A Region of *Bacillus subtilis* CodY Protein Required for Interaction with DNA

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***Bacillus subtilis* CodY protein is the best-studied member of a novel family of global transcriptional regulators found ubiquitously in low-G+C gram-positive bacteria. As for many DNA-binding proteins, CodY appears to have a helix-turn-helix (HTH) motif thought to be critical for interaction with DNA. This putative HTH motif was found to be highly conserved in the CodY homologs. Site-directed mutagenesis was used to identify amino acids within this motif that are important for DNA recognition and binding. The effects of each mutation on DNA binding in vitro and on the regulation of transcription in vivo from two target promoters were tested. Each of the mutations had similar effects on binding to the two promoters in vitro, but some mutations had differential effects in vivo.**

The *Bacillus subtilis* CodY protein controls the expression of several hundred genes that are generally involved in the response to nutrient limitation, including genes that encode extracellular degradative enzymes, transport systems, intracellular catabolic systems, chemotaxis, motility, genetic competence, and sporulation (1, 5, 9, 19–21, 26, 27, 37). CodY is highly active as a repressor in rapidly growing cells in rich medium. Repression of CodY target genes is relieved as cells make the transition from rapid exponential growth to stationary phase.

Two types of small-molecule effectors are now known to modulate the activity of CodY. GTP binds to CodY and activates it as a repressor (33); the branched-chain amino acids (BCAAs) isoleucine and valine also interact with CodY and enhance its binding to target sites (39). Genes under CodY control are, therefore, repressed when intracellular pools of GTP and isoleucine-valine are high and derepressed when these pools are depleted by nutrient limitation.

In *Lactococcus lactis*, CodY has been shown to control expression of intracellular peptidase, peptide transport, and aminotransferase genes in response to BCAA availability (4, 12, 13). In fact, close homologs of CodY can be found encoded in the genomes of most of the low-G+C gram-positive bacteria (33). It is likely that CodY plays similar roles in many of these bacterial species.

CodY is an unusual transcription factor in that it shows no significant overall similarity to any other types of transcriptional regulators characterized to date. The mechanism by which CodY is able to recognize and bind to target promoters is unknown at this time. No consensus sequence common to the regulated promoters has yet been discerned. CodY-controlled promoters are very A+T-rich, and at least one (the *dpp*

promoter) has an intrinsic DNA bend (43). Serrano and Sonenshein (38) suggested that CodY might recognize a three-dimensional structure in the DNA rather than a linear nucleotide sequence. On the other hand, a putative helix-turn-helix (HTH) region (3, 7) was identified near the C terminus of CodY; an in-frame deletion of this region rendered the protein unable to bind to the *dpp* promoter in vitro (38). The same HTH deletion caused derepression of the *dpp* promoter in vivo. The apparent role of this HTH domain in DNA binding suggests that CodY may be a sequence-specific DNA-binding protein, even though no consensus sequence has been found.

The HTH motif is a DNA-binding domain frequently found in bacterial transcriptional regulators (3, 15, 31, 32). X-ray crystallographic and two-dimensional nuclear magnetic resonance studies of bacterial and phage transcription factors have revealed that the basic HTH motif spans approximately 20 amino acids and that the two α -helices are placed at an angle of about 120°. The helices are typically linked by a flexible region of 3 or 4 amino acids. The amino-terminal helix 1 (stabilizing helix) sits above the major groove, near the DNA backbone, and the flexible turn region allows the carboxy-terminal helix 2, called the recognition helix, to form sequence-specific interactions with DNA in the major groove (31). However, the interactions necessary for DNA binding are not limited to helix 2. In some cases, residues of helix 1 (e.g., in the *Escherichia coli* Trp repressor) and amino acids flanking the HTH motif (e.g., helix III of catabolite gene activator protein, the N-terminal arm of λ repressor) participate in DNA binding (15). Thus, recognition and specificity of HTH-mediated protein-DNA interactions may depend on the context of the HTH motif in DNA-binding domains.

The amino acid sequence from residues 203 to 222 of CodY protein resembles a typical HTH motif (Fig. 1A). By comparison with the HTH regions of well-characterized transcription factors, such as phage λ and 434 repressors and λ Cro protein (32), residues arginine-214 (R214), serine-215 (S215), and valine-218 (V218) of CodY helix 2 might be expected to contact target DNA. In addition, alanine-207 (A207) of helix 1 might

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FIG. 1. The putative CodY helix-turn-helix motif. (A) The region encompassing residues 200 to 224 of the *B. subtilis* CodY protein is drawn as a double-barrel structure, showing the amino acid substitutions created in the various mutants used in this study. The positions of these and others residues in the full protein sequence are indicated in parentheses. A hypothetical interaction between residue 5 of helix 1 (A207) and residue 15 of helix 2 (I217) (31), is indicated by a dashed line. (B) Alignment of amino acid sequences of the putative HTH of CodY homologs using the Clustal W algorithm (44). Residues completely conserved in all CodY homologs are indicated with an asterisk at the bottom of the figure.

be implicated in a hypothetical hydrophobic interaction with isoleucine-217 (I217) of helix 2 (Fig. 1A). The putative CodY HTH motif is highly conserved among the CodY homologs ($\geq 80\%$ identity; Fig. 1B), suggesting that CodY homologs recognize and bind target promoters in a similar way. Although crystals of CodY protein have been obtained (2), the three-dimensional structure of CodY has not yet been determined. Despite the absence of such information, it should be possible to identify key interactions between CodY protein and target DNAs by in vitro binding studies and mutational analysis. Since *B. subtilis* CodY was the first member of a novel family of transcriptional regulators identified, potential DNA-interacting and helix-stabilizing residues of the putative CodY HTH region were subjected to site-directed mutagenesis. We report the effects of such mutations on DNA binding and oligomerization capabilities of CodY in vitro and on the regulation of target promoters in vivo. Interestingly, some mutations had differential effects on different target sites.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* and *E. coli* strains used in this study are described in Tables 1 and 2, respectively.

Culture media and DNA manipulations. *E. coli* strains were grown in Luria-Bertani (LB) medium (25a) in the presence of ampicillin ($50 \mu\text{g ml}^{-1}$) or chloramphenicol ($25 \mu\text{g ml}^{-1}$) when appropriate. Plasmid DNA was isolated from *E. coli* by the method of Sambrook et al. (34) or by using a QIAprep spin miniprep kit (QIAGEN). Restriction enzyme digestions and ligations were carried out in accordance with instructions provided by the suppliers. DNA fragments were purified from agarose gels by using a GeneClean kit (Bio 101) or a QIAquick gel extraction kit (QIAGEN). Preparation and transformation of electrocompetent *E. coli* cells were as described previously by others (8). Elec-

trations were performed with a Gene Pulser apparatus (Bio-Rad Laboratories) or a BTX TransPorator Plus. *B. subtilis* chromosomal DNA isolation, competent-cell preparation, and transformation were done as described previously (10). *B. subtilis* cells were grown in DS medium (10). To monitor β -galactosidase expression on plates, 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) was added at $80 \mu\text{g ml}^{-1}$.

