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Roadblock repression of transcription by *Bacillus subtilis* CodY

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Abstract

CodY is a global transcriptional regulator that is known to control, directly or indirectly, expression of more than one hundred genes and operons in *Bacillus subtilis*. Using a combination of mutational analysis and DNase I footprinting experiments, we identified two high affinity CodY-binding sites that contribute to repression of the *ybgE* gene and appear to act independently. One of these sites, located 80 bp downstream of the transcription start site, accounted for the bulk of *ybgE* repression. Using *in vitro* transcription experiments, we demonstrated that in the presence of CodY a shorter-than-expected *ybgE* transcript was synthesized that terminates at the downstream CodY-binding site. Thus, CodY binding to the downstream site represses transcription by a roadblock mechanism. Similar premature termination of transcription was observed for *bcaP* and *yufN*, two other CodY-regulated genes with binding sites downstream of the promoter. In accord with the roadblock mechanism, CodY-mediated repression at downstream sites was partly relieved if transcription-repair coupling factor Mfd was inactivated.

Keywords

Bacillus subtilis; branched-chain aminotransferase; CodY-binding sites; gene expression; roadblock repression

INTRODUCTION

CodY is a global transcriptional regulator in *Bacillus subtilis* that controls expression of many metabolic genes, most of them negatively [1; 2; 3; 4; 5]. CodY homologs are present in most other low G+C Gram-positive bacteria and have been shown to play a global role in metabolic regulation and in coordinating expression of virulence-associated and metabolic genes [6; 7](see also [8; 9] and references therein).

B. subtilis CodY is a dimeric protein that uses a winged helix-turn-helix motif to bind to DNA [10; 11; 12]. CodY binding requires the presence of a 15-bp motif, AATTTTCWGAAAATT [13; 14; 15]. The DNA-binding activity of CodY is increased by interaction with two types of effectors, branched-chain amino acids [isoleucine, leucine, and valine (ILV)] [16; 17; 18; 19] and GTP [2; 18; 20; 21].

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CodY achieves its maximal activity in media enriched with amino acids when the pools of its effectors are high [18; 22; 23; 24; 25; 26; 27]. Under these conditions the synthesis of ILV becomes superfluous, and therefore it makes physiological sense that all genes involved in ILV biosynthesis are under negative CodY control [2; 28; 29; 30](http://www.genome.jp/kegg/expression/). The *ybgE* gene encodes one of the two branched-chain amino acid aminotransferases of *B. subtilis* [31; 32]. *ybgE* is highly repressed by CodY as detected by DNA-microarray analysis, *lacZ* fusions, and other approaches [2; 28; 33]. Because of the *ybgE* involvement in the ILV synthesis and because *ybgE* is one of the most highly CodY-regulated genes, we have been interested in the mechanism of its regulation.

The exact molecular mechanism of CodY-dependent repression or activation of transcription remains unknown. In most known cases, CodY-binding sites lie in the vicinity of the promoters of the target genes [15], and CodY likely acts by affecting important steps in transcription initiation.

In the present work, we demonstrate by genetic and biochemical approaches that the *ybgE* regulatory region has two CodY-binding sites that both contribute to repression, the downstream site being the more important of the two. Binding of CodY to the upstream site appears to inhibit transcription initiation. However, CodY binding to the *ybgE* downstream site, as well as to downstream sites of the *bcaP* and *yufN* genes, contributes to regulation by preventing elongation of transcription via a roadblock mechanism.

RESULTS

CodY-dependent regulation of the *ybgE* gene

We constructed a transcriptional fusion (*ybgE292-lacZ*) containing a 292-bp fragment that includes the entire intergenic region upstream of the coding sequence (Fig. 1); expression of the fusion was highly repressed by CodY (Table 1). Under conditions of maximal CodY activity, in a glucose-ammonium minimal medium containing ILV and a mixture of 13 other amino acids (referred to here as the 16 amino acid-containing medium), fusion activity in a *codY* null mutant strain (BB2771) was 380-fold higher than in the wild-type strain (BB2770). In the wild-type strain, activity of the fusion was derepressed about 10-fold in 13 amino acid-containing medium, i.e., when ILV were omitted, and was further increased 1.6-fold in the absence of all amino acids. Addition of ILV alone to glucose-ammonium medium reduced expression from the *ybgE* promoter about 5.5-fold (Table 1). This pattern of expression is common for CodY-repressed genes [15; 18; 22; 23; 34]. However, regulation of *ybgE* is unusual in that: i) the gene is highly repressed by CodY (22-fold) even in glucose-ammonium medium in the absence of any exogenous amino acids, a condition in which CodY activity is very low; and, ii) expression is only moderately affected by addition of the 13 amino acid mixture alone. This amino acid-independent repression was previously detected in microarray experiments (http://www.genome.jp/kegg/expression/) and by Northern blot analysis [28]. In a *codY* null mutant strain, there was only a 1.6-fold effect of the medium composition on *ybgE* expression, indicating that CodY itself or a CodY-dependent factor is the major regulator of *ybgE* under the conditions tested (Table 1, strain BB2771).

The transcription start point and CodY-binding sites

A primer extension experiment established that the 5′ end of the *ybgE* mRNA is located 127 bp upstream of the initiation codon (Fig. 2A). The sequences TTCATC and TATAAT, with three and no mismatches to the −35 and −10 regions of σ^A-dependent promoters, respectively, and a 17-bp spacer region, can be identified upstream of the location of the 5′ end, suggesting that this position corresponds to the transcription start point (Fig. 1B).

Only one 15-bp sequence strongly resembling the CodY-binding consensus motif, AATTTTCWGAAAATT, and located between positions +10 and +24 with respect to the transcription start point, can be detected in the *ybgE* regulatory region [15]. However, a DNase I footprinting experiment showed that in the presence of the effectors ILV and GTP, CodY protected three sites, Ia, I, and II, of the template DNA strand corresponding to positions −44 to −25, −4 to +22, and +80 to +112 with respect to the transcription start point, respectively (Fig. 1B and 2B). Binding to the upstream site Ia was seen only at high CodY concentrations, but binding to the other two sites occurred with high affinity (see also below). As expected, binding to all three sites was strongly dependent on the presence of CodY effectors (Fig. 2B).

Site I of the *ybgE* gene overlaps the previously recognized motif 1, that has only 2 mismatches with respect to the CodY-binding consensus motif (Fig. 1B and Table 2)(we use the terms “site” and “motif” to describe an experimentally determined location of CodY binding and a 15-bp sequence that is similar to the CodY-binding consensus, respectively). Site II overlaps with three interdigitated versions of the 15-bp sequence, motifs 2, 3, and 4, which have 4 to 5 mismatches each with respect to the CodY-binding consensus motif. The upstream CodY-binding site Ia partly overlaps a sequence, motif 1a, with 5 mismatches with respect to the CodY-binding consensus (Fig. 1B and Table 2).

Roles of different CodY-binding sites

To find out to what extent the three CodY-binding sites detected *in vitro* contribute to regulation of *ybgE*, we created additional *lacZ* fusions containing truncated versions of the *ybgE* regulatory region lacking part of CodY-binding site Ia (*ybgE182-lacZ*) or site II (*ybgE190-lacZ)* or both *(ybgE80-lacZ)* (Fig. 1). (A point mutation in site I will be discussed later). The *ybgE190-lacZ* fusion deprived of the downstream CodY-binding site II was still repressed 6-fold by CodY in the 16 amino acid-containing medium (strains BB2808 and BB2818)(Table 1). The *ybgE182-lacZ* and *ybgE80-lacZ* fusions lacking part of the upstream CodY-binding site Ia (and most of the overlapping motif 1a) were regulated at levels similar to those of their respective counterparts *ybgE292-lacZ* and *ybgE190-lacZ* that carry the full upstream site (Table 1, compare strains BB2806, BB2770, BB2807, BB2808 and their *codY* derivatives). Thus, site Ia does not appear to contribute to regulation. On the other hand, site I does play a role, but, by itself, is responsible for only a fraction (6-fold) of total repression of *ybgE*. Hence, the downstream site II is required for maximal repression and is likely to be the main site of CodY-mediated repression. To prove some of these points, we constructed a fusion (*ybgE162-lacZ)* that retained site Ia but lacked site II and most of site I (Fig. 1). This fusion was not regulated by CodY (strains BB3026 and BB3028)(Table 1).

