# Interactive Regulation by the *Bacillus subtilis* Global Regulators CodY and ScoC

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## SUMMARY

CodY and ScoC are *Bacillus subtilis* transcriptional regulators that control the expression of dozens of genes and operons. Using *scoC-lacZ* fusions and DNA-binding experiments, we show here that *scoC* is directly repressed by CodY. This effect creates multiple forms of cascade regulation. For instance, expression of the *dtpT* gene, which is directly and negatively controlled by ScoC and encodes a putative oligopeptide permease, was activated indirectly by CodY due to CodY-mediated repression of *scoC*. The *opp* operon, which encodes an oligopeptide permease that is essential for sporulation and genetic competence development, proved to be a direct target of repression by both ScoC and CodY, but was not significantly affected in *codY* or *scoC* single mutants. The combined actions of CodY and ScoC maintain *opp* repression when either one of the regulators loses activity, but limit the level of repression to that provided by one of the regulators acting alone. Under conditions of nitrogen limitation, repression by ScoC of *dtpT* and *opp* was partly prevented by TnrA. Thus, the functioning of ScoC is determined by other transcription factors via modulation of its expression or DNA binding.

### Keywords

*Bacillus subtilis*; CodY; ScoC; TnrA; oligopeptide permease

## INTRODUCTION

CodY, a global regulatory protein first identified in *Bacillus subtilis* (Slack *et al.*, 1995), controls directly or indirectly the transcription of more than 200 genes, many of which encode metabolic pathways involved in nutrient acquisition by macromolecular degradation, nutrient transport, and intracellular catabolism, as well as utilization of nutrients for

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biosynthesis (Barbieri *et al.*, 2015; Belitsky and Sonenshein, 2013; Brinsmade *et al.*, 2014; Molle *et al.*, 2003; Sonenshein, 2005; Sonenshein, 2007). Homologs of CodY 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 (Sonenshein, 2007) [(see also refs. (Zhang *et al.*, 2014), (Roux *et al.*, 2014) and references therein)].

The DNA-binding affinity of CodY from *B. subtilis* and most other species is increased by interaction with two types of ligands, the branched-chain amino acids [isoleucine, leucine, and valine (ILV)] (Guedon *et al.*, 2001; Petranovic *et al.*, 2004; Shivers and Sonenshein, 2004) and GTP (Brinsmade and Sonenshein, 2011; Handke *et al.*, 2008; Molle et al., 2003; Ratnayake-Lecamwasam *et al.*, 2001; Shivers and Sonenshein, 2004). Most genes regulated by CodY have in their regulatory or coding sequences sites of direct CodY binding (Belitsky and Sonenshein, 2013; Molle et al., 2003). In these cases, there are multiple mechanisms by which CodY alters gene expression (Belitsky, 2011): they include binding within or near a promoter site to activate or block RNA polymerase binding, competing with a positive regulator for binding near the promoter region, and binding within a coding sequence in order to act as a roadblock to RNA polymerase.

Interestingly, genome-wide analyses have revealed that some genes whose expression is affected by a *codY* null mutation do not have CodY-binding sites and that expression of some genes that have CodY-binding sites is not affected by *codY* mutations (Belitsky and Sonenshein, 2013; Molle et al., 2003). These findings could potentially be explained by complex regulatory schemes in which CodY regulates genes indirectly by controlling the synthesis of the direct regulators or in which CodY and other proteins regulate the same genes redundantly. In fact, established or potential targets of CodY regulation in *B. subtilis* include several genes encoding known or probable transcriptional regulators (Belitsky and Sonenshein, 2013; Brinsmade et al., 2014; Molle et al., 2003), creating the potential for regulatory cascades involving two or more transcriptional regulators. For instance, the negative effect of CodY on expression of ComK, an activator of genetic competence genes, has been previously described (Serror and Sonenshein, 1996a; Smits *et al.*, 2007).

The *B. subtilis scoC* gene (formerly known as *hpr* or *catA)* codes for a transcriptional regulator that negatively controls expression of multiple genes, including some that encode extracellular proteases and oligopeptide permeases (Caldwell *et al.*, 2001; Dod *et al.*, 1978; Higerd *et al.*, 1972; Kallio *et al.*, 1991; Koide *et al.*, 1999). In cells growing in rich media, many of these genes are expressed more strongly during the transition from exponential growth phase to stationary phase (Strauch and Hoch, 1993). ScoC is also involved in the regulation of sporulation (Dod and Balassa, 1978; Perego and Hoch, 1988).

ScoC is a 203-amino acid member of the MarR family of transcriptional regulators (Kallio et al., 1991; Perego and Hoch, 1988; Perera and Grove, 2010). It is a dimeric (or possibly tetrameric) protein containing a winged helix-turn-helix DNA-binding motif. The three-dimensional structure of ScoC (PDB 2FXA) resembles that of many other proteins of the MarR family despite the low level of sequence similarity (http://www.rcsb.org/pdb/explore/explore.do?structureId=2FXA). ScoC is longer by about 40 amino acids than most members

of the MarR family; the role of this C-terminal extension in ScoC activity remains unknown. ScoC is present in most *Bacillus* species and in bacteria of several related genera.

Global analyses of gene expression have indicated that expression of the *B. subtilis scoC* gene is increased ~3-fold in a *codY* null mutant (http://www.genome.jp/kegg/expression/) (Brinsmade et al., 2014), although *scoC* was not detected as a direct CodY target in ChIP-to-chip experiments (Molle et al., 2003) and was not on the list of the strongest CodY-binding sites detected by IDAP-Seq (Belitsky and Sonenshein, 2013). However, a weaker CodY-binding site, just beyond the arbitrary cut-off that defines the strongest sites, was in fact detected in the regulatory region of *scoC* by IDAP-Seq [Dataset S2 of ref. (Belitsky and Sonenshein, 2013)].

In this work, we showed that ScoC expression is indeed under direct CodY control and that CodY-mediated repression of *scoC* leads to an underestimation of the potential repressive effect of ScoC under conditions when CodY is active. CodY appears to employ ScoC as part of a regulatory cascade to maintain or increase repression of certain genes under conditions in which CodY loses activity. Moreover, some genes are subject to direct negative control by both ScoC and CodY, which creates a feed-forward regulatory loop. In addition, the functioning of ScoC proved to be influenced by the global nitrogen metabolism regulator, TnrA, indicating that *B. subtilis* has evolved complex mechanisms for determining the level of expression of certain genes under a variety of nutritional states.

## RESULTS

### CodY-dependent regulation of the scoC gene

Using a transcriptional fusion, *scoC561-lacZ,* containing a 561-bp fragment that includes the entire intergenic region upstream of the *scoC* gene, the 3' end of the upstream *yhaH* gene and 102 bp of the *scoC* coding region (Fig. 1A), we determined that expression of the fusion under conditions of maximal CodY activity in minimal (TSS) glucose-ammonium medium containing a mixture of 16 amino acids (hereafter referred to as the TSS + 16 aa medium) was 4.6-fold higher in the *codY* null mutant strain GB1041 than in the wild-type strain GB1039 (Table 1). This result is in accord with the results of genome-wide analyses of the CodY regulon (http://www.genome.jp/kegg/expression/) (Brinsmade et al., 2014).

Because it is common for transcriptional regulators to be autoregulated, we tested expression of the *scoC561-lacZ* fusion in a *scoC* null mutant. Indeed, expression of the fusion was increased 1.3- to 1.8-fold in *codY*[+] and *codY* mutant strains, respectively, indicating a low level of negative autoregulation. The autorepression appears to increase in a *codY* mutant strain, consistent with the higher level of ScoC expression in this strain (Table 1). The negative autoregulation by ScoC should moderate the effect of *scoC* derepression in a *codY* mutant. In a *codY scoC* double mutant, expression of the *scoC561-lacZ* fusion was 8.2-fold higher than in a wild-type strain, consistent with the conclusion that effects of the*codY* and *scoC* mutations are additive (Table 1, strain GB1063). Thus, the results show that the two proteins control *scoC* independently.

ScoC was reported to be under positive control by AbrB, an important transition state regulator (Perego and Hoch, 1988; Strauch and Hoch, 1993). However, little or no effect of an *abrB* null mutation on *scoC561-lacZ* expression was observed in TSS + 16 aa medium (Table 1).

The *scoC350-lacZ* fusion, containing a similar fragment of the *scoC* regulatory region, but truncated by 211 bp from the 5' end, had nearly the same activity as the longer fusion in all strains tested, indicating that all regions important for expression and regulation were retained within the shorter fusion (Table 1, strain BB3890 and derivatives). However, a *scoC181-lacZ* fusion that lacks an additional 169 bp from the 5' end and retains only 42 bp upstream of the *scoC* transcription start point (Fig. 1A), lost the ability to be regulated by either CodY or ScoC (Table 1, strain BB3898 and derivatives). Under fully derepressing conditions, in a *scoC codY* double mutant, the expression level of the *scoC181-lacZ* fusion was ~4-fold less than that of the other two fusions (Table 1), indicating that this fusion construct contains a *scoC* promoter with reduced activity, presumably because the fusion lacks the sequence just upstream of the −35 region (Fig. 1A).

In gel shift experiments, purified CodY bound to a DNA fragment containing the *scoC* regulatory region with moderate affinity (apparent $K_D$ 25 nM; the equilibrium dissociation constant was estimated as the protein concentration needed to shift 50% of DNA fragments under conditions of vast protein excess over DNA) (Fig. 1B). In general, non-specific binding of CodY in gel shift experiments is observed only at 400–800 nM CodY (Belitsky and Sonenshein, 2011a; Belitsky and Sonenshein, 2011b).

DNase I footprinting experiments showed that CodY protects a region from positions −58 to −31 with respect to the *scoC* transcription start point (Fig. 1A and 1E). This region corresponds very well to the core CodY-binding site, from positions −51 to −31, as determined by the global IDAP-Seq analysis (Fig. 1A and 2A)(Belitsky and Sonenshein, 2013); a core binding site is defined as a sequence that is limited by the 5' and 3' nucleotides that are essential for CodY binding. The *scoC* CodY-protected site includes two 15-bp sequences, motif I (from positions −53 to −39) and motif II (from positions −44 to −30)*,* that have 4 and 5 mismatches, respectively, with respect to the CodY-binding consensus motif, AATTTTCWGAAAATT (Belitsky and Sonenshein, 2008; den Hengst *et al.*, 2005; Guedon *et al.*, 2005) (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) (Fig. 1A). The two motifs overlap each other by 6 bp as is common for many CodY-binding sites (Belitsky and Sonenshein, 2013; Wray and Fisher, 2011). A third CodY-binding motif with 5 mismatches with respect to the consensus is located at positions −65 to −51 and also overlaps the binding site determined by footprinting; a fourth motif with 4 mismatches almost fully overlaps motif I (from positions −45 to −31).

Only low-affinity binding of purified ScoC (apparent $K_D$ 150 nM) to the *scoC* regulatory fragment could be observed in gel shift experiments (Fig. 1D). In our hands, despite the presence of a large excess of salmon sperm DNA in the binding buffer, ScoC had a propensity to bind non-specifically to several randomly chosen DNA fragments with an

apparent $K_D$ of 200–400 nM (data not shown), making interpretation of *scoC* gel shift experiments difficult. Low-affinity binding of ScoC from positions −7 to +7 with respect to the *scoC* transcription start point was detected in DNase I footprinting assays (Fig. 1F). Therefore, it is likely that the low-level negative autoregulation of ScoC reflects a direct effect.

