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Dissecting Complex Metabolic Integration Provides Direct Genetic Evidence for CodY Activation by Guanine Nucleotides[▽]

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The global regulator CodY controls the expression of dozens of metabolic genes and genes mediating adaptation to nutrient availability in many low-G+C Gram-positive bacteria. Branched-chain amino acids L-isoleucine, L-leucine, and L-valine (ILV) activate CodY both *in vivo* and *in vitro*, and genes that direct their synthesis (*ilv*, *ybgE*, and *ywaA*) are highly repressed by CodY, creating a potential negative feedback loop. The nucleoside triphosphate GTP also activates CodY *in vitro*, but the evidence for activation by GTP *in vivo* is limited and indirect. We constructed a *Bacillus subtilis* strain (*ybgE bcd ywaA*) that is unable to convert branched-chain α-keto acids to ILV or to use ILV as a precursor for branched-chain fatty acid synthesis. Unexpectedly, the strain was not viable on rich medium. Supplementing rich medium with short, branched-chain fatty acids or derepressing expression of genes for *de novo* ILV synthesis bypassed the original lethality, restoring growth and showing that the lack of viability was due to insufficient intracellular production of the precursors of branched-chain fatty acids. Spontaneous extragenic suppressor mutants that arose in the triple mutant population proved to have additional mutations in *guaA* or *guaB* or *codY*. Expression of ILV biosynthetic genes in *codY* mutants was increased. The *gua* mutations caused guanine/guanosine auxotrophy and led to partial derepression of direct CodY-repressed targets, including ILV biosynthetic genes, under conditions similar to those that caused the original lethality. We conclude that a guanine derivative, most likely GTP, controls CodY activity *in vivo*.

Microbes respond to fluctuations in multiple environmental conditions, such as temperature and nutrient availability, to realize their full metabolic and physiological potential. Global regulatory proteins provide an effective link between environmental cues and adaptive genetic programs by sensing small molecules and altering the expression of critical genes. The result can have profound physiologic consequences for the cell and, in microbes that cause disease, can indicate transit to and from the host.

The global transcriptional regulatory protein CodY controls the expression of dozens of metabolic genes. Many targets of CodY encode products that allow bacterial cells to adapt to changes in the nutrient availability in each of several low-G+C Gram-positive bacterial genera, including *Bacillus*, *Listeria*, *Staphylococcus*, *Clostridium*, *Lactococcus*, and *Streptococcus* (8, 15, 16, 23, 25, 26, 29, 36, 45, 57). A number of these bacteria are of major industrial importance, and others are recalcitrant human pathogens that use CodY to coordinate the expression of important virulence genes with the exhaustion of nutrients. Therefore, detailed knowledge of the mechanism of action of CodY will allow us to determine how multiple gene expression programs are connected through metabolism.

The CodY proteins of *Bacillus subtilis*, *Staphylococcus aureus*, *Clostridium difficile*, and *Listeria monocytogenes* are activated for DNA binding *in vitro* by the presence of GTP and the branched-chain amino acids (BCAAs; L-isoleucine, L-leucine, and L-valine [ILV]) (Fig. 1A) (8, 17, 36, 49). *Lactococcus lactis* and *Streptococcus pneumoniae* CodY proteins appear to be activated only by BCAAs (25, 43). Analysis of the crystal structure of CodY shows that the C-terminal portion of the protein contains a winged helix-turn-helix (wHTH) domain needed for binding to DNA (33), whereas the N-terminal region of the protein contains a GAF domain that binds the BCAA class of effector molecules. Changes in the rate of ILV synthesis modulate CodY activity *in vivo* (12), and binding of ILV *in vitro* causes a conformational change that is thought to potentiate the DNA-binding activity of CodY (32). Additional increases in CodY activity *in vivo* are achieved in the presence of other exogenously added amino acids (3, 6, 12).

GTP also binds to CodY *in vitro* and enhances binding of CodY to DNA targets (24, 46). The effects of ILV and GTP are synergistic. That is, the affinity of CodY for a given target is higher when exposed to both ILV and GTP than it is in the presence of either effector alone (24, 49). The evidence that GTP is a physiologically meaningful effector of CodY in living cells is indirect and is based on a reduction in CodY-dependent repression in conjunction with conversion of GTP to guanosine tetra/pentaphosphate [(p)ppGpp] during the stringent response (51) or as a result of inhibition of GMP synthetase by the nucleoside analog decoyinine (8, 27, 28, 34, 40, 46, 54). Decoyinine and the accumulation of (p)ppGpp may have additional, incompletely defined effects on cellular physiology.

The common purine biosynthetic pathway generates IMP, a branch point that provides the skeleton for both adenine and guanine nucleotides (Fig. 1B). IMP is converted to xanthine 5′-monophosphate (XMP) by IMP dehydrogenase (encoded by *guaB*). This first committed step in guanine nucleotide synthesis is inhibited by (p)ppGpp (42). XMP is aminated to GMP by the action of GMP synthetase, the product of *guaA* (34).

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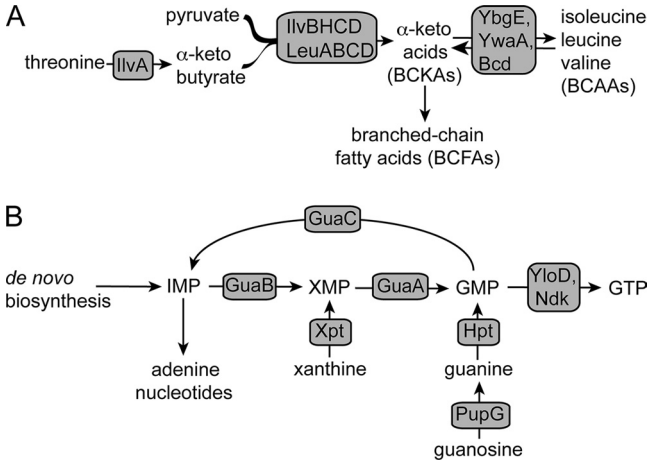

FIG. 1. Pathways for BCAA, BCFA, and guanine nucleotide synthesis in *B. subtilis.* (A) Pyruvate and threonine serve as sources of carbon skeletons for branched-chain fatty acids and branched-chain amino acids. IlvA, threonine deaminase; IlvBH, acetolactate synthase; IlvC, ketol-acid reductoisomerase; IlvD, dihydroxy acid dehydratase; LeuA, 2-isopropylmalate synthase; LeuB, 3-isopropylmalate dehydrogenase; LeuCD, isopropylmalate isomerase. YbgE and YwaA are BCAA aminotransferases, and Bcd is leucine dehydrogenase. (B) IMP is the branch point for adenine and guanine nucleotide synthesis. IMP is converted to xanthine 5′-monophosphate (XMP) by GuaB (IMP dehydrogenase), followed by conversion to GMP by GuaA (GMP synthetase). The putative guanylate kinase YloD and nucleoside diphosphokinase Ndk act sequentially to synthesize GTP. GuaC, GMP reductase; Hpt, hypoxanthine-guanine phosphoribosyltransferase; PupG, guanosine/deoxyguanosine-inosine/deoxyinosine phosphorylase; Xpt, xanthine phosphoribosyltransferase.

The synthesis of GTP from GMP proceeds in two steps by the actions of guanylate kinase (presumably encoded by *yloD*) and nucleoside diphosphokinase (encoded by *ndk*) (52).

De novo synthesis of the BCAAs requires enzymes encoded by *ilvA*, *ilvD*, and the *ilv-leu* operon (here referred to as the *ilvB* operon), leading to the synthesis of the branched-chain α-keto acids (BCKAs; 3-methyl-2-oxopentanoate, 2-oxoisovalerate, and 4-methyl-2-oxopentanoate) (Fig. 1A) (19). The BCKAs are then transaminated to their corresponding amino acids by the combined effects of YbgE and YwaA (Fig. 1A) (10, 53).

The α-keto acids are also the precursors for synthesis of branched-chain fatty acids (BCFAs) (Fig. 1A). BCFAs serve as the principal fatty acids in *B. subtilis* membranes under standard growth conditions and also tailor membrane integrity by altering ratios of BCFAs in response to thermal perturbation, a multifaceted process known as homeoviscous adaptation (1, 14, 38, 44, 50). To synthesize BCFAs, BCKAs are converted first to their acyl-coenzyme A (acyl-CoA) derivatives and then to their carboxylic acid derivatives by functions encoded by the *bkd* operon (13, 20). BCAAs can also be precursors of BCFAs. Leucine dehydrogenase (encoded by *bcd*, a gene located at the *bkd* locus and whose expression is triggered by isoleucine and valine [13]) converts BCAAs to BCKAs, which can then be used as substrates for BCFA synthesis (Fig. 1A) (13, 20).