The maximal expression of the *ybgE182-lacZ* and *ybgE80-lacZ* fusions was reduced 5-fold compared to that of their respective counterparts *ybgE292-lacZ* and *ybgE190-lacZ* that carry additional upstream sequences (from positions −153 to −43) (Fig. 1, Table 1, strain BB2816 versus BB2771 and strain BB2817 versus BB2818). Part of the deleted sequence may serve as an UP element for the *ybgE* promoter [35; 36].

Affinities of CodY for sites I and II were very similar whether these sites were present on the same fragment of DNA or on separate fragments (Fig. 3A). Thus, binding of CodY to these two sites occurs independently. The weak binding to site Ia was much reduced by the deletion of motif 1a and had no effect on CodY binding to the nearby site I, consistent with our expression data in vivo (Fig. 3B).

In gel-shift experiments, CodY bound to DNA fragments containing only site I (with or without complete site Ia) or only site II with apparent dissociation constants (K_D) of ≈8–10 nM or ≈2 nM, respectively, compared with ≈2 nM for the full-length fragment (Fig. 4).

Binding to site Ia was observed only at very high concentrations of CodY (200 to 400 nM) (Fig. 4D). The main complex between the full-length *ybgE* DNA fragment and CodY likely contains CodY molecules bound to both sites I and II. However, a second faint DNA-CodY complex apparently reflecting binding of CodY to only one of the sites could be detected (Fig. 4A).

Mutations in *ybgE* sites I and II

To confirm the role of site I in *ybgE* regulation and to quantify directly the contribution of site II, we changed the very highly conserved A10 residue of CodY-binding motif 1 to G (the p1 down mutation) (Table 2) and compared the resulting phenotype in the presence or absence of site II. Whereas the *ybgE190p*$^{+}$*-lacZ* fusion, lacking site II, was still repressed 6-fold by CodY, the *ybgE190p1-lacZ* fusion lost all ability to be repressed, indicating that the p1 mutation completely inactivated site I as a target of CodY-dependent regulation (Table 1, strains BB2808 and BB3277 and their *codY* derivatives). However, the *ybgE292p1-lacZ* fusion, containing the mutated site I and wild-type site II, was still repressed 50-fold by CodY, consistent with our suggestion that site II is responsible for the major part of CodY-dependent regulation and that site I is required for maximal repression (Table 1, strains BB3301 and BB3302). Since site I alone accounts for ~6-fold repression, site II alone provides ~50-fold repression, and the two sites together give ~380-fold repression, sites I and II act independently in an additive manner.

To analyze further the role of site II in interaction with CodY, we introduced four substitutions (collectively, the p11 up mutation) within motif 3 (the motif that is completely contained within site II) to create a perfect match to the consensus (Table 2). (Alteration of motif 3 by the p11 mutation introduced an additional mismatch in the overlapping motif 2 but did not affect the sequence of the overlapping motif 4.) When the p11 mutation was present simultaneously with the down mutation p1 in site I, expression of the *ybgE292p1/11-lacZ* fusion was reduced 5-fold in the 16 amino acid-containing medium compared to the fusion carrying the p1 mutation only, indicating that the native site II is not fully occupied by CodY under these conditions (Table 1, strains BB3301 and BB3400) (we have not tested the effect of the p11 mutation alone).

CodY represses *ybgE* transcription *in vitro*

CodY efficiently repressed transcription from the *ybgE* promoter *in vitro* and did so in an ILV-dependent manner (Fig. 5A). In addition to the run-off transcript of 177 nucleotides, shorter transcription products of about 60, 84, 94, and 153 nucleotides were observed in most experiments when the *ybgE* regulatory region was used as a template, indicating the presence of several strong RNA polymerase pause sites in the region (Fig. 5A and B). CodY repressed the *ybgE* promoter much more strongly than the *B. subtilis veg* promoter or the semi-synthetic P*spac1/2* promoter [37], which we used as controls since they are not regulated by CodY and do not bind CodY *in vitro* (Fig. 5C and D).

CodY as a transcriptional roadblock

In addition to greatly reducing overall transcription from the *ybgE* promoter *in vitro*, the presence of CodY led to the appearance of two novel transcription products that were shorter than the full-length run-off transcript but did not correspond to any of the paused transcripts seen in the absence of CodY. This doublet transcript was the size expected for RNA molecules that terminate at the location of the downstream CodY-binding site II (Fig. 5A and B, Table 3). The presence of CodY did not cause the appearance of any additional transcripts from the *veg* or P*spac1/2* promoters (data not shown). Inactivation of the *ybgE* promoter by the *ybgEp2* mutation that changed the −10 region sequence from TATAAT to CATAAT prevented the formation of all transcripts, including the transcript that appeared

only in the presence of CodY (data not shown). Thus, the novel transcript originated from the *ybgE* promoter and resulted from a pause/stop site that functions only in the presence of CodY.

The prematurely terminated products could be detected only in a narrow range of CodY concentrations at which total *ybgE* transcription was much reduced. We hypothesized that the appearance of the premature termination product would be more easily detectable if we reduced the repressive effect of CodY at the level of transcription initiation, e.g., by inactivating the CodY-binding site I that overlaps the transcription start point. Indeed, the premature termination products appeared at a lower CodY concentration when a site I mutant template (*ybgEp1)* was used and did not disappear as fast as the CodY concentration was raised (Fig. 5B). Increasing the strength of CodY binding to site II by introducing the p11 mutation did not noticeably affect the efficiency of premature termination (Fig. 5B).

Our previous analysis of the *B. subtilis* genome showed that two other CodY-regulated genes, *bcaP (yhdG)* and *yufN* may possess two CodY-binding sites located widely apart in the corresponding regulatory regions [15]. In fact, we recently showed that, similar to the situation for *ybgE*, the two CodY-binding sites of the *B. subtilis bcaP* regulatory region both contribute independently to repression [34]. In that work, we hypothesized that binding of CodY to site I of the *bcaP* gene serves to inhibit initiation of transcription, and binding of CodY to site II, located 70 bp downstream of the transcription start point may cause premature termination of transcription. In fact, in the presence of CodY two novel *bcaP* transcription products were observed that corresponded in size to the expected product of premature termination of transcription at the downstream CodY-binding site II (Fig. 6, Table 3). These products were barely detectable unless the upstream CodY-binding site I was inactivated by the p2 mutation, disappeared if the downstream site II was made weaker by the p14 mutation, and were not affected by the p15 mutation that makes site II stronger [34] (Fig. 6).

In addition, a novel shortened transcription product was observed in the presence of CodY when the *yufN* promoter was transcribed *in vitro* (Fig. 7). The size of this transcript corresponded well to the size expected for a transcript that terminates at the location of the *yufN* downstream CodY-binding site II (Table 3). As in case of *bcaP*, this product was reproducibly detected only if the *yufN* upstream CodY-binding site I was inactive. Unlike the case for *ybgE* and *braP*, increasing the strength of the *yufN* downstream CodY-binding site II by the p11 mutation increased the efficiency of premature termination (Fig. 7).

