). The signals for the 1.5-kb transcript were rather exaggerated probably due to a synergistic effect of non-specific hybridization to 16S rRNA under the low-stringency conditions. In contrast, appearance of the 3.7-kb transcript depended solely on degU32 in 1A95 and TM016 cells and was not present in strains 168 and TM015 (degLI32::cat), indicating that this novel 3.7-kb transcript may enhance the function of RocG in 1A95 cells.

The 3.7-kb transcript was weakly but substantially detected under low-stringency conditions using either sivA (formerly yweA) or rocA probes in 1A95 and TM016 cells (Figure 2B and C), suggesting coverage of rocG, sivA, and rocA. A very strong 0.6-kb sivA transcript was observed in all four strains, suggesting transcription from a constitutive promoter upstream of sivA (Figure 2B). The probe for rocA detected another 1.5-kb transcript in 168, 1A95, and TM015 cells, but not in TM016 cells, suggesting dependence on rocR (Figure 2C). Even under high-stringency conditions, the 3.7-kb transcript was clearly
detected using the rocG probe in 1A95 and TM016 cells, but was absent after disruption of sivA in TM018 cells
(degU32 sivA::pMutin2; Figure 2D). The 5.0- and 1.5-kb rocG transcripts observed under low-stringency conditions
(Figure 2A) were not detectable under high-stringency conditions in 1A95 cells (Figure 2D), suggesting that the
3.7-kb transcript could be the major rocG transcript in 1A95 cells and is dependent on degU32. Primer extension
analyses revealed a transcription start site at 97 bases upstream of the translation start site of sivA (Figure 3).
Thus, the corresponding promoter may comprise the −35 (TTTACT) and −10 (TAGATT) regions with a 17-bp spacer and may be a typical SigA-dependent one. The constitutive 0.6-kb transcript may be the main sivA transcript, and is produced independently of degU32 or rocR (Figure 2B). Accordingly, the present northern analyses (Figure 2B) and a previous study indicate that transcription from the sivA promoter may be independent of both SigL and RocR [22].

Control of the 3.7-kb rocG transcript by CcpA-dependent CCR
A previous study indicated that rocG is regulated by CcpA-dependent CCR, involving a putative cre site in the intergenic region between sivA and rocG [20]. Moreover, the sivA transcript may be read through to cover downstream rocG [22]. CcpA-dependent CCR involves not only repression of transcriptional initiation at promoter sites but also a roadblock mechanism that aborts ongoing transcription [17]. Along with previous results, the present data suggest that the 3.7-kb transcript is produced by transcription through the main 0.6-kb sivA and elongation to cover both rocG and rocA, and thus escapes CcpA-dependent CCR. Although a Rho-independent terminator (ΔG = −16.90 kcal/mol, Figure 3C) is present and may terminate transcription from the sivA promoter, it may allow some read through. Moreover, CcpA-dependent CCR may arrest the read via the roadblock mechanism in 168 cells but not in 1A95.
Figure 3 The promoter and terminator regions of *sivA*. (A) Primer extension analyses of the *sivA* promoter region; RNAs were prepared from 168 (lane 1) and 1A95 (*degU32*, lane 2) cells, and were reverse transcribed to generate cDNAs corresponding to the 5'-terminus of *sivA* transcripts. Lanes G, A, T, and C contained dideoxy sequencing ladders. The partial nucleotide sequence of the coding strand is shown on the right side, where the putative −10 region and the transcription start site (+1) are shown in enlarged uppercase characters. (B) Schematic presentation of the *sivA* promoter region. The nucleotide sequence of the upper strand of the promoter region is shown, and putative −35 and −10 regions, the transcription start site (+1), and the beginning of the *sivA* open reading frame are shown with enlarged uppercase characters. (C) Nucleotide sequence of the intergenic region between *sivA* and *rocG*. The C-terminal end of *sivA*, the Rho-independent terminator, SigL-dependent *rocG* promoter (+35 and −10 regions), its transcription start site (+1), the *cre* site, and the N-terminal end of *rocG* are indicated.
cells. It was previously shown that rocABC composed a single 5.0-kb transcriptional unit depending on both SigL and RocR [23]. Another northern blotting analysis revealed that a rocB specific probe detected the 5.0-kb transcript but failed to detect the 3.7-kb one (Additional file 1: Figure S1). Recently an internal terminator was found just behind rocA [24,25], and this terminator could function in this case to generate the 3.7-kb transcript covering sivA, rocG, and rocA. The SigL-dependent rocG promoter and its corresponding transcription start site are located upstream of the cre site [26]. Therefore, the CcpA-dependent roadblock might also control SigL-dependent rocG transcription.

When ccpA was inactivated (ccpA::neo) in strain 168, the 3.7-kb transcript containing rocG was produced as in 1A95 cells (Figure 4; note that the 5.0- and 1.5-kb transcripts were not detected under high-stringency conditions). These observations indicated that the 3.7-kb transcript was repressed by CcpA-dependent CCR in 168 cells and was likely “induced” in the 1A95 (degU32) strain, allowing proactive abolition of the CcpA-dependent CCR roadblock at the cre in the intergenic region between sivA and rocG (Figure 3C). However, the ccpA mutation in 168 produced an additional 2.7-kb transcript that remains uncharacterized.