While studying the transaminases that interconvert BCKAs and BCAAs, we discovered, quite surprisingly, that knocking out the aminotransferase genes *ybgE* and *ywaA* and the leucine dehydrogenase gene *bcd* resulted in mutant cells (*ybgE bcd ywaA*) that could not grow on rich medium containing a relatively high concentration of ILV and other amino acids. In this report, we show that the growth defect is due to insufficient BCFA synthesis and that additional mutations in the genes coding for guanine nucleotide synthetic enzymes or CodY suppress the growth defect.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains constructed during this study are derivatives of *Bacillus subtilis* strain SMY and are listed in Table 1. Lennox (L) medium (31) lacking glucose or titration with NaOH was used as rich medium to routinely cultivate nonsynthetic lethal *B. subtilis* strains, except for transformation crosses plated onto rich medium, where DS medium (21) was used. Transformation crosses for strains not viable on rich medium were plated on TSS medium (2) supplemented with 0.02% (wt/vol) $MgSO_4$ and 0.04 mg ml^{-1} each of $FeCl_3$-$6H_2O$ and trisodium citrate. Glucose (28 mM) and NH_4Cl (37.5 mM) were used as the sole sources of carbon and nitrogen, respectively. When necessary, media were solidified with agar at 15% (wt/vol) for DS medium and at 17% (wt/vol) for TSS medium. To maintain antibiotic selection, media were supplemented with antibiotics at the following concentrations: neomycin (Nm), 2.5 μg ml^{-1}; spectinomycin (Spc), 50 μg ml^{-1}; and erythromycin/lincomycin (Em), 1 μg ml^{-1}/12.5 μg ml^{-1}. Growth behavior analyses of guanine/guanosine and amino acid auxotrophs were performed in TSS medium with the indicated supplements in microtiter plate format. Briefly, single colonies were used to inoculate culture tubes containing 2 ml of minimal glucose-ammonium medium supplemented with 1.5 mM ILV and, if necessary, 200 μM guanosine. During exponential growth, cells were collected by centrifugation, washed, and resuspended in medium lacking ILV and guanosine to an optical density at 600 nm (OD_{600}) of 1.0. Each well of a Cellstar 96-well plate (Greiner Bio-One) was filled with 190 μl of fresh medium and 10 μl of inoculum at an OD_{600} of 1.0, giving an initial OD of 0.05. Experiments were performed in a computer-controlled Synergy HT plate reader (Bio-Tek) set to 37°C. Kinetic parameters included vigorous shaking (intensity of 3), and the optical density was read every 15 min, with shaking between readings. Growth was monitored as an increase in absorbance at 600 nm.

For shake flask experiments, precultures were inoculated with the strain of interest in DS medium supplemented with 0.1 mM short, branched-chain fatty acids (sBCFAs; isovaleric, isobutyric, and 2-methylbutyric acids) and, when indicated, 1 mM guanosine. Precultures were incubated overnight at 37°C and were subsequently diluted into 25 ml of fresh medium in 125-ml Erlenmeyer flasks to give an OD_{600} of 0.05. Experimental cultures were incubated at 37°C with vigorous agitation (setting 7; ~280 rpm) in a G76 Gyrotory shaking water bath (New Brunswick Scientific). Growth was monitored using an Ultrospec II UV-visible spectrophotometer (LKB Biochrom). At the end of each experiment, cells were plated on DS medium with and without sBCFAs to verify phenotypes and to ensure that no additional extragenic suppressor mutants were present in the cultures.

Petri plate images were acquired at a 1,200-dpi resolution using a computer-controlled Perfection 1200 flatbed scanner (Epson) and processed using Image Capture (version 6.0.1; Apple) and Photoshop (version CS2; Adobe) software.

DNA manipulations and transformations. All molecular biology techniques, including *E. coli* transformations, were performed as described by Sambrook and Russell (48). Preparation of *B. subtilis* chromosomal DNA and *B. subtilis* transformations with plasmid or chromosomal DNA were performed as previously described (7). Primers were obtained from Integrated DNA Technologies, and all cloned fragments and mutant alleles were verified by sequencing by the Tufts University Nucleic Acids and Protein Core Facility.

Plasmid constructions. (i) pSRB19. The region encompassing positions −601 to +6 relative to the putative *ybgE* translational start was amplified from *B. subtilis* SMY chromosomal DNA using primers oSRB28 (5′-ATG AAG AAG TGG TAC CAT AT-3′ and oSRB29 (5′-ATT CAA TCT AAT CAG CAC ACC TTT CAC ATA ATT G-3′). Underlined bases denote a KpnI site engineered into the primer. In addition, the region encompassing the *ybgE* stop codon and 550 bp of downstream sequence was amplified from the SMY chromosome using primers oSRB30 (5′-**GTG TGC TGA TTA GAT TGA ATT** GAA AAT CGA AAA AGA ACC TG-3′) and oSRB31 (5′-GAT CCT GTC CGC GGC TAA TT-3′). Underlined bases denote a SacII site engineered into the primer, and boldfaced bases denote a region of complementarity for overlapping PCR (58). The upstream and downstream flank-containing fragments were purified to remove primers, mixed in equal proportions, and used as a template in a third PCR with primers oSRB28 and oSRB31 to create an ~1.1-kb overlap PCR product con-

TABLE 1. Strains used during this study

Strain	Genotype	Reference or source
B. subtilis strains		
168	*trpC2*	
Derivatives of 168		
1A619	*trpC2 liv-1*::Tn*917* (*erm*)	*Bacillus* Genetic Stock Center
MGNA-A917	*trpC2* pMUTIN4 (*yebA′ lacI lacZ erm*)	National BioResource Project
MGNA-B889	*trpC2* pMUTIN4 (*yaaH′ lacI lacZ erm*)	National BioResource Project
PS258	*trpC2 codY*::*erm*	P. Serror
SMY	Prototroph, 168 lineage	P. Schaeffer
Derivatives of SMY		
BB2833	*lacA*::*tet amyE*::*spc codY*(*R61A*)	5
BB2834	*lacA*::*tet amyE*::*spc codY*(*R61K*)	5
BB2836	*lacA*::*tet amyE*::*spc codY*(*F71A*)	5
BB2837	*lacA*::*tet amyE*::*spc codY*(*F71R*)	5
SRB4	SMY	P. Schaeffer
SRB10	*codY*::(*erm*::*spc*)	12
SRB44	Δ*ybgE1* Ω pSRB19 (Δ*ybgE1 neo*)	SRB4 × DNA pSRB19
SRB50	Δ*guaA4*::*erm*	SRB4 × DNA pSRB23
SRB58	Δ*ybgE1*	Second crossover recombinant of SRB44
SRB74	Δ*guaA4*::*erm codY*::(*erm*::*spc*)	SRB10 × DNA SRB50
SRB94	*ilvBp4*	12
SRB102	*liv-1*::Tn*917* (*erm*)	SMY × DNA 1A619
SRB109	Δ*flgB2*::*erm*	SRB4 × DNA pSRB30
SRB118	Δ*bcd1*	SRB4 × DNA pSRB32
SRB142	Δ*ywaA2*::*spc*	12
SRB146	*ybgE1* Δ*ywaA2*::*spc*	SRB58 × DNA SRB142
SRB150	*ilvDp*ΔCBS Ω pSRB36 (*ilvDp*ΔCBS *neo*)	12
SRB155	Δ*bcd1* Ω pSRB19 (Δ*ybgE1 neo*)	SRB118 × DNA pSRB19
SRB159	Δ*bcd1* Δ*ybgE1*	Second crossover recombinant of SRB155
SRB163	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc*	SRB159 × DNA SRB142
SRB166	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-1*	Spontaneous suppressor of SRB163
SRB167	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-2*	Spontaneous suppressor of SRB163
SRB179	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-2* Ω pMUTIN4 (*yaaH′ lacI lacZ erm*)	SRB167 × DNA MBNA-B889
SRB189	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-3*	Spontaneous suppressor of SRB163
SRB190	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-4*	Spontaneous suppressor of SRB163
SRB191	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-5*	Spontaneous suppressor of SRB163
SRB192	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-6*	Spontaneous suppressor of SRB163
SRB253	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc codY*::*erm*	SRB163 × DNA PS258
SRB263	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-7*	Spontaneous suppressor of SRB163
SRB264	Δ*bcd1* Δ*ybgE1 liv-1*::Tn*917* (*erm*)	SRB159 × DNA 1A619
SRB265	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-7* Ω pMUTIN4 (*yebA′ lacI lacZ erm*)	SRB263 × DNA MGNA-A917
SRB268	Δ*flgB2*::*erm codY*::(*spc*::*erm*)	SRB109 × DNA SRB10
SRB277	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-1* Δ*flgB2*::*erm*	SRB166 × DNA pSRB30
SRB278	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-3* Δ*flgB2*::*erm*	SRB189 × DNA pSRB30
SRB279	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-4* Δ*flgB2*::*erm*	SRB190 × DNA pSRB30
SRB288	Δ*bcd1* Δ*ybgE1 ilvDp*ΔCBS Ω pSRB36 (*ilvDp*ΔCBS *neo*)	SRB159 × SRB150
SRB294	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-1* Δ*flgB2*::*erm*	SRB163 × DNA SRB277
SRB295	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-3* Δ*flgB2*::*erm*	SRB163 × DNA SRB278
SRB296	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-4* Δ*flgB2*::*erm*	SRB163 × DNA SRB279
SRB298	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc* Ω pMUTIN4 (*yaaH′ lacI lacZ erm*)	SRB163 × DNA SRB179
SRB299	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-2* Ω pMUTIN4 (*yaaH′ lacI lacZ erm*)	SRB163 × DNA SRB179
SRB300	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc* Ω pMUTIN4 (*yebA′ lacI lacZ erm*)	SRB163 × DNA SRB265
SRB301	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-7* Ω pMUTIN4 (*yebA′ lacI lacZ erm*)	SRB163 × DNA SRB265
SRB305	Δ*bcd1* Δ*ybgE1 ilvDp*ΔCBS	Second crossover recombinant of SRB288
SRB308	Δ*bcd1* Δ*ybgE1 ilvDp*ΔCBS *liv-1*::Tn*917* (*erm*)	SRB305 × DNA 1A619
SRB311	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc* Δ*flgB2*::*erm*	SRB163 × DNA SRB109
SRB312	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-6* Δ*flgB2*::*erm*	SRB192 × DNA pSRB30
SRB313	Δ*bcd1* Δ*ybgE1 ilvBp4*	SRB264 × DNA SRB94
SRB314	Δ*bcd1* Δ*ybgE1 ilvBp4 ilvDp*ΔCBS	SRB308 × DNA SRB94
SRB315	Δ*bcd1* Δ*ybgE1 ilvBp4* Δ*ywaA2*::*spc*	SRB313 × DNA SRB163
SRB316	Δ*bcd1* Δ*ybgE1 ilvBp4 ilvDp*ΔCBS Δ*ywaA2*::*spc*	SRB314 × DNA SRB163
SRB319	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc sld-6* Δ*flgB2*::*erm*	SRB163 × DNA SRB312
SRB351	*amyE*::*spc codY*(*R61A*) Δ*flgB2*::*erm*	BB2833 × DNA pSRB30
SRB352	*amyE*::*spc codY*(*R61K*) Δ*flgB2*::*erm*	BB2834 × DNA pSRB30
SRB353	*amyE*::*spc codY*(*F71A*) Δ*flgB2*::*erm*	BB2836 × DNA pSRB30
SRB355	*amyE*::*spc codY*(*F71R*) Δ*flgB2*::*erm*	BB2837 × DNA pSRB30
SRB356	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc codY*(*R61A*) Δ*flgB2*::*erm*	SRB163 × DNA SRB351
SRB357	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc codY*(*R61K*) Δ*flgB2*::*erm*	SRB163 × DNA SRB352
SRB358	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc codY*(*F71A*) Δ*flgB2*::*erm*	SRB163 × DNA SRB353
SRB359	Δ*bcd1* Δ*ybgE1* Δ*ywaA2*::*spc codY*(*F71R*) Δ*flgB2*::*erm*	SRB163 × DNA SRB355
E. coli strains		
DH5α	*supE44* Δ*lac U169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	48
JM107	*supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q}ZΔM15)	48
One Shot Top10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen

TABLE 2. Primers used for real-time quantitative RT-PCR

Primer	Sequence	Target	Reference[a]
oSRB83	5′-ATT TAC GAT AAG CTA TAC AAT TCA-3′	*ilvB*	12
oSRB94	5′-AAT CAA TCA TGG CAT CAG CAA-3′	*ilvB*	12
oSRB116	5′-AAT GCA GGA AGA TTA AAC AGG GTA-3′	*bcaP*	
oSRB117	5′-TGG GAT TTA ATT TTA GAA TAC ATG CT-3′	*bcaP*	
oSRB170	5′-TCT TGA AGC TCG TTT TCG TTG A-3′	*ilvD*	12
oSRB171	5′-TCG CAA TGG GGC ATA TCG GTA T-3′	*ilvD*	12
oSRB194	5′-CCG TTC ATG GTC TTT TGG TG-3′	*rpoC*	
oSRB195	5′-TTT AGC CCG TGT TAC TTC GAC-3′	*rpoC*	

[a] Unless specified, primers were designed during this investigation.

taining the allele Δ*ybgE1*. The fragment was cut with KpnI and SacII, gel extracted, and ligated to the same sites of pBB544 (4). This plasmid of ~5.4 kb was named pSRB19.

(ii) pSRB23. The region encompassing positions −501 to +3 relative to the *guaA* translational start was amplified from the strain SMY chromosome using primers oSRB15 (5′-AGT CCT CCT GGT ACC AGC TGT ATT-3′) and oSRB16 (5′-ATA TAT ATA TAT GTC GAC CAT GGT TGT CAC CTA ATC TCC T-3′). Underlined bases denote KpnI and SalI sites engineered into the primers, respectively. In addition, the region encompassing the *guaA* stop codon and 476 bp of downstream flanking sequence was amplified from the strain SMY chromosome using primers oSRB17 (5′-ATA TAT ATA TAT GGA TCC TAA GAA TCA ATT AAT GGA AAC CA-3′) and oSRB18 (5′-ACA AAA TAT ATG CCG CGG ATA AAA AAG TCG TTA AT-3′). The underlined bases denote BamHI and SacII sites engineered into the primers, respectively. Each 0.5-kb fragment was cloned in pCR2.1 (Invitrogen), according to the manufacturer's instructions. Fragments were then cloned sequentially in pBB544 to yield a Δ*guaA* allele. This plasmid was cut (within Δ*guaA*) with BamHI and SalI, to which a 1.45-kb BamHI/SalI fragment of pJPM14 (J.P. Mueller and A. L. Sonenshein, unpublished data) containing the *erm* cassette conferring Em^r was ligated, generating allele Δ*guaA4*::*erm*. This plasmid of ~6.6 kb was named pSRB23.

(iii) pSRB28. Regions upstream and downstream of the *B. subtilis flgB* coding sequence were amplified from the strain SMY chromosome using primers oSRB108 (5′-AAT GAA AGG TAC CTA TAT CA-3′) and oSRB109 (5′-TTT TTT GTC GAC TTA CCT CCA TTT-3′) and primers oSRB110 (5′-**TGG AGG TAA GTC GAC AAA AAA** TTT TTA GGA TCC TGA TGA CAG CTT TTC ATA-3′) and oSRB111 (5′-TGA CAT TCA CCG CGG ATG ATC TAG T-3′). Underlined bases denote the KpnI, SalI, BamHI, and SacII sites engineered into the primers, respectively, and boldface bases denote a region of complementarity for overlapping PCR. The upstream and downstream fragments (458-bp and 432-bp, respectively) were purified using the Qiagen PCR cleanup kit, mixed 1:1 (vol/vol), diluted appropriately, and used in an overlapping PCR using primers oSRB108 and oSRB111. The resulting ~900-bp fragment containing the Δ*flgB1* allele was digested with KpnI and SacII and ligated to the same sites of pBB544. The resulting plasmid of ~5.2 kb was named pSRB28.

(iv) pSRB30. Plasmid pSRB28 was cut with BamHI and SalI, to which a 1.45-kb BamHI/SalI fragment of pJPM14 containing the *erm* cassette conferring Em^r was ligated, generating allele Δ*flgB2*::*erm*. This ~6.6-kb plasmid was named pSRB30.

(v) pSRB32. Regions upstream and downstream of the *bcd* coding sequence were amplified from strain SMY DNA using primers oSRB121 (5′-TCG TCA ATG AAT TCT GCT CCT T-3′) and oSRB122 (5′-CAT GTT CGT TCC TCC TAA TGT GTG TA-3′) as well as oSRB123 (5′-**ACA TTA GGA GGA ACG AAC ATG** TAA GAA ATT GAT CTG GAG GTT-3′) and oSRB124 (5′-ATA TAC GAA GGG ATC CTC AGG CCG TCA-3′). Underlined bases denote the EcoRI and BamHI sites engineered into the primers, respectively. Boldfaced bases denote a region of complementarity for overlapping PCR. The 503-bp and 437-bp fragments were purified, mixed 1:1 (vol/vol), and diluted appropriately for use in an overlapping PCR using outer primers oSRB121 and oSRB124. The resultant 940-bp fragment carrying allele Δ*bcd1* was cut with EcoRI and BamHI, gel extracted, and ligated to the same sites of pBB544, generating pSRB32 (~5.2 kb).