Site-directed mutagenesis of the CodY HTH region and overexpression of mutant CodY proteins in *E. coli*. Three related protocols were used to create point mutations in the CodY HTH region. In all cases, an overlap PCR procedure adapted from the method of Mikaelian and Sergeant (25) was used. In protocol 1 (used for strains with A207D, S215F, S215T, R214K, R214E, and V218D mutations), the template plasmid used for mutagenesis was pPS40 (38). To create each mutation, two separate PCRs were carried out. One was primed with a mutagenic oligonucleotide (listed in Table 3) and OMRL5 (5'-CGCCA AGCTCGAAATTAACCC-3'); the other was primed with OMRL4 (5'-CTTG TCCGAACCTCTCAAAAAGGGTTC-3') and OMRL3 (5'-GGCCGCGGATCC TGAGGAATGTTTAGGAGG-3'). OMRL3 annealed upstream of the *codY* coding sequence and contained a BamHI restriction enzyme recognition site (indicated in boldface) at the 5' end. The products of these reactions were mixed, denatured, reannealed, and amplified using OMRL3 and OMRL5. Following the final PCR step, the amplified mutant *codY* gene products were digested with restriction endonucleases BamHI (at a site located in the OMRL3 sequence) and HindIII (at a site located immediately downstream of the *codY* gene) and ligated to the BamHI- and HindIII-digested pT7cat6 vector (16). The resultant recombinant plasmids were introduced into *E. coli* strain JM107 (48) by electroporation. The inserts were sequenced (35) to verify the presence of the expected mutation.

To introduce the mutations into the *codY* gene in the *B. subtilis* chromosome, the mutant *codY* alleles were released from their pT7cat6 derivatives by digestion with BamHI and HindIII and ligated to pJPM1, a plasmid that replicates in *E. coli* but not in *B. subtilis* (28). Each pJPM1 derivative (pMRL7, pMRL8, pMRL9, pMRL10, pMRL11, and pMRL15) (Table 2) was then used to transform competent cells of *B. subtilis* strains carrying a *dpp-lacZ* fusion (PS59), generating merodiploid strains carrying both mutant and wild-type alleles of *codY*. The chromosomal DNA of strain PS56 was then used to transform competent cells of the merodiploid strains to introduce the *abrB* null mutation. The sequence of

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype or other relevant characteristics	Source or reference
FJS107	<i>trpC2</i>	40
PS37	<i>trpC2 gid::spc ΔcodY</i>	42
PS56	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet</i>	33
PS59	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo)</i>	FJS107 × DNA PS56
PS83	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet gid::spc ΔcodY</i>	33
PJB1	<i>trpC2 codY::pJP9 cat</i>	FJS107 × pJP9
PJB2	<i>trpC2 codY::pJP10 cat</i>	FJS107 × pJP10
PJB32	<i>trpC2 codY::pJP54 cat</i>	FJS107 × pJP54
MRLB3	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) codY::pMRL7 cat</i>	PS59 × pMRL7
MRLB4	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) codY::pMRL8 cat</i>	PS59 × pMRL8
MRLB6	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) codY::pMRL9 cat</i>	PS59 × pMRL9
MRLB7	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) codY::pMRL10 cat</i>	PS59 × pMRL10
MRLB51	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) codY::pMRL11 cat</i>	PS59 × pMRL11
MRLB9	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) codY::pMRL15 cat</i>	PS59 × pMRL15
MRLB35	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pMRL7 cat</i>	MRLB3 × DNA PS56
MRLB36	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pMRL8 cat</i>	MRLB4 × DNA PS56
MRLB37	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pMRL9 cat</i>	MRLB6 × DNA PS56
PJB40	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pMRL10 cat</i>	PS56 × DNA MRLB7
PJB41	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pMRL11 cat</i>	PS56 × DNA MRLB51
PJB31	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pMRL15 cat</i>	PS56 × DNA MRLB9
PJB3	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pJP9 cat</i>	PS56 × DNA PJB1
PJB4	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pJP10 cat</i>	PS56 × DNA PJB2
PJB34	<i>trpC2 ΔamyE::(Φdpp'-lacZ neo) abrB::cat::tet codY::pJP54 cat</i>	PS56 × DNA PJB32
PJB14	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm)</i>	FJS107 × pRPS10
PJB16	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) gid::spc ΔcodY</i>	PJB14 × DNA PS37
PJB22	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pJP10 cat</i>	PJB14 × DNA PJB2
PJB23	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pMRL15 cat</i>	PJB14 × DNA MRLB9
PJB24	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pMRL8 cat</i>	PJB14 × DNA MRLB4
PJB25	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pMRL9 cat</i>	PJB14 × DNA MRLB6
PJB26	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pMRL11 cat</i>	PJB14 × DNA MRLB51
PJB27	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pMRL10 cat</i>	PJB14 × DNA MRLB7
PJB28	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pMRL7 cat</i>	PJB14 × DNA MRLB3
PJB29	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pJP9 cat</i>	PJB14 × DNA PJB1
PJB33	<i>trpC2 ΔamyE::(ΦilvBΔT'-lacZ erm) codY::pJP54 cat</i>	PJB14 × pJP54

each mutant *codY* allele in the merodiploid strains was verified by amplifying the *codY* gene using OPJ1 (5'-GGGGGAGCTCGCTCGGAACGATAGCC-3') and OMRL5 primers and *B. subtilis* chromosomal DNA as the template. Primer OPJ1 anneals upstream of the *codY* gene and primer OMRL5 to the integrated pJPM1 sequence. The *codY* mutations in the merodiploid strains were transferred to strain PJB14 (*ilvBΔT-lacZ*) by transformation.

To produce pure CodY for in vitro analysis, the mutant *codY* genes were specifically amplified by PCR in two steps. First, PCR was performed using OPJ1 and OMRL5 primers to amplify the promoter-proximal *codY* allele. The resulting PCR product was then used as a template for amplification with OKT1 and OKT2 primers (21). OKT1 anneals upstream of the *codY* gene and includes the *codY* Shine-Dalgarno sequence and a SacI restriction site. OKT2 appends six histidine codons to the 3' end of the *codY* gene, followed by a stop codon and an SphI restriction site. The PCR products were digested with SacI and SphI and ligated to SacI-and-SphI-digested pBAD30 (14) and introduced into *E. coli* strain JM107 by electroporation. As a result, the *codY* gene was placed under the control of the *araBAD* promoter (21). The integrity of each cloned *codY* gene was verified by sequencing using vector-specific primers OBB116 (5'-CTCCATACC CGTTTTTTGG-3') and OBB117 (5'-CTCTCATCCGCCAAAACAG-3').

In protocols 2 and 3, the template plasmid used for mutagenesis was pKT1, a pBAD30 derivative harboring the wild-type *codY* gene with a six-histidine codon C-terminal extension (21). In protocol 2, used to create the mutation A207V, two separate PCRs were carried out, one with mutagenic primer OMRL14 (Table 3) and OBB117 and the other one with OKT1 and OKT2 primers. The two resulting PCR products were mixed, denatured, reannealed, and amplified using OKT1 and OBB117 primers. In protocol 3, used to create the mutations V218A and S215A, one PCR was carried out with the mutagenic primer OPJ2 or OPJ49 (Table 3) and OKT1 and the other one with mutagenic primer OMRL6 or OPJ48 and OKT2. The two PCR products were mixed, denatured, reannealed, and amplified using OKT1 and OKT2 primers. The final PCR products in both cases were digested with SacI and SphI and cloned in pBAD30, generating

plasmids pJP2, pJP8, and pJP53. The sequence of each mutated *codY* gene was verified as indicated above.