Intracellular FBP levels were reduced earlier in degU32 mutant cells due to enhanced expression of lctE

To investigate abolition of CcpA-dependent CCR in 1A95 (degU32) cells, intracellular intermediates of central carbon metabolism were determined by capillary electrophoresis–mass spectrometry (CE–MS) analysis and compared with those in 168 cells (Figure 5). FBP levels decreased during the growth in both strains, and the decrease occurred faster in 1A95 cells. Glucose 6-phosphate (G6P) levels also decreased in 1A95. In contrast, 3-phosphoglyceric acid (3PG), phosphoenolpyruvate (PEP), and pyruvate (PYR) levels were consistently lower in 1A95 cells than in 168 cells. Thus, glycolysis might proceed more efficiently in 1A95 than in 168 cells, and the faster reduction in the FBP levels led to an earlier alleviation of CcpA-dependent CCR. On the other hand, in both strains, levels of intermediates of the pentose phosphate pathway, including 6-phosphogluconate (6PG), ribulose 5-phosphate (Ru5P), and sedoheptulose 7-phosphate (S7P), were generally lower in 1A95 cells than in 168 cells. Potentially, glycolysis might consume glucose more efficiently in 1A95 cells, as reflected by lower levels of 6PG, Ru5P, and S7P.

Transcriptome analyses were performed to compare 168 and 1A95 during transition from the logarithmic to the stationary phase. Among 25 selected genes of glycolysis, pentose phosphate, and branching fermentation pathways for lactate, acetoin, and acetate production, lctE, ackA, and gntZ, were enhanced by 5.44-, 5.86-, and 4.53-fold, respectively, in 1A95 cells compared with 168 cells (Table 1). lctE for lactate dehydrogenase was
prominently expressed in 1A95 cells and its inactivation led to disappearance of the 3.7-kb transcript containing rocG (Figure 6) and maintenance of high intracellular FBP levels (Figure 7). Thus, degU32 in 1A95 cells may enhance the expression of lctE through unknown mechanisms. Because PYR is the end product of glycolysis, rapid clearance of PYR due to its conversion into lactate by lactate dehydrogenase (LctE) might accelerate glycolysis, and lower intracellular FBP levels might cancel CcpA-dependent CCR earlier. gntZ for 6PG dehydrogenase is part of the gntRKPZ operon for gluconate catabolism [27], which is induced specifically in the presence of gluconate and repressed with glucose via CcpA-dependent CCR [28]. There is an additional promoter upstream of gntZ within the gntP coding region, which allows transcription of gntZ independently [27]. Although this
specific transcription is not under CCR, it might be involved in the enhanced expression.

The $\text{degU32}$ allele affects global CcpA-dependent CCR

As described above, expression of $\text{degU32}$ lowered intracellular FBP levels earlier, potentially reflecting an effect on global CcpA-dependent CCR. Accordingly, transcriptome analyses revealed that expression levels of some other genes that are regulated by CcpA-dependent CCR [32] were enhanced in 1A95 cells in the transition state (Table 1). Thus, $\text{ptsGHI}$, $\text{licRBCA}$, and $\text{deoR}$-$\text{dra}$-$\text{nupC}$-$\text{pdp}$ were examined in specific northern blotting analyses using RNAs from transitioning cells grown in the soy–glucose medium (Figure 8).

The $\text{degU32}$ allele affects global CcpA-dependent CCR

As described above, expression of $\text{degU32}$ lowered intracellular FBP levels earlier, potentially reflecting an effect on global CcpA-dependent CCR. Accordingly, transcriptome analyses revealed that expression levels of some other genes that are regulated by CcpA-dependent CCR [32] were enhanced in 1A95 cells in the transition state (Table 1). Thus, $\text{ptsGHI}$, $\text{licRBCA}$, and $\text{deoR}$-$\text{dra}$-$\text{nupC}$-$\text{pdp}$ were examined in specific northern blotting analyses using RNAs from transitioning cells grown in the soy–glucose medium (Figure 8).

The $\text{ptsGHI}$ operon encodes the glucose-specific PTS sugar transport system and is induced following interactions between the terminator immediately downstream of the promoter of $\text{ptsG}$ and the specific antiterminator GlcT, which is activated by glucose [33]. In a previous report, a putative cre site located upstream of the translation initiation point of $\text{ptsG}$ did not function in CCR in cells grown in LB medium [34]. However, the present northern blotting analyses of $\text{ptsG}$ revealed a 4.6-kb transcript in TM023 ($\text{ccpA}$::$\text{neo}$), 1A95 ($\text{degU32}$), and in TM024 ($\text{degU32}$ $\text{ccpA}$::$\text{neo}$) cells, but not in 168 or TM015 ($\text{degU32}$::$\text{cat}$) cells (Figure 8A). This 4.6-kb transcript is of sufficient size to cover the entire operon, and appeared in a similar manner to the 3.7-kb transcript containing $\text{rocG}$.

The $\text{lic}$ operon encodes another PTS sugar transport system for the polysaccharide lichenan [35]. A 2.0-kb transcript containing $\text{licC}$ appeared in both 168 and 1A95 cells, whereas another 4.0-kb transcript was present in the strains carrying $\text{degU32}$ or $\text{ccpA}$::$\text{neo}$, and then disappeared upon inactivation of $\text{degU32}$ (Figure 8B). This 2.0-kb transcript may correspond with $\text{licBCA}$, and the 4.0-kb transcript may reflect