***B. subtilis* strain constructions.** *B. subtilis* Δ*guaA*, Δ*flgB*, Δ*ybgE*, and Δ*bcd* strains were constructed by introducing *E. coli* strain JM107-propagated (*recA*$^+$), plasmid-borne mutant alleles into strain SMY by transformation. For marked *guaA* and *flgB* alleles, we selected for double-crossover recombinants using resistance conferred by *erm* (Em^r) and then scored for loss of the plasmid-encoded *neo* gene. We generated markerless, in-frame deletions of *ybgE* and *bcd* as previously described (12). Briefly, we selected for the inheritance of the plasmid as single-crossover recombinants (Nm^r). Recombinants were PCR screened for merodiploids with two copies of the mutant allele (due to homogenotization). Strains were then serially passaged in rich medium lacking neomycin and plated on L medium. Colonies were replica printed on DS medium with and without Nm and screened for loss of Nm^r to obtain clones that had undergone a second homologous recombination event. To construct strain SRB316 (triple mutant strain derivative with *ilvBp4* and *ilvDp*ΔCBS alleles), we began with strain SRB159 (*ybgE bcd*), transformed it to Nm^r using chromosomal DNA from SRB150 (*ilvDp*ΔCBS Ω pSRB36 [*ilvDp*ΔCBS *neo*]), and followed the method described above for generating markerless mutations. We then moved allele *liv-1*::Tn*917* (*erm*) into this strain by transformation using chromosomal DNA from strain 1A619 as the donor. The resulting strain was an ILV auxotroph and Em^r. Using chromosomal DNA from strain SRB94 as the donor (*ilvBp4*; linked to *liv-1*), we transformed the auxotroph to ILV$^+$ by selecting for prototrophy on TSS plates and scored for Em^s. We then moved Δ*ywaA2*::*spc* into this recipient strain by selecting Spc^r on TSS-plus-ILV plates with Spc. We constructed strain SRB315 (*ybgE bcd ywaA ilvBp4*) similarly.

***codY* allele sequencing.** The *codY* coding sequences were amplified using primers oKK30 (5′-TGT CGA AGA AAA GCT CGG-3′) and oKK31 (5′-CAT AGA AAG ACT TTC AAC-3′). PCR products (~1 kb) were purified and sequenced as described above.

RNA sampling, preparation, and cDNA synthesis. Samples were collected and processed as previously described (12), carefully ensuring that shake flask cultures were in steady state. Cells were collected after at least three generations and within approximately one generation of one another. RNA was prepared as described previously (12), except that we used the High Pure RNA isolation kit (Roche Applied Science). RNaseOUT recombinant RNase inhibitor (200 units; Invitrogen) was added after purification to prevent RNA degradation. RNA preparations were treated with the Turbo DNA-*free* DNase treatment and removal kit (Ambion) to reduce contaminating DNA to insignificant levels. Treated RNAs were assessed for quality and integrity using agarose gel electrophoresis and ethidium bromide staining. cDNAs were prepared from each 250-ng sample of total RNA using SuperScript II reverse transcriptase (Invitrogen) with random primers (Promega) per the manufacturer's instructions.

Real-time qPCR. We performed quantitative PCR (qPCR) using the LightCycler 480 system and associated SYBR green I chemistry (Roche Applied Science) to analyze transcript abundance from prepared cDNA samples. Each 25-μl reaction mixture contained either 600 nM *ilvB*-, *ilvD*-, or *bcaP*-specific primers or 300 nM *rpoC*-specific primers (Table 2). Thermal cycling proceeded as per the LightCycler 480 SYBR green I template protocol, except that we used annealing temperatures of 50°C and 55°C as the minimum temperatures for melting curve analysis. Standard curves were generated for each target using gel-purified, PCR-amplified fragments. Serial dilutions spanning at least 6 orders of magnitude were analyzed, allowing for absolute quantification of 125 to 1.25×10^7 copies of the *bcaP* transcript, 273 to 2.73×10^7 copies of the *ilvB* transcript, 146 to 1.46×10^7 copies of the *ilvD* transcript, and 203 to 2.03×10^8 copies of the *rpoC* transcript. Standard curves as well as standard PCR controls (including no-template and no-reverse transcriptase reactions) were run on each plate along with test reactions. All qPCRs proceeded with at least 85% efficiency, and initial transcript concentrations were calculated using the second-derivative maximum analysis algorithm. Points lay within polynomial (nonlinear) regression. Single amplification products were verified by melting curve analysis (melting temperature (T_m)-calling algorithm). Data are presented as copies of target transcript per copy of *rpoC* transcript multiplied by 100. The *rpoC* transcript, which codes for the β′ subunit of the DNA-dependent RNA polymerase, was

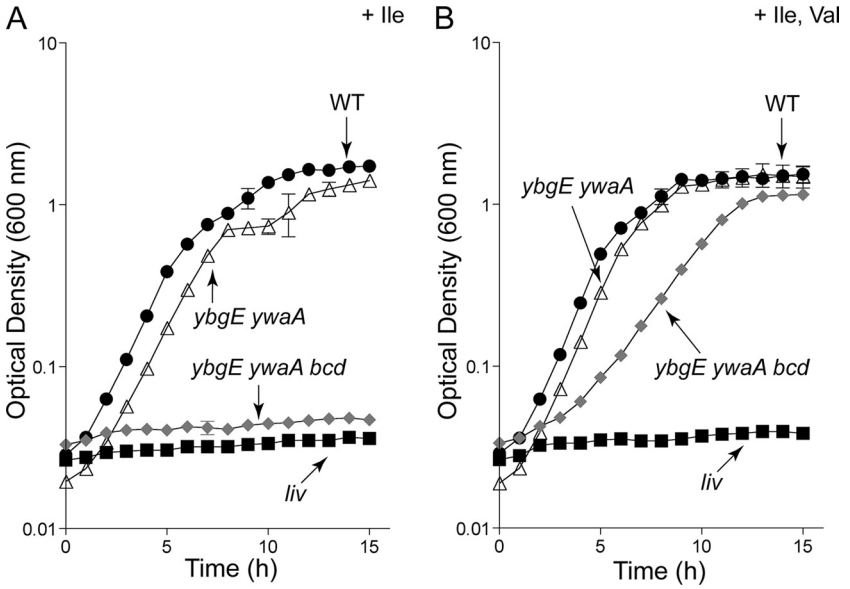

FIG. 2. Growth behavior of *B. subtilis* strains lacking BCAA aminotransferases. Growth behavior was performed in microtiter plate format as described in Materials and Methods. Although readings were taken every 15 min, only data points corresponding to hourly readings are displayed to enhance clarity. Error bars denote standard errors of the mean optical density (OD_{600}) at each time point for at least two technical replicates. When error bars are not visible, they are smaller than the edge of the symbol. Curves are representative of at least two independent experiments. Wild type (WT; SRB4), black circles; *liv*, transposon insertion mutant carried by the *ilvB* operon (SRB102), black squares; *ybgE ywaA* strain (SRB146), open triangles; *ybgE ywaA bcd* strain (SRB163), gray diamonds. (A) Growth in minimal glucose-ammonium medium supplemented with 1.5 mM isoleucine (Ile). (B) Growth in minimal glucose-ammonium medium supplemented with 1.5 mM (each) isoleucine (Ile) and valine (Val).

used for normalization because its abundance was not expected to change significantly under the conditions tested.

Statistical analyses. We used unpaired, two-tailed Student's *t* tests with 95% confidence intervals to test for statistical significance. The comparisons were made using Prism version 4.0a software (GraphPad). Outliers were detected using Dixon's Q-test and associated critical Q values (18, 47).

RESULTS

The *ybgE bcd ywaA* strain is not viable on rich medium. As part of a project seeking to control intracellular ILV pools, we sought *B. subtilis* strains defective in BCAA biosynthesis. Since *ybgE* and *ywaA* had previously been identified as redundant genes coding for BCAA aminotransferases (10, 53), we constructed a *ybgE ywaA* double mutant strain. Unexpectedly, the *ybgE ywaA* strain was not fully auxotrophic for the BCAAs. The double mutant strain required isoleucine, but not valine or leucine, for growth in a minimal glucose-ammonium medium. The growth rate of the double mutant strain was as high as the growth rate of the wild-type (WT) strain in isoleucine-containing medium (Fig. 2A and B, compare the wild type and *ybgE ywaA*). In contrast, we saw no growth of a strain that harbors a Tn*917* transposon insertion in the *ilvB* operon unless we added all three BCAAs to the medium (data not shown). We hypothesized that leucine dehydrogenase, the product of the *bcd* gene and an enzyme known to be involved in synthesis of BCFAs from BCAAs (13), might contribute to the final, transamination step in valine and leucine biosynthesis.

We attempted to construct the *ybgE ywaA bcd* triple mutant strain but, to our surprise, in multiple trials, we failed to obtain transformants when plating on rich (DS) medium. We did, however, obtain transformants on minimal glucose-ammonium-Spc medium supplemented with ILV to fulfill the expected nutritional requirement for the triple mutant strain. This strain was still not a total BCAA auxotroph and required both isoleucine and valine (but not leucine) for growth in a minimal glucose-ammonium medium. The growth rate for the triple mutant strain in the absence of leucine was lower, however, compared to that of the wild-type or *ybgE ywaA* strain (Fig. 2A and B, compare the wild type or *ybgE ywaA* and *ybgE ywaA bcd*), indicating that inactivation of *bcd* reduces but does not abolish the synthesis of leucine.

We speculated that the repeated failure of our selection on rich medium revealed a synthetic lethal genetic combination. In fact, upon prolonged incubation, two types of colonies arose on DS medium—large, rough colonies typical of *B. subtilis* and small, smooth colonies. These apparent suppressor mutants were purified on DS medium and maintained their characteristic appearance. Each mutant was identified with the allele designation *sld* (*s*uppressor of *l*ethality on *D*S medium) (Fig. 3).

