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Additional Targets of the *Bacillus subtilis* Global Regulator CodY Identified by Chromatin Immunoprecipitation and Genome-Wide Transcript Analysis

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Additional targets of CodY, a GTP-activated repressor of early stationary-phase genes in *Bacillus subtilis*, were identified by combining chromatin immunoprecipitation, DNA microarray hybridization, and gel mobility shift assays. The direct targets of CodY newly identified by this approach included regulatory genes for sporulation, genes that are likely to encode transporters for amino acids and sugars, and the genes for biosynthesis of branched-chain amino acids.

Bacteria have evolved a variety of mechanisms to accommodate gene expression to changes in nutritional availability. Some of these mechanisms are specific to a particular gene or operon. In other cases, regulatory proteins control large groups of genes of related function, such as the nitrogen metabolism genes regulated by the Ntr system in enteric bacteria (43) and by TnrA in *Bacillus subtilis* (17) and the carbon metabolism genes regulated by CcpA in gram-positive bacteria (13) and catabolic gene activator protein-cyclic AMP complex in gram-negative bacteria (59). Even broader forms of regulation are mediated by the leucine-responsive protein (Lrp) of gram-negative bacteria and the sigma-B protein of *B. subtilis*. Lrp and sigma-B control the transcription of operons that have diverse functions but have a common need to be expressed under a particular set of environmental conditions (50, 54). Lrp regulates the biosynthesis of leucine, isoleucine, valine, serine, glycine, and glutamate; the degradation of serine and threonine; transport of peptides, amino acids, and sugars; and production of fimbriae in response to the availability of leucine and serine (50). Sigma-B activates transcription of a host of genes when cells are exposed to excessive heat, ethanol, salt, or acid (54). Sigma-B responds through a complex, multibranched signal transduction pathway.

The *B. subtilis* CodY protein also has broad effects on gene expression. CodY is a GTP-binding repressor of several genes that are normally quiescent when cells are growing in a rich medium (57). A high concentration of GTP activates CodY as a repressor (57). When the growth rate of *B. subtilis* slows down because of limitation of the carbon or nitrogen or phosphorus source, the GTP level drops (39, 40), CodY loses repressing activity, and targets of CodY repression are transcribed. The known targets of CodY in *B. subtilis* include the genes that encode transport systems for dipeptides (*dpp*) (65) and γ-aminobutyrate (*gabP*) (16); catabolic pathways for acetate (*acsA*) (S. H. Fisher, personal communication), urea (*ureABC*) (71), histidine (*hut*) (18), arginine (*rocABC* and *rocDEF*) (B. Belitsky, personal communication), and branched-chain keto acids (the *bkd* operon) (12); an enzyme of surfactin synthesis (*srfAA*) (63); the transcription factor for DNA uptake genes (*comK*) (63); a ComA aspartyl phosphate phosphatase and its inhibitor *(rapC-phrC*) (37); motility and chemotaxis (*hag*, *fla*/*che*) (45; F. Bergara, C. Ibarra, J. Iwamasa, R. Aguilera, and L. M. Màrquez-Magaña, submitted for publication); and aconitase (*citB*) (33). CodY also regulates its own synthesis (56). Moreover, CodY is a highly conserved protein in the low-G+C group of gram-positive bacteria (57). In *Lactococcus lactis*, CodY represses expression of extracellular and intracellular peptidases and a peptide uptake system (23, 24). This range of targets suggests that CodY has a broad role in repressing, during rapid exponential growth phase, those genes whose products would allow the cell to adapt to poor nutritional conditions by swimming to a better environment, by taking up potential nutrients, and by metabolizing those nutrients to support continued growth. If so, it seems likely that many additional genes are under CodY control. Direct interaction of CodY with the regulatory regions of target genes has been demonstrated only for the *dpp* (62), *srfAA* (63), *comK* (63), *cod* (56), and *citB* (33) transcription units, however.

Some as yet unidentified CodY target genes in *B. subtilis* are likely to be involved in spore formation. When *B. subtilis* cells enter stationary phase, they have two choices. They can remain in a slow-growth or no-growth state or they can initiate sporulation (67). The onset of sporulation is dependent on nutrient limitation (60) and a consequent drop in the pool of GTP (40). Remarkably, CodY appears to be a major component of this regulation as well. Thus, sporulation of wild-type cells is inhibited in a medium that is highly enriched, but a *codY* null mutant grown in the same medium sporulates at high efficiency (57). The effect of a *codY* mutation can be mimicked by treating cells with a drug that causes a drop in the intracellular pool of GTP (20, 46), implying that in response to GTP excess,

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

Strain	Genotype	Source or reference
PS29	*trpC2 gid*::*spc*	65
PS37	*trpC2 gid*::*spc* Δ*codY*	63
PS56	*trpC2 abrB*::(*cat*::*tet*) Δ*amyE*::(Φ*dpp′-lacZ neo*)	P. Serror
PS83	*trpC2 abrB*::(*cat*::*tet*) *gid*::*spc* Δ*codY* Δ*amyE*::(Φ*dpp′-lacZ neo*)	PS56 × DNA PS37
FU382	*trpC2 ilvB*::pMUTIN2	This study
FU383	*trpC2 ilvD*::pMUTIN2	This study
YBGEd	*trpC2 ybgE*::pMUTIN2	JAFAN[a]
YUFNd	*trpC2 yufN*::pMUTIN2	JAFAN
YUFOd	*trpC2 yufO*::pMUTIN2	JAFAN
BFS1337	*trpC2 yurP*::pMUTIN2	Micado[b]
BFS1251	*trpC2 yurN*::pMUTIN2	Micado
BFS1807	*trpC2 ykfA*::pMUTIN2	Micado
YHDGd	*trpC2 yhdG*::pMUTIN2	JAFAN
FU384	*trpC2 gid*::*spc ilvB*::pMUTIN2	This study
FU385	*trpC2 gid*::*spc* Δ*codY ilvB*::pMUTIN2	This study
FU386	*trpC2 gid*::*spc ilvD*::pMUTIN2	This study
FU387	*trpC2 gid*::*spc* Δ*codY ilvD*::pMUTIN2	This study
FU388	*trpC2 gid*::*spc ybgE*::pMUTIN2	This study
FU389	*trpC2 gid*::*spc* Δ*codY ybgE*::pMUTIN2	This study
FU390	*trpC2 gid*::*spc yufN*::pMUTIN2	This study
FU391	*trpC2 gid*::*spc* Δ*codY yufN*::pMUTIN2	This study
FU392	*trpC2 gid*::*spc yufO*::pMUTIN2	This study
FU393	*trpC2 gid*::*spc* Δ*codY yufO*::pMUTIN2	This study
FU394	*trpC2 gid*::*spc yurP*::pMUTIN2	This study
FU395	*trpC2 gid*::*spc* Δ*codY yurP*::pMUTIN2	This study
FU396	*trpC2 gid*::*spc yurN*::pMUTIN2	This study
FU397	*trpC2 gid*::*spc* Δ*codY yurN*::pMUTIN2	This study
FU398	*trpC2 gid*::*spc ykfA*::pMUTIN2	This study
FU399	*trpC2 gid*::*spc* Δ*codY ykfA*::pMUTIN2	This study
FU400	*trpC2 gid*::*spc yhdG*::pMUTIN2	This study
FU401	*trpC2 gid*::*spc* Δ*codY yhdG*::pMUTIN2	This study
FU407	*trpC2 gid*::*spc amyE*::(*cat* P*yufN-lacZ*)	This study
FU408	*trpC2 gid*::*spc* Δ*codY amyE*::(*cat* P*yufN-lacZ*)	This study

[a] JAFAN, Japan Functional Analysis Network for *B. subtilis* (http://bacillus genome.ad.jp/).
[b] Micado, Microbial Advanced Database Organization (http://locus.jouy.inra.fr/micado).

CodY represses at least one gene whose normal function is required for sporulation.

To assess the breadth of the CodY regulon, we used DNA microarray analysis to compare the pattern of transcripts found in a *codY* mutant to the pattern found in wild-type cells. Hundreds of genes organized in dozens of operons appeared to be directly or indirectly controlled by CodY. We then used antibody to CodY to detect segments of the *B. subtilis* chromosome that could be cross-linked to CodY in vivo. Combining the results of these two approaches, we identified many genes as candidates for direct targeting by CodY. For several of these candidates, we have confirmed the microarray results by assays of *lacZ* fusions to the promoter regions and have shown that CodY binds to the regulatory regions in vitro. The confirmed targets surprisingly include the operons for biosynthesis of branched-chain amino acids.