Finally, we introduced 6 closely spaced substitution mutations in the sequence of the *B. subtilis gudB* gene [38] to create a perfect 15-bp CodY-binding motif starting 124 bp downstream of the transcription start point (the *gudBp2* allele). The *gudBp2* DNA fragment bound CodY with high affinity (≈3 nM), ≥150-fold stronger than the *gudBp*$^+$ fragment (data not shown). The presence of increasing CodY concentrations in the *in vitro* transcription system led to the formation of several truncated transcripts only when the modified *gudB* template was used. The sizes of these transcripts (from 125 to 136 nucleotides) corresponded to the expected sizes of transcripts blocked due to CodY binding (Fig. 8, Table 3).

Role of Mfd in CodY-mediated repression at downstream binding sites

Transcription-repair coupling factor Mfd [39] promotes dissociation of RNA polymerase stalled at DNA lesions of various nature or at protein roadblocks [40; 41; 42; 43]. Mfd enhances the efficiency of roadblocks formed *in vivo* by binding of *B. subtilis* CcpA or *Escherichia coli* LacI to sites downstream of the promoter [42; 44]. Apparently, RNA polymerase molecules impeded by proteins bound to DNA can occasionally resume elongation, given

enough time, due to the dynamic nature of interactions between the proteins and their binding sites. However, Mfd, if present, promotes dissociation of RNA polymerase, eliminating their chance to overcome the impediment. Inactivation of Mfd caused several-fold higher expression of the *ybgE*-, *bcaP*-, and *yufN-lacZ* fusions that contained downstream sites II as the only or principal region able to interact with CodY (Table 4). The positive effect of the *mfd* null mutation on expression of the fusions required the presence of CodY and was much reduced in *codY* null mutants. The remaining ≤2-fold positive effect of *mfd* null mutation (Table 4) was detected previously for other *lacZ* fusions [44]. Mfd did not significantly affect the regulation of the *ybgE*-, *bcaP*-, and *yufN-lacZ* fusions that contained upstream sites I as the only region able to interact with CodY (Table 4). The observed role of Mfd in CodY-dependent regulation at the downstream sites confirms that CodY molecules bound to these sites prevent progression of RNA polymerase.

DISCUSSION

CodY-binding sites of the *ybgE* gene

We have identified two high affinity CodY-binding sites within the *ybgE* regulatory region. Both sites contribute to repression but do so independently. Site I of the *ybgE* gene is associated with a CodY-binding motif of relatively high similarity (2 mismatches) to the consensus motif, AATTTTCWGAAAATT. In contrast, site II contains three overlapping CodY-binding motifs all of which have poor similarity to the consensus. This overlapping arrangement of CodY-binding motifs resembles the sequence of the CodY-binding site of the *dpp* operon [10; 15] and likely causes higher affinity for CodY. In neither of these two cases, however, has the contribution of individual motifs to CodY binding or regulation been analyzed.

Multiple mechanisms of CodY-mediated regulation

CodY regulates directly several dozen transcriptional units [2]. In the case of positively regulated genes, CodY binds upstream of the −35 promoter region [45](our unpublished data). In most cases of negatively regulated genes, CodY-binding sites overlap the promoter or are located immediately upstream of the −35 region [15]. The CodY-binding site I of the *ybgE* gene overlaps the transcription start point, and therefore CodY binding to this site most likely inhibits transcription by interfering with RNA polymerase binding.

The CodY-binding site II of the *ybgE* gene begins 80 bp downstream of the transcription start point and extends a further 32 bp. Our *in vitro* experiments showed that CodY binding to site II of *ybgE* inhibits RNA polymerase elongation, i.e., represses transcription by a roadblock mechanism. Our previous work identified two independently active CodY-binding sites for the *B. subtilis bcaP* gene, one of which is located downstream of the promoter [34]. The present work shows that CodY binding to the *bcaP* downstream site and a similar downstream site of the *B. subtilis yufN* gene also inhibits transcription by a roadblock mechanism. It is likely that other genes in *B. subtilis* and other bacteria are regulated in a similar way.

Thus, CodY is a versatile transcriptional regulator that can affect transcription in at least three different ways: activation (apparently through direct interaction with RNA polymerase), repression at the level of transcription initiation, and repression of RNA polymerase elongation by a roadblock mechanism. The ability of CodY to repress transcription *in vivo* through binding to downstream sites is enhanced by the transcription-repair coupling factor Mfd, which promotes dissociation of RNA polymerase molecules stalled at roadblocks positioned at ≥30–40 bp downstream of a transcription start point [46; 47]. On the one hand, this observation reinforces our conclusion that CodY binding

to downstream sites generates a roadblock for transcription. On the other hand, it shows that the role of Mfd in regulation of transcription elongation is likely more widespread than is reflected in the literature [41; 44; 46; 48].

Role of CodY-binding sites in *ybgE* regulation

From the expression analysis of the truncated and mutant fusions, it is apparent that site II of the *ybgE* gene is responsible for most of the repression in the absence of exogenous amino acids, i.e., under conditions when CodY activity is the lowest, and for the additional repression provided by the presence of ILV in glucose-ammonium medium (Table 1). Site I appears to contribute to *ybgE* repression only when cells are grown in 16 amino acid-containing medium, i.e., when CodY is most active. This is consistent with site II having higher affinity for CodY than does site I. The strong correlation between affinity and contribution to regulation for the two *ybgE* CodY-binding sites is in contrast to the situation for the *bcaP* gene, however. In the latter case, the higher affinity downstream site II contributes to repression no more than or even less than the lower affinity upstream site I [34]. We can conclude that CodY-mediated repression by a roadblock mechanism is not intrinsically less efficient than repression at the level of transcription initiation. At the same time, the extent to which affinity, as measured *in vitro*, correlates with strength of regulation *in vivo* is still unclear. Whatever the mechanism of repression, the presence of two binding sites with different affinities for CodY permits a promoter to respond to a wider range of intracellular concentrations of effectors [34].

Low threshold for repression of *ybgE* expression

The *ybgE* gene is unusual among CodY-repressed genes because it is highly repressed in glucose-ammonium medium in the absence of exogenous ILV and amino acids. Most other genes negatively regulated by CodY are expressed in the absence of amino acids in the medium at a level of 30–100% of their maximal expression levels. Only one other gene, *yurP*, is repressed more than 10-fold under such growth conditions [15]. Apparently, the *ybgE* regulatory region is able to interact with CodY very efficiently even when CodY is poorly active due to low pools of the effectors.

It is likely no coincidence that *ybgE*, encoding the enzyme catalyzing the last step of ILV biosynthesis, is apparently the gene that is most efficiently repressed gene by poorly active population of CodY molecules and that gets fully derepressed only when CodY is completely inactive. The lowest activity of CodY is apparently achieved only under conditions of ILV limitation. These are the growth conditions when the cell needs to maximize ILV biosynthesis in order to maintain growth. In case of *ybgE*, this is achieved by completely relieving the gene from CodY-mediated repression. Interestingly, a similar goal is achieved by an entirely different strategy by the *ilvB* operon that contains seven other genes involved in ILV biosynthesis. A transcription antitermination mechanism is called into play only during leucine limitation. If ILV supply is sufficient, the resulting termination allows only moderate expression of the *ilvB* operon [33; 49]. Expression of both *ybgE* and *ilvB* is further reduced in the presence of exogenous ILV and other amino acids in the medium through the action of CodY [2]. Thus, in case of the *ilvB* operon, two different regulatory mechanisms are responsible for the adjustment of its expression to conditions of ILV feast and famine. In case of *ybgE*, CodY plays both of these roles, rather unusually for a regulator whose main function is cell adaptation to growth in nutrient-replete conditions.