To introduce mutations A207V, V218A, and S215A into the chromosomal *codY* gene, the mutated *codY* gene from plasmids pJP2, pJP8, and pJP53 was amplified using primers OPJ3 and OPJ4. OPJ3 (5'-GGGGAATTCACAAGAA TTATTAACCTCCATGCTGCAAGC-3') annealed to the *codY* coding sequence 18 bp after the initiating ATG codon and contained an EcoRI recognition site (indicated in boldface). OPJ4 (5'-GGGGCTGCAGTTAATGAGATTTTATAGAT TTTCTAATTC-3') contains the 3' end of the *codY* coding sequence and an PstI restriction site (indicated in boldface). The PCR products were digested with EcoRI and PstI and ligated to EcoRI-and-PstI-digested pJPM1 and introduced into *E. coli* strain JM107 by electroporation. The derivative plasmids, pJP9, pJP10, and pJP54, were used to transform *B. subtilis* strain FJS107. The chromosomal DNA of the resultant *B. subtilis* *codY* HTH mutant strains PJB1, PJB2, and PJB32 was then used to transform *B. subtilis* strain PS56 carrying the *dpp-lacZ* fusion and an *abrB* null mutation, creating strains PJB3, PJB4, and PJB34, respectively. The chromosomal DNA of strains PJB3 and PJB4 was then used to transform strain PJB14, carrying the *ilvBΔT-lacZ* fusion, to introduce the HTH mutations into the *codY* gene. The plasmid pJP54 was used to transform competent cells of strain PJB14, generating strain PJB33. In each case, the *B. subtilis* chromosomal alleles of the *codY* mutants were verified by DNA sequencing after amplification by PCR with primers OMRL5 and OPJ1 as described above.

Purification of CodY and mobility shift DNA binding assays. *E. coli* strains carrying plasmid pBAD30 in which the different versions of the *codY* gene were cloned (Table 2) were grown until the optical density at 600 nm (OD_{600}) reached ≈ 0.6 . Arabinose (0.2% final concentration) was added, and growth was continued for 4 to 5 h. Cells were then harvested by centrifugation and broken by sonication. CodY proteins were purified by cobalt affinity chromatography as previously described (21, 39) using 75 mM imidazole for elution. Elution frac-

TABLE 2. *E. coli* strains used in this study

Strain	Genotype or other relevant characteristics	Source or reference
JM107	F' <i>traD36 lacI^a Δ(lacZ)M15 proA⁺B⁺e14⁻ (mcrA) Δ(lac-proAB) thi gyrA96</i> (Nal ^r) <i>endA1 hsdR17 (r_K⁻ m_K⁺) relA1 supE44</i>	48
JM107(pBAD30)	Carrying the pBAD30 expression vector of <i>E. coli</i>	14
JM107(pKT1)	Wild-type <i>codY</i> cloned in the pBAD30 vector	21
JM107(pJP1)	<i>codY</i> (V218D) cloned in pBAD30	This study
JM107(pJP2)	<i>codY</i> (V218A) cloned in pBAD30	This study
JM107(pJP3)	<i>codY</i> (S215T) cloned in pBAD30	This study
JM107(pJP4)	<i>codY</i> (S215F) cloned in pBAD30	This study
JM107(pJP53)	<i>codY</i> (S215A) cloned in pBAD30	This study
JM107(pJP5)	<i>codY</i> (R214E) cloned in pBAD30	This study
JM107(pJP6)	<i>codY</i> (R214K) cloned in pBAD30	This study
JM107(pJP7)	<i>codY</i> (A207D) cloned in pBAD30	This study
JM107(pJP8)	<i>codY</i> (A207V) cloned in pBAD30	This study
JM107(pRPS10)	pHK23 containing the <i>ilvB</i> AT promoter region fused to <i>lacZ</i>	39
JM107(pRPS5)	pHK23 containing the <i>ilvB</i> promoter region fused to <i>lacZ</i>	39
JM107(pJP9)	<i>codY</i> (V218A) cloned in pJPM1	This study
JM107(pJP10)	<i>codY</i> (A207V) cloned in pJPM1	This study
JM107(pMRL7)	<i>codY</i> (V218D) cloned in pJPM1	This study
JM107(pMRL8)	<i>codY</i> (R214E) cloned in pJPM1	This study
JM107(pMRL9)	<i>codY</i> (R214K) cloned in pJPM1	This study
JM107(pMRL10)	<i>codY</i> (S215T) cloned in pJPM1	This study
JM107(pMRL11)	<i>codY</i> (S215F) cloned in pJPM1	This study
JM107(pJP54)	<i>codY</i> (S215A) cloned in pJPM1	This study
JM107(pMRL15)	<i>codY</i> (A207D) cloned in pJPM1	This study

tions were free of detectable contaminating proteins as determined by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels.

A 256-bp *dpp* promoter-containing DNA (from positions -172 to +84 relative to the transcription start site) was PCR amplified using vector-specific primers T3 and T7 (Stratagene) and the plasmid pFS48 (40) as the template. A 453-bp *ilvB* promoter-containing probe (from positions -306 to +147 relative to the transcription start site) was generated by PCR amplification using plasmid pRPS5 as the template and primers ORPS6 and ORPS7 (39). The purified PCR products were labeled using [γ -³²P]ATP and T4 polynucleotide kinase (Invitrogen) as described by the manufacturer. The labeled PCR products were purified on a 8% (wt/vol) nondenaturing polyacrylamide gel by using the Qiaex II gel extraction kit (QIAGEN). Mobility shift DNA binding assays were performed as described previously (27, 39) in the presence of 2 mM GTP and 10 mM each of isoleucine, leucine, and valine. The samples were loaded on a 10% nondenaturing polyacrylamide gel, and electrophoresis was carried out in 35 mM HEPES-43 mM imidazole electrophoresis buffer (pH 7.4) as described previously (39). Gels were dried under vacuum and exposed to a phosphorimager screen before analysis with a Molecular Dynamics Storm 860 Imager and ImageQuant version 1.2 Macintosh software.

DNase I footprinting assays. The *ilvB* DNA fragment was PCR amplified from plasmid pRPS5 using ORPS7 and ³²P-labeled ORPS6 as primers. The *dpp* DNA fragment was PCR amplified from plasmid pFS48 using the T7 primer and ³²P-labeled T3 primer. T3 and ORPS6 primers were labeled using T4 polynucleotide kinase (Invitrogen) and [γ -³²P]ATP as described previously (22). Conditions used for protein binding were the same as for gel shift assays but in a 20- μ l reaction mixture volume. After incubation for 30 min at room temperature, 6 mM MgCl₂, 6 mM CaCl₂, and RQ1 DNase (Promega; 0.059 and 0.13 unit for *dpp* and *ilvB* fragments, respectively) were added to each mixture. After 1 min at room temperature, the reactions were stopped by the addition of EDTA to 25 mM and transferred to a dry ice-ethanol bath. Samples were extracted with phenol-chloroform, precipitated in ethanol, and resuspended in 5 μ l of sequencing gel loading buffer (34). The samples were incubated for 5 min at 80°C and subsequently analyzed on a 6% urea-polyacrylamide DNA sequencing gel prepared in TBE (89 mM Tris, 89 mM borate, 2 mM EDTA). Sanger sequencing reactions for the *dpp* promoter were performed with the T3 primer on plasmid pFS48 using the Sequenase kit (United States Biochemical Corp.) and [α -³⁵S]dATP. The dried gels were analyzed as described above.