### Table 1 Transcriptome analyses of 168 and 1A95 cell

| Gene   | Signal in 168 | Signal in 1A95 | Ratio (1A95/168) |
|--------|--------------|---------------|------------------|
| $\text{ptsG}$ | 868.57       | 5437.79       | 6.26             |
| $\text{ackA}$ | 392.94       | 2301.16       | 5.86             |
| $\text{lctE}$ | 1031.06      | 5603.86       | 5.44             |
| $\text{gntZ}$ | 209.03       | 947.25        | 4.53             |
| $\text{sacP}$ | 194.83       | 680.86        | 3.49             |
| $\text{dra}$  | 405.07       | 1210.64       | 2.99             |
| $\text{ykkJ}$ | 339.75       | 991.7         | 2.92             |
| $\text{restB}$ | 802.05       | 2072.13       | 2.58             |
| $\text{rbsC}$ | 54.75        | 133.97        | 2.45             |
| $\text{rocG}$ | 220.35       | 470.1         | 2.13             |
| $\text{kdgA}$ | 87.12        | 161.59        | 1.85             |
| $\text{tpe}$  | 657.61       | 1190.12       | 1.81             |
| $\text{pahA}$ | 2110.16      | 3796.04       | 1.80             |
| $\text{rbsK}$ | 167.65       | 278.05        | 1.66             |
| $\text{pltA}$ | 2557.11      | 4158.71       | 1.63             |
| $\text{rbsA}$ | 94.2         | 153.12        | 1.63             |
| $\text{rbsR}$ | 178.33       | 280.61        | 1.57             |
| $\text{pyk}$  | 2613.65      | 4060.8        | 1.55             |
| $\text{licB}$ | 137.4        | 210.68        | 1.53             |
| $\text{rbsD}$ | 55.17        | 83.13         | 1.51             |
| $\text{ccpC}$ | 369.74       | 534.18        | 1.44             |
| $\text{cydA}$ | 332.81       | 436.13        | 1.31             |
| $\text{acuA}$ | 364.04       | 452.95        | 1.24             |
| $\text{tpiA}$ | 3565.89      | 4427.84       | 1.24             |
| $\text{eno}$  | 4240.4       | 5052.53       | 1.19             |
| $\text{cccA}$ | 417.01       | 476.84        | 1.14             |
| $\text{xynP}$ | 32.02        | 36.61         | 1.14             |
| $\text{pgm}$  | 5078.95      | 5724.25       | 1.13             |
| $\text{yqIC}$ | 81.09        | 91.33         | 1.13             |
| $\text{iolB}$ | 44.32        | 48.99         | 1.11             |
| $\text{pgk}$  | 5422.94      | 5971.13       | 1.10             |
| $\text{ywIF}$ | 2421.27      | 2526.84       | 1.04             |
| $\text{zwf}$  | 2672.83      | 2708.68       | 1.01             |
| $\text{fbaA}$ | 5096.63      | 4798.53       | 0.94             |
| $\text{gapA}$ | 7210.27      | 6348.19       | 0.88             |
| $\text{pta}$  | 2607.11      | 2276.82       | 0.87             |
| $\text{acoA}$ | 62.03        | 53.74         | 0.87             |
| $\text{tkt}$  | 3921.21      | 3212.77       | 0.82             |
| $\text{ywH}$  | 4166.18      | 3188.41       | 0.77             |
| $\text{fbp}$  | 739.27       | 516.33        | 0.70             |
| $\text{alsS}$ | 5558.12      | 3623.65       | 0.65             |
| $\text{alsD}$ | 6286.45      | 3471.86       | 0.55             |

### Table 1 Transcriptome analyses of 168 and 1A95 cell

(Continued)

| Gene   | Signal in 168 | Signal in 1A95 | Ratio (1A95/168) |
|--------|--------------|---------------|------------------|
| $\text{pgi}$ | 5106.18      | 2565.71       | 0.50             |
| $\text{gapB}$ | 258.35       | 99.25         | 0.38             |
| $\text{acsA}$ | 1836.8       | 450.84        | 0.25             |

*Detailed results are available in the ArrayExpress database [63] under accession number E-MTAB-2944.

*Some representative genes under direct CcpA-dependent CCR [32], and selected genes (italic) of glycolysis, pentose phosphate, and branching fermentation pathways to lactate, acetoin, and acetate, are listed in descending order of ratio (1A95/168).
licRBCA. Moreover, a cre site in the intergenic region between licR and licB [35] may be involved in CcpA-dependent CCR in 168 cells.

The dra-nupC-pdp operon is required for catabolism of deoxyribonucleoside [36], and although its transcription in 168 cells is strongly inhibited by glucose, it can be restored by introducing point mutations in the cre site within the reading frame of dra [37]. As shown in Figure 8C, both 3.4- and 4.3-kb transcripts containing dra appeared in the presence of degU32 and ccpA::neo. A smaller 0.6-kb transcript might correspond to the monocistronic dra transcript, which was also dependent on degU32 but not on ccpA::neo by unknown reason.

Taken together, the present northern blotting analyses of ptsGHI, licRBCA, and deoR-dra-nupC-pdp operons suggest that degU32 affects rocG and other CcpA-dependent CCR targets.

Discussion

Numerous studies of CCR have been performed using the B. subtilis strain 168 as a model of gram-positive bacteria, and the mechanisms of CcpA-dependent CCR reportedly involve formation of a regulatory cre-site-binding complex by CcpA and P-Ser-HPr. CcpA-dependent CCR occurs naturally in 168 cells grown in the soytone–glucose medium containing 2% glucose. In contrast, DegU-P accumulated in 1A95 cells due to the degU32 mutation, and the presence of a novel 3.7-kb transcript containing rocG suggested that CcpA-dependent CCR was proactively abolished.

Most of the described genes that are regulated by the DegS–DegU system are related to flagella formation, secretion of degradative enzymes such as protease and amylase, and biofilm formation [38,39]. Although few studies reported that DegS–DegU regulated genes for intracellular metabolic enzymes, depletion of nitrogen sources reportedly increased transcription of degU through an interaction between TnrA and GlnA, which affected metabolism of nitrogen sources [40].