### Mutagenesis of the scoC CodY-binding site

We introduced separately three single or double substitution mutations into the CodY-binding site of the *scoC561-lacZ* fusion in such a way as to reduce the similarity of one or both of the CodY-binding motifs I and II to the consensus (Fig. 1A). All three mutations abolished or strongly reduced the derepressing effect of a *codY* null mutation (Table 1). In fact, expression of the fusions containing the stronger p1 or p3 mutations in a *codY* mutant strain was lower than in a wild-type strain, probably indicating stronger negative autoregulation by the elevated level of ScoC (Table 1). (Note that in all these strains ScoC protein is expressed from a wild-type promoter). In gel shift experiments, a *scoC* fragment containing the p3 mutation interacted with CodY ~8-fold more weakly than did a corresponding wild-type fragment and did not form a complex with an intermediate mobility (Fig. 1C), confirming that the mutation severely impaired the interaction between CodY and the *scoC* promoter. Taken together, the *in vivo* and *in vitro* results indicate that CodY binding to the *scoC* promoter region is directly responsible for regulation.

The mutations also reduced the derepressed level of fusion expression in a *codY scoC* double mutant (Table 1), suggesting that all three mutations directly affect the intrinsic activity of the *scoC* promoter. This outcome is likely due to the positions of the mutations within the AT-rich sequence (a probable UP element) upstream of the −35 promoter region (Helmann, 1995; Meijer and Salas, 2004). However, we cannot explain why the single mutation (A-44C) in the p3 mutant promoter had a much stronger negative effect on *scoC* expression than did the double mutation (A-44C, A-43C) in the p1 mutant promoter.

### Cascade regulation of the dtpT (yclF) gene by CodY and ScoC

Most previous studies aimed at analyzing ScoC function were performed in amino acid-containing media (Caldwell et al., 2001; Dod et al., 1978; Koide et al., 1999), i.e., under growth conditions that allow CodY to be active and repress *scoC*. Repression of *scoC* by CodY may have caused an underestimation of the potential role of ScoC in gene regulation. To test the extent to which CodY modulates ScoC-mediated regulation, we sought a reporter gene that is regulated by ScoC but is not subject to direct CodY-mediated regulation.

The *dtpT* gene (also called *yclF*) is the gene most highly (6-fold) negatively regulated by ScoC during exponential growth in rich nutrient broth medium (Caldwell et al., 2001). It encodes a protein of uncharacterized function but with strong similarity to certain oligopeptide permeases (Doki *et al.*, 2013; Kunji *et al.*, 1993). Genome-wide analysis of CodY binding to *B. subtilis* chromosomal DNA failed to show any binding of CodY in the vicinity of *dtpT* either *in vivo* or *in vitro* (Belitsky and Sonenshein, 2013; Molle et al., 2003).

In gel shift experiments using a fragment containing the entire 279-bp intergenic region upstream of *dtpT* (Fig. 3A), CodY only bound at a concentration of 200 nM or higher, which is unlikely to be of physiological significance (Fig. 3B). By contrast, ScoC bound the *dtpT* regulatory region with an apparent $K_D$ of ~25 nM, i. e., with a 6- to 16-fold higher affinity than that for the regulatory regions of *scoC* and several other genes (Fig. 3C). This binding appears to be specific, although formation of multiple protein-DNA complexes may reflect a non-specific component of ScoC binding. DNase I footprinting experiments showed that ScoC protects a 21-bp region, site I, from positions −28 to −8 with respect to the transcription start point (see below), an appropriate location for repression (Fig. 3E). Another weak ScoC-binding site, site II, is located from positions −171 to −152 (Fig. 3E).

A *dtpT279-lacZ* fusion, containing the same promoter fragment, was created and shown to be derepressed 6.9-fold in a *scoC* mutant in TSS + 16 aa medium (Table 2, strains BB3928 and BB3929). Importantly, expression of the *dtpT279-lacZ* fusion in this medium, which provides for high CodY activity, was reduced a further 6.7-fold in a *codY* mutant (Table 2, strain BB3930). A double *scoC codY* mutant expressed the *dtpT279-lacZ* fusion at the same high level as did the single *scoC* mutant (Table 2, strain BB3931). These results indicate that CodY is a strong activator of *dtpT,* but exerts control indirectly through ScoC. This is fully consistent with our prediction that, in a *codY* mutant, ScoC expression should increase and provide a higher level of ScoC-mediated repression (superrepression). It also shows that the full potential of ScoC-mediated regulation (47-fold) can be revealed only when CodY is inactive. The maximal effect detected in microarray experiments was 21-fold, reflecting apparently the partial loss of activity by CodY in the stationary phase of growth (Caldwell et al., 2001).

Our results are in accord with and explain the 6-fold reduction in *dtpT* expression that was observed in a *codY* null mutant in the global RNA-Seq analysis of the CodY regulon (Brinsmade et al., 2014) [microarray analysis did not detect *dtpT* as a target of positive regulation by CodY (http://www.genome.jp/kegg/expression/)].

The positive effect of CodY was almost completely lost if the chromosomal *scoC* allele was engineered to be expressed from the CodY-insensitive *scoCp1* promoter (Table 2, strains BB4057 and BB4059). Simultaneously, expression from the *dptT* promoter in *codY*^{+} cells was decreased 4.3-fold, apparently due to higher, unregulated expression of ScoC. These results prove that the CodY-mediated superrepression is indeed indirect and mediated through regulation of ScoC expression. As a corollary, the results confirm that, as predicted, the low-affinity CodY binding to the *dtpT* promoter detected in the gel shift experiments (Fig. 3B) has no physiological significance.

## Mutational and deletion analysis of the dtpT ScoC-binding site

Three pairs of mutations were introduced separately into the strong *dtpT* ScoC-binding site I (Fig. 3A). The p1 version of the promoter retained almost fully the ability to be repressed by ScoC; however, the p3 and p4 mutant promoters lost all repression by ScoC (Table 2). Moreover, the mutations in the p3 and p4 promoters abolished the superrepression of *dtpT* expression observed in the *codY* mutant; such superrepression was not affected by the p1 promoter mutation (Table 2). In gel shift experiments, the affinity of the p3-containing

regulatory region for ScoC was decreased ~3-fold (Fig. 3D)(other mutant regulatory regions were not tested). No binding of ScoC to the *dtpTp3* fragment was detected by DNase footprinting (Fig. 3F). These results prove that the regulatory effect of ScoC is due to its direct interaction with the *dtpT* promoter.

Another pair of mutations, p2, located immediately downstream of ScoC-binding site I (Fig. 3A), almost completely wiped out expression of the *dtpT279p2-lacZ* fusion (Table 2). Considering that p2 affects the last two nucleotides of the sequence TATGAT, which is preceded at a distance of 17 bp by the sequence TTGACA, it is likely that the p2 version of the *dtpT* promoter has a defective −10 region (the two hexanucleotide sequences have one and zero mismatches, respectively, to the consensus −10 and −35 regions of $\sigma^A$-dependent promoters of most bacteria). For convenience, we assumed that the A nucleotide, located 5 bp downstream from the presumed −10 region is the transcription start point of the *dtpT* gene; this position is only 5 bp downstream from the transcription start point estimated by tiling DNA microarray experiments (Nicolas *et al.*, 2012). Based on this assumption, the mutations in the p1, p3, and p4 promoters are located at positions −15 and −14, −12 and −11, and −18 and −17, with respect to the *dtpT* transcription start point, respectively. Therefore, it is not surprising that these three pairs of mutations also altered, to different extents (1.5- to 2.6-fold in a *scoC* single mutant strain), expression from the *dtpT* promoter.

Nine-bp sequences with one mismatch to the proposed ScoC-binding consensus, AATAnTATT (Kallio et al., 1991), are located from positions −173 to −165 and −12 to −4 with respect to the *dtpT* transcription start point and overlap the experimentally determined ScoC-binding sites (Fig. 3A). The p3 mutations are within the downstream 9-bp sequence, but the p4 mutations are located several nucleotides upstream.

To test the role of the weak upstream ScoC-binding site II in *dtpT* regulation, we constructed a *dtpT84-lacZ* fusion, which lacks this site due to the deletion of 195 nt from the 5' end of the *dtpT279-lacZ* fusion. The *dtpT84-lacZ* fusion lost almost completely ScoC-mediated regulation (Table 2). We conclude that site II is also essential for efficient *dtpT* repression though it is located far upstream of the transcription start point. Similar roles of upstream ScoC-binding sites were previously reported for the *aprE* and *nprE* genes (Henner *et al.*, 1988; Kallio et al., 1991; Ogura *et al.*, 2004; Toma *et al.*, 1986). A possible explanation would be that interaction between ScoC molecules bound to the two sites is required for efficient repression.

### Paradoxical regulation of genes controlled by both CodY and ScoC

The global analyses of CodY-binding sites revealed that several ScoC-repressed genes or operons, such as *aprE, nprE, opp,* and *app*, contain strong CodY-binding sites (Belitsky and Sonenshein, 2013; Molle et al., 2003). However, only one of the operons, *app*, was derepressed in a *codY* null mutant strain in DNA microarray or RNA-Seq experiments; expression of the three other genes/operons did not change significantly (Brinsmade et al., 2014; Molle et al., 2003). Based on the results presented above, we considered the possibility that increased repression of these genes by the elevated level of ScoC in a *codY* mutant masks the loss of direct CodY-mediated repression.

The *oppABCDF* operon (formerly known as *spo0K*) encodes an ABC-type oligopeptide permease (Perego *et al.*, 1991; Rudner *et al.*, 1991). It is responsible for transporting signaling oligopeptides that are essential for sporulation and genetic competence development (Lazazzera *et al.*, 1997; Perego, 1997; Solomon *et al.*, 1995). In gel shift experiments, CodY bound a fragment containing ~0.4 kb upstream of the *oppA* coding region with high affinity (apparent $K_D$ 8 nM) (Fig. 4A and 4B). DNase I footprinting experiments showed that CodY strongly protects a region (site I) (Fig. 4A and 4D) from positions +78 to +104 with respect to the transcription start point (Irnov *et al.*, 2010). A weaker CodY-binding region (site II) was detected by footprinting at positions −70 to −36 and a third, even weaker, region (site III) at positions +31 to +59 (Fig. 4D and 4E). A shorter 323-bp *oppA* fragment was used for footprinting in Fig. 4E to resolve better the upstream binding site II; it follows that binding to site II, at least *in vitro*, is independent of binding to other sites. The first two sites correlated very well with the core binding sites determined by IDAP-Seq (from positions +85 to +105 and upstream of position −41); the upstream boundary of site II and location of the weak third site could not be determined by IDAP-Seq due to the signal interference from the strongest binding site I (Fig. 2B, 2C and 4A) (Belitsky and Sonenshein, 2013).

The *oppA* regulatory region contains four 15-bp sequences (each with four mismatches) that resemble the consensus CodY-binding motif. Two motifs, overlapping by 6 bp, are located at positions +81 to +95 and +90 to +104 with respect to the transcription start point; another sequence is located at positions +32 to +46 (Fig. 4A). These two locations correspond very well to the strongest (site I) and weakest (site III) CodY-binding sites detected by footprinting. No candidate CodY-binding motif with fewer than six mismatches could be detected for the upstream binding site of intermediate strength (site II).