MATERIALS AND METHODS

Bacterial strains and culture media

The *B. subtilis* strains created in this study were all derivatives of strain SMY [50] and are described in Table 5 and in the text. *E. coli* strain JM107 [51] was used for isolation of plasmids. Cells growth was as described [34]

DNA manipulations

Methods for common DNA manipulations, transformation, primer extension, DNA sequencing, gel shift experiments, DNase I footprinting, and sequence analysis were as previously described [15; 34; 52]. Chromosomal DNA of *B. subtilis* strain SMY or plasmids containing appropriate promoters were used as templates for PCR if not noted otherwise. All oligonucleotides used in this work are described in Table 6. All cloned PCR-generated fragments were verified by sequencing by the Tufts University Core Facility.

Construction of transcriptional *lacZ* fusions

To create a full-length *ybgE* transcriptional fusion, the 0.54-kb PCR product was synthesized by using *ybgE*-specific oligonucleotides, oRPS11 and oRPS12, as the forward and reverse primers, respectively. The 0.29-kb fragment of this PCR product containing the entire intergenic region upstream of the *ybgE* gene was cut at the intrinsic MfeI and HindIII sites, and cloned between the EcoRI and HindIII sites of the integrative plasmid pHK23 [15] to create pBB1506 (*ybgE292p*$^+$*-lacZ*). pBB1517 (*ybgE182p*$^+$*-lacZ*) carrying a 182-bp version of the *ybgE* regulatory region truncated by 110 bp at the 5′ end was created in a similar manner but using oligonucleotide oBB355 as the forward primer and cutting the 5′ end of the product at the EcoRI site that was incorporated into oBB355. pBB1519 (*ybgE190p*$^+$*-lacZ*) and pBB1581 (*ybgE162p*$^+$*-lacZ*) carrying 190-bp and 162-bp versions of the *ybgE* regulatory regions, respectively, truncated at the 3′-end were created in a manner similar to pBB1506 but with oligonucleotides oBB356 or oBB410 containing the HindIII site as the reverse primers, respectively. pBB1518 (*ybgE80p*$^+$*-lacZ*) carrying a 80-bp version of the *ybgE* regulatory region truncated both at the 5′- and 3′-end was created in a manner similar to pBB1517 but with oligonucleotide oBB356 as the reverse primer.

B. subtilis strains carrying various *lacZ* fusions at the *amyE* locus (Table 5) were isolated after transforming strain BB2511 (*amyE::spc*) with the appropriate plasmids, by selecting for resistance to erythromycin conferred by the plasmids, and screening for loss of the spectinomycin-resistance marker, which indicated a double crossover, homologous recombination event.

Mutations in the promoter and CodY-binding sites

The p1 mutation in the *ybgE190* regulatory region (pBB1638) was introduced by using the mutagenic primer oBB469 as a forward primer for PCR. The p1 mutation in the *ybgE292* regulatory region was introduced by two-step overlapping PCR. In the first step, a product containing the 5′-part of the *ybgE* regulatory region was synthesized by using oligonucleotide oRPS11 as the forward primer and mutagenic oligonucleotide oBB475 as the reverse primer. In a similar manner, a product containing the 3′-part of the *ybgE* regulatory region was synthesized by using mutagenic oligonucleotide oBB476 as the forward primer and oligonucleotide oRPS12 as the reverse primer. The two PCR products were used in a second, splicing step of PCR mutagenesis as overlapping templates to generate a modified fragment containing the entire *ybgE* regulatory region; oligonucleotides oRPS11 and oRPS12 served as the forward and reverse PCR primers, respectively. The spliced PCR product was digested with MfeI and HindIII and cloned in pHK23, to create pBB1645 (*ybgE292p1-lacZ)*.

The p2 mutation in the *ybgE292p1* regulatory region was introduced by two-step overlapping PCR as described above using mutagenic oligonucleotides oBB543 and oBB544, pBB1645 (*ybgE292p1-lacZ)* as template and oBB67 and oBB102 as flanking primers.

The p11 mutation in the *ybgE292p1* regulatory region (pBB1675) was introduced by two-step overlapping PCR as described above using mutagenic oligonucleotides oBB499 and oBB500, pBB1645 (*ybgE292p1-lacZ)* as template and oBB67 and oBB253 as flanking primers.

The *ybgE104* PCR fragment lacking CodY-binding site I was synthesized using oBB364 and oBB102 as primers.

The p2 mutation in the *gudB* gene was introduced by two-step overlapping PCR as described above using mutagenic oligonucleotides oBB441 and oBB442, pBB933 (*gudBp*$^{+}$-*lacZ)* [38] as template and oBB67 and oBB102 as flanking primers. The spliced PCR product was cut at the HindIII and EcoRI sites, originating from the vector, and cloned in pJPM82 [53], to create pBB1620 (*gudBp2-lacZ)*.

Labeling of DNA fragments

The PCR products containing the regulatory region of the *ybgE* gene were synthesized using vector-specific oligonucleotide oBB67 as the forward primer and *lacZ*-specific oligonucleotide oBB102 as the reverse primer (Table 6). The reverse primer for each PCR reaction (which would prime synthesis of the template strand of the PCR product) was labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP. oBB67 starts 112 bp upstream of the EcoRI site used for cloning, and oBB102 starts 36 downstream of the HindIII site that serves as a junction between the regulatory regions and the *lacZ* part of the fusions.

In vitro transcription

Reactions were performed in a 10 μl total volume that contained 40 mM Tris-Cl (pH 8.0)-10 mM $MgCl_2$-5% glycerol-0.1 mM EDTA-1 mM dithiothreitol-0.1 mg/ml BSA, 2 un RNaseOUT (Invitrogen), 200 μM ATP, CTP, and GTP, 10 μM UTP, 0.5 to 1 μCi α-^{32}P-UTP, a mixture of *B. subtilis* RNA polymerase holoenzyme and σ^A factor preincubated for 30 min at 4°C (final concentrations 0.02 μM and 0.4 μM, respectively), and various amounts of CodY. 10 mM ILV was added to the reactions if not specified otherwise. His-tagged RNAP and σ^A factor were purified from *B. subtilis* cells asdescribed [54; 55].

Different PCR-generated fragments containing the regulatory regions were used as templates (~50 nM). The *ybgE*, *bcaP*, and *yufN* fragments for *in vitro* transcription were created using plasmids containing corresponding *lacZ* fusions as templates and oligonucleotides oBB67 and oBB102 that flank the promoter inserts. The *veg* and P*spac1/2* promoters were amplified using oligonucleotides specific to their sequences and chromosomal DNA of strain SMY or pBB1375 [37] as template, respectively. The *gudB* PCR fragments were obtained using oligonucleotides oBB67 and oBB465 and pBB933 [38] or pBB1620 as templates.

The reactions were preincubated at 37° C for 15 min, initiated by addition of the nucleotide mixture, incubated for 20 min, and terminated by addition of 4 μl of the 20 mM EDTA-95% formamide dye solution and subsequent heating of the samples at 80°C for 5 min. The samples were analyzed without further purification using 5% polyacrylamide DNA sequencing gels containing 7 M urea; the radioactive bands were detected and quantified using storage screens, an Applied Biosystems Phosphor Imager, and ImageQuant software (GE Healthcare).

Purification of CodY

Wild-type CodY was purified to near homogeneity as described previously [15].

Enzyme assays

β-Galactosidase specific activity was determined as described previously [38].

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Fig. 1. Plasmid maps and the sequence of the *ybgE* regulatory region

A. Schematic maps of the *ybgE* fragments used in this work. The location of the transcription start point is indicated by the bent arrow. CodY-binding motifs are shown as rectangles. The coordinates indicate the boundaries of different fragments with respect to the transcription start point. The repression ratio is the ratio of expression values for the corresponding *lacZ* fusions in *codY* null mutant and wild-type strains in the 16 amino acid-containing medium.

