Supporting Information

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SI Materials and Methods

***Bacillus subtilis* Strain Construction.** Site-directed mutagenesis of *codY* was performed using overlap PCR (1), and fragments were cloned in pBB1579 (2) for allelic exchange. All plasmids were introduced into *B. subtilis* strain SMY by transformation after passaging through *Escherichia coli* strain JM107 (*recA*$^+$). The mutations *codY62* and *codY59* (CodY^{F71Y} and CodY^{R61H}) were generated by allelic exchange. Briefly, we selected for inheritance of the plasmid as a single-crossover recombinant [neomycin resistant; blue pigmentation due to BgaB-dependent cleavage of 5-bromo-4-chloro-3-indolyl β-D-galactoside (XG)] and screened by PCR for merodiploids having undergone homogenotization to carry two copies of the mutant allele. The merodiploid strain was then cultivated in Luria (L) broth lacking antibiotic and plated on L agar containing XG. White colonies were verified as neomycin-sensitive (due to loss of plasmid after undergoing a second homologous recombination event) and by sequencing using oligonucleotides oKK30 and oKK31 (3). All unmarked *codY* alleles were moved to other strains as was previously done by cotransformation with the linked Δ*flgB2::erm* marker (3). Sequencing services were provided by the Tufts University Nucleic Acids and Protein Core Facility.

Immunoblot Analysis. Immunoblotting was performed essentially as described (4). Briefly, strains were cultivated in a defined glucose-ammonium medium (TSS) supplemented with 16 amino acids. Twelve milliliters of culture was collected during steady-state exponential growth; cells were pelleted and stored at −80 °C until needed. Cells were resuspended in buffer A [50 mM Tris·Cl (pH 7.5), 5% (vol/vol) glycerol, and 1 mM PMSF] and broken by sonication under continuous cooling (5 °C) with a Branson 450 digital sonifier and jacketed high-intensity cup horn for 3 min using 60% amplitude with 10-s bursts, with 5-s pauses between bursts. Cell debris was removed by centrifugation at 18,800 × *g* for 5 min at 4 °C, and total protein concentration was determined using the Coomassie Protein Assay kit (Thermo Scientific) according to the manufacturer's instructions. Twelve micrograms of total protein from each sample was loaded onto 12% (wt/vol) polyacrylamide gels (nondenaturing or denaturing) and electrophoresed at 50 V for 30 min, followed by 150 V for 1.5 h. Proteins were transferred to nitrocellulose (Thermo Scientific) for 1 h at 100 V. Membranes were blocked overnight using 1% (wt/vol) nonfat dry milk. Membranes were incubated for 1 h with rabbit anti-CodY primary antibody (1:10,000 dilution), followed by donkey anti-rabbit IgG conjugated to HRP (1:10,000 dilution). CodY proteins were detected using the ECL Prime kit and the LAS4000 Mini Luminescent Image Analyzer (both from GE Healthcare Life Sciences) according to the manufacturer's recommendations.