Binding of ScoC to the *oppA* regulatory region has never been assayed. In gel shift experiments, ScoC bound the *oppA* promoter region with an affinity similar to that of the *dtpT* gene (apparent $K_D \approx 25$ nM) (Fig. 4C). In DNase I footprinting experiments, ScoC protected two regions, sites II and I, from positions −179 to −157 and −45 to −22 with respect to the *oppA* transcription start point, respectively (Fig. 4E). We conclude that the negative effect of ScoC on the *oppA* promoter is direct. A 9-bp sequence with no mismatches to the proposed ScoC-binding consensus is located from positions −42 to −34 with respect to the *oppA* transcription start point and within the experimentally determined ScoC-binding site I; no such sequence with 2 mismatches to the consensus can be found at the location of site II.

All CodY-binding sites and ScoC-binding site I are located at positions appropriate for repression of *oppA* transcription through interference with either initiation or elongation of transcription. The downstream ScoC-binding site I partially overlaps the upstream CodY-binding site II (Fig. 4A and 4E). As a result, each protein in increasing concentrations was able to displace the other from *oppA* DNA (Fig. 5).

A transcriptional *oppA421-lacZ* fusion, containing the 421-bp *oppA* promoter region used for DNA binding, was constructed. Despite strong binding of CodY *in vitro*, no significant effect of a *codY* mutation on expression of the fusion was detected (Table 3), consistent with

the results of global analyses of the CodY regulon (Brinsmade et al., 2014; Molle et al., 2003). A transcriptional *oppA735-lacZ* fusion, containing the entire long intergenic region upstream of *oppA* was tested previously [Table S1 of Ref. (Belitsky and Sonenshein, 2013)]. The level of its expression was almost identical to that of the *oppA421-lacZ* fusion both in wild-type and *codY* mutant strains.

In accord with a previous report (Koide et al., 1999), expression of the *oppA421-lacZ* fusion increased about 2.6-fold in a *scoC* mutant (Table 3). Importantly, in a *codY scoC* double mutant, expression from the *oppA* promoter increased a further 5.1-fold (Table 3). We conclude that CodY is indeed a direct repressor of the *opp* operon but the effect of a *codY* mutation can be observed only in the absence of ScoC. This result supports the prediction that increased expression of ScoC, resulting from the absence of CodY, can compensate for the loss of CodY-mediated repression. Thus, paradoxically, in addition to being a direct negative regulator of the *opp* operon, CodY acts as an indirect positive regulator by repressing the synthesis of the second negative regulator, ScoC.

The negative, 6.3-fold, effect of CodY was also revealed in a *scoCp1* strain in which the chromosomal *scoC* allele cannot be repressed by CodY (Table 3, strains BB4058 and BB4060). In $codY^+$ *scoCp1* cells, expression from the *oppA* promoter was 3.3-fold lower than in $codY^+$ $scoC^+$ cells, apparently due to higher, unregulated expression of ScoC (Table 3). In fact, these results may underestimate the role of ScoC since the *scoCp1* mutation appears to reduce expression from the *scoC* promoter (Table 1); indeed, the higher expression from the *oppA* promoter in *codY scoCp1* cells, compared with *codY* $scoC^+$ cells (Table 3), apparently results from the lower level of ScoC expressed from the *scoCp1* allele.

The model summarizing the functional interaction between CodY and ScoC at the *oppA* promoter and its comparison to the regulation of the *dtpT* promoter is presented in Fig. 6. Competition between CodY and ScoC at the level of DNA binding is also likely to contribute to the regulation of the *oppA* promoter.

Both CodY and ScoC contributed to repression of the *opp* operon in a wild-type strain under our growth conditions. The high, derepressed level of ScoC in a *codY* mutant strain caused more efficient *opp* repression (15.5-fold) than that provided by the CodY-repressed level of ScoC (2.6-fold) or by CodY (5.1-fold, as detected in a *scoC* mutant strain). The combined effect of the two regulators in a wild-type strain was less than a sum of their maximal effects, reflecting their complex roles in *opp* regulation (Table 3).

To test the role of different CodY-binding sites in *oppA* regulation, we constructed an *oppA323-lacZ* fusion, which lacks sites I and III due to the deletion of 98 nt from the 3' end of the *oppA421-lacZ* fusion. The *oppA323-lacZ* fusion lost half of CodY-mediated regulation (2.3-fold versus 5.1-fold) (Table 3). We conclude that both downstream and upstream CodY-binding sites contribute to *oppA* regulation.

To test the roles of ScoC-binding sites in *oppA* regulation, we constructed an *oppA203-lacZ* fusion, which lacks the upstream site II due to the deletion of 218 nt from the 5' end of the *oppA421-lacZ* fusion. The *oppA203-lacZ* fusion lost completely ScoC-mediated regulation

(Table 3), though *in vitro* ScoC was still able to bind to the *oppA203* fragment (data not shown). We conclude that, as for other genes, the far upstream ScoC-binding site II is essential for *oppA* repression.

### Role of TnrA in ScoC-mediated dtpT and oppA regulation

To determine whether the activity of ScoC varies under different growth conditions, we altered the composition of the medium, using the ScoC-regulated *dtpT279p⁺-lacZ* fusion as a reporter. In a wild-type strain in TSS glucose-ammonium medium without added amino acids, CodY has low activity (Belitsky and Sonenshein, 2008; Slack et al., 1995) and, therefore, transcription of the *scoC* gene was derepressed (Table 4, strain GB1039); as a result, the *dtpTp⁺-lacZ* fusion was expressed at the same low level as in a *codY* mutant strain in TSS + 16 aa medium (Tables 2 and 5, strain BB3928). However, if glutamate, a poor nitrogen source, was substituted for ammonium as the sole nitrogen source in TSS medium, expression from the *scoC* promoter remained largely unchanged (Table 4), but expression of the *dtpTp⁺-lacZ* fusion increased 4.7-fold (Table 5). As expected in the absence of other exogenous amino acids, a *codY* null mutation had only a small effect on *dtpT* expression either in glucose-ammonium or in glucose-glutamate medium (Table 5, strain BB3930).

Because TnrA, a transcriptional regulator of many nitrogen metabolism genes in *B. subtilis*, is known to be activated in glucose-glutamate medium (Fisher, 1999; Yoshida *et al.*, 2003), we tested expression of the *dtpTp⁺-lacZ* fusion in a *tnrA* null mutant strain. Indeed, in the absence of TnrA, expression from the *dtpTp⁺* promoter was reduced in glucose-glutamate medium to the level seen in glucose-ammonium medium, indicating that glutamate-mediated activation of the *dtpTp⁺-lacZ* fusion is due to altered TnrA activity (Table 5, strains BB3992 and BB3960). Interestingly, no effect of glutamate or a *tnrA* mutation on the derepressed level of *dtpT* expression was observed in a *scoC* mutant (Table 5, strains BB3929 and BB3969). Similarly, no effect of glutamate was detected on expression of the *dtpTp3-lacZ* fusion containing a mutation in the ScoC-binding site (Table 5, strain BB3971). Thus, TnrA is not able to activate the *dtpT* promoter by itself, indicating that the role of TnrA is to interfere with ScoC-mediated repression.

In contrast to the *dtpT* gene, which was not previously known to be regulated by TnrA, *opp* expression has been shown to be under direct positive TnrA control (Yoshida et al., 2003). In accord with this result, in TSS glucose-glutamate medium, expression of the *oppA421-lacZ* fusion was 3.7-fold higher in *tnrA⁺* cells compared to that in *tnrA* mutant cells (Table 6). However, no effect of a *tnrA* mutation was observed in a *scoC* mutant strain, indicating that *oppA* activation by TnrA, like that of the *dtpT* gene, is apparently due to interference with repression by ScoC (Table 6). In both cases, TnrA reduced the ability of ScoC to repress the *dtpT* or *oppA* promoter only partially, because the level of expression of the corresponding fusions in TSS glucose-glutamate medium in a *tnrA* mutant did not reach that seen in *scoC* mutant cells (Tables 5 and 6).

It was reported that *scoC* may be a target of direct repression by TnrA (Abe *et al.*, 2009). We have tested the effect of TnrA on *scoC* expression in glucose-glutamate medium, which supports high activity of TnrA, but it was very small if any (Table 4). Therefore, it is

unlikely that the positive effect of TnrA on *dtpT* or *oppA* expression is mediated through reduced *scoC* expression.

## DISCUSSION

### Functional interaction between CodY and ScoC

The results presented here establish that *B. subtilis* ScoC expression is under direct, negative control by CodY. The *Bacillus anthracis scoC* gene (BA1045) also appears to be repressed by CodY (van Schaik *et al.*, 2009). Therefore, ScoC-regulated genes, such as *dtpT*, are potentially subject to cascade regulation that depends on the level of CodY activity (Fig. 6). Under growth conditions in which CodY activity is high, CodY should act as an indirect positive regulator of ScoC-repressed genes by reducing the level of ScoC if those genes do not require other factors for expression under the growth condition used and if the relatively low level of ScoC in wild-type cells is not already sufficient for maximal repression. In addition to *dtpT*, several other genes and operons, such as *rbs* and *yppF*, that are negatively regulated by ScoC (Caldwell et al., 2001)(Caldwell et al., personal communication) have been catalogued as genes positively regulated by CodY (Brinsmade et al., 2014) but lack any associated CodY-binding sites (Belitsky and Sonenshein, 2013). It is likely that for these genes and possibly other targets of ScoC the effect of CodY is indirect and mediated through ScoC.

Our results also describe a second, seemingly paradoxical form of CodY-ScoC regulatory interaction seen for genes that are negatively regulated by both CodY and ScoC. For such genes and operons, e.g., *opp*, CodY and ScoC form a feed-forward regulatory loop, an arrangement in which two regulatory proteins control expression of the same target gene and one of the regulators controls expression of the other (Alon, 2007; Mangan and Alon, 2003) (Fig. 6). In this case, when CodY activity decreases due to nutrient exhaustion, an increase in expression of the target gene will only occur if the increased expression of ScoC is insufficient to maintain repression; in other cases, it might lead to enough accumulation of ScoC to decrease expression even further. Thus, the extent to which any target gene responds to this feed-forward loop depends on the independent efficiencies of repression of that gene by CodY and ScoC. Depending on the parameters of interaction between each protein and DNA, feed-forward loops may affect the kinetics of the target gene response when the upstream regulator gains or loses activity (Mangan and Alon, 2003; Wall *et al.*, 2005).

In the case of the *opp* operon, the direct negative and indirect positive effects of CodY are complemented by the competition with ScoC at CodY-binding site II and appear to balance each other almost perfectly; consequently, no increase in expression was seen when the *codY* gene was inactivated. Only weak *opp* repression by ScoC was achieved in *codY*^{+} cells due to low *scoC* expression. Both *codY* and *scoC* genes had to be inactivated simultaneously in order to fully derepress *opp* transcription under the steady-state growth conditions used. Thus, the combined actions of CodY and ScoC serve to maintain a significant level of *opp* repression even when one of the regulators is not active or not expressed but also avoid overly strong repression of the operon by fully active regulators.

More generally, the presence of CodY and ScoC in the same cell allows different kinds of outcomes when CodY loses activity due to nutrient exhaustion. Genes directly repressed solely by CodY should show increased expression. Genes directly repressed by ScoC, but not by CodY, should have reduced expression due to the increased level of ScoC, whereas genes controlled negatively and directly by both CodY and ScoC can have varying responses depending on the concentrations of the active forms of the proteins needed to bind to the regulatory regions of the genes in question and the strength of the corresponding binding sites.