MATERIALS AND METHODS

Bacterial strains and their construction. *B. subtilis* strains used in this study are listed in Table 1. Strains FU382 and FU383 were constructed by using plasmid pMUTIN2 (70) and primer pairs (GCCGAAGCTTGGATTCAGCATCTGCCGAAT/GCGCAGATCTCGGCAATGAATCAATCATGG and GCCGAAGCTTGATCACACAAGGAATCGATAG/GCGCAGATCTGATACAACCGTTTCCACAGA; coding sequences from the *ilvB* and *ilvD* genes, respectively, are underlined) as described previously (72). Isogenic *codY*$^+$ and Δ*codY* strains, each carrying pMUTIN-integrational disruptions, were constructed as follows. Strains PS29 (*codY*$^+$) and PS37 (Δ*codY*) were separately transformed with DNAs of the pMUTIN disruptants for *ilvB*, *ilvD*, *ybgE*, *yufN*, *yufO*, *yurP*, *yurN*, *ykfA*, and *yhdG*, selecting for erythromycin-resistant colonies (at 0.3 μg/ml) on tryptose-blood-agar base plates containing 10 mM glucose. The presence of *gid*::*spc* (a marker linked to *codY*), pMUTIN integration, and Δ*codY* in the transformants was confirmed by resistance to spectinomycin (60 μg/ml) and erythromycin (0.15 μg/ml) and by the appearance of a PCR product in Δ*codY* strains that is shorter by 250 bp than that of *codY*$^+$ strains when amplified with the primer pair CCGGAATTCAATATGAGGAATGTTTAGGAGG/CGCGGATCCAACCCGAGAAATAAAGCTTATTG.

B. subtilis strains FU407 and FU408 were constructed as follows. The *yufN* promoter region was amplified by PCR by using chromosomal DNA of strain 168 as a template and the primer pair 5′-AGTCGCTAGTCTAGAGAAAACGCACTGCTTGC-3′ and 5′-GATCGCGGATCCTTAGATCACAAGTGACATCG-3′ (the underlined sequences are upstream and downstream from the promoter region, respectively). The PCR product was doubly digested with *Xba*I and *Bam*HI and then ligated to the large *Xba*I-*Bam*HI fragment of plasmid pCRE-test2 (47) from which P*spac* had been removed. The ligated DNA was used for transformation of *E. coli* strain DH5α to ampicillin resistance (50 μg/ml). After the sequence of the cloned DNA was confirmed, the plasmid was linearized with *Pst*I and used to transform strains PS29 (*codY*$^+$) and PS37 (Δ*codY*) to chloramphenicol resistance. In the resulting transformants, the *yufN-lacZ* fusion had integrated at the *amyE* locus by double-crossover recombination.

Growth of cells and extraction of RNA for microarray analysis. Strains PS29 (*codY*$^+$) and PS37 (Δ*codY*) were grown in minimal medium (72) supplemented with glucose (0.5%), glutamine (0.2%), and a mixture of 16 other amino acids (only histidine, tyrosine, and asparagine were omitted) (1) until the optical density at 600 nm (OD_{600}) reached 1.0. To examine the effects of the 16-amino-acid mixture on gene expression in wild-type cells, strain 168 (*trpC2*) was grown in the glucose- and glutamine-supplemented minimal medium (minimal glucose-glutamine medium), as described above, with and without the 16 amino acids, until the OD_{600} reached 0.5.

Extraction of RNA from 100-ml portions of the cultures (73), preparation of fluorescently labeled cDNA (52), and hybridization to microarrays (35, 73) were as described previously. The arrays were spotted with PCR products corresponding to 4,005 *B. subtilis* open reading frames as well as the human β-actin gene and calf thymus DNA as negative controls (73). Fluorescence intensity was determined by using the GMS 418 Array Scanner (Affymetrix) and ImaGene software (version 3) (BioDiscovery, Inc.). Each spot was tested in duplicate, and the hybridization results were averaged for the two samples. Background was defined as the average intensities of eight spots of calf thymus DNA and four spots of the β-actin gene.

Cultures of strains PS56 (*abrB*) and PS83 (*abrB* Δ*codY*) were grown at 37°C in DS medium (19), a nutrient broth-based medium in which cells grow rapidly and then sporulate after entering stationary phase. Samples were removed when the absorbance at 600 nm reached 0.5 (mid-exponential phase). (AbrB is a repressor of early stationary-phase gene expression [68] whose targets overlap with those of CodY [65].) Additional samples were harvested during early stationary phase. RNA was harvested from each culture and prepared for hybridization as described by Britton et al. (5). The arrays were spotted with 4,074 PCR products corresponding to *B. subtilis* open reading frames as well as with four *Escherichia coli* genes as negative controls (5). The hybridization results were scanned by using a GenePix 4000B scanner (Axon Instruments, Inc.) and analyzed with GenePix 3.0 software (Axon Instruments, Inc.) (5). The entire procedure was carried out four times, and the results were averaged.

Chromatin immunoprecipitation-microarray (ChIP-to-chip) experiments. An overnight culture of *B. subtilis* strain PS56 (*abrB*) on Luria-Bertani agar was used to inoculate a 50-ml culture in DS medium to give an initial OD_{600} of 0.05. Cross-linking with formaldehyde, extraction and shearing of DNA, and immunoprecipitation generally followed the protocol of Quisel et al. (55) with differences in details noted. When the cells growing at 37°C had reached an OD_{600} of 0.4 to 0.6, the cultures were treated for 30 min with formaldehyde (1% final concentration) in 10 mM sodium phosphate buffer (pH 7.0). Glycine was added to 125 mM final concentration, and the cultures were incubated for an additional 5 min. Cells were washed twice with 40 ml of phosphate-buffered saline (pH 7.3) (2), resuspended in 1 ml of IP buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.5% Triton X-100) supplemented with 50 μl of 1× protease inhibitor cocktail (Roche) and 10 mg of lysozyme, and incubated at 37°C for 20 min. DNA in the lysate was sheared by sonication (Branson 250 microtip sonicator) to give an average fragment size of 300 to 1,000 bp. Ten microliters of the supernatant fluid of subsequent centrifugation was removed and saved for later analysis (total DNA). The remainder of the supernatant fluid was precleared by incubation with one-tenth volume of 50% protein A-Sepharose slurry (Sigma) for 1 h at 4°C. After centrifugation, CodY and CodY-DNA complexes in the supernatant fluid were immunoprecipitated overnight at 4°C by using a rabbit antibody that is highly specific to CodY (57), followed by incubation with 50 μl of a 50% protein A-Sepharose slurry (1 h at 4°C). Complexes were washed four times (15 min each) with 1 ml of IP buffer. The slurry was resuspended in 150 μl of elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% sodium dodecyl sulfate). The 10-μl total-DNA sample was mixed with 150 μl of elution buffer. To reverse formaldehyde-induced cross-links, the immunoprecipitated and total-DNA samples were incubated at 65°C overnight. Supernatants were collected and treated at 37°C for 2 h with 150 μl of Tris-EDTA buffer (TE) containing glycogen (0.27 mg/ml) and proteinase K (100 μg/ml). The DNA was purified by phenol-chloroform extraction, precipitated with isopropanol, and washed with 70% ethanol. Immunoprecipitated DNA was resuspended in 25 μl of TE, and the total DNA sample was resuspended in 100 μl of TE.

PCR amplification of DNA, differential fluorescence labeling, hybridization to microarrays, and array scanning were done according to the protocols at http://microarrays.org/protocols.html. The entire procedure was carried out five times, and the results were averaged. The enrichment factor for a given gene was calculated as the ratio of hybridized immunoprecipitated DNA to hybridized total DNA, normalized by using Resolver software (Rosetta).