NA – not applicable. The *ybgE104* fragment does not contain the promoter and therefore was not tested as part of a fusion.

B. The sequence of the coding (non-template) strand of the *ybgE* regulatory region. The likely initiation codon, −10 and −35 promoter regions, transcription start site and CodY-binding motifs (15-bp sequences similar to the proposed CodY-binding consensus) 1a, 1, 2, and 4 are in bold. CodY-binding motifs 3 is boxed. The direction of transcription and translation is indicated by the arrows. The sequences protected by CodY (sites Ia, I, and II) in DNase I footprinting experiments on the template strand of DNA are underlined. The boundaries of DNA fragments used in this work are indicated by vertical arrows. The coordinates of the 5′ and 3′ end of the sequence with respect to the transcription start point are shown in parenthesis.

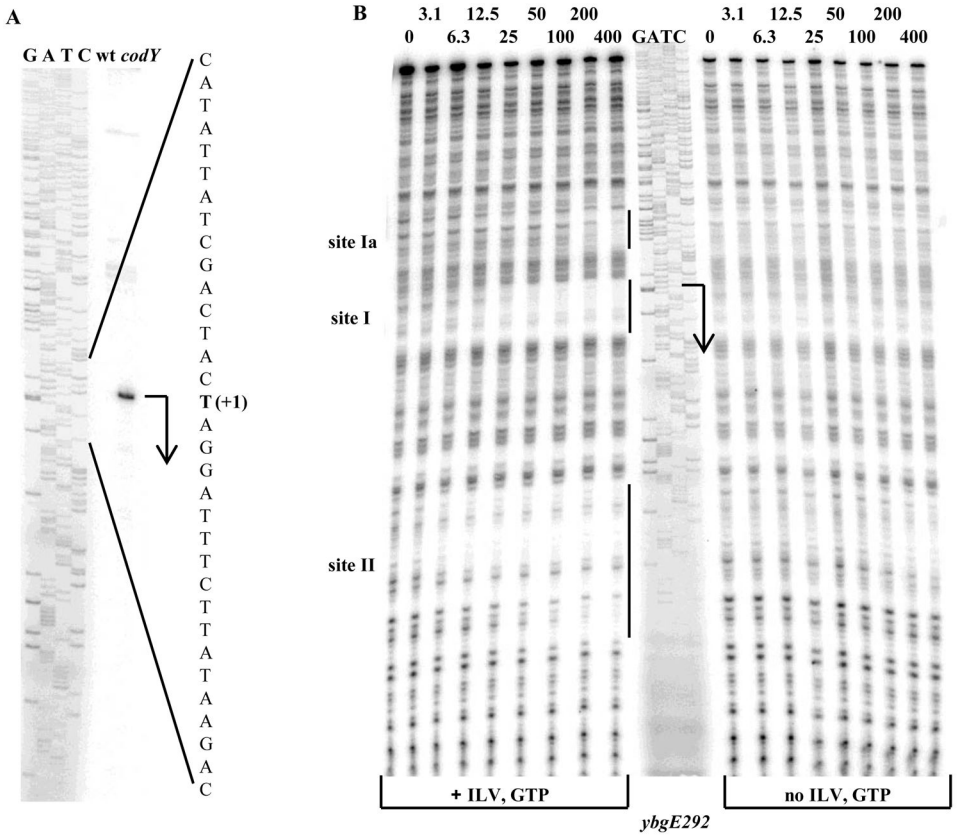

Fig. 2. Determination of the *ybgE* transcription start point and CodY-binding regions

A. Primer extension analysis of the *ybgE* mRNA. Primer oBB102 annealing to the *lacZ* gene of the *ybgE292-lacZ* fusion containing the entire *ybgE* regulatory region was extended with reverse transcriptase using as the template total RNA from fusion-containing strains BB2770 (wt) and BB2771 (*codY*) grown in the 16 amino acid-containing medium. The sequence of the template strand of the *ybgE* fragment from pBB1506 determined from reactions primed with oBB102 is shown to the left. The apparent transcription start site of the *ybgE* gene is in bold and marked by the +1 notation. A bent arrow indicates the direction of transcription. Additional faint bands observed in the primer extension lanes reflect unspecific binding of oBB102 to DNA and were present even when total RNA used for reverse transcription was isolated from a strain that did not contain the *ybgE-lacZ* fusion (data not shown).

B. DNase I footprinting analysis of CodY binding to the *ybgE* regulatory region. The *ybgE292p*$^+$ DNA fragment labelled on the template strand was incubated with increasing amounts of purified CodY in the presence of 10 mM ILV and 2 mM GTP or in their absence and then with DNase I. The sequence of the *ybgE* region was determined by using pBB1506 as the template and oBB102 as the primer and is shown in the middle. The apparent transcription start site and direction of *ybgE* transcription are shown by the bent arrow. The protected areas are indicated by the vertical lines. CodY concentrations used (nM of monomer) are indicated above each lane.

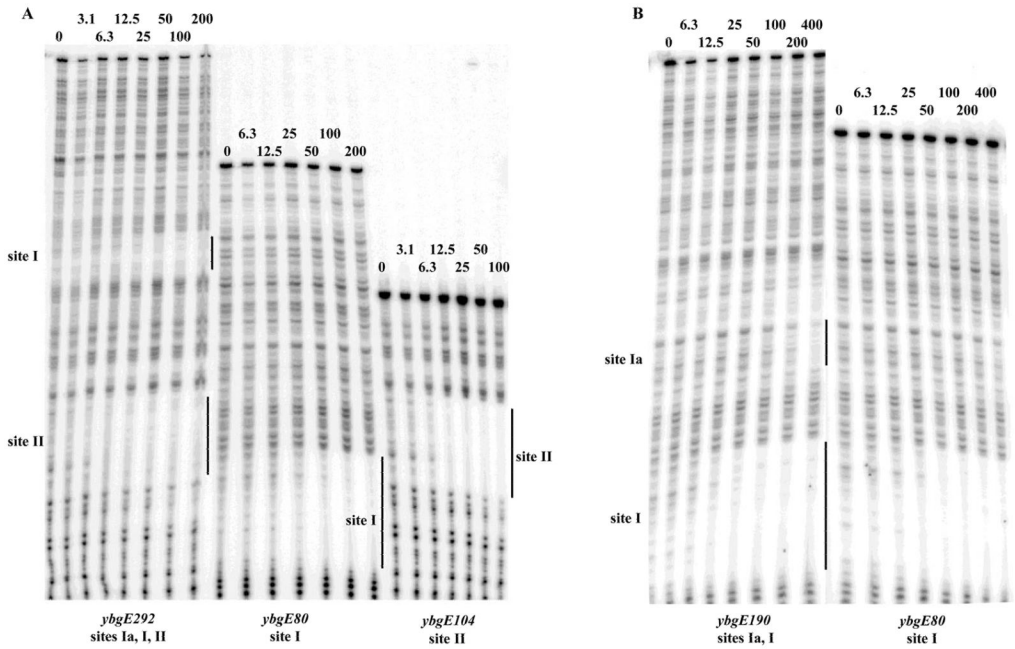

Fig. 3. Independent binding of CodY to the *ybgE* sites I and II

Various labelled *ybgE* PCR fragments were analyzed by DNase I footprinting in the presence of 10 mM ILV and 2 mM GTP as described for Fig. 2B.

