A Comprehensive, CRISPR-based Functional Analysis of Essential Genes in Bacteria

Graphical Abstract

Comprehensive analysis of essential gene function

Bacillus subtilis essential gene CRISPRi library

Drug targets

Chemical phenotypes

Gene networks

Growth characteristics

Morphology

Terminal phenotypes

dCas9 + sgRNA library → B. subtilis strains

Highlights

- CRISPRi knockdown of essential genes enables discovery of direct antibiotic targets
- An essential gene network reveals functional connections between core processes
- A cell morphology screen identifies essential genes intimately tied to cell shape
- A majority of essential genes show morphological defects as a terminal phenotype

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In Brief

A systematic analysis of all essential genes in Bacillus subtilis using a CRISPR-based knockdown approach established the essential gene network, identified modes of action for antibiotics, and discerned fundamental underpinnings of growth and morphological characteristics of cells.

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A Comprehensive, CRISPR-based Functional Analysis of Essential Genes in Bacteria

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SUMMARY

Essential gene functions underpin the core reactions required for cell viability, but their contributions and relationships are poorly studied in vivo. Using CRISPR interference, we created knockdowns of every essential gene in Bacillus subtilis and probed their phenotypes. Our high-confidence essential gene network, established using chemical genomics, showed extensive interconnections among distantly related processes and identified modes of action for uncharacterized antibiotics. Importantly, mild knockdown of essential gene functions significantly reduced stationary-phase survival without affecting maximal growth rate, suggesting that essential protein levels are set to maximize outgrowth from stationary phase. Finally, high-throughput microscopy indicated that cell morphology is relatively insensitive to mild knockdown but profoundly affected by depletion of gene function, revealing intimate connections between cell growth and shape. Our results provide a framework for systematic investigation of essential gene functions in vivo broadly applicable to diverse microorganisms and amenable to comparative analysis.

INTRODUCTION

Essential gene functions underpin core cellular processes. Interrogating the relationships among essential gene functions is critical for understanding how bacterial growth is controlled and for facilitating drug development. Yet, few approaches can assess essential gene function in vivo to elucidate their connections. Neither gene-deletion libraries (Baba et al., 2006; Winzeler et al., 1999) nor saturating transposon mutagenesis (Goodman et al., 2009; van Opijnen et al., 2009) can be used to study essential genes, as cells cannot survive without their functions (Christen et al., 2011). Several high-throughput approaches have been used to identify or perturb essential genes in eukaryotes, including destabilizing the 3′ UTR of mRNAs (DaMP alleles; Breslow et al., 2008), CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 gene editing (Blomen et al., 2015; Wang et al., 2015), and CRISPR/dCas9 transcriptional regulation technologies (Gilbert et al., 2014). The only study screening essential genes in bacteria used antisense RNA knockdowns to screen for antibiotic sensitivities (Xu et al., 2010), a method of limited utility due to variable efficacy (Forsyth et al., 2002).

Here, we establish a CRISPR interference (CRISPRi) framework for systematic phenotypic analysis of essential genes in bacteria. CRISPRi uses a nuclease-deactivated variant of Streptococcus pyogenes Cas9 (dCas9) paired with a single guide RNA (sgRNA) to sterically hinder transcription at the sgRNA base-pairing genomic locus (Qi et al., 2013) and is a specific and efficient approach for knockdown, with demonstrated applicability in bacteria. We generated a comprehensive essential gene-knockdown library in the Gram-positive model bacterium Bacillus subtilis and used the library to enable drug target discovery, establish a functional network of essential gene processes, characterize how cell morphology and growth rate respond to reductions in essential gene expression, and dissect essentiality in a highly redundant genetic pathway. Our study provides a framework for comprehensive,
high-throughput analysis of essential gene functions applicable to diverse bacteria.

RESULTS

CRISPRi Is Effective, Specific, and Titratable in B. subtilis

We established a CRISPRi system in B. subtilis, consisting of S. pyogenes dCas9 driven by a xylose-inducible promoter (P<sub>xyl</sub>) and sgRNAs expressed from a strong, constitutive promoter (P<sub>veg</sub>), both transferred to the chromosome via integrating plasmids (Figure 1A). This system is very efficient, exhibiting 3-fold repression of red fluorescent protein (RFP) without induction and 150-fold repression with full dCas9 induction (Figures 1B and S1A). Although we also report a system with no basal repression based on a weak isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter (Figure S1B), we exclusively used P<sub>xyl</sub> throughout to consistently sensitize the library with slight knockdown and avoid inconsistencies resulting from slight variations in IPTG induction.