Large-colony suppressor mutations map to *codY*. The involvement of the mutated genes in BCAA biosynthesis and the amino acid-responsive phenotype of the triple mutant strain raised the possibility that the activity of the pleiotropic global transcriptional regulator CodY might be involved in the mutant phenotype. We therefore tested the hypothesis that the suppressor mutant strains harbored lesions in the *codY* gene by introducing chromosomal DNA carrying the closely linked Δ*flgB2*::*erm* allele by transformation (as shown below, the mutation in *flgB*, which encodes a component of the flagellar hook-basal body complex, homologous to the *Salmonella enterica* protein [60], did not affect CodY-dependent regulation). In fact, most Em^r derivatives (selected on minimal glucose-

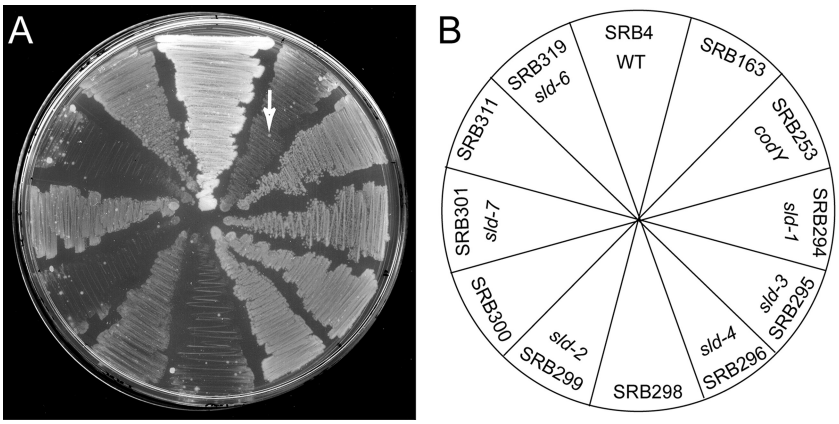

FIG. 3. Growth behavior of *B. subtilis* strains on DS medium. (A) Suppression of the lethality of strain SRB163 on DS medium by mutations in *codY* (*sld-1*, *sld-3*, *sld-4*, and *sld-6*), *guaA* (*sld-7*), and *guaB* (*sld-2*). SRB4 (wild type) serves as the positive control, and SRB253 is the CodY-deficient triple mutant strain. Strains SRB298, -300, and -311 are each isogenic control strains harboring markers linked to *sld*. Strains were streaked and incubated at 37°C. The arrow indicates an exemplary extragenic suppressor mutant. (B) Plate schematic depicting the arrangement of strains on DS medium.

ammonium ILV medium) of four independent large-colony suppressor mutants (*sld-1*, *sld-3*, *sld-4*, and *sld-6*) lost the ability to form colonies when streaked on DS medium, indicating that the lesions necessary for suppression are tightly linked to *flgB*. Plasmid pSRB30 (which carries the Δ*flgB2*::*erm* allele but not the *codY* gene) was introduced by transformation into the *sld* strains SRB166, -189, -190, and -192 to place the Em^r marker near the *sld* alleles without affecting *codY*. Chromosomal DNA from the resulting strains was used as the donor in transformation crosses, using the *ybgE bcd ywaA* strain as the recipient. In a separate experiment, we knocked out *codY* in the triple mutant strain. These reconstructed strains and the triple mutant strain with an authentic null allele of *codY* (strain SRB253) grew on DS medium, whereas a control strain harboring the *ybgE*, *ywaA*, and *bcd* mutations, but not the *sld* mutation, did not (Fig. 3), suggesting that *sld* alleles were necessary and sufficient for suppressing lethality. We amplified the *codY* gene from the chromosome of the *sld-1*, *sld-3*, *sld-4*, and *sld-6* strains and identified point mutations in the coding sequence for CodY, creating the variants CodY(L126P), CodY(A227V), CodY(E101K), and CodY(S12P), respectively.

***sld-2* and *sld-7* alleles encode defective enzymes for purine synthesis.** Two small-colony variant suppressor mutants isolated on DS medium failed to grow on minimal glucose-ammonium-ILV medium. We inferred that the allele(s) conferring the ability to grow on rich medium created a secondary auxotrophy. Knowing that guanine auxotrophs are among a limited number of auxotrophs that form small colonies on DS medium, we tested whether the GMP precursor xanthine or guanosine would stimulate growth of our mutants on minimal medium. On minimal glucose-ammonium-ILV plates, the strain carrying the *sld-2* allele grew when the medium was supplemented with xanthine or guanosine. The strain carrying the *sld-7* allele grew only when the medium was supplemented with guanosine. These results suggested that the lesions were in *guaB* and *guaA* for *sld-2* and *sld-7*, respectively (Fig. 1B). Using available strains in the *B. subtilis* mutant collection (National BioResource Project [NIG, Japan]), we obtained markers linked to *guaB* (Ω pMUTIN4 [*yaaH′ lacI lacZ erm*]) or *guaA* (Ω pMUTIN4 [*yebA′ lacI lacZ erm*]) and used those linked mutations to transform the original triple mutant strain (*ybgE bcd ywaA* strain) to Em^r. The *sld* mutations were separable from but linked to the *erm* markers, resulting in isogenic pairs of strains. We analyzed the growth behavior of these strains. Derivatives of the triple mutant strain lacking *sld* alleles grew in the absence of added purine, with a doubling time of 57.0 min, similar to that of the wild-type parent strain (Fig. 4A, compare the WT with the *ybgE bcd ywaA yaaH* strain and the WT with the *ybgE bcd ywaA yebA* strain in TSS-plus-ILV medium). In contrast, addition of xanthine or guanosine stimulated growth of the isogenic *sld-2* strain, but only guanosine permitted growth of the isogenic *sld-7* strain. An authentic *guaA* null allele (strain SRB50, Δ*guaA4*::*erm*) yielded similar results (Fig. 4A to C). All strains derived from SRB163 failed to grow when ILV was omitted from the medium (data not shown). The growth phenotypes of the *sld-2* and *sld-7* strains as well as their genetic linkages suggest that they have mutations in *guaB* and *guaA*, respectively. Indeed, sequencing revealed single point mutations coding for the variant proteins GuaB(T310K) and GuaA(Q325K).

Derepressing *ilv* synthesis or supplying short, branched-chain fatty acids bypasses the conditions of the original lethality. We reasoned that under the original conditions that caused lethality, CodY was either activating a gene that prevents growth on rich medium or repressing the expression of a gene that is needed for growth. Furthermore, since DS medium contains ILV, the inability of the triple mutant strain to form colonies on DS medium was not caused by ILV auxotrophy *per se*. Rather, since the biosynthesis of BCFAs is essential for growth in *B. subtilis* (59) and the cells could not efficiently convert exogenously supplied ILV to their α-keto acid precursors due to lack of YbgE, Bcd, and YwaA, we hypothesized that cells were dependent on *de novo* BCFA precursor synthesis by the Ilv enzymes. We suspected that insufficient enzymes were being made to support the demand, due to CodY-dependent repression of the *ilvB* operon and the *ilvD* and *ilvA* genes in the presence of excess amino acids (35, 49, 56). To see if relieving CodY-mediated repression of the *ilvB* operon or the

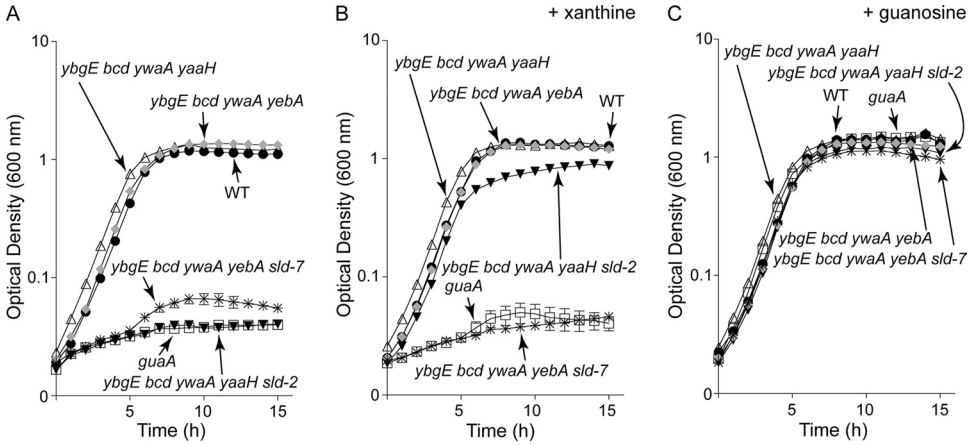

FIG. 4. *sld-2* and *sld-7* alleles encode defective Gua enzymes. Growth behavior was performed in microtiter plate format as described in Materials and Methods. Although readings were taken every 15 min, only data points corresponding to hourly readings are displayed to enhance clarity. Error bars denote standard errors of the mean optical density at each time point for at least two technical replicates. When error bars are not visible, they are smaller than the edge of the symbol. Curves are representative of at least two independent experiments. Wild type (WT; SRB4), black circles; *guaA* (SRB50), open squares; *ybgE bcd ywaA yaaH* strain (SRB298), open triangles; *ybgE bcd ywaA yaaH sld-2* strain (SRB299), black inverted triangles; *ybgE bcd ywaA yebA* strain (SRB300), gray diamonds; *ybgE bcd ywaA yebA sld-7* strain (SRB301), asterisks. (A) Growth in minimal glucose-ammonium-ILV medium. (B) Growth in minimal glucose-ammonium-ILV medium with 200 μM xanthine. (C) Growth in minimal glucose-ammonium-ILV medium with 250 μM guanosine.