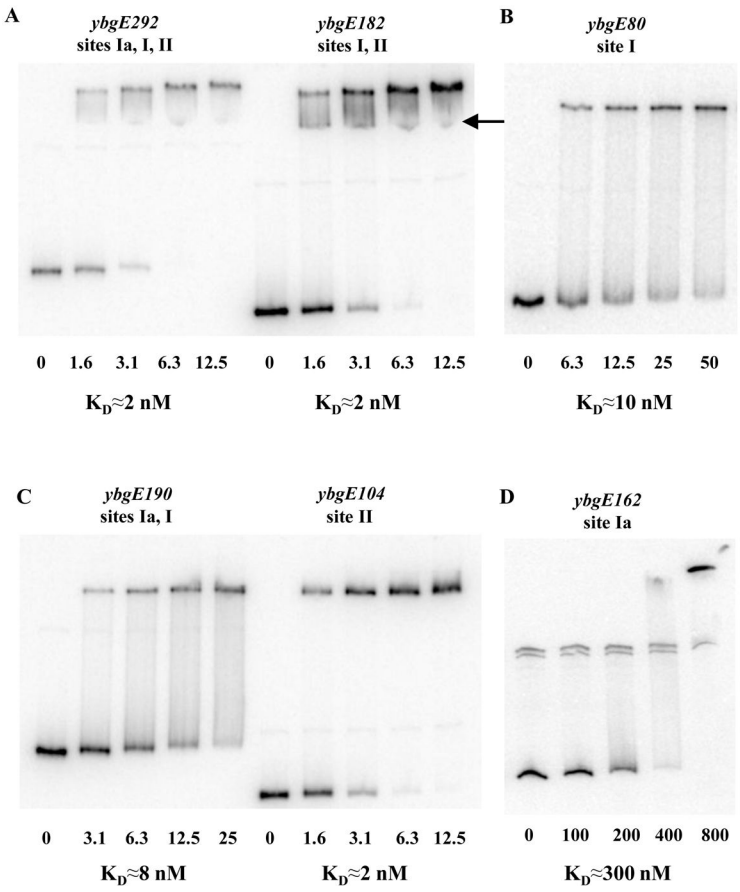

Fig. 4. Gel-shift assay of CodY affinity for *ybgE* DNA fragments

Various labelled *ybgE* DNA fragments were incubated with increasing amounts of purified CodY in the presence of 10 mM ILV and 2 mM GTP. CodY concentrations used (nM of monomer) are indicated below each lane. The arrow indicates a likely complex in which CodY is bound only to one binding site as opposed to two sites simultaneously. K_D, the apparent equilibrium dissociation constant, reflects the CodY concentration needed to shift 50% of DNA fragments under conditions ofvast CodY excess over DNA.

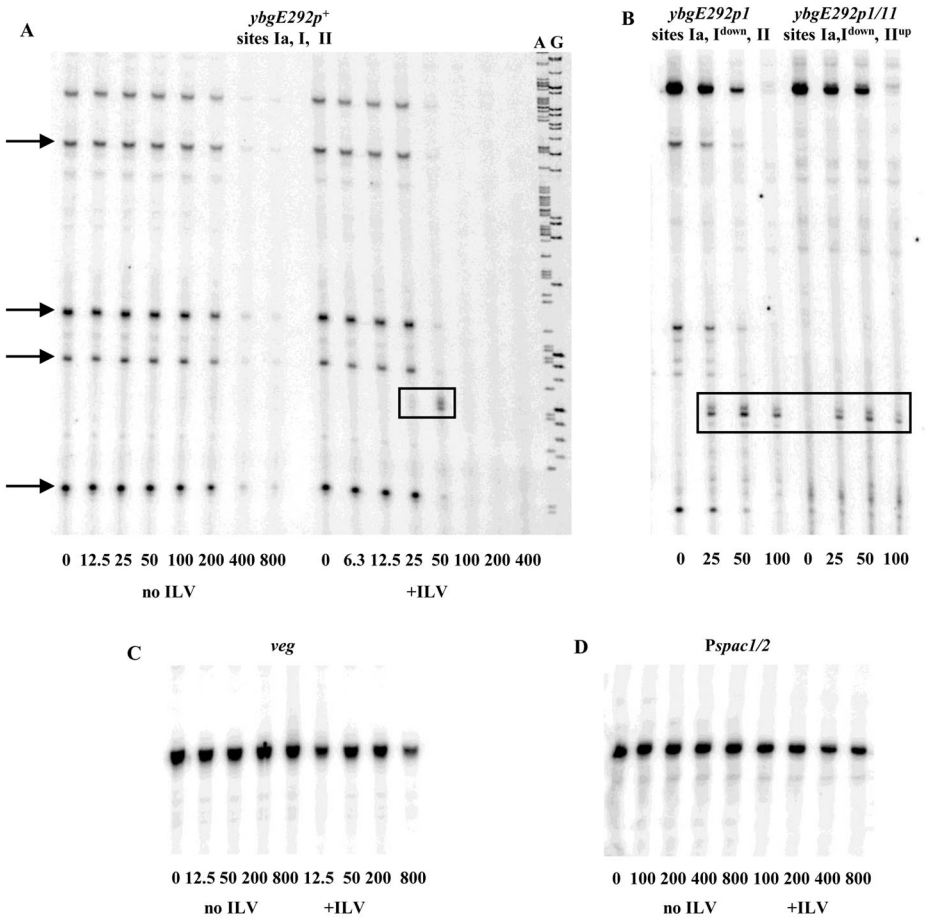

Fig. 5. Repression of *ybgE* expression *in vitro*

A. The 441-bp *ybgE292p*$^+$ PCR fragment was transcribed *in vitro* using purified *B. subtilis* RNA polymerase in the presence of increasing amounts of CodY with or without 10 mM ILV. GTP concentration was 0.2 mM in all samples. The A and G sequencing reactions used for sizing the transcripts are shown to the right. CodY concentrations used (nM of monomer) are indicated below each lane. Strong RNA polymerase pause sites are indicated with arrows. The roadblock transcription products are boxed.

B. The same as Fig. 5A using the *ybgE292p1* and *ybgE292p1/11* PCR fragments and 10 mM ILV. A down or up notation indicates the presence of a down or up mutation in the CodY-binding site.

C. The same as Fig. 5A using the 376-bp PCR fragment containing the *veg* promoter.

D. The same as Fig. 5A using the 494-bp PCR fragment containing the P*spac1/2* promoter.

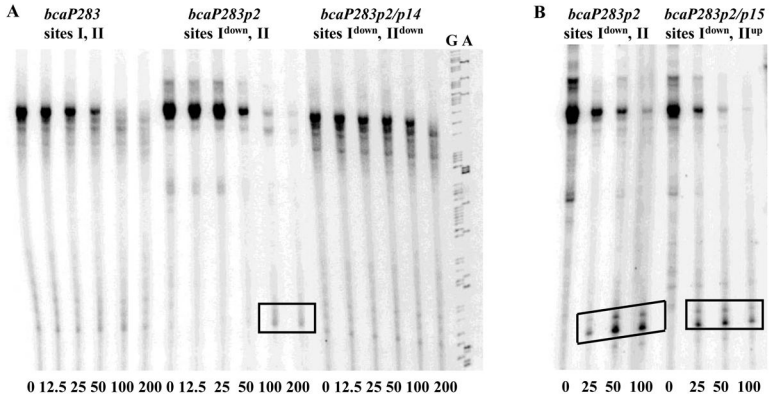

Fig. 6. CodY acts as roadblock for transcription from the *bcaP* promoter *in vitro*

428-bp PCR fragments containing various versions of the *bcaP* promoter were transcribed *in vitro* as described in Fig. 5B. The G and A sequencing reactions used for sizing the transcripts are shown to the right. Panels A and B show results from two independent experiments.