Our P<sub>xyl</sub>-based CRISPRi system is titratable, with unimodal repression at the single-cell level across sub-saturating inducer concentrations (Figures 1B and S1C), and high specificity, repressing only RFP expression at saturating inducer concentrations (Figure 1C). We used NET-seq to identify the genomic positions of transcribing RNA polymerase (Larson et al., 2014), showing that CRISPRi sterically blocks transcription in an operon showing equivalent knockdown (Figure 1E), and also reduces expression of upstream genes in the operon (Figure S1E). Thus, CRISPRi technology is suitable for examining gene function at the operon level.

A CRISPRi Knockdown Library of Essential Genes

We constructed an arrayed library of B. subtilis strains expressing computationally optimized sgRNAs (Supplemental Experimental
CRISPRi-Based Essential Gene Phenotyping and Drug Target Discovery

The ∼3-fold repression of our knockdown library without induction (basal repression; Figures 1B and S1A) sensitized strains to various chemicals. This enabled us to define essential gene phenotypes via chemical-genomic analysis by measuring colony size against 35 unique compounds (Supplemental Experimental Procedures). We achieved high reproducibility, as measured by correlated colony sizes ($R = 0.89$; Figure S2A). We converted colony sizes to chemical-gene scores (Figures S2A–S2D; Table S2; Nichols et al., 2011) and identified significant chemical-gene phenotypes (false discovery rate ≤ 5%; Nichols et al., Schujman et al., 2001), not essential due to a gene duplication (Thomaides et al., 2007), another knockdown in the pathway (fabG) was sensitized (Table S2). We conclude that our CRISPRi platform effectively identifies known drug–gene interactions.

We tested our essential knockdown library as a platform for drug target discovery by screening against MAC-0170636, an antibiotic that upregulates the cell-wall-damage-responsive promoter $P_{ywac}$ (Czarny et al., 2014) by an unknown mechanism. Undecaprenyl pyrophosphate synthetase ($uppS$) was the most sensitized knockdown (Figure 2A; Table S2), and we confirmed its sensitivity in liquid (Figure 2B). Conversely, $uppS$ overexpression increased MAC-0170636 resistance relative to wild-type (WT) cells (Figure 2B). Purified B. subtilis UppS activity was inhibited by MAC-0170636 with an IC$_{50}$ of 0.79 μM (Figure 2C), indicating that UppS is the direct target of MAC-0170636. UppS purified from another Firmicute, Staphylococcus aureus, was completely resistant to MAC-017063 (Figure S2E), as was S. aureus itself. Likewise, B. subtilis expressing only S. aureus $uppS$ was resistant (Figure 2D). These results highlight the utility of our knockdown library for identifying direct targets of uncharacterized compounds; CRISPRi portability suggests its utility in future organism-specific drug-discovery efforts.

Functional Analysis of the Essential Gene Network

Highly correlated responses of gene knockdowns across chemical conditions (phenotypic signatures) indicate functional connections...
Nichols et al., 2011). We established a network of gene-gene connections using statistically significant correlations among the phenotypic signatures of our essential gene knockdowns, based on direct or indirect effects of drug-gene interactions (Figures 3A, S2B–S2D, and S3; Table S3; Supplemental Experimental Procedures). The network was rich in known biological connections among genes in related processes, such as cell-wall biosynthesis and cell division (Figure 3B).
We quantitatively validated the network with a receiver operating characteristic (ROC) curve. Because CRISPRi exhibits polarity, this analysis was based on operons containing essential genes (“essential operons,” n = 203), rather than on the individual genes themselves (Table S3; Supplemental Experimental Procedures). We compared high-confidence gene–gene connections in the STRING database (http://string-db.org/; Szkarczyk et al., 2015) with correlations between essential operons in our chemical-genomics dataset that met our correlation threshold (R = 0.572; Figure 3C; Supplemental Experimental Procedures). Our network showed excellent agreement with STRING, with a ~20-fold ratio of true- to false-positive rates (Figure 3C, inset).