RNA-Sequencing Library Construction. RNA was prepared as described (3, 5). Nucleic acid (8.6 μg) was treated with TURBO DNA-free DNase (Ambion). Treatment was considered successful if no *rrnA* PCR product was detected after 30 cycles of amplification using the Light Cycler 480 System (Roche Applied Science) and an RNA integrity number >8 was obtained, as assessed on a Bioanalyzer and RNA Pico chip (both from Agilent Technologies). rRNA was depleted from DNA-depleted RNA preparations using RiboZero (Gram-positive kit; Epicentre), and nucleic acid concentrations were determined in an HT Synergy III plate reader (BioTek) using RiboGreen dye for RNA (Invitrogen). mRNAs were fragmented using the NEBNext RNA Fragmentation Module (New England Biolabs). Depletion and fragmentation were assessed using an RNA Pico chip and the Bioanalyzer. Fragmented RNA was purified and concentrated using the RNA Clean & Concentrator-5 kit (Zymo Research Corporation) before first-strand cDNA synthesis using SuperScript III reverse transcriptase (Life Technologies, Inc.). We included actinomycin D (8 μg) in each reaction to prevent spurious second-strand synthesis. The first-strand cDNAs were purified using the RNA Clean & Concentrator-5 kit and subjected to second-strand cDNA synthesis with 13.3 units of DNA polymerase I and 3.3 units each of *E. coli* DNA ligase and RNase H using dUTP in place of dTTP in the reaction mixture. The ds cDNAs were blunted using the Quick Blunting Kit (New England Biolabs) and A-tailed using the Klenow fragment (3′-to-5′ exo minus), to which the universal adaptor Olj331/Olj543 (Table S2) was ligated in a 10:1 (adaptor/fragment) molar ratio. The libraries were purified and size-selected using AMPure XP SPRI beads (Agencourt) as directed, eluting in 1× low TE buffer [10 mM Tris·Cl (pH 8), 0.1 mM EDTA]. Quantifluor dsDNA dye (Promega) was used to quantify ds cDNA and DNA. The second strand was selectively degraded using 1 unit of USER enzyme (New England Biolabs) before library enrichment and barcoding using Phusion HiFi polymerase (New England Biolabs) and oligonucleotides containing unique 6-bp barcodes. Final library size distributions were analyzed using a DNA1000 chip on the Bioanalyzer or a fragment analyzer before pooling samples. Samples were loaded into single lanes of a HiSeq 2500 instrument (Illumina) in the Tufts University Genomics Core and sequenced in multiplex (single-end 50-bp reads) using v3 chemistry.

MS-Based Metabolomics. ***1. Sample preparation and liquid chromatography-MS.*** Sample preparation and liquid chromatography-TOF MS (LC-MS) analyses were performed using methods similar to those previously detailed by Weisenberg et al. (6). Single colonies of each strain streaked on L plates were picked into 5 mL of chemically defined TSS medium containing glucose, NH_4Cl, and a mixture of 16 amino acids, including isoleucine, leucine, and valine, and incubated overnight. Experimental cultures were created by diluting preadapted overnight cultures into 125-mL Delong flasks (Bellco Glass, Inc.) containing 25 mL of fresh medium to an OD_{600} of 0.05. We grew the cultures at 37 °C with vigorous aeration at ∼180 RPM in a G76 gyrotory shaking water bath (New Brunswick Scientific). At an OD_{600} approximating 1.0, ∼13 mL of this liquid culture was then rapidly filtered onto 2-μm nitrocellulose filters (Millipore) under vacuum and washed with 5 mL of PBS (pH 7.5) to remove excess media and noncellular material (7). Biological replicates of bacteria-laden filters were then metabolically quenched by immersion into acetonitrile/methanol/H_2O (40:40:20) supplemented with 0.1 M formic acid and precooled to −40 °C. Metabolites were extracted by mechanical lysis of the entire solution with 0.1-mm zirconia beads in a mini-Beadbeater-1 (BioSpec Products) for four cycles of 30 s each at 4,800 RPM with 2-min cooling intervals on dry ice. Lysates were clarified by centrifugation at 14,000 × *g* for 10 min at 4 °C and extracted into acetonitrile mixed 50:50 with 0.2% (vol/vol) formic acid. Intracellular metabolites were analyzed by LC-MS as previously described (8, 9), with the modification that 0.2% (vol/vol) formic acid was used in place of 0.2% (vol/vol) acetic acid for the mobile phase. Experiments were performed in triplicate to ensure reproducibility.

2. LC-MS data processing and analysis. Metabolites were searched for by chemical formula. Identities of specific metabolites were confirmed against pure chemical standards (where available) by molecular mass (mass tolerance <0.01 Da) and retention times. Where

chemical standards were not available, provisional identifications were made by matching against a database of accurate mass retention time pairs. Peak heights of all detected metabolites were imported into an Excel (Microsoft Corp.) data sheet and adjusted for bacterial biomass by residual protein content analysis, as previously described (10). Normalized levels for each metabolite across replicates from independent experiments were generated by dividing the adjusted abundance for each by the average adjusted abundance in the control group (Dataset S6). Heat maps were created using Excel after log(2) transformation of the average normalized abundance of each metabolite in each isolate (average of replicates across independent experiments).