Cell growth and β-galactosidase assays. *B. subtilis* cells (*codY*$^+$ and Δ*codY*) with *lacZ* fusions integrated in the target genes or integrated at the *amyE* locus were grown overnight at 30°C on tryptose-blood-agar base plates containing 10 mM glucose and erythromycin (0.3 μg/ml) and spectinomycin (60 μg/ml). Strains FU407 and 408 were grown in the same medium with chloramphenicol (5 μg/ml) and spectinomycin. The overnight cultures were used to inoculate 50 ml of minimal glucose-glutamine medium with 16 amino acids, as described above, and were incubated with shaking at 37°C. At various times, 1-ml samples were withdrawn and β-galactosidase activity was determined as previously described (72).

Purification of CodY and gel mobility shift assays. *E. coli* strain KS272 carrying pKT1, a plasmid in which a C-terminal, six-histidine-tagged version of the *codY* gene is under the control of the *araBAD* promoter (33), was grown in Luria broth containing ampicillin (50 μg/ml) until the absorbance at 600 nm reached 0.7. L-Arabinose was added to give a final concentration of 0.2%, and incubation was continued for an additional 4 to 5 h. After sonication, the soluble extract was treated with streptomycin sulfate (62) to remove ribosomes and nucleic acids, and the soluble fraction was mixed with Talon Co^+ beads (Clontech). After several washes, CodY-His_6 protein was eluted with increasing concentrations of imidazole. The preparation was free of contaminating proteins, as determined by Coomassie blue staining of a sodium dodecylsulfate-polyacrylamide gel.

The regulatory regions of genes to be tested were amplified by PCR by using *B. subtilis* chromosomal DNA as a template and two primers, one of which was radioactively labeled. The PCR products ranged from 199 to 525 bp in length. Primer labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP has been described previously (34). In some cases, enough was known about the transcription unit to design a probe that would be sure to include any likely regulatory sites. When there was insufficient knowledge, we prepared probes that extended from the beginning of the coding sequence to a position several hundred base pairs upstream that overlapped with the end of the neighboring coding sequence.

Labeled DNA was mixed with increasing amounts of CodY protein in a 10-μl reaction mixture that contained 20 mM Tris-Cl [pH 8.0], 50 mM sodium glutamate, 10 mM $MgCl_2$, 5 mM EDTA, 0.05% (vol/vol) Nonidet P-40 (Igepal; Sigma Chemical Co.), 5% (vol/vol) glycerol, and 250 ng of calf thymus DNA. Where indicated, GTP was also present at 2 mM. After 20 min of incubation at room temperature, the samples were loaded on a 12% polyacrylamide gel that was running at 110 V. Subsequent electrophoresis in 35 mM HEPES and 43 mM imidazole (pH 7.4) was at 150 V for 90 to 150 min. The 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.

RESULTS

Transcript analysis. CodY was originally defined as a factor that exerts repression of the *dpp* and *hut* operons in a minimal medium containing an excess of glutamine and 16 other amino acids (1, 18, 65). For cells grown in such a medium, we identified, by whole genome microarray analysis, 124 genes located in about 70 apparent transcription units that were overexpressed by a factor of 3 or more in a *codY* null mutant compared to a wild-type strain. The 54 genes whose transcript level was most highly derepressed by the *codY* mutation (as well as other genes previously identified as being under CodY control) are listed in Table 2. (Genes whose highest level of hybridization was less than twofold greater than the background were excluded from this analysis.) Most of these genes were repressed in wild-type cells by addition of the amino acid mixture (Table 2), but not all genes repressed by the amino acids were derepressed by a *codY* mutation (data not shown). Notably, the biosynthetic pathways for arginine, cysteine, methionine, and the branched-chain amino acids were strongly repressed by the amino acid mixture (reference 42 and data not shown), but of these, only the pathway for branched-chain amino acid biosynthesis (and one of two genes for asparagine synthetase [*asnH*]) were derepressed in a *codY* mutant (Table 2). Another 27 genes located in 20 transcription units appeared to be dependent on CodY for their expression. A few of those genes are included in Table 2. There is no prior evidence for positive regulation by CodY. The results of these microarray experiments are available at the KEGG Expression Database website (http://www.genome.ad.jp/kegg/expression/).

For cells grown in the nutrient broth-based DS medium, 187 genes in 84 apparent transcription units were overexpressed in a *codY* mutant during exponential phase and an additional 79 genes in 43 apparent transcription units were overexpressed in a *codY* mutant only during stationary phase (when CodY is less

TABLE 2. Selected results of transcript analysis[a]

Gene designation	Transcript ratio			ChIP enrichment factor[b]	Functional assignment[c]
	Δ*codY*/*codY*$^+$, MM + AAs	Wild type in MM/wild type in MM + AAs	Δ*codY*/*codY*$^+$, DS medium		
Previously known targets					
acsA	4.4	2.3	2.4	2.10	Acetyl CoA synthetase
dppA	55	7.1	7.1	2.78	D-Ala-aminopeptidase
dppB	65	7.1	4.8	~1.0[e]	Dipeptide permease
dppC	20	5.4	6.4	~1.0	Dipeptide permease
dppD	26	7.5	5.7	~1.0	Transport ATPase
dppE	28	7.0	9.2	~1.0	Dipeptide binding protein
srfAA	1.0	3.6	0.55	~1.0	Surfactin synthetase, competence factor
comK	2.5	1.5	1.3	1.63	Competence transcription factor
ureA	3.7	5.4	1.4	1.40 (*ywmG*)	Urease subunit
ureB	2.6	2.4	2.8	1.23	Urease subunit
ureC	0.9	0.7	2.5	1.25	Urease subunit
hag	1.0	1.5	0.38	~1.0	Flagellin
bkdR	0.4	0.9	ND[d]	~1.0	Regulatory protein for branched-chain, keto acid dehydrogenase operon
ptb	1.3	0.4	ND	~1.0	Phosphate butyryltransferase
bcd	1.1	0.9	ND	~1.0	Leucine dehydrogenase
buk	0.4	0.7	ND	~1.0	Butyrate kinase
lpdV	1.3	0.8	ND	~1.0	BCKA dehydrogenase subunit
bkdAA	1.1	0.9	ND	~1.0	BCKA dehydrogenase subunit
bkdAB	0.9	0.6	ND	~1.0	BCKA dehydrogenase subunit
bkdB	0.6	0.9	ND	~1.0	BCKA dehydrogenase subunit
hutP	11.1	1.3	1.3	~1.0	Histidine utilization regulator
rapA	2.8	2.5	0.9	2.24	Spo0F~P phosphatase
rapC	0.7	0.8	0.9	~1.0	ComA~P phosphatase
rocA	12	0.4	0.6	~1.0	Pyrolline-5-carboxylate dehydrogenase
rocB	37	2.2	0.9	1.65	Probable citrullinase[f]
rocC	24	1.4	1.0	~1.0	Arginine permease
rocD	11	1.1	0.53	~1.0	Ornithine transaminase
rocE	4.0	1.0	0.43	~1.0	Arginine permease
rocF	2.0	0.8	0.47	~1.0	Arginase
gabP	1.0	1.2	1.5	1.31	γ-Aminobutyrate permease
codV	1.0	0.8	2.5	2.56	Recombinase
clpQ	1.0	0.7	2.0	~1.0	Chaperone-type ATPase
clpY	1.3	1.5	1.0	~1.0	Protease
codY	1.3	1.2	2.9	~1.0	GTP-dependent regulatory protein
citB	1.4	11.3	1.2	~1.0	Aconitase
Newly identified targets					
appD	17	22	1.2	1.54	Transport ATPase
appF	28	27	1.2	~1.0	Transport ATPase
appA	18	54	2.1	~1.0	Oligopeptide binding protein
appB	30	22	1.5	~1.0	Oligopeptide permease
appC	3.7	5.9	1.2	~1.0	Oligopeptide permease
glpF	0.19	0.4	0.9	~1.0	Glycerol uptake facilitator
glpT	0.20	0.6	0.40	~1.0	Glycerol-3-phosphate permease
guaB	0.28	1.0	0.24	1.86	IMP dehydrogenase
guaC	0.23	0.32	ND	~1.0	GMP reductase
ilvB	38	21	21	2.1 (*ysnD*)	Acetolactate synthase subunit
ilvH	44	11	ND	~1.0	Acetolactate synthase subunit
ilvC	49	25	11	~1.0	Ketol-acid reductoisomerase
leuA	47	26	12	1.16	2-Isopropylmalate synthase
leuB	46	38	51	~1.0	3-Isopropylmalate dehydrogenase
leuC	46	16	51	~1.0	3-Isopropylmalate dehydratase subunit
leuD	2.4	1.9	6.6	1.17	3-Isopropylmalate dehydratase subunit
ilvD	20	11	3.6	3.2 (*ypgR*)	Dihydroxy-acid dehydratase
ilvA	5.1	4.2	1.8	~1.0	Threonine dehydratase
kinB	7	2.8	3.3	1.76	Spo0F kinase