We investigated the relationship between network connections and gene function by assigning essential operons to functional groups using the SubtiWiki online resource (http://subtiwiki.uni-goettingen.de/; Michna et al., 2014). Of the 61 non-overlapping functional groups, the 35 with two or more essential operons were assessed for connectivity within the group (intra-connectivity). Mean intra-connectivity was ~2.4, compared with a background mean connectivity of ~0.3 (p < 10^{-3}), with ten groups showing connectivity among ≥3 essential operons (Table S3). Indeed, the ROC analysis showed strong specificity and sensitivity for recapitulating existing intra-process connections in STRING (Figure S4A), with excellent recovery of connections within functional groups (Figure S4B). Connections within the peptidoglycan (PG) cell-wall biosynthesis (18) and DNA replication (9) functional groups were most dense, likely reflecting convergence of PG precursor biosynthesis and localized cell-wall assembly (Tumer et al., 2014) and re-plisome protein-protein interactions (Sanders et al., 2010), respectively.

Next, we compared connectivity between functional groups (inter-connectivity) in our network with those in STRING, finding common connections and important distinctions (Figures 3D and 3E). First, the balance of intra- and inter-process connections differed; 46.2% of connections in STRING were inter-process versus 59.0% in our network (p < 10^{-3}). Second, STRING inter-process connections were biased toward extensively studied processes, e.g., 39% of STRING, but only 4.3% of our inter-process connections were between ribosomal proteins and translation factors. Moreover, 84% (113/134) of connections unique to our network were between processes (Table S3), highlighting the ability of our open-ended approach to detect such connections. Finally, our network revealed many connections between operons in distant functional groups. Using the hierarchical annotation levels from SubtiWiki (Michna et al., 2014) as an approximation of “annotation distance,” we found that novel connections in our network were skewed toward processes furthest apart in annotation, whereas those in STRING were predominantly between related processes (Figure 3D). Some distant connections were intuitive, such as DNA replication (holB) and folate biosynthesis (folC and the sul-folB-folK operon), likely reflecting the folate requirement for deoxythymidine triphosphate (dTTP) production (Hardy et al., 1987). Other connections were unexpected, such as those between peptidoglycan (PG) biosynthesis/cell division and DNA replication/modification (e.g., ftsL and dnaX, murC and gyrB, ddl-murF and yidO); these connections may be involved in failsafe mechanisms that link division and DNA replication (Arijes et al., 2014).

We explored the unexpected connection between transcription (rpoB) and cell division (ftsL), which is based on shared sensitivities to DNA intercalators and cell-wall antibiotics (Figure S2D). Using RNaseq, we found that basal knockdown of rpoB reduced the rpoB-rpoC mRNA level by two-fold. Among the few other substantial expression changes (Figure S4D), we found several downregulated envelope genes including manA (4-fold), which is involved in cell-wall integrity (Elbaz and Ben-Yehuda, 2010), and sigW (1.5-fold to 2-fold), a master regulator of envelope stress (Cao et al., 2002) (Table S3). Selective reduction of several envelope functions due to rpoB knockdown could result in cell-wall defects that mimic those caused by knockdown of late-acting cell-division genes.

We also identified novel connections to essential genes of unknown function (Figures 3A, S3, and S4E). For example, resistance to cell-wall-targeting antibiotics drove strong correlations among the largely uncharacterized gene ylaN (Figure S2F) (Hunt et al., 2006; Xu et al., 2007), iron-sulfur cluster biogenesis, and isoprenoid biosynthesis, the latter of which depends on the iron-sulfur cluster enzyme IspH (Gräwert et al., 2004; Wolff et al., 2003). These results suggest that defects in cellular iron homeostasis underlie the connections to ylaN. Indeed, in follow up experiments, we determined that ylaN is non-essential with added iron(III) (Figure S4F), further highlighting the ability of our unbiased approach to identify novel connections among essential processes.

**Growth Characteristics of the Essential Gene Knockdown Library**

We measured apparent lag, maximum growth rate, and saturating density of growth curves for all basal knockdowns in lysogeny broth (LB) medium (Figures 4A and 4B; Supplemental Experimental Procedures). Almost all knockdowns (~80%) had a maximal growth rate equivalent to the control (Figure 4B, inset); ribosomal proteins were the only functional category with slower growth rates relative to other knockdowns (p = 0.0008, t test). However, most knockdowns (95%) had longer apparent lag times (Figure 4A, inset), and cell-wall synthesis genes were enriched for the longest lags (p = 0.0013, Mann-Whitney U test; Table S4). CRISPRi repression was maintained in stationary phase (Figure S4G).