3. Statistical analyses. a) Two-group comparisons. Biomass-adjusted, normalized ion counts for each metabolite in each experiment were plotted to determine normality of distribution, which was confirmed by the Shapiro–Wilk test for normality (11), and equality of variance was determined by a two-group variance comparison test. For the comparison of WT *B. subtilis* (SRB109) with the *codY* null mutant (SRB268), a Mann–Whitney *U* test was used to determine significant differences in intracellular abundance. Results of these analyses were then adjusted for multiple hypothesis testing by the Benjamini–Hochberg procedure (12).

b) Multigroup comparisons. Biomass-adjusted, normalized ion counts were analyzed across more than two groups by Cuzick's nonparametric test for trend (13) and adjusted for multiple testing by the Benjamini–Hochberg procedure (12). All statistical analyses were performed using Stata 10.1 (StataCorp). Unless otherwise cited, metabolites were ascribed to pathways by comparison against the Kyoto Encyclopedia of Genes and Genomes Database (14).

1. Vallejo AN, Pogulis RJ, Pease LR (2008) PCR Mutagenesis by Overlap Extension and Gene SOE. *CSH Protoc* 2008:pdb.prot4861, 10.1101/pdb.prot4861.
2. Belitsky BR, Sonenshein AL (2011) CodY-mediated regulation of guanosine uptake in *Bacillus subtilis*. *J Bacteriol* 193(22):6276–6287.
3. Brinsmade SR, Sonenshein AL (2011) Dissecting complex metabolic integration provides direct genetic evidence for CodY activation by guanine nucleotides. *J Bacteriol* 193(20):5637–5648.
4. Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Plainview, NY), 3rd Ed.
5. Brinsmade SR, Kleijn RJ, Sauer U, Sonenshein AL (2010) Regulation of CodY activity through modulation of intracellular branched-chain amino acid pools. *J Bacteriol* 192(24):6357–6368.
6. Weisenberg SA, Butterfield TR, Fischer SM, Rhee KY (2009) Suitability of silica hydride stationary phase, aqueous normal phase chromatography for untargeted metabolomic profiling of *Enterococcus faecium* and *Staphylococcus aureus*. *J Sep Sci* 32(13):2262–2265.
7. Brauer MJ, et al. (2006) Conservation of the metabolomic response to starvation across two divergent microbes. *Proc Natl Acad Sci USA* 103(51):19302–19307.
8. de Carvalho LP, et al. (2010) Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chem Biol* 17(10):1122–1131.
9. Pesek JJ, Matyska MT, Loo JA, Fischer SM, Sana TR (2009) Analysis of hydrophilic metabolites in physiological fluids by HPLC-MS using a silica hydride-based stationary phase. *J Sep Sci* 32(13):2200–2208.
10. Stich TM (1990) Determination of protein covalently bound to agarose supports using bicinchoninic acid. *Anal Biochem* 191(2):343–346.
11. Shapiro SS, Wilk MB (1965) An analysis of variance test for normality (complete samples). *Biometrika* 52(3-4):591–611.
12. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 57(1): 289–300.
13. Cuzick J (1985) A Wilcoxon-type test for trend. *Stat Med* 4(1):87–90.
14. Kotera M, Hirakawa M, Tokimatsu T, Goto S, Kanehisa M (2012) The KEGG databases and tools facilitating omics analysis: Latest developments involving human diseases and pharmaceuticals. *Methods Mol Biol* 802:19–39.