In the present study, the degU32 mutation led to accumulation of DegU-P and elevated expression of lctE, which encodes a lactate dehydrogenase that may enhance lactic acid synthesis in B. subtilis. Under anaerobic conditions, growing cells produce ATP and consume glycolytic intermediates by alternative respiration using the electron acceptor nitrate instead of oxygen or by fermentation to produce acetone, lactic acid, and acetolactate.
However, under the present aerobic conditions, cells did not need to produce lactic acid for energy production. Therefore, the counter-intuitive increase in lactic acid synthesis may promote clearance of glycolytic intermediates, including FBP, and may reflect inactivation of HPrK, reduced CcpA/P-Ser-HPr activity, and CCR blockade. Schilling and colleagues [42] compared intracellular metabolites of aerobically cultured *B. subtilis* in glucose minimal medium with and without succinic acid and glutamic acid supplementation. The supplementation strongly lowered oxaloacetate synthesis but increased lactic acid synthesis significantly. Thus, the presence of organic acid in addition to glucose may induce *lctE* transcription and expression of lactate dehydrogenase, which plays an important role in the control of the intracellular redox state.

Transcriptional control of *lctE* has predominantly been examined under anaerobic conditions, and another two-component system involving ResD–ResE [43], the intracellular NAD⁺ and NADH redox sensor Rex [44], the two ECF sigma factors SigW and SigV [45], and a global regulator CodY [46] have been identified to be involved. The ResD–ResE system functions in global regulation of aerobic and anaerobic respiration and activates *resA*, *ctaA*, *qcrABC*, and *fnr* [47,48]. Transcription of *lctE* is controlled directly by Fnr, which is an integral part of the regulatory cascade required for adaptation of bacteria to low oxygen tension [49]. *B. subtilis* Fnr differs structurally from that in other bacteria such as *E. coli*, and its unique functions have not been fully characterized [50]. Nonetheless, mutations in the two Fnr-binding sites within the *lctE* promoter region disabled induction of the promoter activity [41], indicating that *lctE* may be regulated by the ResD–ResE system through Fnr. DegU-P recognizes and binds DNA carrying the relatively poorly conserved sequence motif AGAA-N11-TTCAG, and corresponding sequences are found within the promoter regions of a number of genes that are regulated by *degU*, including *wapA*, *sacB*, *sacXY*, *aprE*, *degR*, *degQ*, *srfAB*, *comC*, *comG*, *comEA*, and *mecA* [16,51,52]. In contrast, non-phosphorylated DegU reportedly binds DNA with inverted repeat sequences with the consensus ATTTA-N7-TAAAT [53], which is similar to sequences in the promoter regions of *fnr* and *lctE* (data not shown). However, further studies are required to determine whether *lctE* is regulated by non-phosphorylated *degU*. Moreover, at present we are unable to explain the mechanism how *lctE* was induced upon hyperphosphorylation of DegU.

Our transcriptome analyses revealed that *ackA* and *gntZ* were induced for 5.86- and 4.53-fold in 1A95 cells.
respectively. Especially, the induction of ackA seemed contradictory, because ackA is under positive regulation of CcpA/P-Ser-HPr and CcpA/P-Ser-Crh [31] and thus could be down regulated as FBP levels get lower. However, it is also known that ackA is induced by CodY in the presence of branched-chain amino acids [46]. 1A95 secretes extracellular proteases efficiently [14], and they could degrade soybean peptides in soytone–glucose medium to provide more branched-chain amino acids, which thus might trigger the CodY-dependent induction. On the other hand, gntZ is the last gene of the gntRK[PZ operon for gluconate catabolism [27], which is under the control of two promoters; the one is the gnt promoter induced specifically in the presence of inducer gluconate and repressed with glucose via CcpA-dependent CCR [28], and the other just upstream of gntZ allowing its independent transcription [27]. Probably because of the absence of gluconate, the other gnt genes were scarcely transcribed, indicating that the gnt promoter was not active. Therefore, the latter promoter might be involved in the induction but is known not under CCR [27]. It might be worthwhile to investigate its specific induction upon hyperphosphorylation of DegU.

On the genetic background of the laboratory strain 168, degU32 caused accumulation of DegU-P and production of a novel 3.7-kb transcript containing rocG, suggesting that CcpA-dependent CCR was proactively abolished. In our preliminary experiments in the natto starter strain NAFM5, similar phenomena occurred as in 1A95 (the results would be described elsewhere). In 168 cells, degQ is not efficiently transcribed due to a mutation in its cognate promoter [10]. Thus, DegU-P levels remain lower in these cells, whereas in wild-type strains such as natto starter strains, DegQ may enhance DegU phosphorylation instead of degU32 transcription. Potential to abort CCR with hyperphosphorylation of DegU may have offered survival advantages in many other bacteria that utilize various carbon sources in environmental competition, but could have been lost during domestication to breed the laboratory strain 168. The DegS–DegU system interacts with the ComP–ComA two-component system that is involved in natural competence. Upon increases in cell density, ComA is phosphorylated and activates DegQ [7], suggesting a shared function of DegQ in the ComP–ComA and DegS–DegU systems. However, unphosphorylated DegU is essential for the expression of the competence transcription factor encoded by comK [4]; and thus, hyperphosphorylation of DegU may restrict natural competence as unphosphorylated DegU decreased. Because strain 168 was selected for its high natural competence, the degQ promoter may have been mutated to avoid accumulation of DegU-P enhanced by DegQ following activation of the ComP–ComA system.