Continued on following page

TABLE 2—*Continued*

Gene designation	Transcript ratio			ChIP enrichment factor[b]	Functional assignment[c]
	Δ*codY*/*codY*$^+$, MM + AAs	Wild type in MM/wild type in MM + AAs	Δ*codY*/*codY*$^+$, DS medium		
ybgE	17	1.3	42	2.63	Branched-chain amino acid Aminotransferase
yccC	15	ND	1.5	~1.0	Similar to asparaginase
ycgA	13	1.9	2.7	1.6	Unknown
ycgM	6.3	3.4	0.63	2.9	Proline oxidase
yhdG	116	26	11	1.86	Amino acid transporter
yhjC	92	4.3	6.3	1.57	Unknown
ykfA	13	2.8	5.6	~1.0	Similar to microcin
ykfB	16	4.5	8.5	~1.0	L-Ala-D/L-Glt epimerase
ykfC	14	4.4	8.4	~1.0	γ-D-glutamyl-L-amino acid peptidase
ykfD	14	4.9	5.6	~1.0	Transporter
yufN	21	8.2	13	1.65	Substrate binding protein
yufO	55	15	14	~1.0	Probable transport ATPase
yufP	14	4.7	8.7	~1.0	Permease
yufQ	14	9.4	13	~1.0	Permease
yuiC	23	2.0	4.7	1.72	Unknown
yuiB	18	3.3	6.9	2.32	Unknown
yurJ	120	1.1	8.5	~1.0	Probable transport ATPase
yurP	312	7.2	20	1.52	Similar to glutamine-fructose transaminase
yurO	346	5.4	41	~1.0	Similar to sugar binding protein
yurN	723	3.7	33	~1.0	Similar to sugar permease
yurM	58	1.7	18	1.94	Similar to sugar permease
yurL	55	1.9	13	~1.0	Similar to ribokinase
yusC	0.30	4.8	0.61	1.85	Similar to transport ATPase
yxbC	45	5.6	4.0	1.96	Unknown
yxbB	65	11	10	1.74	Unknown
yxbA	99	5.7	7.3	~1.0	Unknown
yxnB	78	10	10	~1.0	Unknown
asnH	5.6	2.5	7.6	~1.0	Asparagine synthetase
yxaM	51	4.2	5.9	~1.0	Similar to antibiotic resistance protein

[a] Previously known and newly identified targets of CodY and genes whose transcript level was highly affected by a *codY* mutation during growth in minimal medium (MM) with or without added amino acids (AAs) or in DS medium.
[b] ChIP enrichment data were taken from the data of Table 3 and from additional data not shown. They are presented here for comparative purposes.
[c] Functional assignments are from SubtiList (http://genolist.pasteur.fr/SubtiList/) or from literature cited in the text. CoA, coenzyme A.
[d] ND, transcript level was not significantly above background in either the mutant or wild-type sample.
[e] ~1.0, enrichment factor for precipitation of specific genes by antibody to CodY (see text and Table 3) was not statistically different from 1.0.
[f] B. Belitsky (personal communication).

active). One hundred thirty-two genes in 62 transcription units were underexpressed in a *codY* mutant during exponential growth phase. The full data set for these experiments can be viewed at the website of the Losick laboratory (http://mcb.harvard.edu/losick/). For the experiments on cells grown in DS medium, both the *codY*$^+$ and Δ*codY* strains carried a deletion in the *abrB* gene to avoid missing genes whose transcription is repressed by AbrB as well as by CodY. In fact, the vast majority of the genes that were overexpressed in a *codY* mutant in medium containing amino acids were also overexpressed in a *codY abrB* double mutant in DS medium. An apparent discrepancy in the behavior of the *rocABC* and *rocDEF* operons in the two different growth conditions can be rationalized. Both of these operons are dependent for their expression on RocR, a positive regulator that is activated by arginine or ornithine (21). The defined medium contains a high concentration of arginine, but DS medium does not. For genes that were underexpressed in a *codY* mutant, we saw very little correlation between the results obtained in minimal glucose-glutamine medium with 16 amino acids and DS medium.

The microarray analysis did not identify all targets of CodY. Of the previously known CodY targets, only the *acsA* gene and the *dpp* and *ure* operons were overexpressed in *codY* mutant cells grown both in minimal medium containing amino acids and broth medium. Other known targets either were not expressed above background levels in cells grown in one of the media tested or were not overexpressed in a *codY* mutant in one or both media. These genes included *srfAA*, *comK*, *hag*, the *bkd* cluster, *hutP*, *rapA*, *rocABC*, *rocDEF*, *gabP*, *rapC*, the *cod* operon, and *citB*. Several of these transcription units require positive regulators that might not be active under the conditions tested. For instance, *bkd* expression requires BkdR (12), *gabP* requires TnrA (16), *srfAA*, *comK*, and *rapA* require ComA~P (25, 48, 49), and *hag* requires sigma-D (45). The *citB* gene, on the other hand, is strongly repressed by CcpC in cells in glucose-glutamine-containing medium and during rapid exponential growth phase in DS medium (31, 33, 34); the effect of a *codY* mutation on *citB* expression can be detected only in a *ccpC* mutant strain (33).