In addition to ScoC, synthesis of another important regulator, ComK, as well as the ComS protein, which controls ComK degradation (Hamoen *et al.*, 1995), were shown to be directly repressed by CodY in *B. subtilis* (Serror and Sonenshein, 1996a; Smits et al., 2007). ComK is a master regulator of genetic competence and its expression is under the control of several additional transcriptional regulators (Hamoen *et al.*, 2003a; Hamoen *et al.*, 2003b); however, none of them is known to be regulated by CodY.

The *sinIR* operon was reported to be under direct, negative ScoC control (Kallio et al., 1991; Shafikhani *et al.*, 2002). SinR is a master regulator of biofilm formation in *B. subtilis* whose activity is inhibited by SinI (Bai *et al.*, 1993; Kearns *et al.*, 2005). This finding implies that at least some SinR targets (Chu *et al.*, 2006) may be subject to a two-step cascade regulation mediated by CodY, ScoC, and SinR, but, since *sinI* and *sinR* are both repressed by ScoC, it remains unclear whether the contribution of ScoC to SinR activity is negative or positive.

### Regulation of ScoC activity

We have detected a low level (two- to three-fold or less) of negative autoregulation by ScoC that apparently serves to moderate the effect of CodY-mediated regulation on expression of the *scoC* gene. That is, expression of *scoC* increases when CodY activity decreases, but the maximum extent of ScoC accumulation is limited by its self-repression.

CodY is the only DNA-binding regulator known to affect expression of ScoC directly. Expression of *scoC* is moderately increased in nutrient broth medium in a *spo0A* mutant strain, which is consistent with activation of *scoC* by AbrB, an important transition state regulator (Perego and Hoch, 1988)(B. R. Belitsky and A. L. Sonenshein, data not shown). However, we did not observe any significant effect of an *abrB* null mutation on *scoC-lacZ* expression under our growth conditions and attempts to demonstrate specific binding of AbrB to the *scoC* regulatory region have been unsuccessful (Strauch *et al.*, 1989). A global study of sites that are bound by AbrB *in vivo* also failed to detect *scoC* as a target (Chumsakul *et al.*, 2010). SalA and multicopy SenS have also been reported to negatively affect expression of *scoC*, but the molecular mechanisms of action of these proteins are not known (Kawachi *et al.*, 2005; Ogura et al., 2004).

Although many transcriptional regulators of the MarR-family respond to low molecular-weight effectors (Perera and Grove, 2010; Wilkinson and Grove, 2006), no effectors of ScoC activity are known. Expression of the ScoC-regulated *dtpT* gene varies to a limited extent (less than 1.7-fold) over a very wide range of growth conditions (Nicolas et al., 2012), indicating that activity of ScoC was not drastically altered in those experiments. Although

we found that *dtpT* expression is activated in glucose-glutamate medium, this effect is due to TnrA-mediated interference with ScoC functioning. Thus, the activity of ScoC appears to be determined principally, if not solely, by modulation of its expression or DNA-binding by other transcription factors.

In other studies, ScoC was reported to be phosphorylated by the protein arginine kinase McsB at the Arg3 position (Elsholz *et al.*, 2012). In our hands, neither an *mcsB* null mutation nor a null mutation in the *ywlE* gene, encoding protein arginine phosphatase, affected expression of the *dtpTp⁺-lacZ* fusion used as a reporter for ScoC activity in a *codY⁺* or *codY* mutant strain (data not shown).

**Oligopeptide permeases as targets of complex transcriptional regulation**

Though the function of the DtpT protein has never been established experimentally, its sequence strongly suggests that DtpT is an oligopeptide permease. Three other peptide permeases of the ABC transporter type and encoded by the *dpp-ykf, opp*, and *app* operons are present in *B. subtilis* (Barbe *et al.*, 2009). Interestingly, three of the four oligopeptide permeases, DtpT, Opp, and App, have been shown to be negatively regulated by ScoC in this work and previously (Caldwell et al., 2001; Koide et al., 1999). Dpp, Opp, and App oligopeptide permeases are also direct targets of repression by CodY (Belitsky and Sonenshein, 2013; Molle et al., 2003; Serror and Sonenshein, 1996b; Slack et al., 1995)(this work). Thus, ScoC and CodY cooperate in the regulation of *B. subtilis* oligopeptide uptake either by binding to the promoter regions or through CodY-mediated repression of ScoC or both. Expression of the *dpp-ykf* and *app* operons is also known to be negatively regulated by AbrB; in contrast, the *opp* operon appears to be under positive regulation of AbrB (Koide et al., 1999; Slack *et al.*, 1991).

As noted above, CodY serves as an indirect positive regulator of the ScoC-repressed *dtpT* gene and *opp* operon. Interestingly, under growth conditions when CodY is inactive, another transcription regulator, TnrA, which is active only under conditions of nitrogen limitation, acts as a positive regulator of the same two oligopeptide permeases via interference with ScoC action. This role of TnrA is consistent with its main function, which is to increase the supply of nitrogen-containing nutrients under conditions of nitrogen limitation (Fisher, 1999). The TnrA-binding sites of the *dtpT* and *oppA* genes have been identified recently by a ChIP-on-chip experiment (Mirouze *et al.*, 2015). They do not overlap the ScoC-binding sites of these genes, and the mechanism(s) of interaction between TnrA and ScoC remains unknown. Because *scoC* expression was reported to be negatively affected by TnrA (Abe et al., 2009), we cannot exclude that TnrA, like CodY, affects *dtpT* and *oppA* expression both directly and indirectly.

In summary, our results indicate that ScoC's function as a transcriptional repressor may be determined by the activities of two other transcriptional regulators, CodY and TnrA. Both CodY and ScoC act as direct repressors of the *opp* operon, forming a rare feed-forward regulatory loop. It also follows that a complete picture of the ScoC regulon can be revealed only when both CodY and TnrA are inactive. Although in most previous experiments the growth media used contained an excess of amino acids, thereby activating CodY and inactivating TnrA, in some experiments amino acids could have been partially exhausted

and the level of ScoC would have increased due to CodY inactivation. Whether the resulting concentration of ScoC would have reached the level needed for efficient binding to all of its targets remains unknown, as does the full impact of ScoC on gene expression.

## Experimental procedures

### Bacterial strains and culture media

The *B. subtilis* strains constructed and used in this study were all derivatives of strain SMY (Zeigler *et al.*, 2008) and are described in Table 7 or in the text. *Escherichia coli* strain JM107 (Yanisch-Perron *et al.*, 1985) was used for isolation of plasmids. Bacterial growth in TSS 0.5% glucose medium supplemented with 0.2% $NH_4Cl$ or sodium glutamate or DS nutrient broth medium was as described (Belitsky and Sonenshein, 2011b).

### DNA manipulations

Methods for common DNA manipulations, transformation, and sequence analysis were as previously described (Belitsky and Sonenshein, 1998). All oligonucleotides used in this work are described in Table 8. Chromosomal DNA of *B. subtilis* strain SMY or plasmids constructed in this work were used as templates for PCR. All cloned PCR-generated fragments were verified by sequencing.

### Construction of transcriptional fusions

Plasmid pGB15 (*scoC561p⁺-lacZ*) was created by cloning the XbaI- and HindIII-treated PCR product, containing the entire *scoC* regulatory region, in an integrative plasmid pHK23 (*erm*) (Belitsky and Sonenshein, 2008). The 0.57-kb *scoC* PCR product was synthesized with oGB26 and oGB28 as primers; the resulting fusion is identical to one described earlier (Kawachi et al., 2005). Plasmids pBB1812 (*scoC350p⁺-lacZ*) or pBB1813 (*scoC181p⁺-lacZ*), containing the same *scoC* regulatory region but truncated from the 5' end, were constructed in a similar way by cloning the 3' part of the same PCR fragment after its digestion with BstYI and HindIII or ApoI and HindIII in pHK23, treated with BamHI and HindIII or EcoRI and HindIII, respectively.

Plasmid pBB1819 (*dtpT279p⁺-lacZ*) was created by cloning the EcoRI- and HindIII-treated PCR product, containing the entire *dtpT* regulatory region, in pHK23. The 0.29-kb *dtpT* PCR product was synthesized with oBB697 and oBB698 as direct and reverse primers, respectively. Plasmid pBB1833 (*dtpT84p⁺-lacZ*), containing the *dtpT* regulatory region truncated from the 5' end, was constructed as pBB1819, but using oBB712 as the direct PCR primer. Plasmid pBB1741 (*oppA735p⁺-lacZ*) was created by cloning the EcoRI- and BglII-treated PCR product, containing the entire *oppA* regulatory region, in pHK23. The 0.74-kb *oppA* PCR product was synthesized with oBB576 and oBB577 as direct and reverse primers, respectively. Plasmid pBB1742 (*oppA421p⁺-lacZ*), containing the *oppA* regulatory region truncated from the 5' end, was constructed in a similar way by cloning the 0.42-kb 3' part of the same PCR fragment after its digestion with MfeI and BglII. Plasmid pBB1827 (*oppA323p⁺-lacZ*), containing the *oppA* regulatory region truncated from the 3' end, was constructed as pBB1742, but using oBB708 as the reverse PCR primer. Plasmid pBB1831

(*oppA203p$^+$-lacZ*), containing the *oppA* regulatory region truncated further from the 5' end, was constructed as pBB1741, but using oBB711 as the direct PCR primer.

*B. subtilis* strains carrying various *lacZ* fusions at the *amyE* locus (Table 7) were isolated after transforming strain BB2511 (*amyE::spc lacA*) 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. Strain BB2511 and all its derivatives have very low endogenous β-galactosidase activity due to a null mutation in the *lacA* gene [Daniel, 1997 #33].

## Mutations in the CodY- and ScoC-binding sites

Plasmids pGB18 (*scoC561p1-lacZ*), pGB22 (*scoC561p2-lacZ*), and pBB1817 (*scoC561p3-lacZ*), containing 1-bp or 2-bp substitution mutations in the CodY-binding site, were constructed as described for pGB15 using fragments generated by two-step overlapping PCR. In the first step, a product containing the 5' part of the *scoC* regulatory region was synthesized by using oligonucleotide oGB26 as the forward primer and mutagenic oligonucleotide oGB39, oGB41 or oBB695 as the reverse primer. A product containing the 3' part of the *scoC* regulatory region was synthesized by using mutagenic oligonucleotides oGB38, oGB40 or oBB696 as the forward primer and oligonucleotide oGB28 as the reverse primer. The PCR products were used in a second, splicing step of PCR mutagenesis as overlapping templates to generate a modified fragment containing the entire *scoC* regulatory region; oligonucleotides oGB26 and oGB28 served as the forward and reverse PCR primers, respectively.

Plasmids pBB1821 (*dtpTp1-lacZ*), pBB1822 (*dtpTp2-lacZ*), pBB1823 (*dtpTp3-lacZ*), and pBB1825 (*dtpTp4-lacZ*), containing 2-bp substitution mutations in the ScoC-binding site, were constructed as described for pBB1819, using mutagenic oligonucleotides oBB701, oBB702, oBB703 or oBB704 as the reverse PCR primer, respectively.

## Construction of the scoCp1 mutant

The 0.64-kb XbaI-SalI PCR fragment of pGB18 (*scoC561p1-lacZ*), containing the entire *scoC* insert, was cloned in the integrative plasmid pBB1579 (*neo bgaB*) (Belitsky and Sonenshein, 2011a). The resulting plasmid, pBB1830, was introduced by a single-crossover homologous recombination event into the *scoC* chromosomal locus of strain BB1888 (*lacA*). White Neo$^s$ colonies indicating excision of pBB1830 from the chromosome were searched for on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), the colored substrate of *bgaB*-encoded β-galactosidase. The presence of the *scoCp1* mutation in strain BB4042 was confirmed by sequencing of the chromosomal *scoC* allele.