The present northern blotting analyses of the rocG locus suggested constitutive transcription from the sivA promoter (Figure 2B). However, this transcription is not completely terminated at the Rho-independent terminator (Figure 3C) and is usually aborted at the downstream cre site via the CcpA-dependent CCR roadblock mechanism [17]. Thus, upon alleviation of CCR, rocG may be induced immediately, leading to formation of the 3.7-kb transcript shown in 1A95 cells, which may represent a novel mode of CcpA-dependent regulation to induce the target gene. In this study, it was implied that hyperphosphorylation of DegU induced lctE through unknown mechanisms to decrease intracellular FBP levels earlier and thus proactively cancelled the CCR roadblock. Potentially, this mode of regulation may operate at the global level, as suggested by transcriptome (Table 1) as well as the other northern blotting analyses (Figure 8). The physiological significance of this mechanism requires further investigation.

Conclusions

The 3.7-kb transcript covering sivA, rocG, and rocA may be tightly controlled under physiological conditions by a roadblock mechanism involving P-Ser-HPr/CcpA in 168 cells, while in 1A95 cells the tight control was alleviated. Accumulation of DegU-P in 1A95 cells affects central carbon metabolism involving lctE enhanced by unknown mechanisms, downregulates FBP levels earlier, and inactivates HPrK to allow the 3.7-kb transcription. Similar events may occur in other catabolite repressive loci.

Methods

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 2. Strains of B. subtilis were routinely maintained on TBABG plate medium containing 3.3% tryptose blood agar base (Becton, Dickinson and Company, NJ, USA) and 0.18% glucose. To induce experimental growth, cells were precultured on TBABG plates for 16 h at 37°C, and fresh colonies were then inoculated into 30–40 ml of soytone–glucose medium containing 1.0% Bacto-soytone (Becton, Dickinson and Company), 0.5% Bacto yeast extract (Becton, Dickinson and Company), 1.0% NaCl, and 2.0% glucose (pH 7.0) in a 500-ml shaking flask at OD600 of 0.05, and were grown at 37°C with shaking at 150 rpm. The Escherichia coli strain was grown in LB medium. When required, cells were cultured in the presence of 100 μg/ml ampicillin, 0.5 μg/ml erythromycin, 50 μg/ml kanamycin, 10 μg/ml chloramphenicol, 100 μg/ml spectinomycin, or 15 μg/ml neomycin.

Construction of strains

The strains TM013 and TM014 were constructed from respective parental strains 168 and 1A95 by transformation
with pGP958 plasmid DNA [54] harboring a chloramphenicol resistance gene cassette inactivating rocG. To construct TM015, 1A95 cells were transformed with chromosomal DNA from WFT28 [8] carrying a chloramphenicol resistance gene cassette inactivating degU32. To construct TM016, 1A95 cells were transformed with DNA from the strain ΔrocR (FW15) [55], which harbored a kanamycin resistance gene cassette inactivating rocR. TM017 and TM018 strains were constructed by transformation of 168 and 1A95 cells with DNA from the strain BFS3227 (National BioResource Project; NIG, Japan; ΔrocR (FW15)) [56]. TM023, TM024, and TM024 strains were constructed by transformation of 168 and 1A95 cells with DNA from the strain FU402 [57], which harbored a neomycin resistance gene cassette inactivating ccpA.

MY02 and KI004 were constructed as follows: two DNA fragments (approximately 500-bp long) corresponding to the flanking regions upstream and downstream of lctE were amplified using chromosomal DNA from 168 cells as a PCR template and the respective primers lctE-R2/lctE-F1 and lctE-R2/lctE-F1 (Table 3). Another PCR fragment containing the spectinomycin resistance gene cassette was amplified using DNA from the strain FU341 [58] and the primers lctE-spec-R and lctE-spec-F (Table 3). A mixture of the three DNA fragments was used as a template for PCR, and the primer pair lctE-R2/lctE-F1 was used to generate a recombinant fragment of the spectinomycin resistance gene sandwiched between the flanking regions upstream and downstream of lctE. This recombinant fragment was used to transform 168 and 1A95 cells with the spectinomycin resistance gene and inactivated lctE to obtain MY02 and KI004 strains, respectively.

RNA analysis

RNA samples were prepared as previously described [59] with some modifications. Briefly, cells were grown to the transition state from logarithmic and stationary phases (4 h after inoculation). Subsequently, 20-ml aliquots of the culture were centrifuged, and cell pellets were resuspended in 1 ml of LETS buffer containing 1% SDS, 100 mM LiCl, 10 mM EDTA, and 10 mM Tris–HCl (pH 7.4). After addition of 500 μl of glass beads (φ0.5 mm) and 1 ml of phenol:chloroform:isoamyl alcohol (25:24:1), cells were disrupted with vigorous shaking and were then centrifuged. Finally, purified RNA was precipitated from the upper aqueous phase using ethanol.

Northern blotting and primer extension analyses were performed as described previously [60]. Briefly, probes for northern blotting analyses were prepared using in vitro transcription with T7 RNA polymerase from template DNAs of PCR fragments that were generated
using the following respective primer pairs (Table 2): rocG, NrocG-F/NrocG-R-T7; sivA, NwyeA-F/NwyeA-R-T7; rocA, NrocA-F/NrocA-R-T7; ltcE, NlctE-F/NlctE-R-T7; ptsG, NptsG-F/NptsG-R-T7; lieC, NlieC-F/NlieC-R-T7; and dra, Ndra-F/Drna-R-T7. Hybridization was performed for 16 h at 50°C (lower stringency) or 55°C (higher stringency). In primer extension experiments for the sivA promoter region, RNA samples were subjected to reverse transcription with the specific primer PEywA-R (Table 2), which was 5’-end labelled with [γ-32P] ATP (GE Healthcare Bio-Sciences, NJ, USA) using a MEGALABEL kit (Takara Bio Inc., Otsu, Japan). Sequence ladders were produced from the same primer using dideoxy sequencing reactions, and the template PCR fragment was amplified from the 168 strain using the primers PEywA-F and PEywA-R (Table 3).