The *hutP* gene was overexpressed in a *codY* mutant in min-

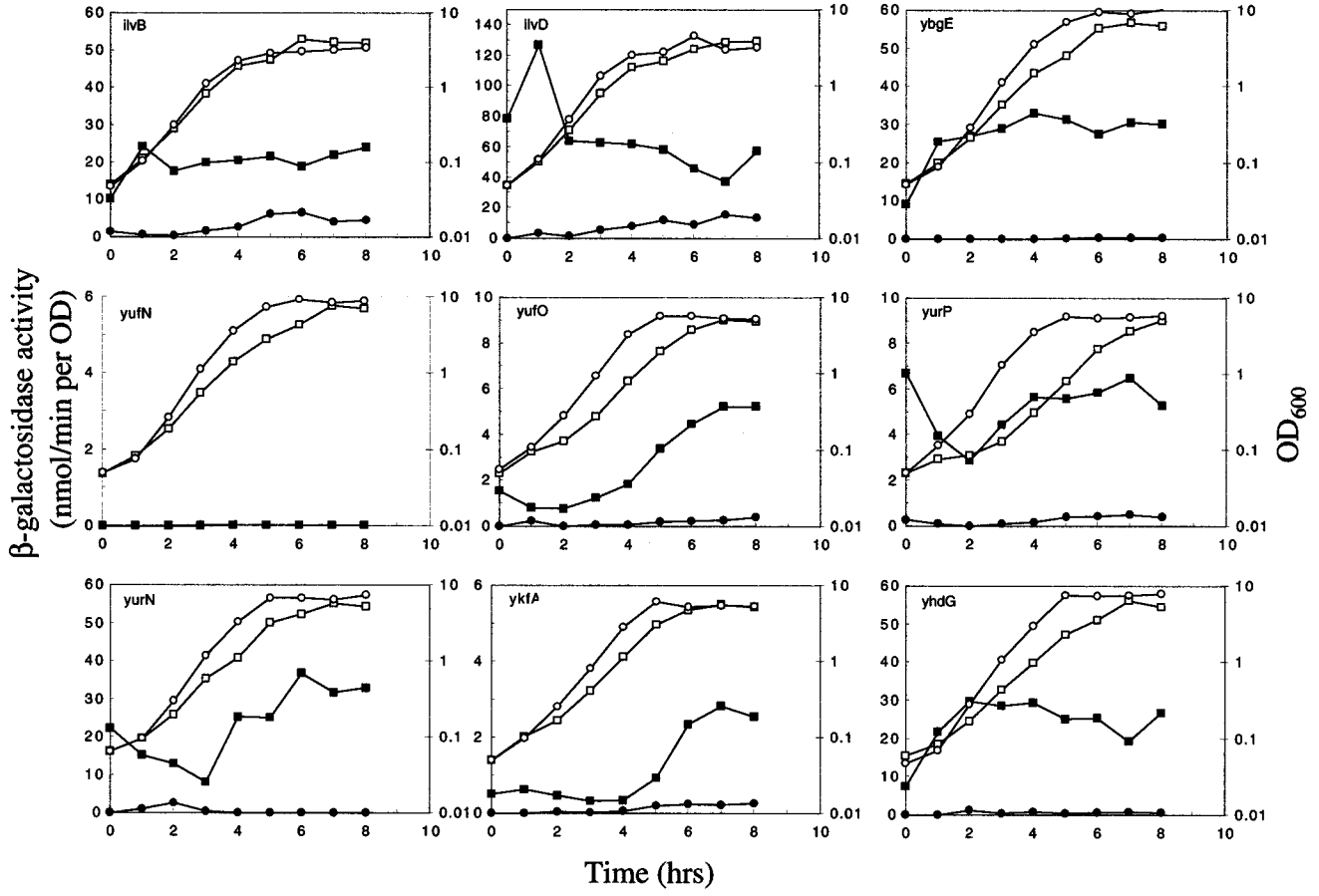

FIG. 1. Expression of *lacZ* fusions to promoters of putative CodY target genes in *codY*$^+$ and Δ*codY* strains. Strains were grown in minimal glucose-glutamine medium containing a mixture of 16 additional amino acids (see Materials and Methods), and samples were removed at indicated times after inoculation for assays of β-galactosidase activity. Isogenic *codY*$^+$ and Δ*codY* strains carrying each gene disruption were FU384 and FU385 for *ilvB*, FU386 and FU387 for *ilvD*, FU388 and FU389 for *ybgE*, FU390 and FU391 for *yufN*, FU392 and FU393 for *yufO*, FU394 and FU395 for *yurP*, FU396 and FU397 for *yurN*, FU398 and FU399 for *ykfA*, and FU400 and FU401 for *yhdG*. Circles and squares denote *codY*$^+$ and Δ*codY* strains, respectively, whereas open and closed symbols represent the OD_{600} and β-galactosidase activity, respectively.

imal medium containing amino acids (Table 2), but the other genes of the *hut* operon were not detectably transcribed (data not shown). Transcription of genes downstream of *hutP* depends on an antitermination event that requires histidine (51), one of three amino acids not present in the mixture used.

Among the previously unsuspected targets of CodY, the most highly affected by a *codY* mutation included the *appDFABC*, *ykfABCD*, and *ilvBHC-leuABCD* operons, the *ilvD*, *ilvA*, *ybgE*, and *yhdG* genes, the *yufNOPQ* cluster, and the *yurPONML* cluster (Table 2). Experiments described below indicate that some but not all of these genes and operons are direct targets of CodY binding.

Expression of *lacZ* fusions. To confirm the transcript analysis for a subset of the newly identified potential target genes (*ilvB*, *ilvD*, *ybgE*, *yhdG*, *ykfA*, *yufN*, *yufO*, *yurP*, and *yurN*), we constructed isogenic *codY*$^+$ and Δ*codY* strains carrying integrational disruptions created through single-crossover recombination with plasmid pMUTIN2 derivatives possessing short coding regions from the 5′ ends of the genes. Such integration resulted in the transcriptional fusion of the upstream region of each disrupted gene to the *E. coli lacZ* gene. To monitor gene expression, samples taken at various times during growth in minimal glucose-glutamine medium with 16 amino acids were assayed for β-galactosidase activity. As shown in Fig. 1, *lacZ* fusions to the *ilvB*, *ilvD*, *ybgE*, *yhdG*, *ykfA*, *yufO*, *yurP*, and *yurN* promoter regions were all at least partially derepressed in Δ*codY* strains during exponential growth and stationary phase. (RNA for the DNA microarray analysis of cells in defined medium containing a mixture of amino acids was prepared from cells harvested at an OD_{600} of 1.0, i.e., near the end of the rapid exponential growth phase.)

By contrast, the strain carrying a pMUTIN2-derived *lacZ* fusion to *yufN* (and a *yufN* disruption) seemed to behave anomalously. No β-galactosidase activity was detected in either the *codY*$^+$ or Δ*codY* strain at any stage of growth (Fig. 1). To test the possibility that expression of the *yufN* gene depends on its own product, we constructed *codY*$^+$ and Δ*codY* strains carrying at the *amyE* locus a *lacZ* transcriptional fusion to the intergenic region upstream of *yufN*. In this case, the *yufN-lacZ* fusion was clearly expressed and derepressed in the Δ*codY* background (Fig. 2). Thus, the results of the *lacZ* fusion experiments with respect to *ilvB*, *ilvD*, *ybgE*, *yhdG*, *ykfA*, *yufN*, *yufO*, *yurP*, and *yurN* coincided well with those of the DNA microarray analysis.

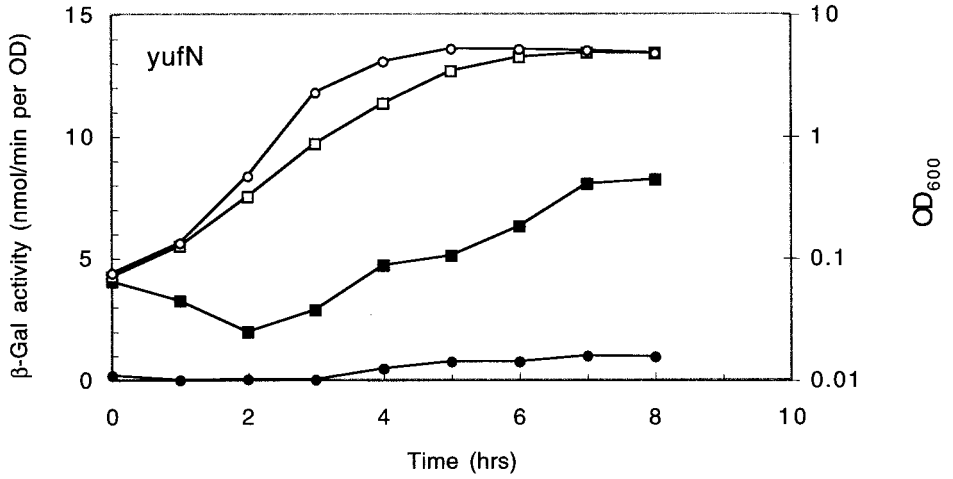

FIG. 2. Expression of a *yufN-lacZ* fusion integrated at the *amyE* locus. Strains FU407 (*codY*$^+$) and FU408 (Δ*codY*) were grown in minimal glucose-glutamine medium containing 16 additional amino acids (see Materials and Methods). Samples removed at the indicated times after inoculation of the culture were assayed for β-galactosidase activity. Circles and squares denote *codY*$^+$ and Δ*codY* strains, respectively, whereas open and closed symbols represent the OD_{600} and β-galactosidase activity, respectively.

Immunoprecipitation of CodY-DNA complexes. Formaldehyde-induced in vivo cross-linking of protein to DNA has been used with immunoprecipitation by specific antibodies to demonstrate binding of proteins to specific DNA regions in both eukaryotes and prokaryotes (38, 66). The power of this method can be extended by combining the selectivity of chromatin immunoprecipitation (ChIP) with the global analysis provided by a DNA microarray (chip) (29, 36, 58). To identify segments of *B. subtilis* DNA that interact with CodY in vivo, we treated cells with formaldehyde to create protein-DNA cross-links and then used antibody to CodY to select those DNA regions that were specifically cross-linked to CodY (see Materials and Methods). The putative binding sites were then revealed by hybridization to a DNA microarray of the *B. subtilis* genome (ChIP-to-chip). A calculated enrichment factor denotes the extent to which the relative abundance of a given gene was augmented by immunoprecipitation.