## Purification of CodY and ScoC

CodY-His$_5$ was purified to near homogeneity as described previously (Belitsky and $_6$-ScoC was purified to near homogeneity in the same manner after induction plasmid-containing *E. coli* BL21(DE3) pLysS cells (Studier and Moffatt, 1986) with 0.1% IPTG for 4 hours. Elution from the Ni$^{2+}$-affinity column (His·Bind resin; Novagen) was with a buffer containing 385 mM imidazole. The plasmid pRSETA-scoC, containing the *scoC* open

reading frame cloned between the BamHI and EcoRI sites of pRSET A (Life Technologies, Thermo Fisher Scientific), was obtained from U. Gerth. The resulting tagged protein contained the peptide MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS as its N-terminal extension.

## Labeling of DNA fragments

The PCR products containing the regulatory region of the *scoC* or *dtpT* or *oppA* gene were synthesized using vector-specific oligonucleotides oBB67 and oBB102 as the forward and reverse primer, respectively. 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 96 bp or 112 bp upstream of the XbaI or EcoRI site used for cloning, respectively, and oBB102 starts 36 bp or 65 bp downstream of the HindIII or BglII site that served as a junction between the promoters and the *lacZ* part of the fusions, respectively.

## Gel shift assays and DNase I protection experiments

Incubation of CodY or ScoC with the $^{32}$P-labeled promoter fragments was performed in a binding buffer containing 20 mM Tris-Cl (pH 8.0) - 50 mM KCl - 2 mM MgCl2 - 5% glycerol - 0.5 mM EDTA - 1 mM DTT - 0.05% Nonidet P-40 - 25 μg/ml sonicated salmon sperm DNA. Samples (11 μl) containing varying amounts of proteins and less than 1 fmole of DNA were incubated for 16 min at room temperature and separated on 8% nondenaturing 50 mM Tris - 384 mM glycine - 1 mM EDTA polyacrylamide gels in 35 mM Hepes - 43 mM imidazole buffer. In some experiments, 10 mM ILV were present in the incubation mixture and gel buffers.

For DNase I protection experiments, samples containing 20–40 fmoles of labeled DNA were incubated with proteins as described above. One μl of the binding buffer containing 0.1–0.2 U RQ1 DNase I (Promega), 10 mM MgCl2 and 20 mM CaCl2 was then added, followed by addition, after 1 min, of 4 μl of 20 mM EDTA-95% formamide dye solution and subsequent heating of the samples at 80°C for 5 min. The samples were loaded without further purification on 7 M urea - 6% polyacrylamide DNA sequencing gels. The G+A sequencing ladder, generated according to a published procedure by boiling the appropriate samples of labeled DNA for 20 min (Liu and Hong, 1998), served to locate precisely the protected region.

The gels were dried, and the radioactive bands were detected and quantified using storage screens, an Applied Biosystems PhosphorImager, and ImageQuant software (GE Healthcare).

## Enzyme assays

β-Galactosidase specific activity was determined as described previously (Belitsky and Sonenshein, 1998).

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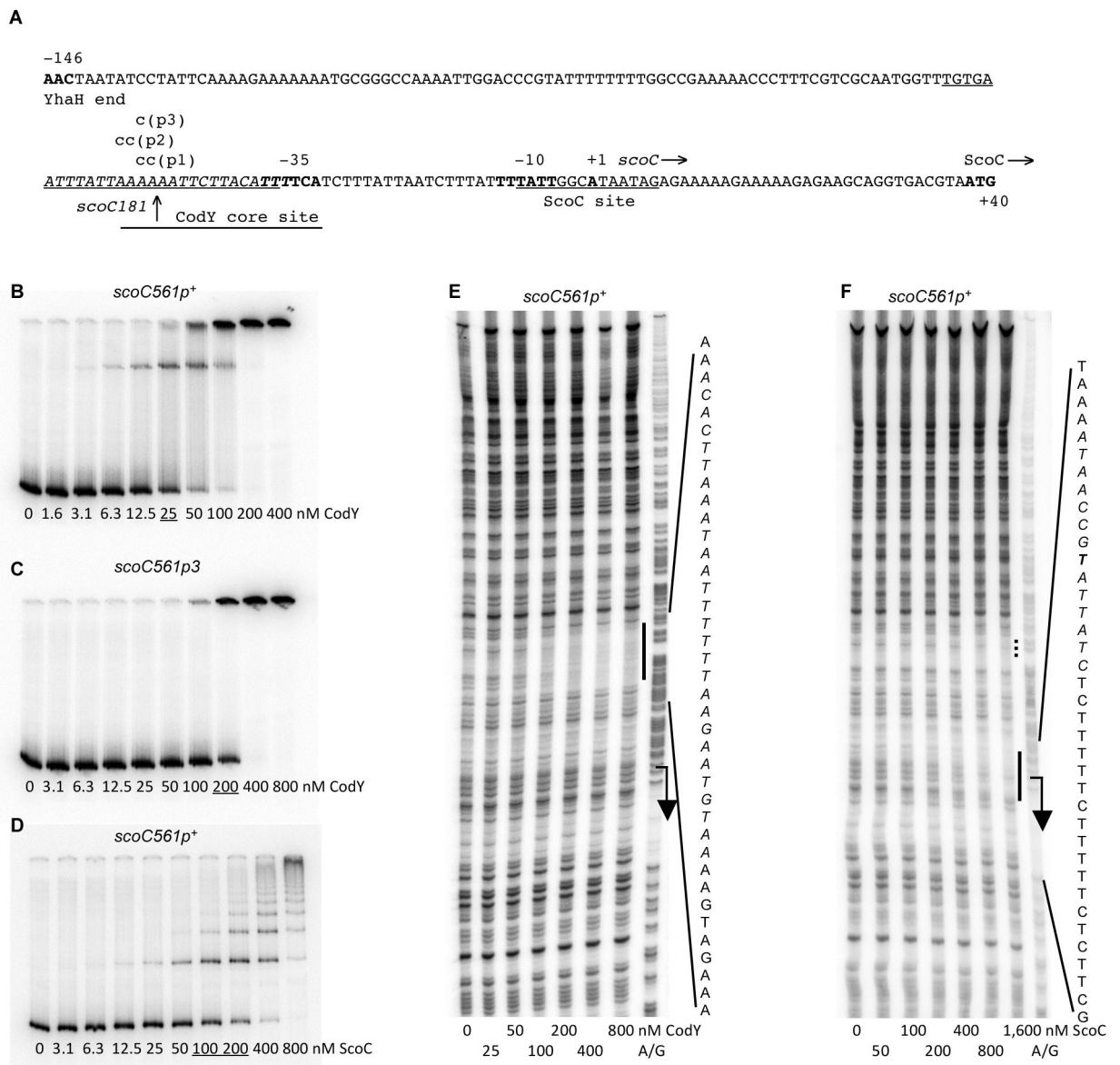
**Fig. 1.**

Binding of CodY and ScoC to the *scoC* regulatory region.

**A.** The sequence (5' to 3') of the coding (non-template) strand of the *scoC* regulatory region. Coordinates are reported with respect to the transcription start point (Abe et al., 2009). The upstream endpoints of inserts within the *scoC561* and *scoC350* fusions are at positions −422 and −211, respectively. The upstream boundary of the *scoC181-lacZ* fusion at position −42 is indicated by a vertical arrow. The downstream boundary for all fusions is at position +139. The likely translation initiation codon (ATG), −10 and −35 promoter regions, and transcription start point are in bold. The directions of transcription and translation are indicated by the horizontal arrows. The mutated nucleotides are shown in lowercase above the sequence. The sequences on the template strand that were protected by CodY or ScoC in DNase I footprinting experiments are underlined. The core CodY-binding site identified by

IDAP-Seq (Fig. 2A) is shown by a horizontal line below the sequence. Two overlapping CodY-binding motifs with 4 and 5 mismatches to the consensus are italicized; two additional motifs with 4 and 5 mismatches to the consensus can be found in the promoter region but are not indicated.

**B and C.** Gel shift assays of CodY binding in the presence of 10 mM ILV to radioactively labeled *scoC561p$^+$* (B) or *scoC561p3* (C) PCR fragments obtained with oligonucleotides oBB67 and oBB102. CodY concentrations used (nM of monomer) are reported below each lane; concentrations corresponding to the apparent $K_D$ for binding are underlined.

**D.** Gel shift assay of ScoC binding to the *scoC561p$^+$* PCR fragment. ScoC concentrations (nM of monomer) are reported below each lane; concentrations corresponding to the apparent $K_D$ for binding are underlined.

**E and F.** DNase I footprinting analysis of protein binding to the *scoC* regulatory region. The *scoC561p$^+$* DNA fragment labeled on the template strand and used in panel B was incubated with increasing amounts of purified (E) CodY in the presence of 10 mM ILV or (F) ScoC and then treated with DNase I. The protected areas are indicated by vertical lines and the corresponding sequences are reported; the protected nucleotides are italicized. The transcription start point and direction of transcription are shown by a bent arrow. Protein concentrations used (nM of monomer) are indicated below each lane. The A + G sequencing ladder of the template DNA strand is shown in the right lane in each case.
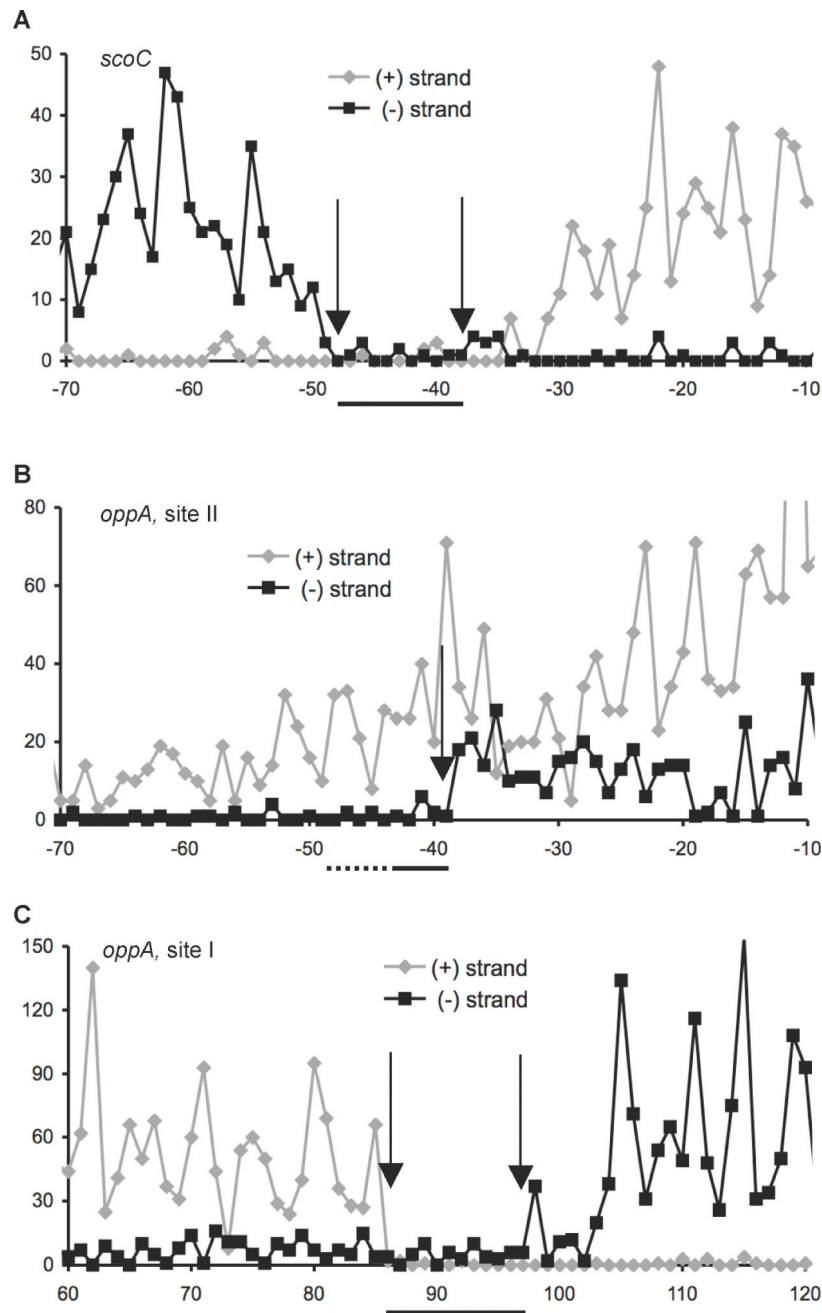
**Fig. 2.**
Coverage maps of CodY-binding sites in the (A) *scoC* and (B and C) *oppA* regulatory regions using strand-specific counting of 5' nucleotides of Illumina sequencing reads obtained in IDAP-Seq experiments. The coverage of the (+) and (−) strands is shown in grey and black, respectively. Arrows indicate boundaries of coverage gaps corresponding to the boundaries of core binding sites at the CodY concentration used. The core site location is also indicated by horizontal lines below x-axes; the 5' end of the *oppA* site II was not determined. Forty nM CodY was used for affinity purification of DNA fragments in presented experiments; the boundaries of the core sites, mentioned in the text, were deduced