Transcriptome analyses were performed using a high-density tiling oligonucleotide chip as described previously [61]. Briefly, cDNAs were synthesized from RNAs and were labelled and hybridized to the oligonucleotide chip using established methods [62]. To compensate for differences in hybridization efficiency of 25-mer oligonucleotide probes on the chip, hybridization intensities of cDNAs were divided with that of total genome DNA. Normalized transcriptional signals were processed and analyzed using the In Silico Molecular Cloning program, Array Edition (In Silico Biology, Yokohama, Japan). The data are available in the ArrayExpress database [63] under accession number E-MTAB-2944.

Measurement of metabolites
Metabolic intermediates of central carbon metabolism were determined in 168 and 1A95 cells grown on the soytone-glucose medium. At the indicated time points, 10-ml extracts were prepared from 168 (lane 1), 1A95 (degU32, lane 2), TM015 (degU32::cat, lane 3), TM016 (degU32 rocR::kan, lane 4), and TM024 (degU32 ccpp4neo, lane 5) cells. The rocR-specific probe was prepared using in vitro transcription with T7 RNA polymerase from the template DNA of PCR fragment that was generated using the primer pairs of NrocB-F (5’-aatacgactcttacttttgcc-3’) and NrocB-R-T7 (5’-aatacgactttacttacctggaggtgcc-3’). RNAs (23S and 16S) on membrane were visualized as a loading control using methylene blue staining.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors’ contributions
KT, KI, TMo, TM, OC, and SI carried out the molecular genetic studies including the transcriptome and CE-MS analyses, and participated in drafting the manuscript. TH supervised the CE-MS analysis. ST, NO, and KY coordinated the collaboration. KY conceived of the study, conducted it, and prepared the final manuscript. All authors read and approved the final manuscript.

Acknowledgements
The plasmid pGP958 was provided by J. Stülke. The strains ΔrocR (FW15), WIT28, FU402, and BFS3227 were kind gifts from M. Débarbouillé, K. Kobayashi, Y. Miwa, and National BioResource Project (NIG, Japan), respectively. We thank S. Kada for measuring ammonia levels in culture media. This work was financially supported by the Ministry of Education, Culture, Sports, Science and Technology, Japan; in parts by Special Coordination Funds for Promoting Science and Technology, Creation of Innovative Centers for Advanced Interdisciplinary Research Areas, by the Advanced Low-Carbon Technology Research and Development Program, by KAKENHI (25660067), and by Grants-in-Aid from the NC-CARP project.

Author details
1Organization of Advanced Science and Technology, Kobe University, Kobe, Hyogo, Japan. 2Department of Agrobiocience, Kobe University, Kobe, Hyogo, Japan. 3Biological Science Laboratories, Kao Corporation, Haga, Tochigi, Japan. 4Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, Japan.

Received: 19 January 2015 Accepted: 4 February 2015
Published online: 22 February 2015

References
1. Stock AM, Robinson VL, Goudreau PN. Two-component signal transduction. Annu Rev Biochem. 2000;69:183–215. doi:10.1146/annurev.biochem.69.1.183.
2. Maddek T, Kunst F, Henner D, Klier A, Rapoport G. Signal transduction pathway controlling synthesis of a class of degradative enzymes in Bacillus subtilis: expression of the regulatory genes and analysis of mutations in degS and degU. J Bacteriol. 1990;172:824–34.
3. Ogura M, Tanaka T, Bacillus subtilis DegU acts as positive regulator for comK expression. FEMS Lett. 1996;397:173–6. doi:10.1016/S0014-5793(96)01170-2.
4. Hamoen LW, van Werkhoven AF, Venema G, Dubnau D. The pleotropic response regulator DegU functions as a priming protein in competence development in Bacillus subtilis. Proc Natl Acad Sci U S A. 2000;97:9246–51. doi:10.1073/pnas.160010597.
5. Márquez LM, Helmann JD, Ferrari E, Parker HM, Ordal GW, Chamberlin MJ. Studies of sigma D-dependent functions in Bacillus subtilis. J Bacteriol. 1990;172:3435–43.
6. Tokunaga T, Rashid MH, Kuroda A, Sekiguchi J. Effect of degS-degU mutations on the expression of sigD, encoding an alternative sigma factor, and autolysin operon of Bacillus subtilis. J Bacteriol. 1994;176:5177–80.
7. Maddek T, Kunst F, Klier A, Rapoport G. DegS-DegU and ComP-ComA modulator-effector pairs control expression of the Bacillus subtilis pleiotropic regulatory gene degQ. J Bacteriol. 1991;173:2366–77.
8. Kobayashi K. DegS-DegU activation of the response regulator DegU controls setal expression of genes for flagellum formation and biofilm formation in Bacillus subtilis. Mol Microbiol. 2007;66:395–409. doi:10.1111/j.1365-2958.2007.05923.x.
9. Mukai K, Kawata-Mukai M, Tanaka T. Stabilization of phosphorylated Bacillus subtilis DegU by DegR. J Bacteriol. 1992;174:7954–62.
10. Stanley NR, Lazzerara BA. Defining the genetic connections between wild and domestic strains of Bacillus subtilis that affect poly-gamma-di-glutamic acid production and biofilm formation. Mol Microbiol. 2005;57:1143–58. doi:10.1111/j.1365-2958.2005.04746.x.