As summarized in Table 3, 68 regions of the chromosome were preferentially selected by immunoprecipitation with antibody to CodY. Forty-two of these regions contained at least one gene whose transcript level was significantly affected by a *codY* mutation when cells were grown in minimal medium or DS medium or both. The nature of the method does not permit unambiguous assignment of the *codY* binding site to a specific gene, however. That is, fragmentation of the DNA may separate the CodY binding site from the coding sequence that it regulates. Since the microarrays were spotted with PCR products corresponding to coding sequences but not intergenic regions, the analysis of the immunoprecipitated DNA might fail to identify some target sites and might identify a neighboring gene in addition to or even instead of the actual target. For instance, in the *ureA-ywmG-ywmF* region, we detected the *ywmG* gene but not *ureA* by immunoprecipitation, even though *ureA* is a known target of CodY (71) (Tables 2 and 3). As a result, we made educated guesses about the likely target gene in some cases (e.g., *ilvB*). On the basis of this analysis, we determined that the *ilvB*, *ilvD*, *ilvA*, *ybgE*, *yhdG*, *yurM*, and *yufN* genes are likely to be direct targets of CodY, whereas the *ykfABCD* and *yufOPQ* operons may be indirectly regulated by CodY. The case of *yurP* was uncertain; the *yurPON* cluster was highly derepressed in a *codY* mutant but the ChIP-to-chip enrichment factor was only 1.52.

The ChIP-to-chip analysis revealed 26 apparent CodY target genes whose transcript level was below detection or did not appear to be influenced dramatically by a *codY* mutation. These genes may depend on a positive regulator that is inactive under the conditions tested or may be expressed in a CodY-dependent manner only under growth conditions other than those used here.

Only a few CodY targets identified by ChIP-to-chip analysis (*gltT*, *guaB*, and *braB*) were consistently and significantly underexpressed in a *codY* mutant. Their regulatory regions may be sites of direct, positive regulation by CodY.

Gel mobility shift assays of CodY binding. To test whether CodY binds directly to the regulatory regions of putative target genes, we prepared radioactive double-stranded DNA probes corresponding to the upstream regions of several of the genes. Gel mobility shift analysis showed that CodY can interact in vitro with probes for the *ilvB*, *ilvD*, *ybgE*, *yufN*, *yhdG*, and *yurP* genes (Fig. 3). By contrast, probes for the *ykfA* and *yufO* genes did not interact with CodY even at very high protein concentrations (Fig. 3). Thus, these two transcription units, which are strongly responsive to a *codY* mutation (Table 1 and Fig. 1), are probably indirect targets of CodY. The in vitro binding results are consistent with the absence of *ykfA* and *yufO* among the genes that were cross-linked to CodY in vivo. A likely explanation is that *ykfA* and *yufO* lie immediately downstream of transcription units that are highly regulated by CodY (*dpp* and *yufN*, respectively). Although the neighboring operons are in both cases separated by an apparent transcription terminator, this terminator must be leaky, allowing read-through into the *ykfA* operon by *dpp* transcription and into the *yufO* operon by *yufN* transcription. The fact that *yufO* and the *ykfA* operon are only indirect targets of CodY does not diminish the physiological significance of their response to CodY (see Discussion).

TABLE 3. Targets of CodY revealed by ChIP-to-chip analysis

Region detected[a]	Enrichment factors[b]	Likely target gene[c]	Transcript ratio of likely target gene[d]		Function of likely target gene/operon
			Δ*codY*/*codY*$^+$, DS medium	Δ*codY*/*codY*$^+$, MM + AAs	
Biosynthesis					
ilvB-ysnD-ysnE	2.1	*ilvB*	21.3	38.1	BCAA biosynthesis
ilvD-ypgR-ypgQ	3.2	*ilvD*	3.6	19.5	BCAA biosynthesis
ybgA-ybgB-ybgE-ybgF	3.54 (*ybgB*), 2.63 (*ybgE*)	*ybgE*	42.2	17	BCAA transaminase
ggaB-ggaA-tagH	3.1	*ggaA*	1.1	0.6	Teichoic acid synthesis
rrnO-yaaC-guaB	2.4 (*yaaC*), 1.86 (*guaB*)	*guaB*	0.24	0.27	IMP dehydrogenase
Catabolism					
tyrS-acsA-acuA	2.1	*acsA*	2.4	4.4	Acetyl CoA synthetase
opuAC-amhX-ycgA	2.27 (*amhX*), 1.6 (*ycgA*)	*amhX*	34.3	2.5	Aminohydrolase
yhjN-aprE-yhfO	1.94 (*aprE*), 1.74 (*yhfO*)	*aprE*	1.5	2.3	Subtilisin E
cgeE-cgeD-cgeC	2.05	*cgeD*	0.9	1.4	Spore coat maturation
gid-codV-clpQ	1.93 (*gid*), 2.56 (*codV*)	*codV*	2.5	1.0	Site-specific recombinase (*cod* operon)
proG-dppA-dppB	3.06 (*proG*), 2.78 (*dppA*)	*dppA*	7.1	55	Dipeptide permease
metE-ispA-ykoB	3.21	*ispA*	1.8	ND[e]	Intracellular protease
yktD-nprE-ylaA	1.73	*nprE*	0.29	0.7	Neutral protease
trpS-oppA-oppB	1.51	*oppA*	0.55	0.8	Oligopeptide permease
rocG-rocA-rocB-rocC	1.65	*rocA*	0.60	11.8	Arginine catabolism
rocR-rocD-rocE	1.52	*rocD*	0.53	4.1	Arginine catabolism
ycgL-ycgM-ycgN	4.18 (*ycgL*), 2.9 (*ycgM*)	*ycgM*	0.63	6.3	Proline catabolism
ureA-ywmG-ywmF	1.4	*ureA*	1.4	3.7	Urea catabolism
uxaC-yjmB-yjmC-yjmD	2.39 (*yjmB*), 2.18 (*yjmC*)	*yjmB*	1.1	ND	Hexuronate catabolism?
yoaB-yoaC-yoaD	1.79 (*yoaC*), (2.81 *yoaD*)	*yoaC*	1.2	ND	Similar to xylulokinase
ycbE-ycbF-ycbG	1.88	*ycbF*	2.4	ND	Glucarate dehydratase?
Regulation					
spoIVB-spo0A-recN	1.92	*spo0A*	1.3	ND	Transcription factor
patB-kinB-kapB	1.76	*kinB*	3.3	7.0	Histidine kinase
yjoB-rapA-phrA	2.24	*rapA*	0.9	2.8	Spo0F∼P phosphatase
yqcI-yqcJ-rapE-phrE	3.16	*rapE*	5.4	5.8	Spo0F∼P phosphatase
ywhK-rapF-phrF	2.32 (*rapF*), 3.17 (*phrF*)	*rapF*	2.2	2.0	Aspartyl∼P phosphatase
yddK-rapI-phrI-yddM	1.8	*rapI*	0.02	ND	Aspartyl∼P phosphatase
yozJ-rapK-phrK	1.84	*rapK*	1.2	3.7	Aspartyl∼P phosphatase
ykuV-rok-yknT	1.83	*rok*	1.0	1.1	Competence regulator
yveK-slr-pnbA	2.65	*slr*	1.5	1.3	Regulatory protein
yrhI-yrhH-yrzI	1.79 (*yrhI*), 2.86 (*yrhH*)	*yrhI*	1.4	ND	Transcription regulator?
xkdA-xre-xkdB-xkdC	1.42 (*xre*), 1.97 (*xkdB*)	*xkdB*	1.2	1.7	PBSX phage regulator?
Transport					
sunA-sunT-yolF	4.18	*sunT*	1.5	ND	Lantibiotic transporter
yrrI-glnQ-glnH	1.77	*glnQ*	1.3	2.8	Glutamine transport
nifZ-braB-ezrA	1.94	*braB*	0.36	0.5	BCAA transport
yjaZ-appD-appF	2.39 (*yjaZ*), 1.54 (*appD*)	*appD*	1.2	17	Oligopeptide transport
gltT-yhfh-yhfI	2.17	*gltT*	0.42	0.4	H^+-Na^+/glutamate symporter
ydiD-gcp-ydiF-ydiG	1.46 (*gcp*), 1.53 (*ydiF*)	*ydiF*	1.5	0.7	Sugar transporter?
ytmM-ytmL-ytmK	3.9 (*ytmM*), 1.95 (*ytmL*)	*ytmL*	0.9	ND	Amino acid transporter?
citA-yhdF-yhdG-yhdH	2.71 (*yhdF*), 1.86 (*yhdG*)	*yhdG*	11.5	116	Amino acid transporter?
yurL-yurM yurN	1.94	*yurM*	18.3	58.7	Sugar permease?
yufM-yufN-yufO	1.65	*yufN*	13.2	21.4	ABC transporter?
Other					
ybbI-ybbJ-ybbK	1.91	*ybbJ*	1.2	ND	Unknown
ydcK-ydcL-ydcM-ydcN	2.24 (*ydcL*), 2.55 (*ydcM*), 2.55 (*ydcN*)	*ydcL*	ND	1.8	Prophage integrase?
yddS-yddT-ydeA	1.79	*yddT*	1.1	ND	Unknown
ydhQ-ydhR-ydhS	1.78	*ydhR*	1.0	1.0	Similar to fructokinase
yfmC-yfmB-yfmA	1.67	*yfmC*	1.8	1.0	Unknown
yfmI-yfmH-yfmG	ND	*yfmG*	5.5	6.5	Unknown
yhjC-yhjD-yhjE	1.57	*yhjC*	6.3	93	Unknown
pit-ykaA-ykbA	3.24	*ykaA*	1.0	ND	Unknown
splB-ykwB-mcpC	2.42	*ykwB*	4.4	25.7	Unknown
ylbO-ylbP-ylbQ	1.85	*ylbP*	1.5	3.0	Unknown
yndJ-yndK-yndL-yndM	1.69 (*yndK*), 1.79 (*yndL*)	*yndK*	1.3	0.8	Unknown