from multiple experiments using a range of CodY concentrations. Non-uniform coverage is likely due to varying position-specific efficiency of DNA shearing during sonication and to non-uniform size of DNA fragments. Coordinates are reported with respect to the transcription start points. The results shown here were deduced from data provided as supplementary material in a previous publication (Belitsky and Sonenshein, 2013) but were not previously analyzed.

**Fig. 3.**

Binding of CodY and ScoC to the *dtpT* regulatory region.

**A.** The sequence (5' to 3') of the coding (non-template) strand of the *dtpT* regulatory region. Coordinates are reported with respect to the transcription start point as deduced from the mutational analysis (see text). The 5' nucleotide of the sequence presented corresponds to the first nucleotide of the *dtpT* insert within the *dtpT279-lacZ* fusions. The vertical arrow above the sequence indicates the 5' nucleotide of the *dtpT84-lacZ* fusion at position −62. The vertical arrow below the sequence indicates the junction point, at position +22, between the *dtpT* and *lacZ* sequences. The likely translation initiation codon (ATG), −10 and −35 promoter regions, and transcription start point are in bold. The directions of transcription and translation are indicated by the horizontal arrows. The mutated nucleotides are shown in lowercase above the sequence. The sequences protected by ScoC in DNase I footprinting experiments on the template strand of DNA are underlined. The ScoC-binding motifs are italicized.

**B, C and D.** Gel shift assay of protein binding to a radioactively labeled *dtpT279p+* (B and C) or *dtpT279p3* (D) PCR fragment, obtained with oligonucleotides oBB67 and oBB102. The proteins tested for binding were (B) CodY in the presence of 10 mM ILV or (C and D) ScoC. Protein concentrations (nM of monomer) are reported below each lane. The underlined concentrations indicate the apparent $K_D$ for binding.

**E and F.** DNase I footprinting analysis of ScoC binding to the *dtpT* regulatory region. The *dtpT279p+* (E) or *dtpT279p3* (F) DNA fragment labeled on the template strand and used in

panels B or D, respectively, was incubated with increasing amounts of purified ScoC and then treated with DNase I. See the legend to Fig. 1E for additional details.
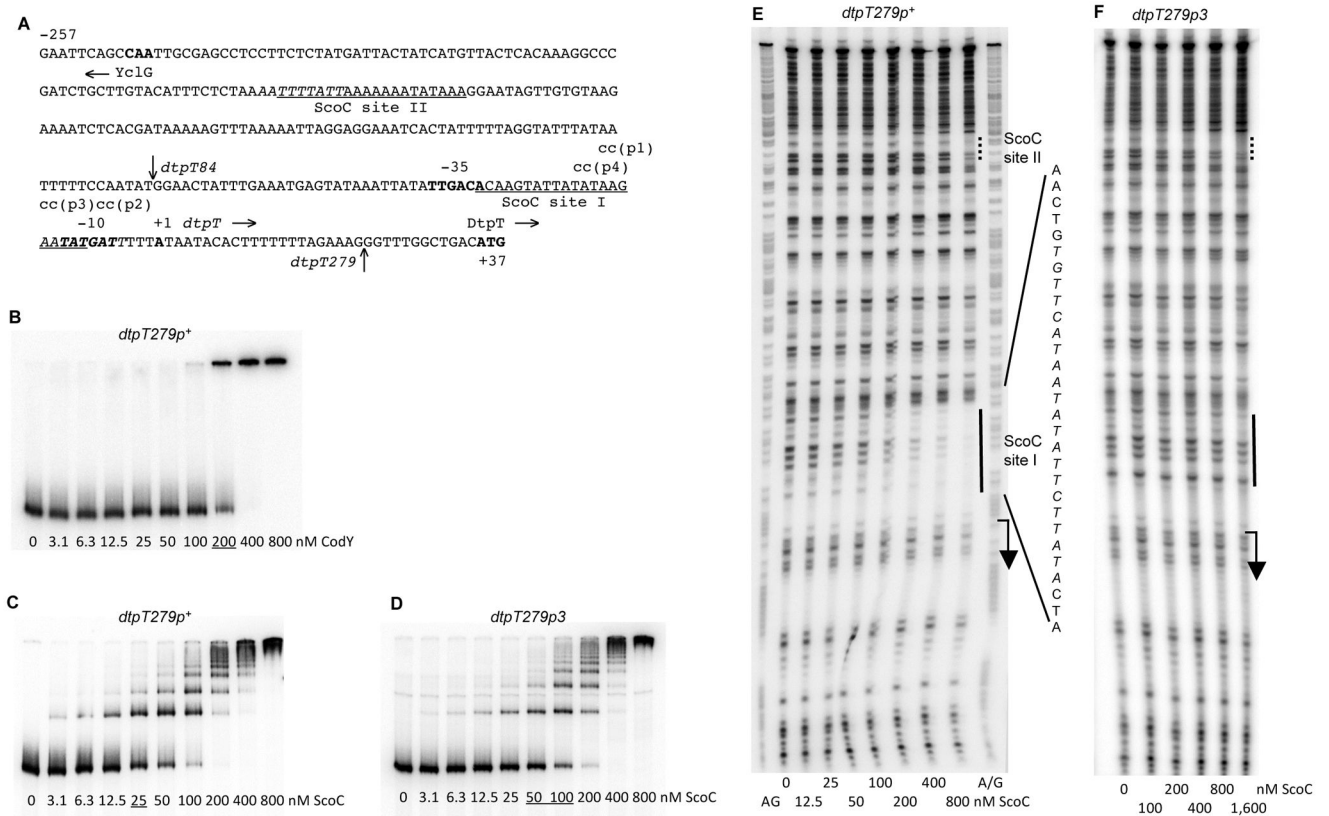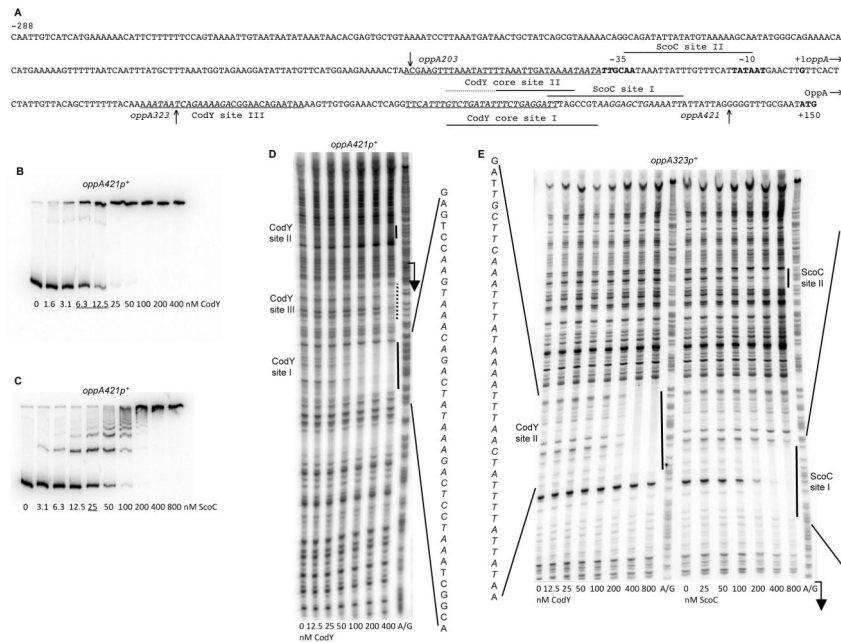
**Fig. 4.**

Binding of CodY and ScoC to the *oppA* regulatory region.

**A.** The sequence (5' to 3') of the coding (non-template) strand of the *oppA* regulatory region. Coordinates are reported with respect to the transcription start point (Irnov *et al.*, 2010). The 5' nucleotide of the sequence presented corresponds to the first nucleotide of the *oppA* insert within the *oppA421*- and *oppA323-lacZ* fusions. The vertical arrow above the sequence indicates the 5' nucleotide of the *oppA203-lacZ* fusion at position −69. The vertical arrows below the sequence indicate the junction points, at positions +36 and +134, between the *oppA* and *lacZ* sequences for the *oppA323* or *oppA421* and *oppA203* fusions, respectively. The likely translation initiation codon (ATG), −10 and −35 promoter regions, and transcription start point are in bold. The directions of transcription and translation are indicated by the horizontal arrows. The sequences protected by CodY in DNase I footprinting experiments on the template strand of DNA are underlined. The core CodY-binding sites I and II identified by IDAP-Seq (Fig. 2B and 2C) and ScoC-binding sites determined by footprinting are shown by horizontal lines below the sequence; the 5' boundary of the core site II is unknown. The CodY-binding motifs overlapping sites I and II and the ScoC-binding motif within site I are italicized; other CodY-binding motifs with 4 and 5 mismatches to the consensus can be found in the promoter region and are not shown.

**B and C.** Gel shift assays of protein binding to a radioactively labeled *oppA421p*[+] PCR fragment, obtained with oligonucleotides oBB67 and oBB102. The proteins tested were (B) CodY in the presence of 10 mM ILV and (C) ScoC. Protein concentrations used (nM of monomer) are reported below each lane; concentrations corresponding to the apparent $K_D$ for binding are underlined.