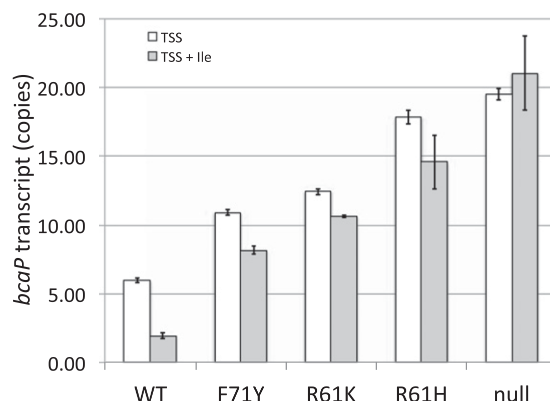

Fig. S1. CodY variants are defective in activation by L-isoleucine. The *bcaP* transcript abundance was assessed by real-time quantitative RT-PCR of cDNAs prepared from *B. subtilis* strains cultivated in TSS medium with and without isoleucine (Ile; gray and white bars, respectively). SRB109, WT; SRB268, *codY* null strain; SRB361, R61K CodY variant; SRB465, R61H CodY variant; SRB468, F71Y CodY variant.

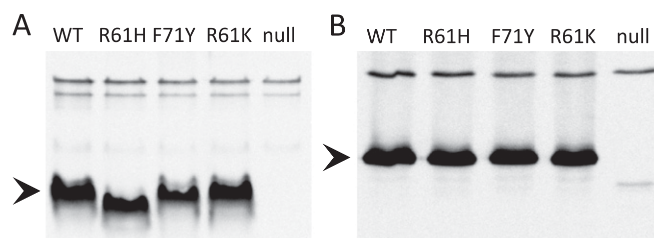

Fig. S2. CodY molecular weight is not significantly affected by amino acid substitution. *B. subtilis* strains SRB109 (WT), SRB268 (*codY* null), SRB361 (R61K), SRB465 (R61H), and SRB468 (F71Y) were cultivated in TSS + 16 amino acids. Soluble protein extracts were probed using antibody to CodY. Arrowheads indicate CodY protein. (*A*) PAGE analysis under nondenaturing conditions. (*B*) PAGE analysis under denaturing conditions.

Table S1. Strains and plasmids used during this study

Bacterial strains	Genotype	Source*
B. subtilis strains		
SMY	WT	P. Schaeffer
Derivatives of SMY		
SRB109	Δ*flgB2::erm*	(1)
SRB268	Δ*flgB2::erm codY::(erm::spc)*	(1)
SRB361	Δ*flgB2::erm codY* (CodY^{R61K})	(1)
SRB465	Δ*flgB2::erm codY59* (CodY^{R61H})	
SRB468	Δ*flgB2::erm codY62* (CodY^{F71Y})	
E. coli strains		
DH5α	*supE44* Δ*lac U169* (φ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(2)
JM107	*supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^{+}$ *lacI*^{q}*Z*ΔM15)	(2)
Plasmids		
pBB1579	*bgaB neo bla*	(3)
pSRB30	*neo bla* Δ*flgB2::erm*	(1)
pSRB59	*bgaB neo bla codY59* (CodY^{R61H})	
pSRB62	*bgaB neo bla codY62* (CodY^{F71Y})	

*Unless otherwise noted, strains and plasmids were designed and created during this study.

1. Brinsmade SR, Sonenshein AL (2011) Dissecting complex metabolic integration provides direct genetic evidence for CodY activation by guanine nucleotides. *J Bacteriol* 193(20):5637–5648.
2. Sambrook J, Russell DW (2001) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Plainview, NY), 3rd Ed.
3. Belitsky BR, Sonenshein AL (2011) CodY-mediated regulation of guanosine uptake in *Bacillus subtilis*. *J Bacteriol* 193(22):6276–6287.