Expression of mutant CodY proteins in *B. subtilis*. *B. subtilis* strains carrying a *codY* HTH mutation (PJB22, PJB23, PJB24, PJB25, PJB26, PJB27, PJB28, and PJB29) or a *codY* deletion mutation (PJB16) or the wild-type *codY* gene (PJB14) were grown in 20 ml of DS medium to an OD₆₀₀ of approximately 1. A 10-ml

sample of each culture was removed, and the cells were pelleted by centrifugation at 13,000 rpm for 3 min at 4°C. The cell pellets were then resuspended in 2 ml solution A (38) containing 1 mM phenylmethylsulfonyl fluoride. The cells were sonicated on ice in 30-second pulses with 30-second breaks between pulses. The lysates were then centrifuged at 13,000 rpm at 4°C. The quantity of total protein present in the supernatant fluid was determined using the Bio-Rad protein assay reagent. Ten micrograms of total soluble protein was loaded on a 12% denaturing polyacrylamide gel after boiling for 3 minutes in Laemmli loading buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.015% bromophenol blue) containing 2.5% β -mercaptoethanol. Electrophoresis was conducted at a constant voltage of 110 V for 1 hour. Proteins were then electrotransferred to Immobilon-P membranes (Millipore) at 4°C under constant voltage of 100 V in electrotransfer buffer (192 mM glycine, 25 mM Tris, 15% methanol). The immunoblotting was performed as described previously (33) using rabbit polyclonal antibody to CodY prepared by Biodesign International (Kennebunkport, ME).

β -Galactosidase assays. *B. subtilis* strains carrying fusions to the *E. coli lacZ* gene were assayed for β -galactosidase activity after growth in DS medium as described previously (41).

In vitro cross-linking of CodY. Using an adaptation of the method of den Hengst et al. (6), we incubated wild-type or mutant CodY proteins (2.5 μ M) for

TABLE 3. Mutagenic oligonucleotides used in this study

Primer	Sequence ^a	Amino acid substitution
OMRL14	5'-GCAAGTAAAATTGTAGACCGTGTC-3'	A207V
OMRL15	5'-GCAAGTAAAATTGACGACCGTGTC-3'	A207D
OMRL8	5'-CGGCATTACCGAATCTGTTATTGTG-3'	R214E
OMRL9	5'-CGGCATTACCAATCTGTTATTGTG-3'	R214K
OMRL10	5'-CGGCATTACCCGTACCGTTATTGTG-3'	S215T
OMRL11	5'-CGGCATTACCCGTTCGTTATTGTG-3'	S215F
OPJ48	5'-CGGCATTACCCGTGCTGTTATTGTG-3'	S215A
OPJ49	5'-CACAAATACAGCAGGGTAATGCCG-3'	S215A
OMRL6	5'-CGTTCTGTTATTGCTAACGCACTC-3'	V218A
OPJ2	5'-GAGTGCCTTAGCAATAACAGAACG-3'	V218A
OMRL7	5'-CGTTCTGTTATTGACAACGCACTC-3'	V218D

^a The mutated codons are underlined.

15 min at room temperature in $1\times$ cross-linking buffer (100 mM KCl, 15 mM Tris-HCl, pH 7.5) in a total volume of 12 μ l. Formaldehyde was then added to the reaction mixture to a final concentration of 1% (vol/vol), followed by a 20-min incubation step at room temperature. Cross-linking with formaldehyde was stopped by the addition of 3 μ l of $5\times$ Laemmli loading buffer containing 2.5% β -mercaptoethanol. The samples were heated at 37°C for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting using rabbit antibody to CodY. As a control, parallel samples were heated at 96°C for 10 min to break the chemical cross-links (45).

Detection of *codY* mRNA by reverse transcription-PCR (RT-PCR). *B. subtilis* strain PJB23, expressing the mutant form of CodY, was grown in 20 ml of DS medium to an OD₆₀₀ of approximately 0.9, and a 1-ml sample of culture was removed to extract total RNA using the QIAGEN RNeasy kit. Total RNA was subjected to vigorous DNase treatment using the TURBO DNA-free kit (Ambion) as described by the manufacturer. Reverse transcription was performed using SuperScript II (Invitrogen) with 1 μ g of total RNA and 2 pmol of OPJ20 primer (5'-GGAGCTCAGGAGGAACCTTTGAAATGG-3') following the manufacturer's protocol. As a control, reactions were also carried out in the absence of SuperScript II to check for genomic contamination. Primers OPJ9 (5'-GATCATCGGAGGCGGGGAAAG-3') and OPJ20 were used to amplify a 427-bp segment of *codY*-coding sequence that would contain the relevant mutation. PCR was performed with Platinum *Taq* DNA polymerase (Invitrogen) and with 1 μ l of cDNA as the template. In all cases, PCR products (500 ng) were subjected to DrrI restriction enzyme treatment and analyzed on a 2.5% agarose gel prepared in TBE.

RESULTS

Site-directed mutagenesis of potential CodY HTH residues.

To test the functional significance of the putative HTH sequence of CodY and to determine whether specific residues in this motif are important in DNA binding, CodY HTH region residues that were expected to contact DNA or to form hydrophobic interactions between helix 1 and helix 2 were targeted (by comparison to well-characterized HTH-containing transcriptional factors such as phage λ repressor and λ Cro protein) for site-directed mutagenesis. Using overlap PCR primed by mutagenic oligonucleotides, each targeted residue (A207, R214, S215, and V218) of the putative CodY HTH region was separately substituted by either a similar or a contrasting (e.g., acidic versus basic, charged versus uncharged) amino acid (see Materials and Methods) (Fig. 1A). The targeted residues are 100% conserved in CodY homologs from 27 bacterial species (Fig. 1B). The mutated *codY* genes with a six-histidine C-terminal tag were cloned in an *E. coli* expression vector downstream of the *araBAD* promoter (14), enabling inducible expression of the various forms of CodY for purification (21).

Effects of CodY HTH mutations on DNA binding activity of CodY in vitro at the *dpp* and *ilvB* promoters. To allow analysis of DNA binding activity in vitro, wild-type and mutant versions of His₆-tagged CodY were purified by cobalt affinity chromatography. Immunoblotting using antibodies raised to CodY protein verified that the introduction of the HTH mutations into the purified His₆-tagged CodY proteins did not affect the stability of the corresponding proteins in *E. coli* (data not shown). The mutants were then tested for interaction with the *dpp* and *ilvB* target promoter regions by electrophoretic mobility shift assays (EMSAs). Since GTP and BCAAs are effectors of CodY that enhance its affinity to target DNAs (27, 39), the EMSAs were conducted in the presence of a mixture of GTP and BCAAs.

The wild-type CodY was found to bind to the *ilvB* promoter with high affinity (Fig. 2). The apparent K_d , based on the CodY concentration needed to shift 50% of the input DNA, was 4 to

8 nM, consistent with previous estimates obtained with an independent protein preparation (39). Multiple bands of different mobilities were observed with increased protein concentrations (Fig. 2a). Shivers and Sonenshein (39) showed, by footprinting experiments, that the appearance of these different shifted bands correlates with binding of CodY to multiple sites of various affinities in the *ilvB* promoter region.

Wild-type CodY was found to bind to the *dpp* promoter with an affinity similar to that seen at the *ilvB* promoter (apparent K_d of ~ 2 to 4 nM). Interestingly, multiple shifted bands were also observed at increased protein concentrations (Fig. 2h). This result could be due to binding of CodY to several sites on the *dpp* promoter, as for the *ilvB* promoter, or to oligomerization of CodY at a single site on the DNA fragment or both. To distinguish between these possibilities, DNase I footprinting experiments were performed. As previously determined by Serror and Sonenshein (38), whose work predated the discovery that binding of CodY is enhanced by GTP and BCAAs, a 36-bp region on the template strand from positions -10 to $+27$ with respect to the transcription start site was protected by CodY in the presence of enhancing effectors (Fig. 3). In the presence of effectors, however, the concentration of CodY necessary to achieve 50% protection of this region was very low (<4 nM) or at least 18-fold lower than that seen in the absence of effectors (38). In addition, we found that at higher protein concentrations, the protected region was extended to position -63 relative to the transcription start site (Fig. 3). This additional region of protection represents binding to a lower-affinity site. Moreover, we found that, at the highest protein concentration used in the assay (256 nM), CodY protected two additional regions upstream of position -63 (Fig. 3). These two regions, representing the lowest-affinity binding sites, were fully protected at a CodY concentration of 1,024 nM (data not shown).