11. Kunst F, Pascual M, Lepesant-Kejzlarova J, Lepesant JA, Billaut A, Dedonder R. Pleiotropic mutations affecting sporulation conditions and the synthesis of extracellular enzymes in Bacillus subtilis 168. Biochimie. 1994;76:481–9.

12. Dahl MK, Maudek T, Kunst F, Rapoport G. Mutational analysis of the Bacillus subtilis DegU regulator and its phosphorylation by the DegS protein kinase. J Bacteriol. 1991;173:2539–47.

13. Dahl MK, Maadek T, Kunst F, Rapoport G. The phosphorylation state of the DegU response regulator acts as a molecular switch allowing either degradative enzyme synthesis or expression of genetic competence in Bacillus subtilis. J Bacteriol. 1992;167:14509–14.

14. Henner DJ, Ferrari E, Perego M, Hoch JA. Location of the targets of the hpr–sacU32 (Hy) and sacQ36 gene products in Bacillus subtilis. Mol Genet Genomics. 2002;268:453–67.

15. Antelmann H, Tjalma H, Voigt B, Ohlmeier S, Bron S, van Dijl JM, et al. A proteomic view on genome-based signal peptide predictions. Genome Res. 2001;11:1494–502. doi:10.1101/gr.182801.

16. Mäder U, Antelmann H, Buder T, Dahl MK, Hecker M, Homuth G. DegU regulator and its phosphorylation by the DegS protein kinase. J Bacteriol. 1996;178:424–34.

17. Fujita Y. Carbon catabolite control of the metabolic network in Bacillus subtilis. Microbiol. 1997;143:2625–37. doi:10.1099/1365-2958.1997.3881754x.

18. Bellitsky BR, Sonenshein AL. An enhancer element located downstream of the major glutamate dehydrogenase gene of Bacillus subtilis. Proc Natl Acad Sci U S A. 1999;96:10290–95. doi:10.1073/pnas.96.19.10290.

19. Gordan R, Rapoport G, Débarbouillé M. Role of the transcriptional activator RocR in the arginine-degradation pathway of Bacillus subtilis. Mol Microbiol. 1997;24:2539–67. doi:10.1111/j.1365-2958.1997.12189.76.07102b.29.

20. Bellitsky BR, Sonenshein AL. Characterization of a novel beta-glucoside utilization system in Bacillus subtilis. J Bacteriol. 1999;181:6992–7000. doi:10.1128/JB.181.20.6992-7000.1999.

21. Ali NO, Jeuette J, Larquet E, Carn EL, Bellitsky B, Sonenshein AL, et al. Specificity of the interaction of RocR with the rocG-rocA intergenic region in Bacillus subtilis. Microbiology. 2003;149:739–50. doi:10.1099/micro.0.26013-0.

22. Bellitsky BR, Kim HJ, Sonenshein AL, CcpA-dependent regulation of Bacillus subtilis glutamate dehydrogenase gene expression. J Bacteriol. 2004;186:1392–8. doi:10.1128/JB.186.4.1392-1398.2004.

23. Calogero S, Gordan R, Glaser P, Schweizer J, Rapoport G, Débarbouillé M, RocR, a novel regulatory protein controlling arginine utilization in Bacillus subtilis, belongs to the NirC/NaF family of transcriptional activators. J Bacteriol. 1998;176:1234–41.

24. Nicolaus P, Mäder U, Derven E, Rochat T, Leduc A, Pigéonneau N, et al. Condition-dependent transcriptome reveals high-level regulatory architecture in Bacillus subtilis. Science. 2012;335:1103–6. doi:10.1126/science.1206848.

25. B. regulon expression Data Browser. INRA-MIG. Gene/Segment: rocLocus Tag: B5U3T78Sb. http://genome.jouy.inra.fr/cg-bin/servlet/detail/p? id=rocA_3879566_3880531_1. Accessed 16 January 2015.

26. Choi SK, Saier Jr MH. Regulation of sgl expression by the catabolite control protein CcpA involves a roadblock mechanism in Bacillus subtilis potential connection between carbon and nitrogen metabolism. J Bacteriol. 2005;187:6856–61. doi:10.1128/JB.187.18.6856-6861.2005.

27. Fujita Y, Fujita T, Miwa Y, Nihashi J, Aratani Y, Organization and transcription of the glutamate operon, gnt, of Bacillus subtilis J. Biol Chem. 1999;2651:1344–53.

28. Miwa Y, Nagai K, Eguchi S, Fukuda H, Fujita Y, Sadaie Y, DNA microarray analysis of Bacillus subtilis sigma factors of extracytoplasmic function family. FEMS Microbiol Lett. 2003;220:155–62. doi:10.1016/S0378-1097(03)00093-9.

29. Shivers RP, Dineen SS, Sonenshein AL. Positive regulation of glutamate racemase expression in Bacillus subtilis by the CcpA protein. J Bacteriol. 1996;178:373–8.

30. Nakano MN, Zuber P, Glaser P, Hulett FM. Two-component regulators of aerobic and anaerobic respiration in Bacillus subtilis. J Bacteriol. 2006;188:1103–12. doi:10.1128/JB.00368-08.

31. Dorais T, Ribeyre F, Ranquet C, Guigon D. The role of energy metabolism in Bacillus subtilis by arpA (arpB). J Bacteriol. 2001;183:6815–21. doi:10.1128/JB.183.22.6815-6821.2001.

32. Larsson JT, Fogstam A, von Wachenfeldt C. Coordinated patterns of cytochrome bd and lactate dehydrogenase expression in Bacillus subtilis. Microbiology. 2005;151:3225–33. doi:10.1099/mic.0.26124-0.