**D and E.** DNase I footprinting analysis of protein binding to the *oppA* regulatory region. The *oppA421p*[+] (D) or *oppA323p*[+] (E) DNA fragment, obtained with oligonucleotides oBB67 and oBB102 and labeled on the template strand, was incubated with increasing

amounts of (D and E) CodY in the presence of 10 mM ILV or (E) ScoC and then treated with DNase I. See the legend to Fig. 1E for additional details.
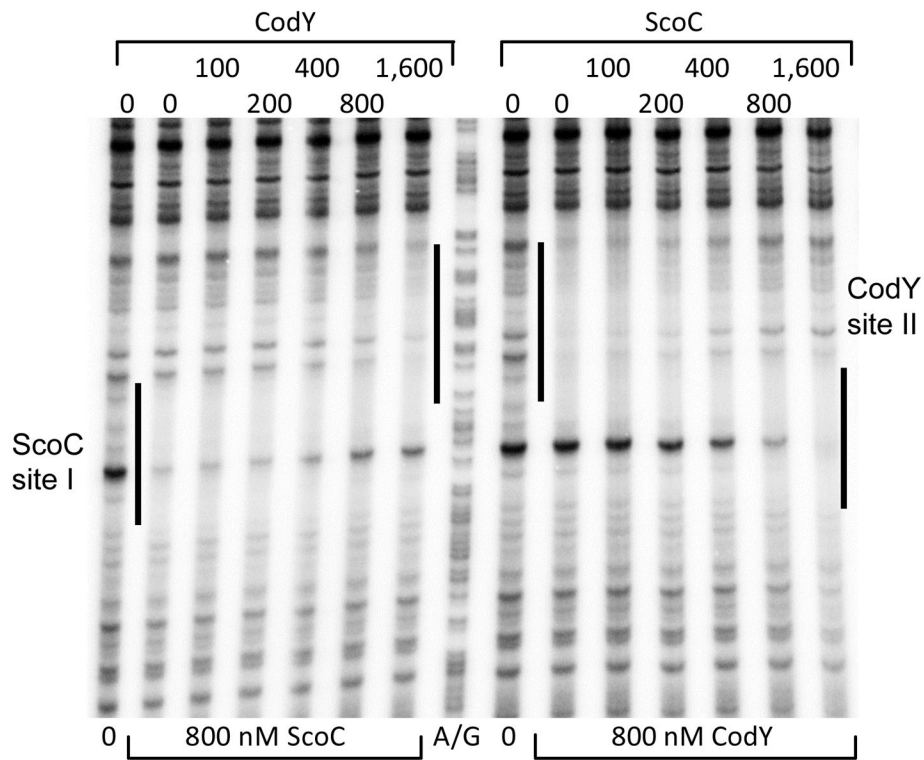
**Fig. 5.**
Competition between CodY and ScoC at *oppA* CodY-binding site II and ScoC-binding site I. The *oppA323p*[+] DNA fragment used in Fig. 4E was preincubated for 16 min with or without 800 nM ScoC or 800 nM CodY in the presence of 10 mM ILV, then incubated for additional 16 min with increasing amounts of either CodY or ScoC, and finally treated with DNase I. The corresponding A+G sequencing ladder is shown in the middle. The protected areas are indicated by vertical lines. Protein concentrations (nanomolar of monomer) used in the first and the second stages of the incubation are indicated below or above each lane, respectively.
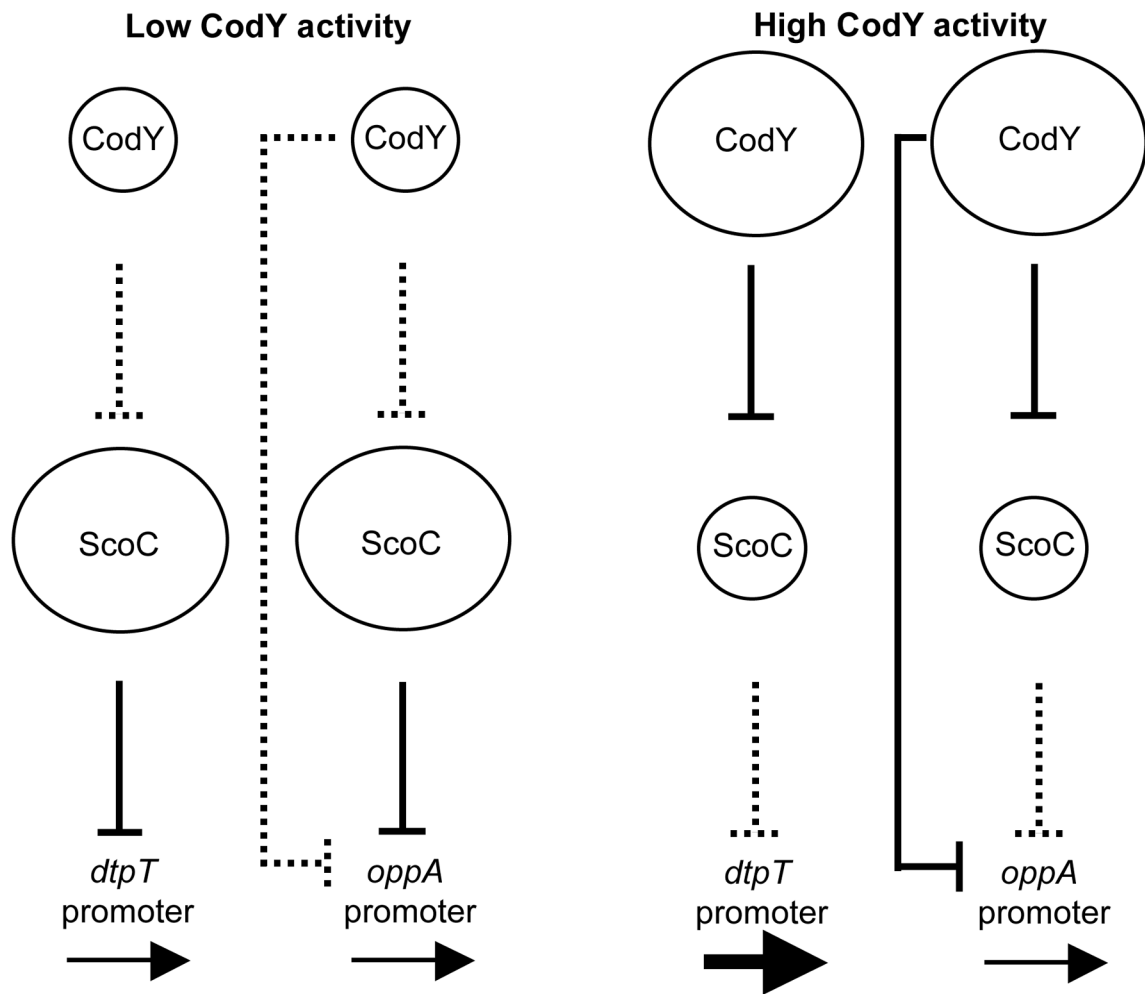
**Fig. 6.**
A model of regulation of the *dtpT* and *oppA* promoters by the combined actions of CodY and ScoC. The sizes of the circles reflect the relative amount of the active form of each protein. The solid vertical lines indicate relatively strong effects on transcription. Dotted lines indicate relatively weak effects on transcription. The boldness of the horizontal arrows indicates the relative strength of transcription of the target genes.

**Table 1**

Expression of *scoC-lacZ* fusions

| Strain | Fusion promoter | Relevant genotype | β-Galactosidase activity | Fold regulation | | |
|---|---|---|---|---|---|---|
| | | | | *codY/codY+* | *scoC/scoC+* | *abrB/abrB+* |
| GB1039 | *scoC561p+* | wild type | 52.0 | 4.6 | 1.3 | 0.95 |
| GB1041 | | *codY* | 241.4 | | 1.8 | |
| GB1061 | | *scoC* | 68.8 | 6.2 | | |
| GB1063 | | *codY scoC* | 427.2 | | | 0.80 |
| BB3897 | | *abrB* | 49.4 | | | |
| BB3907 | | *codY scoC abrB* | 342.4 | | | |
| BB3890 | *scoC350p+* | wild type | 48.6 | 4.7 | 1.8 | |
| BB3902 | | *codY* | 228.4 | | 2.0 | |
| BB3901 | | *scoC* | 87.1 | 5.1 | | |
| BB3908 | | *codY scoC* | 445.4 | | | |
| BB3898 | *scoC181p+* | wild type | 120.4 | 0.94 | 1.0 | |
| BB3904 | | *codY* | 113.5 | | 1.1 | |
| BB3903 | | *scoC* | 120.8 | 0.99 | | |
| BB3909 | | *codY scoC* | 119.8 | | | |
| GB1045 | *scoC561p1* | wild type | 125.5 | 0.61 | 1.8 | |
| GB1046 | | *codY* | 76.7 | | 3.3 | |
| BB4001 | | *scoC* | 236.1 | 1.1 | | |
| BB4002 | | *codY scoC* | 255.3 | | | |
| GB1059 | *scoC561p2* | wild type | 25.1 | 1.4 | 2.6 | |
| GB1060 | | *codY* | 34.0 | | 3.2 | |
| BB4003 | | *scoC* | 68.6 | 1.6 | | |
| BB4004 | | *codY scoC* | 107.5 | | | |
| BB3919 | *scoC561p3* | wild type | 8.64 | 0.55 | 1.2 | |
| BB3925 | | *codY* | 4.71 | | 2.3 | |
| BB3920 | | *scoC* | 10.7 | 1.0 | | |
| BB3926 | | *codY scoC* | 10.8 | | | |

Cells were grown in TSS glucose-ammonium medium with a mixture of 16 amino acids. β-Galactosidase activity was assayed and expressed in Miller units. All values are averages of at least two experiments, and the standard errors of the mean did not exceed 30%.

**Table 2**

Expression of *dtpT-lacZ* fusions

| Strain | Fusion promoter | Relevant[a] genotype | β-Galactosidase activity | Fold regulation | |
|---|---|---|---|---|---|
| | | | | codY/codY+ | scoC/scoC+ |
| BB3928 | dtpT279p+ | wild type | 4.30 | 0.15 | 6.9 |
| BB3929 | | scoC | 29.8 | 1.0 | |
| BB3930 | | codY | 0.64 | | 47.3 |
| BB3931 | | codY scoC | 30.3 | | |
| BB4057 | | scoCp1 | 1.01 | 0.89 | |
| BB4059 | | codY scoCp1 | 0.90 | | |
| BB3954 | dtpT279p1 | wild type | 7.63 | 0.14 | 4.7 |
| BB3958 | | scoC | 36.2 | 0.93 | |
| BB3957 | | codY | 1.08 | | 31.3 |
| BB3959 | | codY scoC | 33.8 | | |
| BB3970 | dtpT279p2 | wild type | 0.41 | | 1.2 |
| BB3973 | | scoC | 0.48 | | |
| BB3971 | dtpT279p3 | wild type | 17.6 | 0.74 | 1.1 |
| BB3975 | | scoC | 20.2 | 0.96 | |
| BB3976 | | codY | 13.0 | | 1.5 |
| BB3982 | | codY scoC | 19.3 | | |
| BB3978 | dtpT279p4 | wild type | 10.5 | 0.94 | 1.1 |
| BB3983 | | scoC | 11.4 | 0.86 | |
| BB3984 | | codY | 9.91 | | 1.0 |
| BB3987 | | codY scoC | 9.93 | | |
| BB4047 | dtpT84p+ | wild type | 47.8 | 0.71 | 1.2 |
| BB4049 | | scoC | 56.0 | 1.1 | |
| BB4048 | | codY | 33.8 | | 1.9 |
| BB4050 | | codY scoC | 62.9 | | |

Cells were grown and β-galactosidase activity was assayed as described in Table 1.

[a] *scoCp1* is a promoter mutation; other mutations are null.