The V218A mutant form of CodY was able to bind to the *ilvB* and *dpp* promoters with an affinity similar to that of wild-type CodY (Fig. 2b and i), while the mutant forms with R214K, S215T, and S215A bound to the two promoters with slightly reduced affinities (four- to eightfold) compared to that of the wild-type CodY (Fig. 2c, d, e, j, k, and l). The A207V and S215F mutants showed a stronger defect in their ability to interact with the two promoters. The affinities of A207V and S215F mutants were reduced 32-fold for the *dpp* promoter (Fig. 2m and n), whereas the affinities were decreased 16-fold for the *ilvB* promoter (Fig. 2f and g). Interestingly, these two mutants also showed a defect in their capacity to form the highest-molecular-weight complexes, suggesting that the CodY A207V and S215F mutants were able to interact only with the high-affinity binding sites in the *ilvB* and *dpp* promoter regions, albeit with lower affinities than that of the wild-type protein.

To confirm this hypothesis, DNase I footprinting experiments were performed with the CodY protein with the A207V substitution [CodY(A207V)] and wild-type CodY protein on the 453-bp *ilvB* probe used in the gel shift assays. Analysis of the footprint showed the four protected regions for the wild-type protein, as previously seen (39) (Fig. 4). Regions I and II correspond to the high-affinity binding sites and regions III and IV to the low-affinity binding sites (Fig. 4). In addition, a fifth region was protected at higher CodY concentrations (Fig. 4). Regions I and II were also protected by CodY(A207V), but

FIG. 2. In vitro DNA binding activities of CodY proteins containing HTH mutations. The abilities of the purified wild-type and HTH mutant proteins to bind to the *dpp* and *ilvB* promoters were determined in EMSAs as described in Materials and Methods. The numbers above each lane indicate the CodY protein concentration expressed in nM. In all cases, the reaction mixtures contained the effectors GTP (2 mM) and isoleucine, valine, and leucine (10 mM each).

only at higher protein concentrations than those needed for the wild-type CodY (Fig. 4). In contrast, regions III to V were not protected by the mutant CodY even at the highest protein concentrations (Fig. 4). Similarly, only the high-affinity binding site in the *dpp* promoter region was found to be protected by CodY(A207V), and again only at higher protein concentrations than those needed for the wild-type protein to bind (Fig. 3). Therefore, the low-molecular-weight complexes seen for CodY(A207V) in gel shift assays (Fig. 2f and m) reflect binding to the high-affinity sites in the *dpp* and *ilvB* promoter regions.

The CodY proteins carrying the nonconservative substitution A207D, R214E, or V218D showed the strongest defects in DNA binding. No binding to either promoter was observed even at very high protein concentrations (apparent K_d of >500 nM) (Fig. 2g and n). Overall, the orders of affinities of the mutant CodY proteins were very similar for the *ilvB* and *dpp* promoter regions. The mutants that retained DNA binding activity were able to respond to the presence of GTP and BCAAs, since their affinities for DNA were increased in the presence of either GTP or BCAAs (Table 4), but the extent of

stimulation by effectors was not always as high as for the wild-type CodY protein (see Discussion).

Introduction of the HTH mutations at the *codY* locus. To study the effects of the CodY HTH mutations in *B. subtilis* cells, each mutant *codY* gene was ligated to an integrative vector carrying a *B. subtilis*-compatible chloramphenicol resistance gene (28), and introduced by single-crossover homologous recombination into the chromosome of a *B. subtilis* strain carrying either a *dpp-lacZ* or an *ilvB-lacZ* fusion at the *amyE* locus. The resulting transformants were partial diploids, carrying both a mutant *codY* gene under the control of the *cod* operon promoter and a wild-type, promoterless copy of *codY* (Fig. 5A). Since all the HTH mutations were located within 100 bp of the 3' end of the *codY* gene, we were successful in introducing each mutation into the expressed copy of the *codY* gene on the *B. subtilis* chromosome at a high frequency. The presence and location of the expected mutations were verified by amplifying the expressed *codY* gene from the chromosome with primers OPJ1 and OMRL5 (Materials and Methods and Fig. 5A) and sequencing the PCR product. Only strains that

FIG. 3. DNase I footprint assay of wild-type CodY and CodY(A207V) with the *dpp* promoter region. A 256-bp DNA fragment (32 P end labeled on the template strand) corresponding to positions -172 to $+84$ relative to the transcription start site was incubated with increasing concentrations (in nM) of purified wild-type CodY or CodY(A207V) in the presence of 2 mM GTP and 10 mM each of isoleucine, leucine, and valine. The footprinting assay was carried out as described in Materials and Methods. Protected regions observed only with wild-type CodY at 128 nM and 256 nM are marked by vertical lines. Sanger sequencing ladders are shown in the leftmost lanes (A, C, G, and T). The -35 and -10 boxes are indicated by vertical lines and the transcription start site by a bent arrow.

had the expected mutation and no other mutations were used for β -galactosidase assays.

Even though the wild-type *codY* copy was promoterless, we still wanted to verify that only the mutant copy was expressed. To do so, we took advantage of the creation of new restriction enzyme recognition sites caused by some HTH mutations. Total RNA was extracted from cultures of recombinant strains and the *codY* mRNA was reverse transcribed using the primer OPJ20, which annealed to the 3' end of the *codY*-coding sequence (Materials and Methods and Fig. 5A). The *codY* cDNA was then amplified by PCR using primers OPJ9 and OPJ20 (Fig. 5A), and the resulting RT-PCR product was then incubated with the appropriate restriction enzyme. An example of the results obtained with strain PJB23 carrying the *codY*(A207D) mutation is shown in Fig. 5A. In this case, if the wild-type *codY* copy were expressed as well as the mutant *codY* was, we would find three bands after digestion with DrdI, an enzyme that can cut only the mutant *codY* allele. One band should correspond to the undigested wild-type copy (427 bp) and two others to the digested mutant copy (273 bp and 154 bp). As shown in Fig. 5A, lane 4, the RT-PCR product subjected to digestion generated only two bands (273 bp and 154 bp) identical in size to those generated by a control PCR

FIG. 4. DNase I footprint assay of wild-type CodY and CodY(A207V) with the *ilvB* promoter region. A 453-bp PCR product, corresponding to positions -306 to $+147$ relative to the transcription start site and labeled at the 5' end of the nontemplate (coding) strand, was incubated with increasing concentrations (in nM) of purified wild-type CodY (WT-CodY) or CodY(A207V) in the presence of 2 mM GTP and 10 mM each of isoleucine, leucine, and valine. The footprinting assay was carried out as described in Material and Methods. Protected regions are marked by the vertical bars, as are the -35 and -10 boxes. The transcription start site is indicated by a bent arrow.

obtained from the amplification of the cloned *codY*(A207D) mutant allele (Fig. 5A, lane 2). As expected, neither the wild-type PCR product obtained from amplification of *codY* cloned in pBAD30 (Fig. 5A, lane 1) nor the wild-type RT-PCR product obtained by amplification of *codY* cDNA from the PJB14 strain (Fig. 5A, lane 3) was cut by the enzyme. These results indicate that the wild-type *codY* copy was not expressed at a detectable level in strain PJB23. The results obtained for several other mutants were all consistent with those shown in Fig. 5A (data not shown).