ilvD gene would overcome lethality, we combined *ilvBp4* (an allele containing two point mutations in the major CodY binding site [CBS] in the *ilvB* promoter region [12]) or *ilvBp4* and *ilvDp*ΔCBS (a deletion of the CodY binding site in the *ilvD* promoter region [12]) alleles (12) with the *ybgE*, *bcd*, and *ywaA* alleles and assessed the ability of the resulting strains to grow on rich medium. While *ilvBp4* alone did not suppress the lethality of the triple mutant strain, introducing both the *ilvBp4* and *ilvDp*ΔCBS alleles (each of which remove CodY-dependent regulation) rescued the strain on DS medium (Fig. 5A and B, compare *ybgE bcd ywaA ilvBp4* and *ybgE bcd ywaA ilvBp4 ilvDp*ΔCBS strains). The wild-type and the triple mutant strain were included as positive and negative controls for growth, respectively. Moreover, in contrast to the wild-type strain, the triple mutant strain failed to grow on DS medium unless we included in the medium 0.1 mM (each) short, branched-chain fatty acids (sBCFAs; isovaleric, isobutyric, and 2-methyl butyric) (Fig. 6A and B). Interestingly, we observed that the triple mutant strain appeared to be cross-fed by a diffusible factor secreted by growing strains on DS medium (Fig. 3, 5, and 6). This factor is likely to be a precursor of the branched-chain fatty acids or the fatty acids themselves.

Reducing CodY activity bypasses lethality by derepressing the BCAA biosynthetic pathway. To quantify the effects of *sld* mutations in *codY* on the BCAA biosynthesis pathway, we performed quantitative, real-time reverse transcription-PCR (RT-PCR) to measure the abundance of *ilvB* and *ilvD* transcripts in the triple mutant and suppressor strains. The *bcaP* gene (formerly *yhdG* [5]), another target of CodY, was also included. The *bcaP*, *ilvB*, and *ilvD* promoters are directly repressed by CodY (5, 39, 49). We cultivated each of the reconstructed strains containing the *codY sld* alleles in DS medium supplemented with 0.1 mM sBCFAs to bypass lethality. In this medium, the wild-type strain grew with a doubling time of 36.8

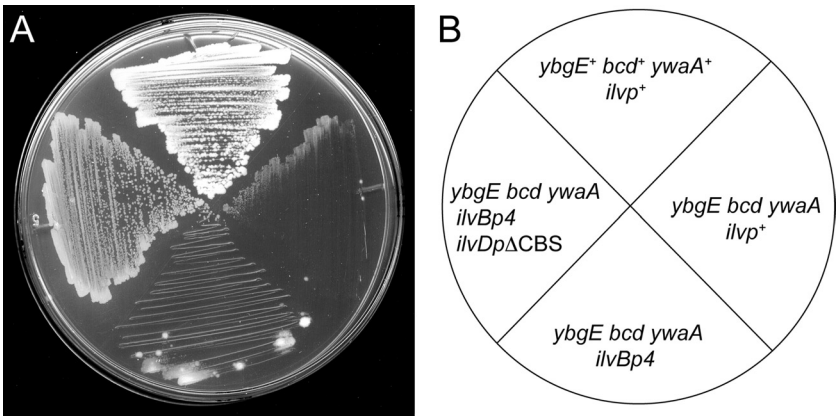

FIG. 5. Derepression of the ILV pathway restores growth of strain SRB163 on DS medium. Strain SRB4 (wild type), *ybgE*$^+$ *bcd*$^+$ *ywaA*$^+$ *ilvp*$^+$; SRB163, *ybgE bcd ywaA ilvp*$^+$; SRB315, *ybgE bcd ywaA ilvp4*; SRB316, *ybgE bcd ywaA ilvp4 ilvDp*ΔCBS. (A) Strains were streaked to DS medium and incubated overnight at 37°C. (B) Plate schematic depicting the orientation of strains on DS medium.

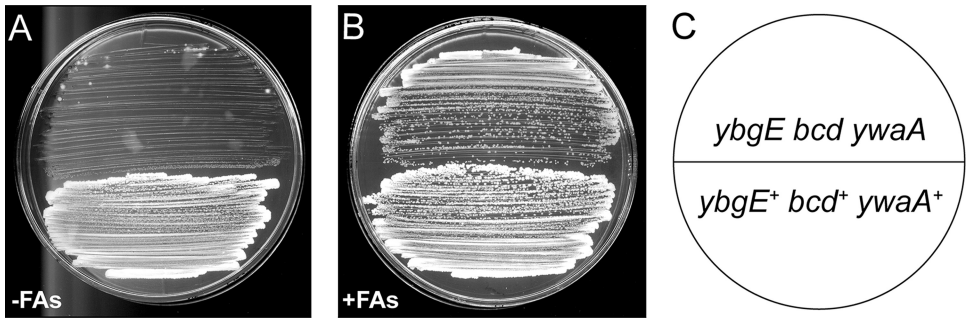

FIG. 6. Supplying short, branched-chain fatty acids bypasses the original lethality of strain SRB163 on DS medium. Strains were streaked to DS media and incubated at 37°C. SRB4 (wild type), *ybgE*$^+$ *bcd*$^+$ *ywaA*$^+$; SRB163, *ybgE bcd ywaA*. (A) DS medium lacking branched-chain fatty acids (−FAs). (B) DS medium supplemented with 0.1 mM isovaleric, isobutyric, and 2-methylbutyric sBCFAs (+FAs). (C) Schematic depicting strain orientation on media.

min, and we observed relatively low transcript abundance for *bcaP*, *ilvB*, and *ilvD* (0.96, 0.59, and 0.53 copies of transcript relative to the *rpoC* transcript, respectively) (Table 3). Transcript abundances in the triple mutant strain (and in the same strain with *flgB* knocked out) were essentially equivalent, though growth was slower. We observed 53-, 45-, and 7-fold derepression of *bcaP*, *ilvB*, and *ilvD*, respectively, when the *codY* gene was knocked out (Table 3, compare the WT and *codY*). Similarly, when we knocked out *codY* in the triple mutant strain, we observed a 44- to 53-fold increase in the *bcaP* transcript, a 19- to 30-fold increase in the *ilvB* transcript, and an approximately 5- to 6-fold increase in the *ilvD* transcript (Table 3, compare *ybgE bcd ywaA* or *ybgE bcd ywaA flgB* and *ybgE bcd ywaA codY*).

Strains harboring *sld* alleles encoding CodY(L126P) and CodY(A227V) exhibited derepression essentially equivalent to that exhibited by the *codY* null mutant strains. However, strains producing CodY(E101K) and CodY(S12P) were derepressed only about 42- and 29-fold for *bcaP*, respectively (Table 3, compare *ybgE bcd ywaA flgB* and *ybgE bcd ywaA flgB sld-4* or *ybgE bcd ywaA flgB sld-6* and compare *ybgE bcd ywaA flgB sld-4* or *ybgE bcd ywaA flgB sld-6* and *ybgE bcd ywaA codY*).

TABLE 3. Transcript analysis of CodY-regulated targets by real-time quantitative RT-PCR