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TABLE 3—*Continued*

Region detected[a]	Enrichment factors[b]	Likely target gene[c]	Transcript ratio of likely target gene[d]		Function of likely target gene/ operon
			Δ*codY*/*codY*$^+$, DS medium	Δ*codY*/*codY*$^+$, MM + AAs	
yoaC-yoaD-yoaE	1.78 (*yoaC*), 2.81 (*yoaD*)	*yoaD*	2.4	ND	Similar to phosphoglycerate dehydrogenase
yobQ-yobR-yobS	1.91	*yobR*	2.3	3.4	Unknown
odhA-yojO-yojN	1.83	*yojO*	1.5	0.8	Unknown
yqgB-yqgA-yqfZ	1.86	*yqgA*	1.1	1.1	Unknown
yqjZ-yqjY-yqjX	1.99	*yqjZ*	1.6	2.5	Unknown
yuaG-yuaF-yuaE	1.78	*yuaF*	1.5	2.1	Unknown
yuiC-yuiB-yuiA-yumB	2.32 (*yuiB*), 3.39 (*yuiA*)	*yuiB*	6.9	17.8	Unknown
yurP-yurQ-yurR	1.52	*yurP*	19.5	312	Unknown
yusC-yusD-yusE	1.85	*yusC*	0.61	0.3	Unknown
yvaV-yvaW-yvaX-yvaY	1.7 (*yvaW*), 3.29 (*yvaX*), 1.67 (*yvaY*)	*yvaX*	0.92	4.2	Unknown
yvdB-yvdA-yvcT	1.76 (*yvdB*), 3.16 (*yvdA*)	*yvdA*	1.3	2.3	Carbonic anhydrase?
yxbC-yxbB-yxbA	1.96 (*yxbC*), 1.74 (*yxbB*)	*yxbC, yxbB*	3.9 (*yxbC*), 10.1 (*yxbB*)	35 (*yxbC*), 65 (*yxbB*)	Unknown Unknown
pepT-yxjJ-yxjI	2.24	*yxjJ*	1.1	ND	Unknown
yycO-yycN-rapG	1.83	*yycN*	1.2	0.7	Unknown

[a] Gene clusters are listed within which the underlined genes were preferentially precipitated by antibody to CodY, after in vivo cross-linking, as detected by hybridization to a genomic array.
[b] The enrichment factor indicates the extent to which a gene was preferentially precipitated compared to its abundance in total DNA.
[c] The likely target gene was either the enriched gene or a neighboring, promoter-proximal gene whose transcription is strongly affected by a *codY* mutation.
[d] Transcript ratios are from the data of Table 2 and additional data not shown; they are included here for comparative purposes. MM + AAs, minimal medium with added amino acids.
[e] ND, transcript not detectable in either mutant or wild-type sample.

Binding of CodY to the *dpp* promoter region is slightly stimulated by GTP (57). (Efficient repression of *dpp* transcription by CodY has a more stringent requirement for GTP [57].) GTP also stimulated binding of CodY to the *ilvB*, *ilvD*, *yufN*, *yhdG*, *yurP*, and *ybgE* regulatory regions (Fig. 2).

DISCUSSION

Our results show that ChIP-to-chip, when combined with global transcriptional analysis, is a powerful method for locating previously unknown, direct targets of a bacterial regulatory protein. An alternative strategy would be to combine ChIP-to-chip results with a sequence scan that searches for a conserved binding site for the regulatory protein (15). In the case of CodY, such a search was not possible, because no conserved binding site is yet known.

Even the combination of ChIP-to-chip analysis and transcript profiling did not identify all CodY targets, however. Transcriptional profiling was expected to miss some targets because many stationary-phase genes regulated by CodY are also subject to control by other regulatory proteins. As a result, multiple mutations are needed in some cases to reveal fully the role of CodY (33, 65). More surprisingly, some genes whose regulatory regions bind CodY in vitro and whose expression is subject to CodY-mediated repression in vivo failed to be enriched for by immunoprecipitation. Examples are *srfAA*, *hutP*, and *citB*. Perhaps these targets have affinities for CodY that are relatively low, or perhaps binding of CodY under the growth conditions tested was prevented by interference by other regulators that bind to overlapping sites.

The newly identified targets of CodY unexpectedly include genes for amino acid biosynthesis (the *ilvB* operon and the *ilvA*, *ilvD*, and *ybgE* genes). All previously recognized and many newly identified CodY targets are genes whose products permit the cell to search for, take up, and metabolize secondary nutritional sources or to sporulate. The cell's rationale in coregulating biosynthesis of amino acids and catabolism of secondary nutrients (including some amino acids) may be the following. During rapid growth in rich medium, cells utilize preformed amino acids and repress the relevant biosynthetic pathways. When the external amino acid supply becomes limited, cells turn on de novo biosynthesis at the same time that they hunt for other carbon and nitrogen sources. This principle should hold for all amino acids and has been at least partially verified experimentally (reference 42, Table 2, and data not shown). However, only the isoleucine-leucine-valine biosynthetic pathway proved to be under CodY control. Either the branched-chain amino acids are preferentially consumed or the cell has evolved to tie its control of stationary-phase gene expression specifically to the availability of branched-chain amino acids. In fact, preliminary experiments establish that the ability of CodY to bind to many of its targets in vitro is stimulated by branched-chain amino acids (R. Shivers and A. L. Sonenshein, unpublished results). This finding fits well with the observation of Guédon et al. (23) that dipeptides containing branched-chain amino acids are particularly effective in activating CodY in vivo in *L. lactis*. These authors, in fact, suggested that the amino acids might be direct effectors of CodY (23). Given the central position of the branched-chain amino acids in cellular metabolism, such regulation would not be surprising and is, in fact, reminiscent of the role of Lrp in *E. coli* (50). The *B. subtilis* genome encodes seven homologs of Lrp, none of which is yet known to be a global regulator (3, 4,