To rule out the possibility that potential effects on gene regulation were due to instability of mutant CodY proteins, each *B. subtilis* strain was tested by immunoblotting using antibodies raised against CodY protein. The results shown in Fig.

TABLE 4. In vitro binding activities of wild-type and mutant versions of CodY and relative expression of the *dpp-lacZ* and *ilvB-lacZ* fusions

Mutation	Apparent K_d (nM) ^a			Relative expression in vivo ^b	
	<i>ilvB</i> (–)	<i>ilvB</i> (+)	<i>dpp</i> (+)	<i>ilvB</i>	<i>dpp</i>
None (<i>codY</i> ⁺)	16–32	4–8	2–4	1	1
$\Delta codY$				44	40
<i>codY</i> (R214K)	64–128	32–64	8	5	2.5
<i>codY</i> (R214E)	>500	>500	>500	30	33.5
<i>codY</i> (S215T)	32–64	16–32	8–16	7	6.5
<i>codY</i> (S215A)	32–64	16–32	8–16	20	12
<i>codY</i> (S215F)	64	32–64	128	48.5	48
<i>codY</i> (V218A)	16–32	4–8	4–8	8	1
<i>codY</i> (V218D)	>500	>500	>500	42	43
<i>codY</i> (A207V)	128	64–128	64–128	44	1
<i>codY</i> (A207D)	>500	>500	>500	42	47

^a The apparent K_d determined in the presence (+) or the absence (–) of both 2 mM GTP and 10 mM BCAAs corresponds to the concentration of CodY that caused a shift of 50% of the input DNA to any more slowly migrating complex.
^b The relative expression values were calculated as the mean of the quotients of the β -galactosidase specific activity of the *codY* HTH mutant strains divided by the β -galactosidase specific activity of the wild-type *codY* strain in samples harvested 30 and 60 min prior to the end of the exponential growth phase.

5B indicate that all CodY mutants were produced as stable proteins in *B. subtilis*. Surprisingly, the protein having the A207D mutation migrated slightly more slowly than did either the wild-type protein or the other mutant proteins (Fig. 5B, lane 4). Single mutations that alter mobility in SDS-polyacrylamide gels have been described for other proteins (23, 24, 29).

Effects of CodY HTH mutations on *dpp* expression in *B. subtilis*. In wild-type cells, the *dpp* operon is induced in early stationary phase, coincident with relief of repression by two regulatory proteins, CodY and AbrB (41, 42). To analyze CodY activity in vivo in the absence of overlapping repression by AbrB, each *codY* HTH mutation was introduced into a *B. subtilis* strain (PS56) carrying a *dpp-lacZ* fusion at the *amyE* locus and an *abrB* mutation. *B. subtilis* strains carrying a *codY* HTH mutation or a *codY* deletion mutation (PS83) or the wild-type *codY* gene (PS56) were then assayed for β -galactosidase activity at various times during growth in DS medium (Fig. 6). The *codY* HTH mutant strains fell into three classes based on their effects on *dpp* expression compared to the parent PS56 strain.

The first class comprises the strains containing the *codY*(V218A) and *codY*(A207V) mutations. These strains displayed a wild-type phenotype as shown in Fig. 6C and D. Thus, the A207V and V218A mutations had no effect on the ability of CodY to repress the *dpp* promoter in vivo. This result is in good agreement with the observation that the V218A mutation had little if any effect on the binding of CodY in vitro (Fig. 2i and Table 4). However, the result was surprising for the A207V mutation, since the CodY(A207V) mutant displayed a significant defect in binding to the *dpp* promoter compared to the wild-type protein (Fig. 2m and Table 4) (see Discussion).

The second class comprises the strains harboring the

FIG. 5. Transcription of *codY* in a merodiploid strain. (A) Introduction of an HTH mutation at the *codY* locus was performed as described in the text and in Materials and Methods. The resulting recombinant strains (e.g., PJB23) carried both a mutant *codY* gene (the mutation is indicated by an asterisk) under the control of the normal *cod* operon promoter (located upstream of *codV* and indicated by a broken arrow) and a wild-type, promoterless *codY* copy downstream of the integrated pJPM1 plasmid. The presence and location of the expected mutation were verified by amplifying the expressed *codY* gene from the chromosome with primers OPJ1 and OMRL5. *codY* mRNA of mutant strain PJB23 was reverse transcribed using primer OPJ20. The *codY* cDNA was then amplified using primers OPJ9 and OPJ20. The resulting RT-PCR product was subjected to digestion with *DrdI* restriction enzyme and loaded on a 2.5% agarose gel (lane 4). The corresponding PCR products obtained from amplification of the wild-type *codY* gene cloned in pBAD30 (lane 1) or the *codY*(A207D) allele cloned in pBAD30 (lane 2), from amplification of wild-type *codY* cDNA from strain PJB14 (lane 3), or from amplification of *codY* from genomic DNA of the PJB23 mutant strain (lane 5) were also subjected to *DrdI* digestion as controls. (B) Expression of CodY HTH mutant proteins in *B. subtilis*. Whole-cell lysates of strain PJB16 carrying a *codY* null mutation (lane 1), wild-type strain PJB14 (lane 2) or *codY* HTH mutant strains PJB22 (lane 3), PJB23 (lane 4), PJB24 (lane 5), PJB25 (lane 6), PJB26 (lane 7), PJB27 (lane 8), PJB28 (lane 9), and PJB29 (lane 10) were assayed by immunoblotting using anti-CodY antibodies as described in Materials and Methods. The CodY-specific band is indicated by an arrow on the right.

codY(R214K), *codY*(S215T), and *codY*(S215A) mutations. These mutations caused a moderate derepression of the *dpp* promoter early during the exponential phase, increasing β -galactosidase activity to a level about 2.5- to 12-fold higher than in the wild-type strain, PS56 (Fig. 6A and B and Table 4). These strains showed an induction of *dpp* expression similar to (Fig. 6A) or higher than (Fig. 6B) that of the wild-type strain when they approached stationary phase. These results indicate that the R214K, S215T, and S215A substitutions prevent full repression of the *dpp* promoter during exponential phase and that this partial repression is relieved when stationary phase is reached. Interestingly, the substitution of Ser-215 by alanine had a greater effect on the ability of CodY to repress the *dpp*

FIG. 6. Effects of CodY HTH mutations on transcription from the *dpp* promoter. *B. subtilis* strains carrying a *dpp-lacZ* fusion were grown in DS liquid medium and tested for β -galactosidase specific activity at the indicated time point. The x axis shows the sampling time relative to the end of exponential growth phase (time zero). The experiment was performed in triplicate, and any variation in data points was less than 15% of the represented values. (A–D) PS56 (open squares, wild-type *codY*) and PS83 (filled squares, $\Delta codY$). (A) MRLB36 [filled circles, *codY*(R214E)] and MRLB37 [open circles, *codY*(R214K)]. (B) PJB41 [filled circles, *codY*(S215F)], PJB40 [open circles, *codY*(S215T)], and PJB34 [open triangles, *codY*(S215A)]. (C) MRLB35 [filled circles, *codY*(V218D)] and PJB3 [open circles, *codY*(V218A)]. (D) PJB31 [filled circles, *codY*(A207D)] and PJB4 [open circles, *codY*(A207V)].

promoter than did the substitution by threonine (Fig. 6B) (see Discussion). The slightly decreased affinities of these CodY mutants for DNA, as measured by gel shift assays (Fig. 2j, k, and l), correlated with partial derepression of the *dpp-lacZ* fusion in vivo (Table 4). Therefore, the inability of the mutants CodY(R214K), CodY(S215T), and CodY(S215A) to repress the *dpp* promoter to the same extent as the wild-type protein is likely due to their reduced ability to bind efficiently to DNA in vivo.