Genotype	Medium[a]	CodY protein	GuaA/GuaB protein	Doubling time (min)	No. of copies of transcript[b]		
					bcaP	*ilvB*	*ilvD*
WT	DSM	WT	WT/WT	36.8 ± 1.8	0.96 ± 0.23	0.59 ± 0.08	0.53 ± 0.09
codY	DSM	—[d]	WT/WT	41.9 ± 1.8	51.29 ± 1.86	26.71 ± 1.08	3.58 ± 0.01
guaA	DSM	WT	—/WT	54.2 ± 3.3	1.23 ± 0.34	4.16 ± 0.10	0.70 ± 0.07
guaA codY	DSM	—[d]	—/WT	74.2 ± 0.3	90.88 ± 12.74	77.86 ± 5.53	4.36 ± 1.16
ybgE bcd ywaA	DSM	WT	WT/WT	48.7 ± 4.7	1.41 ± 0.13	1.81 ± 0.68	0.95 ± 0.26
ybgE bcd ywaA ilvBp4	DSM	WT	WT/WT	40.3 ± 2.4	0.73 ± 0.05	8.51 ± 1.28	0.47 ± 0.05
*ybgE bcd ywaA ilvBp4 ilvDp*ΔCBS	DSM	WT	WT/WT	43.0 ± 3.2	0.34 ± 0.07	5.74 ± 0.29	2.80 ± 0.04
ybgE bcd ywaA flgB	DSM	WT	WT/WT	45.8 ± 1.2	1.16 ± 0.16	1.11 ± 0.23	0.70 ± 0.12
ybgE bcd ywaA flgB sld-1	DSM	L126P	WT/WT	41.0 ± 1.0	64.15 ± 4.24	37.23 ± 4.10	4.72 ± 0.68
ybgE bcd ywaA flgB sld-3	DSM	A227V	WT/WT	40.8 ± 1.2	71.90 ± 12.94	42.87 ± 4.76	6.14 ± 1.11
ybgE bcd ywaA flgB sld-4	DSM	E101K	WT/WT	40.0 ± 0.6	48.35 ± 1.42	24.59 ± 2.82	3.95 ± 0.52
ybgE bcd ywaA flgB sld-6	DSM	S12P	WT/WT	36.5 ± 0.2	33.63 ± 1.75	18.62 ± 2.44	4.00 ± 0.74
ybgE bcd ywaA flgB codY	DSM	F71A	WT/WT	46.1 ± 3.4	7.77 ± 1.61	3.16 ± 0.06	1.96 ± 0.28
ybgE bcd ywaA flgB codY	DSM	R61K	WT/WT	49.6 ± 6.2	17.17 ± 0.32	7.20 ± 0.05	2.88 ± 0.10
ybgE bcd ywaA codY	DSM	—	WT/WT	43.8 ± 3.3	61.79 ± 3.78	33.82 ± 4.00	4.52 ± 0.64
ybgE bcd ywaA yaaH	DSM	WT	WT/WT	45.6 ± 3.6	0.77 ± 0.10	0.87 ± 0.11	0.54 ± 0.14
ybgE bcd ywaA yaaH sld-2	DSM	WT	WT/GuaB(T310K)	71.5 ± 8.0	3.71 ± 0.68	15.22 ± 1.57	1.65 ± 0.18
ybgE bcd ywaA yebA	DSM	WT	WT/WT	47.7 ± 2.3	0.65 ± 0.05	0.77 ± 0.05	0.44 ± 0.05
ybgE bcd ywaA yebA sld-7	DSM	WT	GuaA(Q325K)/WT	57.4 ± 5.8[c]	0.81 ± 0.19[c]	4.02 ± 1.03[c]	0.52 ± 0.08[c]
ybgE bcd ywaA yaaH	DSM+Guo	WT	WT/WT	48.8 ± 0.4	0.67 ± 0.19	0.92 ± 0.20	0.56 ± 0.12
ybgE bcd ywaA yaaH sld-2	DSM+Guo	WT	WT/GuaB(T310K)	48.8 ± 2.8	0.42 ± 0.10	0.36 ± 0.10	0.24 ± 0.08
ybgE bcd ywaA yebA	DSM+Guo	WT	WT/WT	48.2 ± 1.7	0.56 ± 0.14	0.61 ± 0.01	0.34 ± 0.04
ybgE bcd ywaA yebA sld-7	DSM+Guo	WT	GuaA(Q325K)/WT	47.5 ± 0.4	0.62 ± 0.16	0.68 ± 0.06	0.35 ± 0.01

[a] In all cases, the growth medium DS was supplemented with 0.1 mM (each) short, branched-chain fatty acids (isovaleric, isobutyric, and 2-methylbutyric). In some cases, guanosine (Guo) was added to a final concentration of 1 mM.

[b] Data are the means ± standard errors of the means from at least two independent biological replicates. Target transcript abundances are relative to the *rpoC* transcript abundance.

[c] Data are from four independent biological replicates. A fifth biological replicate was deemed an outlier by Dixon's Q-test (18, 47) and was discarded. If all five biological replicates had been included, the doubling time would have been 57.6 ± 4.5 min, and transcript levels would have been 2.03 ± 1.22, 12.04 ± 8.06, and 1.64 ± 1.12 copies for *bcaP*, *ilvB*, and *ilvD*, respectively.

[d] —, no Cody protein in null mutant.

A Student's *t* test suggested that these transcript abundances are significantly different from those of fully derepressed *bcaP* ($P < 0.05$). For the less highly regulated targets *ilvB* and *ilvD*, the level of derepression caused by these *codY* alleles was indistinguishable from full derepression (Table 3, compare *ybgE bcd ywaA flgB sld-4* or *ybgE bcd ywaA flgB sld-6* with *ybgE bcd ywaA codY*). To confirm that full derepression was not necessary for growth on DS medium lacking sBCFAs, we tested the ability of two bona fide partial activity variants of CodY to suppress lethality on DS medium (5). Variant protein CodY(F71A) retained sufficient activity to keep the *ilv* promoters repressed and did not suppress lethality on DS medium. Analysis of transcript abundances showed 7-, 3-, and 3-fold derepression for *bcaP*, *ilvB*, and *ilvD*, respectively (Table 3, compare *ybgE bcd ywaA flgB* and *ybgE bcd ywaA flgB codY* [F71A]). In contrast, the strain producing CodY(R61K) showed 15-, 6-, and 4-fold derepression of *bcaP*, *ilvB*, and *ilvD*. The corresponding reduction in CodY activity was apparently sufficient to promote enough sBCFA synthesis to make cells producing this variant viable on DS medium. For both variants, derepression of *bcaP* and *ilvB* was apparently significantly different from that observed in cells lacking CodY. *ilvD* derepression was statistically indistinguishable from that observed in *codY* null mutant strains (Table 3).

Elimination of endogenous guanine nucleotide synthesis results in partial CodY activity in DS medium. We also assayed *bcaP*, *ilvB*, and *ilvD* transcript abundance in strains carrying lesions in *guaB* and *guaA* (*sld-2* and *sld-7*, respectively) during growth in DS medium. DS medium was the sole source of guanine nucleotides for these guanine/guanosine auxotrophs, and differences in doubling times between *gua* and *gua*$^+$ isogenic strains suggest that guanine nucleotides may be limiting (Table 3, compare doubling times of DSM and DSM plus Guo). Therefore, we hypothesized that lowering the pools of guanine nucleotides in these strains reduces CodY activity. Isogenic *sld*$^+$ derivatives had transcript abundances similar to the wild-type and triple mutant strains (Table 3). We observed approximately a 5-fold increase in the *bcaP* transcript, an 18-fold increase in the *ilvB* transcript, and a 3-fold increase in the *ilvD* transcript in the strain producing the variant GuaB(T310K) (Table 3, compare *ybgE bcd ywaA yaaH* and *ybgE bcd ywaA yaaH sld-2*). These transcript abundances suggest that CodY still retains some activity compared to abundances measured in the absence of CodY (a Student's *t* test yields a *P* value of <0.05). Surprisingly, we saw no effect on the *bcaP* or *ilvD* transcript in the strain producing the GuaA(Q325K) protein (Table 3, compare *ybgE bcd ywaA yebA* and *ybgE bcd ywaA yebA sld-7*) ($P < 0.05$) or in a *guaA* null mutant (Table 3, compare the WT and *guaA*). In fact, only a ~5- to 7-fold increase in the *ilvB* transcript was observed and was sufficient to suppress lethality. While the triple mutant strain requires sBCFAs for growth in rich medium, *guaA* and *guaB* derivatives lacking functional IMP dehydrogenase and GMP synthetase do not. In accord with our hypothesis that CodY activity is lower because the pool of guanine nucleotides is reduced, supplementing the medium with 1 mM guanosine restored full CodY-dependent regulation and lethality (sBCFA requirement) on DS medium (Table 3 and data not shown).

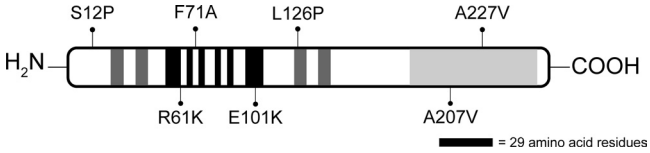

FIG. 7. Single-amino acid substitutions reduce CodY activity. Variant residues are mapped onto a schematic representation of the CodY polypeptide. The regions involved in binding ILV (black), in dimerization (dark gray), and in binding DNA (winged helix-turn-helix [wHTH]; light gray) are depicted as rectangles. The representation is approximate.

DISCUSSION

The global transcriptional regulator CodY is activated as a DNA-binding protein *in vitro* by ILV and GTP. The structure of CodY in complex with the amino acids revealed a conformational change induced by binding of the amino acids that presumably enhances CodY activity (32). In contrast, no crystal structure of CodY in complex with GTP has been obtained to date. Similarly, changes in the rate of ILV synthesis *in vivo* alter CodY activity (12), but proof that the GTP pool is an intracellular signal for CodY has been difficult to obtain. New evidence for the intracellular role of guanine nucleotides in activating CodY, as described here, came unexpectedly from analysis of the growth defect caused by inactivation of three enzymes critical for interconverting BCAAs and BCKAs. Since the only nucleotides that activate CodY *in vitro* are GTP and dGTP (24), it is very likely that one of these molecules is the true intracellular activator of CodY. Since the pool of dGTP is too low to be effective (11), GTP remains the prime candidate.