Table S2. Oligonucleotides used during this study

Oligonucleotide	Used for	Sequence[†,‡]	Source
BC33	Library amplification and barcoding	5′-CAAGCAGAAGACGGCATACGAGAT**CGTGAT**GTGAC-TGGAGTTCAGACGTGTGCTCTTCCGATCT-3′	D. Lazinski
BC34	Library amplification and barcoding	5′-CAAGCAGAAGACGGCATACGAGAT**ACATCG**GTGA-CTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′	D. Lazinski
BC35	Library amplification and barcoding	5′-CAAGCAGAAGACGGCATACGAGAT**GCCTAA**GTGA-CTGGAGTTCAGACGTGTGCTCTTCCGATCT-3′	D. Lazinski
BC36	Library amplification and barcoding	5′-CAAGCAGAAGACGGCATACGAGAT**TGGTCA**GTGAC-TGGAGTTCAGACGTGTGCTCTTCCGATCT-3′	D. Lazinski
BC37	Library amplification and barcoding	5′-CAAGCAGAAGACGGCATACGAGAT**CACTGT**GTGAC-TGGAGTTCAGACGTGTGCTCTTCCGATCT-3′	D. Lazinski
BC42	Library amplification and barcoding	5′-CAAGCAGAAGACGGCATACGAGAT**AAGCTA**GTGAC-TGGAGTTCAGACGTGTGCTCTTCCGATCT-3′	D. Lazinski
oBB272	Overlap PCR	5′-GAGACGaaTTCATACAATACTTGAAC-3′	(1)
oBB278		5′-CAAGCATTTTTTTCATTTCATCATTTTCAATTTG-3′	(1)
oKK30	*codY* allele sequencing	5′-TGTCGAAGAAAAGCTCGG-3′	(2)
oKK31		5′-CATAGAAAGACTTTCACC-3′	(2)
Olj331	Adaptor ligation	5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T-3′	D. Lazinski
Olj139	Library amplification and barcoding	5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA-3′	D. Lazinski
Olj543	Adaptor ligation	5′-pGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGG-TCGCCGTATCATT	D. Lazinski
oSRB83	*ilvB* qPCR[§]	5′-ATTTACGATAAGCTATACAATTCA-3′	(3)
oSRB94		5′-ATTCAATCATGGCATCAGCAA-3′	(3)
oSRB116	*bcaP* qPCR	5′-AATGCAGGAAGATTAAACAGGGTA-3′	(2)
oSRB117		5′-TGGGATTTAATTTTAGAATACATGCT-3′	(2)
oSRB276	*sigA* qPCR	5′-CAGCTTGCCAAAGCCGAAGAAGAA-3′	
oSRB277		5′-TTTGCGATACTGACAACAAGCCGC-3′	
oSRB278	Mutagenesis *codY59* (R61H)	5′-TGAAAATGATCATATGAAAAAAATGCTTG-3′	
oSRB279		5′-CAAGCATTTTTTTCATATGATCATTTTCAATTTG-3′	
oSRB283	Mutagenesis *codY62* (F71Y)	5′-GAGGATCGTCAATATCCTGAAGAATATAC-3′	
oSRB289		5′-ATATTCTTCAGGATATTGACGATCC-3′	
oSRB333	*amhX* qPCR	5′-CTTTGTGCAGCTCGATCTTTG-3′	
oSRB334		5′-GCATCGTTCAGCCTTGATTTG-3′	
oSRB350	*yoyD* qPCR	5′-TTTGCCATTCGTTCAGCTC-3′	
oSRB351		5′-CGGCGTAACAATAATCCACAG-3′	

*Denotes a phosphorothioate linkage.
[†]Unless otherwise noted, oligonucleotides were designed and created during this study.
[‡]Boldface bases denote a unique 6-bp barcode for Illumina multiplexing.
[§]qPCR, quantitative PCR.

1. Villapakkam AC, et al. (2009) Genetic and biochemical analysis of the interaction of *Bacillus subtilis* CodY with branched-chain amino acids. *J Bacteriol* 191(22):6865–6876.
2. Brinsmade SR, Sonenshein AL (2011) Dissecting complex metabolic integration provides direct genetic evidence for CodY activation by guanine nucleotides. *J Bacteriol* 193(20): 5637–5648.
3. Brinsmade SR, Kleijn RJ, Sauer U, Sonenshein AL (2010) Regulation of CodY activity through modulation of intracellular branched-chain amino acid pools. *J Bacteriol* 192(24):6357–6368.

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)
Dataset S4 (XLSX)
Dataset S5 (XLSX)
Dataset S6 (XLSX)