We determined whether longer apparent lag times reflected strain-specific differences or decreased viability. Both the fraction of growing cells on agarose pads (Figure 4C) and plate viability measurements (Figure 4D) of strains spanning the apparent lag times negatively correlated with apparent lag. Apparent lag times also tracked with expectations from the number of live cells present. For example, using the 45-min doubling time in batch culture (Figure 4B, inset), the ~8-fold reduction in mraY knockdown viable cells (Figures 4C and 4D) requires ~3 additional divisions, accounting for the 2.1 hr increase in apparent lag. Moreover, viable mraY knockdown cells and the control had equivalent growth rates after transfer to an agarose pad with fresh LB (Figure 4C, inset). Together, these surprising results suggest that slight reductions in essential gene products affect outgrowth from stationary phase, creating a mixed...
Basal Knockdown of Essential Genes Results in Changes in Cellular Dimensions that Reveal Shape Actuators and Modulators

Our library offers an unprecedented opportunity to examine whether basal knockdown of essential genes affects cell morphology. To provide baseline values, we quantified the dimensions of WT cells. Cells were imaged 3.5 hr after different degrees of dilution into fresh medium (Supplemental Experimental Procedures) and therefore had different culture optical densities (OD). We found that median cell length and width systematically varied with the extent of dilution (Figure 5A), likely reflecting growth phase differences as cells cycle through nutrients in rich medium (LB). Cells diluted less were smaller, reflecting their slower growth rate at the time of imaging, similar to previous reports linking cell size and steady-state growth rate (Schaechter et al., 1958) and consistent with known shortening in the stationary phase (Overkamp et al., 2015).

Next, we determined morphological parameters of the essential gene basal knockdowns to pinpoint proteins for which a small expression change results in altered shape, using high-throughput imaging at 3.5 hr after dilution (Supplemental Experimental Procedures). Because of variable stationary-phase outgrowth (Figures 4C and 4D), culture OD widely varied at the time of imaging. We found a general relationship between growth rate and OD for virtually all knockdowns (Figure S4H), indicating a large range of instantaneous growth rates at imaging. Nonetheless, the length and width of the knockdowns were highly correlated (Figure 5B), similar to WT cells across dilutions (Figure 5A). The fact that cells reach their maximum growth rate at a fixed OD...
rather than at a fixed number of doublings after growth resumption, underscores the importance of cell density and the extracellular milieu in growth rate control.

Basal knockdowns that deviated from the length/width trend-line (Figure 5B; Table S5) could represent proteins involved in either actuating or regulating growth of the shape-determining cell wall. Only cell-envelope genes exhibited significant enrichment of outliers (average median length deviation = 0.99 μm, p < 0.0005 by bootstrapping; Figure 5B; Supplemental Experimental Procedures), as expected because they are largely PG synthesis related and are therefore actuators of cell shape. To validate our identification of outliers and ensure that cell chaining was rare, we used the membrane stain, FM4-64, on a subset of strains with different median cell lengths, finding that length measurements from phase images and peripheral fluorescence were highly correlated (Figure S5A).
Figure 6. Essential Gene Depletion Reveals a Diversity of Terminal Phenotypes

(A) (i) Area-proportional graph of the fraction of essential gene knockdowns that give rise to morphological and growth terminal phenotypes. (ii–viii) Single-cell imaging of common terminal phenotypes of essential-gene knockdowns, with bar graphs depicting the broad functional categories underlying each terminal phenotype.

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Several outliers in other functional groups were intriguing, as they could identify cell-shape regulators. Basal knockdown of tufA, encoding the translation elongation factor Tu, resulted in cells that were substantially shorter than expected (Figure 5B; Table S5); Tu interacts with the bacterial cytoskeleton protein and rod-shape determinant MreB (Defeu Soufo et al., 2010). Knockdowns of several DNA replication genes (dnaX, dnaA, and nrdEF) resulted in longer cells (Figure 5B; Table S5). Moreover, dnaX and rtsL, both of which are large morphologically outliers, were also significantly correlated in our chemical screen, suggesting a functional connection between replication and division, corroborated by multiple independent data types.