The third class comprises the strains carrying the four nonconservative substitutions *codY*(A207D), *codY*(R214E), *codY*(S215F), or *codY*(V218D). The effects of these mutations on *dpp* expression were similar to the effect of a *codY* null mutation (Fig. 6A, B, C, and D). In these strains, the *dpp* promoter was expressed during exponential growth at levels 33- to 48-fold higher than in strain PS56 (Table 4). As shown above, these mutations abolished binding or dramatically reduced the affinity of CodY for the *dpp* promoter in vitro (Fig. 2n).

Effects of CodY HTH mutations on *ilvB* expression in *B. subtilis*. Among the many targets of CodY are the *ilv* genes that encode the enzymes of BCAA biosynthesis (27, 39). To test the effects of CodY mutations in vivo on the regulation of these genes, each mutation was integrated into the chromosome of a *B. subtilis* strain carrying an *ilvB-lacZ* fusion at the *amyE* locus.

The *ilvBΔT-lacZ* fusion used in our experiment was missing the T-box region that causes leucine-responsive transcription termination (11). *B. subtilis* strains carrying a *codY* HTH mutation, a *codY* deletion mutation (PJB16), or a wild-type *codY* gene (PJB14) were then assayed for β -galactosidase activity at various times during growth in DS medium. The *codY* HTH mutant strains fell into two classes based on their effects on *ilvB* expression.

The first class included strains carrying the substitutions R214K, S215T, S215A, and V218A. In these strains, the *ilvBΔT-lacZ* fusion was partially derepressed during exponential growth phase. The *codY*(R214K) and *codY*(S215T) mutations allowed about five- to sevenfold derepression. Partial derepression of the *ilvBΔT-lacZ* fusion in strains carrying these mutations (R214K and S215T) fits well with the partial defect in DNA binding seen for the mutant proteins in vitro (Fig. 2c and d and Table 4). Interestingly, as for the *dpp* promoter, the *codY*(S215A) mutation had a much greater effect on the activity of CodY than did the *codY*(S215T) mutation, causing a 20-fold derepression of the *ilvBΔT-lacZ* fusion (see Discussion). The *codY*(V218A) mutation caused an eightfold derepression of *ilvBΔT-lacZ* expression, a surprising result, since CodY(V218A) bound to *ilvB* DNA in vitro with approximately the affinity of the wild-type protein (Fig. 2b and Table 4) (see Discussion). The strains also showed derepression of the *ilvB*

FIG. 7. Effects of CodY HTH mutations on transcription from the *ilvB* promoter. *B. subtilis* strains carrying an *ilvB-lacZ* fusion were assayed for β -galactosidase specific activity as described in the legend of Fig. 6. (A–D) PJB14 (open squares, wild-type *codY*) and PJB16 (filled squares, $\Delta codY$). (A) PJB24 [filled circles, *codY*(R214E)] and PJB25 [open circles, *codY*(R214K)]. (B) PJB26 [filled circles, *codY*(S215F)], PJB27 [open circles, *codY*(S215T)], and PJB33 [open triangles, *codY*(S215A)]. (C) PJB28 [filled circles, *codY*(V218D)] and PJB29 [open circles, *codY*(V218A)]. (D) PJB23 [filled circles, *codY*(A207D)] and PJB22 [open circles, *codY*(A207V)].

promoter when the cells approached stationary phase and showed, in stationary phase, a slightly higher level of β -galactosidase than in the wild-type strain (Fig. 7).

By comparison, strains carrying mutations *codY*(R214E) (Fig. 7A), *codY*(S215F) (Fig. 7B), *codY*(V218D) (Fig. 7C), *codY*(A207D) and *codY*(A207V) (Fig. 7D) had β -galactosidase activities similar to that of strain PJB16 carrying a *codY* null mutation. That is, introduction of the *codY* HTH point mutations led to approximately 30- to 48-fold derepression of the *ilvB* promoter. We noted again a good correlation between the severely reduced DNA binding affinities of these mutants in vitro and the total derepression of the *ilvB* promoter in vivo (Table 4).

Oligomerization of wild-type and HTH-mutated CodY proteins detected by in vitro cross-linking. Since the functional forms of many transcriptional regulators are dimers or oligomers, we sought to determine whether the HTH mutations affected potential CodY oligomerization. Native gel electrophoresis and light-scattering experiments indicated that purified CodY is primarily in the form of a dimer of approximately 60 kDa (2; K. Matsuno and A. L. Sonenshein, unpublished results). To study CodY oligomerization further, we performed in vitro cross-linking assays with wild-type and mutant CodY proteins.

First, the purified mutant and wild-type proteins were subjected to electrophoresis under nondenaturing conditions (Fig. 8A). Each protein sample migrated as a single species whose mobility varied slightly from sample to sample. Next, the pu-

rified proteins were subjected to formaldehyde cross-linking and SDS-polyacrylamide gel electrophoresis. An immunoblot showed that the monomer form of CodY predominated after denaturation; a small fraction of the protein was cross-linked with formaldehyde to generate forms with the mobilities expected for dimers, trimers, or larger oligomers (Fig. 8B). Although the efficiency of cross-linking was not high, the fractions of wild-type and mutant CodY proteins in the oligomeric forms were similar for the various proteins (Fig. 8B). Under the same experimental conditions, the molecular mass of ovalbumin, a monomeric protein, remained unchanged, indicating the specificity of cross-linking (data not shown). In addition, heating the samples to break the chemical cross-link reduced the oligomers to the monomer form in all cases (data not shown). Since the concentration of CodY in our cross-linking reaction mixtures (2.5 μ M) was similar to that found inside the cell (2 to 4 μ M [R. Shivers, personal communication]), formation of higher-order CodY complexes is probably favored under physiological conditions. We conclude that the HTH mutations tested do not affect significantly the global structure of the protein and that they have no discernible effect on the ability of CodY to oligomerize in solution.

DISCUSSION

Prokaryotic and eukaryotic transcriptional regulators have distinct DNA-binding domains that contain helix-loop-helix,

FIG. 8. (A) Coomassie blue staining of purified wild-type and mutant CodY proteins subjected to nondenaturing polyacrylamide (10%) gel electrophoresis. (B) Cross-linking of purified wild-type and mutant CodY proteins. Formaldehyde was used to cross-link potential oligomers of CodY. After electrophoresis on SDS-polyacrylamide gels, the proteins were visualized by immunoblot analysis using a rabbit polyclonal serum antibody to CodY. The positions of monomers and putative dimers and trimers of CodY are indicated to the left. Lane M contains prestained molecular mass markers (in kDa; GIBCO-BRL). The positions of molecular mass markers (in kDa) are indicated to the right.

zinc finger, leucine zipper, β -ribbon, or HTH motifs (32). Of these discrete motifs, the HTH motif is the best characterized to date. Although not homologous to any previously known family of HTH-containing protein, CodY has a predicted HTH-like motif (3, 7, 38) within which residues critical for repression correspond to residues of other HTH proteins implicated in interacting with DNA or stabilizing the helices.