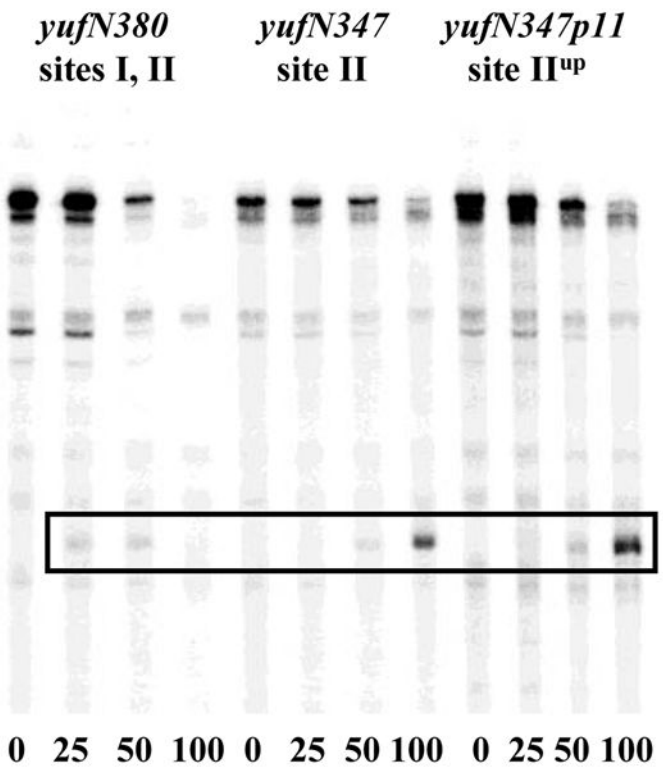

Fig. 7. CodY acts as a roadblock for transcription from the *yufN* promoter *in vitro*

645-bp (*yufN503*) or 489-bp (*yufN347*) PCR fragments, containing various versions of the *yufN* promoter, were transcribed *in vitro* as described in Fig. 5B. Analysis of the CodY-binding sites of the *yufN* gene will be presented separately (Belitsky and Sonenshein, manuscript in preparation).

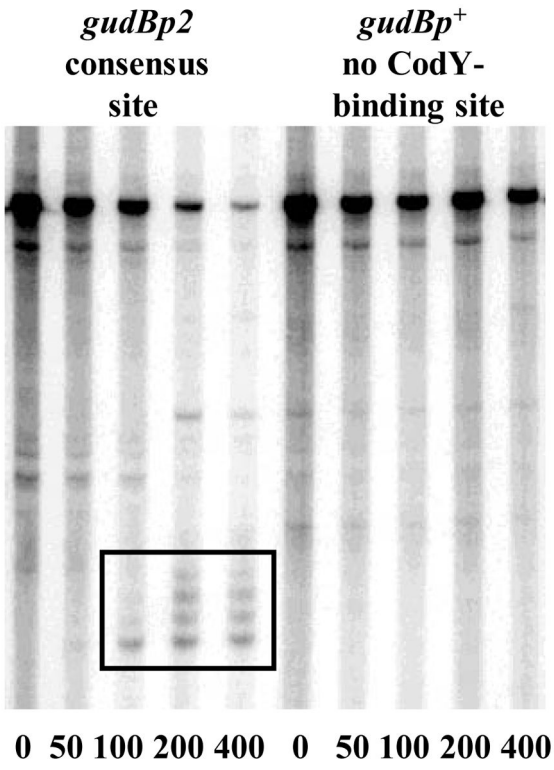

Fig. 8. CodY binding creates a roadblock for transcription from the *gudB* promoter *in vitro*

597-bp PCR fragments, containing the p^+ and p2 versions of the *gudB* promoter, were transcribed *in vitro* as described in Fig. 5B.

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Table 1

Expression of *ybgE-lacZ* fusions[a]

Strain	Relevant genotype [b]	Fusion genotype	Additions β to the medium	-galactosidase activity (MU)	β-galactosidase activity (%)[c]
BB2770	wild-type	*ybgE292p*$^{+}$ (sites Ia, I, II)	none	19.2	4.5
			ILV	3.49	0.8
			13 aa	11.7	2.7
			16 aa	1.12	0.3
BB2771	*codY*		none	268.0	
			16 aa	428.0	100
BB3301	wild-type	*ybgE292p1*^{down}	none	42.3	6.2
			ILV	14.8	2.2
			13 aa	38.9	5.7
			16 aa	13.5	2.0
BB3302	*codY*		none	420.0	
			16 aa	681.0	100
BB3400	wild-type	*ybgE292p1*^{down}/*11*^{up}	none	10.2	1.5
			16 aa	2.52	0.4
BB3415	*codY*		none	326.0	
			16 aa	648.0	100
BB2808	wild-type	*ybgE190p*$^{+}$ (sites Ia, I)	none	78.0	80
			ILV	48.2	49
			13 aa	51.3	52
			16 aa	16.5	17
BB2818	*codY*		none	86.2	
			16 aa	97.8	100
BB3277	wild-type	*ybgE190p1*^{up}	16 aa	101.0	93
BB3278	*codY*		16 aa	109.0	100
BB2806	wild-type	*ybgE182p*$^{+}$ (sites I, II)	none	1.90	2.2
			ILV	0.31	0.4
			13 aa	1.24	1.4
			16 aa	0.14	0.2

Strain	Relevant genotype [b]	Fusion genotype	Additions β to the medium	-galactosidase activity (MU)	β-galactosidase activity (%)[c]
BB2816	*codY*		none	50.1	
			16 aa	87.5	100
BB2807	wild-type	*ybgE80p*$^+$ (site I)	none	12.7	68
			ILV	7.59	41
			13 aa	8.85	47
			16 aa	2.40	13
BB2817	*codY*		none	14.6	
			16 aa	18.7	100
BB3026	wild-type	*ybgE162* (site Ia)	none	204.0	121
			16 aa	199.0	118
BB3028	*codY*		none	176.0	
			16 aa	169.0	100

[a] Cells were grown in TSS glucose-ammonium medium with or without a mixture of 16 amino acids (aa) or the same mixture without ILV (13 aa) or ILV only. β-Galactosidase activity was assayed and expressed in Miller units (MU). All values are averages of at least two experiments, and the mean errors did notexceed 30%.

[b] Strain BB2511 and all its derivatives have very low endogenous β-galactosidase activity due to a null mutation in the *lacA* gene [56].

[c] β-Galactosidase activity of each fusion in the 16 aa-containing medium in a strain containing a *cod*Y null mutation was normalized to 100%.

Table 2

CodY-binding motifs of the *ybgE* gene

Motif	Sequence[a]	Number of mismatches	Score[b]	Location with respect to the transcription start point
Consensus	AATTTTCWGAAAATT	0	13.8–14.1	
ybgE 1a	tgTTTTCTttAAtTT	5	3.7	−56 to −42
ybgE 1	AATaTTCTGAAAtTT	2	10.3	+10 to +24
ybgE 2[c]	AAaagaCTGAAtATT	5	6.5	+81 to +95
ybgE 3	AATaTTtAaAcAATT	4	6.8	+90 to +104
ybgE 4[c]	AATTaTgTGAAAggT	4	4.1	+101 to +115
ybgE 1 p1	AATaTTCTG**g**AAtTT	3	6.5	+10 to +24
ybgE 3 p11	AAT**T**TTT**C**A**G**AA**A**AATT	0	14.1	+90 to +104

[a] Mismatches to the proposed CodY-binding consensus are indicated by lower case letters. Mutations are in bold face.

[b] The scores for individual CodY-binding motifs have been generated using the position-specific weight matrix as described in 15.

[c] Parts of motifs 2 and 4 that overlap with motif 3 are underlined.

Table 3

Sizes of *in vitro* transcripts[a]

Gene	Size of a full-length transcript expected	observed	Distance from the transcription start point to the downstream CodY-binding motif	Size of the shortest terminated transcript
ybgE	175	177	80[b]	76
bcaP	131	133	70	67
yufN	308[c]	309	168	164
gudBp2	235	233	124	120

[a] DNA sequencing ladders were used to determine transcript sizes (in nucleotides). The deduced transcript size was calculated using the coefficient of 0.96, reflecting the average difference in mass between dNTPs and NTPs.