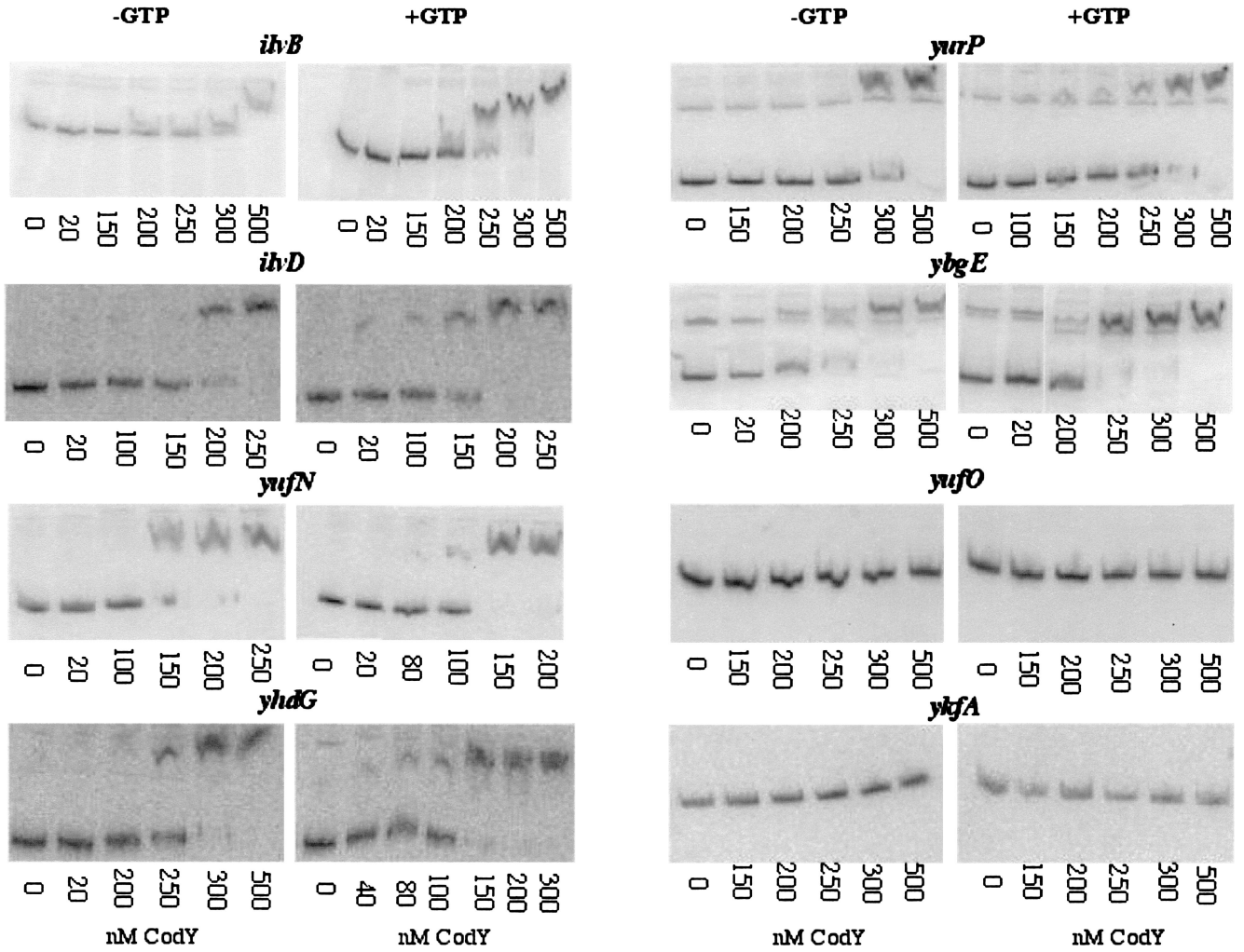

FIG. 3. Gel mobility shift analysis of CodY binding to putative target regulatory regions. The regulatory regions of potential target genes were amplified by PCR by using radioactive primers, incubated with purified CodY-His_6 protein at various concentrations, and analyzed by nondenaturing polyacrylamide gel electrophoresis (see Materials and Methods for details). The lengths of the PCR products in base pairs were as follows: *ilvB*, 453; *ilvD*, 525; *ybgE*, 446; *yhdG*, 345; *yufN*, 492; *yufO*, 199; *ykfA*, 340; and *yurP*, 321. In each panel, the position of unshifted DNA is seen in the leftmost lane, which contained no CodY. Where indicated, GTP was included in the reaction mixture at a concentration of 2 mM.

11). It is conceivable that CodY in gram-positive bacteria is the functional equivalent of Lrp in enteric bacteria. A central role for the branched-chain amino acid biosynthetic pathway in *B. subtilis* metabolism is also suggested by its susceptibility to direct or indirect repression by CcpA, a global regulator that responds to glucose availability (41).

Other newly identified targets of CodY also have interesting features. The *ykfABCD* operon, which seems to be controlled via the *dpp* promoter, encodes an L-alanine-D/L-glutamate epimerase (YkfB), a γ-D-glutamyl-L-diamino acid peptidase (YkfC), and a transport protein (YkfD) (61). YkfA is related to a microcin-resistance protein in *E. coli* (22, 61). These proteins may be involved in recycling of peptidoglycan degradation products. If so, the coregulation of the *ykfABCD* and *dpp* operons would be reasonable, since the *dpp* operon encodes a D-aminopeptidase and a dipeptide uptake system (9, 44).

On the basis of homology searches, the *yufN*, *yufO*, *yufP*, and *yufQ* genes appear to encode, respectively, the lipid-linked substrate binding protein, the ATP-binding component, and the permease proteins of an ABC transporter; the *yurPONML* cluster is likely to be involved in sugar transport and metabolism, and the YhdG protein is similar to amino acid transporters.

Since CodY appears to be responsible for the inhibition of sporulation that occurs when nutrients are in excess (57), we anticipated that one or more key sporulation genes would be revealed as CodY targets. In fact, at least three participants in regulation of early sporulation gene expression through the Spo0A phosphorelay (6, 26) can be found among the CodY targets. The *kinB* gene encodes a membrane-associated histidine kinase that can serve as the first enzyme of the Spo0A phosphorelay (69). This gene was enriched in the ChIP-to-chip analysis and was overexpressed in a *codY* mutant in both minimal medium containing 16 amino acids and DS medium. The *kinB* gene is unlikely to be the only sporulation-related CodY target, however, because *kinB* is not by itself essential for sporulation (69).

The *rapA-phrA* and *rapE-phrE* operons also proved to be

likely CodY targets. The genes of these operons encode Rap phosphatases for Spo0F~P, an intermediate component of the phosphorelay, and inhibitors of the phosphatases (28, 30, 53). These proteins appear to determine the time at which enough Spo0A~P accumulates to cause cells to choose the sporulation pathway (48, 53). PhrA is essential for sporulation (53), indicating that its derepresed expression in a *codY* mutant might be sufficient to unleash sporulation under conditions of nutrient excess.

The *spo0A* region was enriched in the ChIP-to-chip analysis, but its transcription was barely detectable and was not derepressed in a *codY* mutant under the conditions tested. The *spo0A* gene has two promoters, however. A low-level vegetative promoter provides a basal level of *spo0A* mRNA during growth (10). A second, more active promoter is induced when cells enter stationary phase, and transcription from this promoter is essential for sporulation (10, 64). Under the growth conditions we have used, transcription from the vegetative promoter would have predominated. The sporulation promoter of *spo0A* not only requires sigma-H for its activity but is also repressed by Soj, SinR, and ScoC (7). Thus, the lack of any detectable change in *spo0A* expression in a *codY* mutant is not surprising. Preliminary in vitro experiments indicate that the sporulation promoter region of *spo0A* does indeed include a binding site for CodY (K. Tachikawa, M. Ratnayake-Lecamwasam, and A. L. Sonenshein, unpublished). Therefore, *spo0A* may be the critical gene whose repression by CodY ties initiation of sporulation to nutrient depletion.

The CodY regulon partially overlaps with the RelA and ScoC regulons. The *dpp* operon is induced by activation of the stringent response (57), presumably because the activity of RelA (stringency factor) leads to a drop in the GTP pool (27). A survey of global transcription after exposure to norvaline, an inhibitor of isoleucyl- and leucyl-tRNA synthetases, showed RelA-dependent induction of the *ilvB* operon, *appD*, *ureA*, *gabP*, *rapA*, *spo0A*, *yurP*, and *yxbC* (14), all of which are CodY targets (Tables 1 and 2). Other stringency-induced genes may not be targets of CodY.

ScoC (also known as Hpr) negatively regulates extracellular enzyme production and sporulation and positively regulates other genes (32). Caldwell et al. (8) noted some overlap among genes that are regulated by ScoC and CodY. The *hutP*, *comK*, *rapA*, and *hag* genes, the *bkd* cluster, and the *ureABC* operon are all underexpressed in a *scoC* mutant but overexpressed in a *codY* mutant (reference 8 and Tables 1 and 2). On the other hand, the *glnQ* gene is overexpressed in both *scoC* and *codY* mutants. While not all of these genes may be direct targets of either regulatory protein, there are probably cases where the two proteins bind simultaneously to the same regulatory region, either in cooperation or in competition.

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