Positions 12 and 13 of the HTH are commonly involved in sequence-specific interaction with DNA (32). Although we cannot conclude that residues at these positions in CodY make direct contact with DNA, replacement of arginine-214 (position 12) by glutamate (R214E) and serine-215 (position 13) by phenylalanine (S215F) caused a 30- to 48-fold derepression of expression from the *dpp* and *ilvB* promoters. When arginine-214 and serine-215 were replaced with structurally similar residues, lysine (R214K) and threonine (S215T), respectively, repression of the *dpp* and *ilvB* promoters was only partially affected in vivo, and the binding activity in vitro was slightly reduced compared to that of the wild-type protein. A study of the HTH region of the *E. coli* TyrR protein has shown that an alanine substitution at position 12 (histidine-494) results in a substantial derepression of the *tyrP* and *aroF* promoters during exponential phase (17). Histidine-494 in TyrR interacts with a

cytosine and alteration of the base pair or the histidine-494 residue completely abolishes DNA binding activity (17, 18). Substitution by alanine at position 13 (threonine-495) in TyrR has a significant effect on repression, but a serine substitution does not (17, 47). Interestingly, alanine substitution of the corresponding residue in CodY protein, serine-215, has a greater effect on repressor activity at both the *dpp* and *ilvB* promoters than does the substitution by threonine. The hydroxyl group of serine-215 may therefore be involved in interaction with DNA, as has been proposed for the corresponding residue, threonine-495, in TyrR protein (17). In the *Haemophilus influenzae* TyrR nuclear magnetic resonance structure, H494 and T495 are part of a hydrophilic cluster that is accessible to solvent and therefore may be involved in DNA interaction as in *E. coli* TyrR protein (46). In the phage λ and 434 repressors, residues at positions 12 (glutamine in both cases) and 13 (serine and glutamine, respectively) are also known to contact DNA (30, 32). Specifically, the conserved glutamine at position 12 contacts an adenine base in the operator site (30). In the λ repressor, the serine at position 13 makes contact with a guanine, while in the 434 repressor, the glutamine at this position associates with thymine and guanine bases (30).

Position 16 in the phage repressor HTH domains, corresponding to valine-218 in CodY, also contacts DNA (32). Substitution of CodY residue Val218 by a negatively charged residue (V218D) caused total derepression of both the *dpp* and *ilvB* promoters and abolished binding in vitro. The substitution of residue Val218 by alanine (V218A), however, did not affect the ability of CodY to repress the *dpp* promoter in vivo or to bind to this target in vitro at all. The same V218A mutation also had no significant effect on the ability of CodY to bind to the *ilvB* promoter in vitro but caused a partial derepression of an *ilvB* Δ T-*lacZ* fusion in vivo. Since the affinity of CodY (V218A) for DNA is increased in the presence of effectors to the same extent as for wild-type CodY (Table 4), it is unlikely that the partial derepression of the *ilvB* Δ T-*lacZ* fusion is due to a defect in response to the effectors. The lack of effect of the alanine substitution on binding in vitro may indicate that Val218 does not contact DNA directly. However, the methyl groups of valine and isoleucine have been shown to interact with the methyl group of thymine in homeodomain proteins (32) and that type of interaction may be maintained, in the CodY case, by the methyl group of alanine. To explain the in vivo defect in repression of the *ilvB* promoter, we can imagine two possibilities. First, the V218A mutation may affect interaction of CodY with another protein. Alternatively, the mutation could alter the conformation of the HTH motif in such a way as to interfere with the ability of other residues to interact with target promoters.

In typical HTH motifs, hydrophobic interactions between the highly conserved alanine residue at position 5 and isoleucine or valine at position 15 are thought to stabilize the positioning of helices 1 and 2 (3, 31). In the *E. coli* Trp repressor, residue 5 is a lysine, and the HTH elbow is perturbed by its presence (36). Alanine-207 occupies position 5 in the putative helix 1 of CodY. The substitution of Ala207 by a charged residue, aspartate, rendered the protein unable to bind DNA even at very high protein concentrations. Thus, the potential Ala207-mediated structural interactions may indeed be required for target DNA recognition and binding. Substitution

by another nonpolar residue, valine, decreased by 16- to 64-fold the affinities of CodY for the *dpp* and *ilvB* promoters. Interestingly, footprinting experiments showed that, even at high protein concentrations, the mutant CodY(A207V) was able to bind only to the high-affinity binding sites in the *dpp* and *ilvB* promoter regions. Although CodY(A207V) is able to oligomerize in solution to the same extent as wild-type CodY (Fig. 8), we cannot exclude the possibility that higher-order oligomerization of CodY upon binding to DNA, which might be required for binding to the low-affinity sites, is defective in this mutant. The expression of a *dpp-lacZ* fusion in a strain carrying the *codY*(A207V) mutation was similar to that in the wild-type strain, whereas the expression of an *ilvBΔT-lacZ* fusion in a strain carrying the same mutation was similar to that in a strain carrying a *codY* null mutation. These diametrically opposed results are surprising, since the CodY protein carrying the A207V mutation had similarly decreased affinities for the two target promoters in vitro. The high-affinity binding site in the *dpp* promoter region encompasses the transcription start site as well as the -10 region. At the *dpp* promoter, even low-affinity versions of the proteins may be able to bind tightly enough in vivo to repress the promoter. By contrast, binding to the high-affinity sites in the *ilvB* promoter region may not be sufficient to repress the promoter. Also, we cannot exclude the possibility that another regulatory protein competes with CodY for the same binding site (R. P. Shivers and A. L. Sonenshein, Mol. Microbiol., in press). This regulatory protein could be a positive regulator that has a higher affinity for the *ilvB* promoter region than does CodY(A207V) mutant protein, destabilizing the interaction of CodY with DNA. It is interesting that the HTH mutations generally had a much greater effect on the repression of the *ilvBΔT-lacZ* fusion than the *dpp-lacZ* fusion (Table 4).

Although the mutant forms of CodY that retained residual DNA binding activity all responded to the presence of GTP and BCAAs in vitro, the extent of the response was, in some cases, less than that for wild-type CodY. They also presumably responded to effectors in vivo, since their repression activity was reduced as cells made the transition from exponential phase to stationary phase. Whereas all the mutant proteins appear to have the same global structure in solution as that of wild-type CodY, they may be less susceptible to hypothetical conformational changes induced by effectors. The fact that G3, one of the motifs involved in GTP binding, is within the HTH domain (33) may help to explain why certain HTH mutations render CodY less responsive to effectors. The reduced response to effectors does not fully explain the phenotypes of these mutants, however. They also have a clear defect in intrinsic DNA binding, since their affinity for DNA in the absence of effectors is lower than that of the wild type.

In summary, our results show that, although CodY is not a member of any previously described family of transcriptional regulators, its DNA-binding domain is similar to those carried by proteins belonging to the HTH family of DNA-binding proteins. Interestingly, the HTH-like regions of all identified CodY homologs are extremely well-conserved (Fig. 1). However, since no CodY-binding consensus sequence has been discernible, it is conceivable that the CodY HTH motif is not the only part of the protein required for specific binding. Another domain of the protein may interact with a conserved

DNA structural motif (e.g., bent DNA) in target promoters, whereupon the HTH domain may then be utilized to form specific interactions with appropriately located residues exposed on the DNA. If this is the case, CodY-regulated promoters may share a common structure but have variations in their primary nucleotide sequences. X-ray crystallographic studies currently in progress (2) should be very helpful in understanding this interaction in greater detail.

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