[b] The distance to the proximal motif 2 of site II is specified.

[c] The apparent *yufN* transcription start point was determined to be 270 bp upstream of the initiation codon (Belitsky and Sonenshein, manuscript in preparation).

Table 4

Effect of Mfd on CodY-mediated repression at the downstream sites[a]

Strain	Relevant genotype[b]	Fusion genotype[c]	β-galactosidase activity (MU)	Relief of repression in the *mfd* mutant (fold)[d]
BB2808	wild-type	*ybgE190p*$^+$ (site I)	16.5	
BB2818	*codY*		97.8	
BB3554	*mfd*		18.7	1.1
BB3301	wild-type	*ybgE292p1* (site I^{down}, II)	13.5	
BB3302	*codY*		681.0	
BB3555	*mfd*		35.6	2.6
BB3573	*mfd codY*		783.0	1.1
BB3400	wild-type	*ybgE292p1/11* (site I^{down}, II^{up})	2.52	
BB3415	*codY*		648.0	
BB3556	*mfd*		51.9	21.0
BB3574	*mfd codY*		861.0	1.3
BB2764	wild-type	*bcaP235p*$^+$ (site I)	6.49	
BB2767	*codY*		345.0	
BB3551	*mfd*		3.59	0.6
BB2822	wild-type	*bcaP167p*$^+$ (site II)	0.10	
BB2844	*codY*		5.82	
BB3552	*mfd*		2.24	22.0
BB3571	*mfd codY*		10.6	1.8
BB3399	wild-type	*bcaP167p15* (site II^{up})	≤0.03	
BB3414	*codY*		4.42	
BB3553	*mfd*		0.12	≥6.0
BB3572	*mfd codY*		9.18	2.1
BB2809	wild-type	*yufN276p*$^+$ (site I)	0.09	
BB2819	*codY*		6.04	
BB3557	*mfd*		0.17	1.9
BB2899	wild-type	*yufN347p*$^+$ (site II)	1.61	
BB2906	*codY*		10.4	
BB3558	*mfd*		10.0	6.2
BB3575	*mfd codY*		22.5	2.2
BB3401	wild-type	*yufN347p11* (site II^{up})	0.04	
BB3416	*codY*		2.01	
BB3559	*mfd*		0.46	12.0
BB3576	*mfd codY*		3.75	1.9

[a] Cells were grown in TSS glucose-ammonium medium with a mixture of 16 amino acids. β-Galactosidase activity was as described in Table 1.

[b] Strain BB2511 and all its derivatives have very low endogenous β-galactosidase activity due to a null mutation in the *lacA* gene [56].

[c] Construction of the *yufN* fusions will be described separately (Belitsky and Sonenshein, manuscript in preparation). A down or up notation indicates the presence of a down or up mutation in the CodY-binding site.

[d] The ratios of expression levels between *mfd* and wild-type strains or between *mfd codY* and *codY* strains are shown.

TABLE 5

B. subtilis strains used

Strain	Genotype	Source or reference
SMY	wild-type	[50]
PS251	*codY::(erm::spc) trpC2*	P. Serror
SF22CDH-T	*mfd22::Tn10(cat::tet) trpC2*	S. Fisher
BB2511	*ΔamyE::spc lacA::tet*	[15]
BB2770	*ΔamyE::[erm* **Φ***(ybgE292p$^+$-lacZ)] lacA::tet*	BB2511 x pBB1506
BB2806	*ΔamyE::[erm* **Φ***(ybgE182p$^+$-lacZ] lacA::tet*	BB2511 x pBB1517
BB2807	*ΔamyE::[erm* **Φ***(ybgE80p$^+$-lacZ)] lacA::tet*	BB2511 x pBB1518
BB2808	*ΔamyE::[erm* **Φ***(ybgE190p$^+$-lacZ)] lacA::tet*	BB2511 x pBB1519
BB3026	*ΔamyE::[erm* **Φ***(ybgE162p$^+$-lacZ)] lacA::tet*	BB2511 x pBB1581
BB3277	*ΔamyE::[erm* **Φ***(ybgE190p1-lacZ)] lacA::tet*	BB2511 x pBB1638
BB3301	*ΔamyE::[erm* **Φ***(ybgE292p1-lacZ)] lacA::tet*	BB2511 x pBB1645
BB3400	*ΔamyE::[erm* **Φ***(ybgE292p1/11-lacZ)] lacA::tet*	BB2511 x pBB1675
BB3531	*mfd22::Tn10(cat::tet)*	SMY x DNA(SF22CDH-T)
BB3534	*mfd22::Tn10(cat::neo)*	BB3531 x pCm::Nm [57]

Table 6

Oligonucleotides used in this work

Name	Sequence	Specificity
Flanking forward primers		
oRPS11[b]	5′-GGGGGGATCCCCGGATCTGACGGTTAACAAGCTGGCGGGG	*ybgE292*
oSP20[b]	5′-CTGAGAATTGGTATGCC	*veg*
oBB67	5′-GCTTCTAAGTCTTATTTCC	*erm* (pHK23)
oBB211	5′-GAATTCTAGAATGCCCAGTCCAGACTATTC	P*spac1/2* (pBB1375)
oBB355	5′-TCTTTGAATTCAGCTTCATCTGAC	*ybgE182*
oBB364	5′-GCGGCGTATTATTAAAGG	*ybgE104*
Flanking reverse primers		
oRPS12	5′-GGGGGAATTCGCGTAAGCGGTGCGTACGGCGC	*ybgE292*
oSP21	5′-CAGAAGGGTACGTCTCAG	*veg*
oBB102	5′-CACCTTTTCCCTATATAAAAGC	*lacZ* (pHK23)
oBB253	5′-GGTTTTCCCGGTCGAC	*lacZ* (pHK23)
oBB295	5′-ACCGGGACGTCGGATCCTAGAAGCTTATC	pBB1375 (P*spac1/2)*
oBB356	5′-ATACGAAGCTTCACACCTATAAATTTC	*ybgE190*
oBB410	5′-AGAATAAGCTTTAGGATCATCAGC	*ybgE162*
oBB465	5′-CTTTACTGAACCGTCG	*gudB*
oBB469	5′-ATACGAAGCTTCACACCTATAAATT**g**CAG[a]	*ybgE190p1*
Internal mutagenic forward primer		
oBB442	5′-AA**TTT**TCTG**A**AAA**TT**TTGGGATATCCCGAAGA	*gudBp2*
oBB476	5′-ATTCTG**c**AATTTATAGGTGTGAAG[a]	*ybgE292p1*
oBB500	5′-GAAT**T**TT**C**A**G**AA**A**ATTATGTGAAAGGTG[a]	*ybgE292p11*
oBB544	5′-GTGGTG**c**ATAATAGCTGATG	*ybgE292p2*
Internal mutagenic reverse primer		
oBB441	5′-CCCAA**AA**TTT**T**CAGA**AAA**TTATGTATTACGGTTTG	*gudBp2*
oBB475	5′-CACCTATAAATT**g**CAGAATATTC[a]	*ybgE292p1*
oBB499	5′-TTCACATAATT**T**T**C**T**G**AA**A**ATTCAGTC[a]	*ybgE292p11*
oBB543	5′-CATCAGCTATTAT**g**CACCAC	*ybgE292p2*

[a]The altered nucleotides are in bold; those conferring up mutations in the CodY-binding motif are in upper case, those conferring down mutations in the CodY-binding motif or in the promoter are in low case. The EcoRI and HindIII sites are underlined.

[b]oRPS and oSP primers were designed by R. Shivers and S. Picossi, respectively.