**Table 3**

Expression of *oppA-lacZ* fusions

| Strain | Fusion promoter | Binding sites within the promoter | Relevant[a] genotype | β-Galactosidase activity | Fold regulation | |
|---|---|---|---|---|---|---|
| | | | | | *codY/codY+* | *scoC/scoC+* |
| BB3626 | *oppA421p+* | CodY: I, II, III ScoC: I, II | wild type | 51.0 | 0.86 | 2.6 |
| BB3628 | | | *codY* | 43.9 | | 15.5 |
| BB3874 | | | *scoC* | 132.5 | 5.1 | |
| BB3878 | | | *codY scoC* | 679.0 | | |
| BB4059 | | | *scoCp1* | 15.4 | 6.3 | |
| BB4060 | | | *codY scoCp1* | 96.6 | | |
| BB3993 | *oppA323p+* | CodY: II ScoC: I, II | wild type | 251.8 | 0.30 | 1.3 |
| BB3995 | | | *codY* | 74.3 | | 9.9 |
| BB3996 | | | *scoC* | 315.0 | 2.3 | |
| BB3999 | | | *codY scoC* | 735.9 | | |
| BB4029 | *oppA203p+* | CodY: I, II, III ScoC: I | wild type | 172.0 | 4.5 | 1.1 |
| BB4030 | | | *codY* | 775.4 | | 1.2 |
| BB4031 | | | *scoC* | 181.8 | 5.0 | |
| BB4032 | | | *codY scoC* | 915.0 | | |

Cells were grown and β-galactosidase activity was assayed as described in Table 1.

[a] *scoCp1* is a promoter mutation; other mutations are null.

**Table 4**

Role of TnrA in expression of the *scoC561p⁺-lacZ* fusion

| Strain | Relevant genotype | Nitrogen source | β-Galactosidase activity | Fold regulation Glt/NH₄⁺ | Fold regulation tnrA⁺/tnrA |
|--------|-------------------|-----------------|--------------------------|--------------------------|----------------------------|
| GB1039 | wild type | $NH_4^+$ | 180.8 | | 0.88 |
| | | Glt | 131.4 | 0.73 | 0.59 |
| BB3927 | *tnrA* | $NH_4^+$ | 205.1 | | |
| | | Glt | 223.3 | 1.1 | |
| GB1063 | *codY scoC* | $NH_4^+$ | 633.8 | | 1.1 |
| | | Glt | 849.5 | 1.3 | 1.0 |
| BB3967 | *codY scoC tnrA* | $NH_4^+$ | 587.3 | | |
| | | Glt | 809.3 | 1.4 | |

Cells were grown in TSS glucose medium with 0.2% NH4Cl or 0.2% glutamate as sole nitrogen source and β-galactosidase activity was assayed as described in Table 1.

**Table 5**

Role of TnrA in expression of *dtpT-lacZ* fusions

| Strain | Fusion promoter | Relevant genotype | Nitrogen source | β-Galactosidase activity | Fold regulation Glt/NH₄⁺ | Fold regulation tnrA⁺/tnrA |
|--------|-----------------|-------------------|-----------------|---------------------------|----------------------------|-----------------------------|
| BB3928 | *dtpT279p⁺* | wild type | NH₄⁺ | 0.69 | | 1.6 |
| | | | Glt | 3.26 | 4.7 | 7.8 |
| BB3930 | | *codY* | NH₄⁺ | 0.42 | | 1.2 |
| | | | Glt | 2.28 | 5.4 | 6.7 |
| BB3992 | | *tnrA* | NH₄⁺ | 0.43 | | |
| | | | Glt | 0.42 | 1.0 | |
| BB3960 | | *codY tnrA* | NH₄⁺ | 0.34 | | |
| | | | Glt | 0.34 | 1.0 | |
| BB3929 | | *scoC* | NH₄⁺ | 61.7 | | 0.96 |
| | | | Glt | 57.1 | 0.93 | 1.0 |
| BB3969 | | *scoC tnrA* | NH₄⁺ | 64.6 | | |
| | | | Glt | 56.4 | 0.87 | |
| BB3971 | *dtpT279p3* | wild type | NH₄⁺ | 24.5 | | |
| | | | Glt | 26.0 | 1.1 | |

Cells were grown and β-galactosidase activity was assayed as described in Table 4.

**Table 6**

Role of TnrA in expression of the *oppA421-lacZ* fusion

| Strain | Relevant genotype | Nitrogen source | β-galactosidase activity | Fold regulation | |
|---|---|---|---|---|---|
| | | | | Glt/NH$_4^+$ | *tnrA$^+$/tnrA* |
| BB3626 | wild type | NH$_4^+$ | 53.5 | | 0.71 |
| | | Glt | 166.3 | 3.1 | 3.7 |
| BB3979 | *tnrA* | NH$_4^+$ | 75.4 | | |
| | | Glt | 44.8 | 0.59 | |
| BB3874 | *scoC* | NH$_4^+$ | 657.7 | | 0.98 |
| | | Glt | 621.6 | 0.95 | 1.0 |
| BB3980 | *scoC tnrA* | NH$_4^+$ | 669.3 | | |
| | | Glt | 596.2 | 0.89 | |

Cells were grown and β-galactosidase activity was assayed as described in Table 4.

**Table 7**

*B. subtilis* strains used

| Strain | Genotype | Source or reference[a] |
|---|---|---|
| SMY | prototroph | (Zeigler et al., 2008) |
| JH12586 | *abrB::cat trpC2 pheA1* | (Perego *et al.*, 1988) |
| JH14272 | *amyE::[aph Φ(opp-lacZ)] scoC::cat trpC2 pheA1* | (Koide et al., 1999) |
| BB278 | *tnrA62::Tn917* | (Belitsky *et al.*, 2000) |
| BB382 | *abrB::cat* | SMY x DNA(JH12586) |
| BB383 | *abrB::(cat::neo)* | BB382 x pCm::Nm (Steinmetz and Richter, 1994) |
| BB1043 | *codY::(erm::spc)* | (Barbieri et al., 2015) |
| BB1888 | *lacA::tet* | (Belitsky and Sonenshein, 2008) |
| BB2511 | *amyE::spc lacA::tet* | (Belitsky and Sonenshein, 2008) |
| GB1039 | *amyE::[erm Φ(scoC561p⁺-lacZ)] lacA::tet* | BB2511 x pGB15 |
| GB1045 | *amyE::[erm Φ(scoC561p1-lacZ)] lacA::tet* | BB2511 x pGB18 |
| GB1059 | *amyE::[erm Φ(scoC561p2-lacZ)] lacA::tet* | BB2511 x pGB22 |
| BB3543 | *tnrA62::(Tn917::neo)* | BB278 x p917::Nm (Steinmetz and Richter, 1994) |
| BB3890 | *amyE::[erm Φ(scoC350p⁺-lacZ)] lacA::tet* | BB2511 x pBB1812 |
| BB3898 | *amyE::[erm Φ(scoC181p⁺-lacZ)] lacA::tet* | BB2511 x pBB1813 |
| BB3919 | *amyE::[erm Φ(scoC561p3-lacZ)] lacA::tet* | BB2511 x pBB1817 |
| BB3928 | *amyE::[erm Φ(dtpT279p⁺-lacZ)] lacA::tet* | BB2511 x pBB1819 |
| BB3929 | *amyE::[erm Φ(dtpT279p1-lacZ)] lacA::tet* | BB2511 x pBB1821 |
| BB3970 | *amyE::[erm Φ(dtpT279p2-lacZ)] lacA::tet* | BB2511 x pBB1822 |
| BB3971 | *amyE::[erm Φ(dtpT279p3-lacZ)] lacA::tet* | BB2511 x pBB1823 |
| BB3978 | *amyE::[erm Φ(dtpT279p4-lacZ)] lacA::tet* | BB2511 x pBB1825 |
| BB3626 | *amyE::[erm Φ(oppA421-lacZ)] lacA::tet* | BB2511 x pBB1742 |
| BB3993 | *amyE::[erm Φ(oppA323-lacZ)] lacA::tet* | BB2511 x pBB1827 |
| BB4029 | *amyE::[erm Φ(oppA203-lacZ)] lacA::tet* | BB2511 x pBB1831 |
| BB4042 | *scoCp1 lacA::tet* | BB1888 x pBB1830 |
| BB4047 | *amyE::[erm Φ(dtpT84p⁺-lacZ)] lacA::tet* | BB2511 x pBB1833 |

[a]The symbol × indicates transformation by plasmid or chromosomal DNA.

**Table 8**

Oligonucleotides used in this work

| Name | Sequence[a] | Specificity |
|------|-------------|-------------|
| **Flanking forward primers** | | |
| oBB67 | 5'-GCTTCTAAGTCTTATTTCC | *erm* (pHK23) |
| oGB26 | 5'-GGAC<u>TCTAGA</u>GCACCTTCCTCAGGAAAGC | *scoC* |
| oBB576 | 5'-TCGTT<u>GAATTC</u>ATGAAAACATCAACC | *oppA735/421/323* |
| oBB697 | 5'-AATAT<u>GAATTC</u>AGCCAATTGC | *dtpT279* |
| oBB711 | 5'-GAAAA<u>GAATTC</u>GAAGTTTAAATATTTTAAATTG | *oppA203* |
| oBB712 | 5'-TTTTC<u>GAATTC</u>GGAACTATTTGAAATGAG | *dtpT84* |
| **Flanking reverse primers** | | |
| oBB102 | 5'-CACCTTTTCCCTATATAAAAGC | *lacZ* (pHK23) |
| oGB28 | 5'-CGGGA<u>AAGCTT</u>ATCCTTCTCGATCGATTTCC | *scoC* |
| oBB577 | 5'-CGCAA<u>AGATCT</u>AATAATAATTTTCAGCTC | *oppA735/421/203* |
| oBB698 | 5'-GCCAA<u>AAGCTT</u>TCTAAAAAAAGTGTA | *dtpT* |
| oBB701 | 5'-GCCAA<u>AAGCTT</u>TCTAAAAAAAGTGTA<br>TTATAAAAATCATATTCggATATAATAC | *dtpTp1* |
| oBB702 | 5'-GCCAA<u>AAGCTT</u>TCTAAAAAAAGTGTA<br>TTATAAAAATCAggTTCTTATATAA | *dtpTp2* |
| oBB703 | 5'-GCCAA<u>AAGCTT</u>TCTAAAAAAAGTGTA<br>TTATAAAAATCATAggCTTATATAATAC | *dtpTp3* |
| oBB704 | 5'-GCCAA<u>AAGCTT</u>TCTAAAAAAAGTGTA<br>TTATAAAAATCATATTCTTAggTAATAC | *dtpTp4* |
| oBB708 | 5'-TCTTT<u>AGATCT</u>TATTTTTGTAAAAAAGCTG | *oppA323* |
| **Internal mutagenic forward primers** | | |
| oGB38 | 5'-GGTTTGTGAATTTATTAAccAATTCTTACAT | *scoCp1* |
| oGB40 | 5'-GGTTTGTGAATTTATTccAAAATTCTTACAT | *scoCp2* |
| oBB696 | 5'-GGTTTGTGAATTTATTAAcAAATTCTTACAT | *scoCp3* |
| **Internal mutagenic reverse primers** | | |
| oGB39 | 5'-ATGTAAGAATTggTTAATAAATTCACAAACC | *scoCp1* |
| oGB41 | 5'-ATGTAAGAATTTTggAATAAAATTCACAAACC | *scoCp2* |
| oBB695 | 5'-ATGTAAGAATTTgTTAATAAATTCACAAACC | *scoCp3* |

The altered nucleotides conferring down mutations in the CodY- or ScoC-binding site are in lowercase. The restriction sites are underlined.