33. Isai K, Yamaguchi H, Kang CM, Yoshida K, Fujita Y, Sadaie Y. DNA microarray analysis of Bacillus subtilis sigma factors of extracytoplasmic function family. FEMS Microbiol Lett. 2002;203:155–60. doi:10.1016/S0378-1097(03)00093-9.

34. Shivers RP, Dineen SS, Sonenshein AL. Positive regulation of Bacillus subtilis ackA by CodY and CcpA: establishing a potential hierarchy in carbon flow. Mol Microbiol. 2006;62:881–22. doi:10.1111/j.1365-2958.2006.05410.x.

35. Sun G, Shankova E, Chesnut R, Birkey S, Duggan MF, Sorokin A, et al. Regulators of aerobic and anaerobic respiration in Bacillus subtilis. J Bacteriol. 1996;178:3734–85.

36. Reents H, Münch R, Dammeyer T, Jahn D, Härting E. The nar regulon of Bacillus subtilis. J Bacteriol. 2006;188:1103–12. doi:10.1128/JB.188.3.1103-1112.2006.

37. Komori H, Murata S, Nakamura T, Tsuchiya K, Cry crystal structure analysis of Bacillus subtilis ferredoxin-NAPD(+) oxidoreductase and the structural basis for its substrate selectivity. Protein Sci. 2010;19:2729–39. doi:10.1002/pro.508.

38. Datoos V, Débarbouillé M, Kunert F, Rapoport G. Characterization of a novel member of the DegS-DegU response regulator region affected by salt stress in Bacillus subtilis. J Bacteriol. 1998;180:1855–61.

39. Komori H, Murata S, Nakamura T, Tsuchiya K, Cry crystal structure analysis of Bacillus subtilis ferredoxin-NADP(+) oxidoreductase and the structural basis for its substrate selectivity. Protein Sci. 2010;19:2729–39. doi:10.1002/pro.508.

40. Ogura M, Yamaguchi H, Yoshida K, Fujita Y, Tanaka T. DNA microarray analysis of Bacillus subtilis DegU, ComA and PhoP regulons: an approach to comprehensive analysis of B. subtilis two-component regulatory systems. Nucleic Acids Res. 2001;29:3804–13. doi:10.1093/nar/29.18.3804.
53. Ogura M, Tsukahara K. Autoregulation of the Bacillus subtilis response regulator gene degU is coupled with the proteolysis of DegU-P by ClpCP. Mol Microbiol. 2010;75:1244–59. doi:10.1111/j.1365-2958.2010.07047.x.
54. Commichau FM, Herzberg C, Tripal P, Valerius O, Stülke J. A regulatory protein-protein interaction governs glutamate biosynthesis in Bacillus subtilis: the glutamate dehydrogenase RocG moonlights in controlling the transcription factor GltC. Mol Microbiol. 2007;66:642–54. doi:10.1111/j.1365-2958.2007.05816.x.
55. Wegeshoff F, Beckering CL, Debarbouille M, Marahiel MA. Sigma L is important for cold shock adaptation of Bacillus subtilis. J Bacteriol. 2006;188:3130–3. doi:10.1128/JB.188.3130-3133.2006.
56. Garti-Levi S, Eswara A, Smith Y, Fujita M, Ben-Yehuda S. Novel modulators controlling entry into sporulation in Bacillus subtilis. J Bacteriol. 2013;195:1475–83. doi:10.1128/JB.02160-12.
57. Tojo S, Satomura T, Morisaki K, Deutscher J, Hirooka K, Fujita Y. Elaborate transcription regulation of the Bacillus subtilis ilv-leu operon involved in the biosynthesis of branched-chain amino acids through global regulators of CcpA, CodY and TnrA. Mol Microbiol. 2005;56:3150–73. doi:10.1111/j.1365-2958.2005.04635.x.
58. Yoshida K, Fujita Y, Ehrlich SD. Three asparagine synthetase genes of Bacillus subtilis. J Bacteriol. 1999;181:6081–91.
59. Igo MM, Losik R. Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in Bacillus subtilis. J Mol Biol. 1986;191:615–24. doi:10.1016/0022-2836(86)90449-3.
60. Yoshida K, Sanbongi A, Murakami A, Suzuki H, Takenaka S, Takami H. Three inositol dehydrogenases involved in utilization and interconversion of inositol stereoisomers in a thermophile, Geobacillus kaustophilus HTA426. Microbiology. 2012;158:1942–52. doi:10.1099/mic.0.059980-0.
61. Ishikawa S, Ogura Y, Yoshimura M, Okumura H, Cho E, Kawai Y, et al. Distribution of stable DnaA-binding sites on the Bacillus subtilis genome detected using a modified ChIP-chip method. DNA Res. 2007;14:55–68. doi:10.1093/dnares/dsm017.
62. Oshima T, Ishikawa S, Kurokawa K, Aiba H, Ogasawara N. Escherichia coli histone-like protein H-NS preferentially binds to horizontally acquired DNA in association with RNA polymerase. DNA Res. 2006;13:141–53. doi:10.1093/dnares/dsl009.
63. ArrayExpress database. EMBL-EBI. www.ebi.ac.uk/arrayexpress.
64. Hashimoto T, Sanda T, Yamada R, Yoshimura K, Ishii J, Kondo A. Metabolic pathway engineering based on metabolomics confers acetic and formic acid tolerance to a recombinant xylose-fermenting strain of Saccharomyces cerevisiae. Microb Cell Fact. 2011;10:2. doi:10.1186/1475-2859-10-2.