The *ybgE bcd ywaA* strain depends on *de novo* BCFA synthesis for viability. Branched-chain fatty acids are derived from branched-chain α-keto acids produced *de novo* or by degradation of ILV (13). The fact that elimination of three enzymes that interconvert ILV and their α-keto acids led to a growth defect in rich medium suggested that either branched-chain fatty acids or another metabolite synthesized from α-keto acids (i.e., coenzyme A or pantothenate) was limiting for growth. Consistent with this hypothesis, derepression of the biosynthetic pathway for BCKAs (Fig. 5) or supplying short, branched-chain fatty acids (Fig. 6) restored growth of the triple mutant strain, revealing both the identity of the missing factor(s) (BCFAs or BCKAs; they likely cross-feed the triple mutant strain [Fig. 3]) and the relevant target of CodY-dependent regulation (the ILV biosynthetic pathway).

***codY* and *gua* suppressor mutations are loss-of-function mutations that decrease CodY activity.** Relatively large pseudorevertant colonies harbored mutations that mapped to the *codY* gene, creating variant proteins lacking full regulatory activity. The mutations fell within or near known functional domains of CodY (Fig. 7). Interestingly, one allele (*sld-8*) obtained but not analyzed in detail during this investigation encodes variant protein CodY(A207V), which was independently isolated 6 years ago (29). An *ilvB-lacZ* transcriptional fusion was 44-fold derepressed in a strain producing CodY(A207V) (29). An additional allele, *sld-11*, is a 93-bp deletion near the 5′ end of the *codY* gene, resulting in a frameshift and production of a truncated protein containing only 22 N-terminal amino acids (data not shown).

The variants CodY(L126P) and CodY(A227V) appear

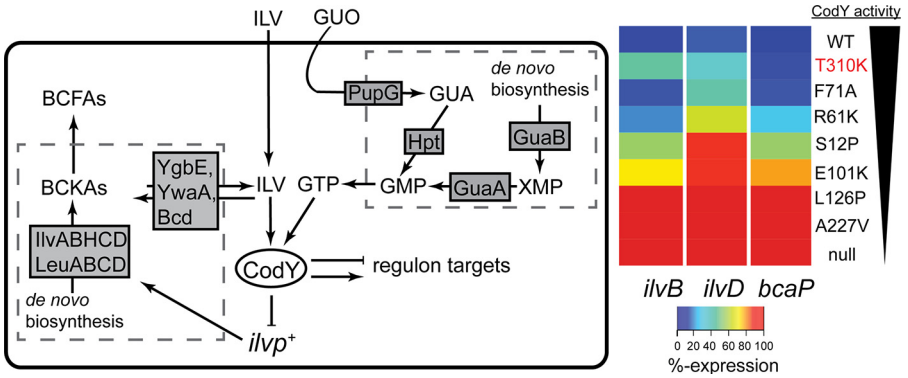

FIG. 8. CodY activity is intimately connected to intracellular BCAA and GTP pools to control major metabolic pathways. (Left) *De novo* precursors provide sources of carbon skeletons for synthesis of ILV and GTP by the Ilv and Gua enzymes. The aminotransferases YbgE and YwaA along with leucine dehydrogenase (Bcd) interconvert ILV and their α-keto acid precursors, which serve as building blocks for branched-chain fatty acids (BCFAs). CodY activity (defined as the ratio of active CodY molecules to total CodY molecules) is modulated by internal pools of ILV and a guanine nucleotide (presumably GTP). CodY-repressed targets (e.g., $ilvBp^+$, $ilvDp^+$, and $bcaPp^+$) are differentially regulated depending on the extent of CodY activity and the strength of CodY binding site. (Right) Heat map depicting transcriptional output for each promoter over the full dynamic range for *sld* alleles, some of which appear to partially deactivate CodY. Minimum expression is governed by maximally activated wild-type CodY (DS medium, purple), and maximum expression occurs in CodY-deficient cells (DS medium, red; 100%). Values are represented as a percentage of the total possible expression in DS medium. CodY proteins are indicated to the right of the heat map, and the GuaB(T310K) variant is indicated by red text. The black wedge depicts the apparent trend in CodY activity. GUA, guanine; GUO, guanosine; Hpt, hypoxanthine-guanine phosphoribosyltransferase; PupG, guanosine/deoxyguanosine-inosine/deoxyinosine phosphorylase.

to lack any significant regulatory activity, but the variants CodY(S12P) and CodY(E101K) appear to retain partial regulatory activity (Table 3). This partial activity of CodY(S12P) and CodY(E101K) is visible only when *in vivo* CodY activity is maximized (i.e., in cells grown in amino acid-containing medium).

Substitutions in GMP synthetase and IMP dehydrogenase [GuaA(Q325K) and GuaB(T310K) variants, respectively] are in conserved regions predicted to be in or near the active sites of these enzymes. We have not biochemically characterized these proteins in any detail, but we presume them to have, at most, low residual activity based on the inability to support the growth of *B. subtilis* in a defined medium in the absence of added guanosine.

We were surprised to observe differences between the *guaA* and *guaB* alleles with respect to transcript abundances of CodY targets. For example, in the *guaB* mutant, we saw ~5-, ~18-, and ~3-fold increases in abundances of the *bcaP*, *ilvB*, and *ilvD* transcripts, respectively. These numbers represent ~6%, ~45%, and ~36% of the level seen in a *codY* null mutant (Table 3 and Fig. 8). In contrast, mutations in *guaA* caused an up to ~7-fold increase in *ilvB* transcript abundance but no significant change in expression of *bcaP* or *ilvD* (Table 3). The simplest explanation for relatively mild derepression in a *guaA* mutant is that reduced IMP dehydrogenase activity has a greater effect on the guanine nucleotide pool than does reduced GMP synthetase activity. Whereas the effect of a *guaB* mutation appears to be mediated through CodY, we conclude from comparing transcript abundances in the *codY* strain and the *codY guaA* strain that there is a positive, CodY-independent *guaA* effect on *ilvB* transcription due to guanine/guanosine limitation (Table 3) (54, 55).

The apparent lack of derepression of *ilvD* in the *guaA* mutant strains seems at odds with our finding that lethality of the triple mutant strain can be bypassed (though growth was apparently not fully restored) by inactivating the CodY binding sites in the *ilvB* and *ilvD* promoters but not by inactivation of the *ilvB* site alone (Fig. 5). Perhaps *ilvD* is slightly overexpressed in *guaA* cells at a level that cannot be detected by our qPCR assay. We know that at least 3-fold derepression is sufficient to suppress lethality (Table 3, compare *ybgE bcd ywaA* and *ybgE bcd ywaA ilvBp4 ilvDp*ΔCBS). Alternatively, in the absence of substantial CodY activity, IlvD enzyme activity may be stabilized by a factor whose gene is normally repressed by CodY. Interestingly, the *yfm* locus, which was shown to be a direct target of CodY in chromatin immunoprecipitation with microarray technology (ChIP-to-chip) experiments (39), encodes proteins that facilitate the uptake of iron and is a target of the Fur (ferric uptake regulator) global regulatory protein (41). IlvD is an iron-sulfur cluster-containing dehydratase whose activity may increase with increased iron uptake.

Distinct regulatory activities afforded by decreases in guanine nucleotides or CodY variants demonstrate hierarchical organization of selected CodY regulon members. Deducing the true *in vivo* effectors of a regulatory protein is one of the greatest challenges in cell physiology. We have presented genetic evidence that a guanine nucleotide controls CodY activity, consistent with previous results based on induction of stringency and treatment of cells with decoyinine (8, 9, 22, 26–28, 30, 37, 46, 54, 55). Figure 8 presents a summary of our results and their interpretation. When the triple mutant strain, which cannot derive BCFAs from ILV, is cultivated in DS medium, the concentration of ILV and other amino acids in the medium must be high enough to activate CodY-dependent repression of the *ilv* and *leu* genes, whose products synthesize the BCKAs that are the precursors of the BCFAs. As a result, the cells are unable to synthesize membrane fatty acids at a rate high enough to support growth. By reducing CodY activity either directly or indirectly (by altering the guanine nucleotide pool), suppressor strains bypass the original lethality by derepressing

the *ilv* promoters. The heat map shown in Fig. 8 reflects transcript abundances of the *ilvB*, *ilvD*, and *bcaP* promoters as a function of CodY activity and illustrates that these CodY targets are differentially sensitive to CodY activity. That is, the *bcaP* promoter requires substantial reduction in CodY activity to be highly expressed. In contrast, the *ilv* promoters—especially *ilvD*—are more sensitive to CodY activity and are maximally expressed with only modest reductions in CodY activity. This graded response to various effector levels is undoubtedly dependent on the various affinities of CodY for its many targets (5). We are currently determining the hierarchy of CodY-regulated gene expression at different levels of nutrient sufficiency. Since CodY controls expression of both metabolic genes and some of the most important virulence genes carried by pathogenic low-G+C Gram-positive bacteria, a comprehensive understanding of the interplay between metabolite pools and global gene expression will give an unprecedented view of how metabolic and virulence gene expression programs are interrelated and interdependent.

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