As cell size is dependent on the degree of dilution prior to regrowth (Figure 5A), direct comparisons of cellular dimensions among different strains require cells to be at the same OD. Large variation in log times prevented us from measuring the entire library by this method. Instead, we examined two actin homologs (mreB and mbl) involved in coordinating PG synthesis (Scheffers and Pinho, 2005) and the mur genes responsible for PG precursor synthesis at OD 0.3 ± 0.05. Both actin homologs were wider than WT, on average, with mreB exhibiting larger standard deviation, suggesting that cell width is particularly sensitive to MreB levels (Figure 5C). The median cell lengths of mur strains were similar to WT (Figure 5D), but widths (population median between 1.04–1.14 μm) were larger than no-sgRNA cells (1.02 μm), validating their classification as outliers (Figure 5D). The murB cell width monotonically increased with dcas9 induction (Figure 5E), indicating that cell width is responsive to the cellular levels of PG precursors.

In summary, our systematic screen for the essential gene products most intimately tied to cell-shape identified genes with known ties to morphology (e.g., mreB/mbl) and cell-wall synthesis, revealed a quantitative relationship between PG precursor gene expression and cell width, and uncovered potential new regulators of cell morphology.

**Single-Cell Characterization of Terminal Phenotypes Provides a Novel View of Essential Gene Function**

We examined whether substantial depletion results in more extreme morphological changes than basal knockdown by imaging the entire library after prolonged (~24 hr) induction of dcas9 (“terminal phenotypes;” Supplemental Experimental Procedures). Remarkably, >60% of the strains (174/289 or 166/258 bona fide essentials) exhibited morphological phenotypes, even eliminating strains with only growth defects (overdivision or growth halting; Figure 6Aii), a number far exceeding the 48 known cell-envelope-related strains. Knockdowns displayed several predominant morphologies (Figures 6Aii–6Aviii and S5B), whereas the control formed a uniform lawn of normal, rod-shaped cells (Figure 6Aii).

We quantified terminal morphologies by manually classifying phenotypes with a 6D phenotype vector, including lysis, bulging, bending, filamentation, overdivision (shorter cells), and extent of growth. We assessed whether functionally related genes mapped to particular phenotypes. Many cell-envelope genes bulged (e.g., tagA/B/FGIO [teichoic acid], mreC [cell shape/PG biosynthesis]) (Figure 6Aii), while many ribosomal genes displayed severe cell bending (Figure 6Aiv). However, in some cases genes in different processes had similar terminal phenotypes, such as growth halting (Figure 6Aii) and bending (Figure 6Aiv). Several metabolic genes (e.g., metK and dxs) exhibited bulging (Table S5), consistent with network connections between these processes and cell-envelope synthesis/division (Figure 3A). Finally, some strains displayed both bulging and lysis (Table S5), indicating that depletions in one process can lead to more than one terminal phenotype.

Phenotypic variability within functional groups and common phenotypes of different groups made it challenging to map phenotype to process with simple visual comparison. Instead, we evaluated the similarities between terminal phenotype vectors for all SubtiWiki annotation pairs (Figure 6B; Supplemental Experimental Procedures), a process conceptually similar to the one we used to identify inter-process connections in the chemical-genomics dataset. Our similarity matrix recapitulated known connections (e.g., PG synthesis/cell division), while providing support for novel network connections (e.g., fatty acid metabolism with both isoprenoid biosynthesis and several central-dogma annotations; Figures 3A, 3B, and 6B). Terminal-phenotype links are consistent with and complement the high level of inter-process connectivity of the essential gene network (Figure 5E).

Variable terminal phenotypes within a group might reflect the rate of gene-product depletion. We tested this hypothesis by inhibiting tRNA charging (Figure 6C), either by valS (ValS aminoacyl-tRNA synthetase) knockdown or by addition of serine hydroxamate to cells. Knockdowns displayed several predominant morphologies (Figure 6C), either by valS aminoacyl-tRNA synthetase knockdown or by addition of serine hydroxamate (tRNA<sup>ser</sup> aminoacylation inhibitor). We observed dose-dependent phenotypes (Figure 6C): shorter cells with slight depletion/inhibition (Figures S5C and S5D), filamentation and bending with intermediate levels, and growth halting at high levels; these phenotypes were also exhibited by other aminoacyl-tRNA synthetase knockdowns (Figures 6Aiv, 6Avii, and S5B). Thus, the rate of inhibition of essential processes can qualitatively affect their terminal phenotypes.

In summary, the majority of essential genes knockdowns display an altered terminal morphological phenotype. Phenotypes group both within and across functional processes, demonstrating the utility of morphology for revealing interactions, and can vary with the kinetics and extent of gene knockdown.
A. CRISPRi multiplexing

CRISPRi multiplexing

8 sgRNAs per cell

dCas9

B. Relative expression

C. Gene expression heatmap

D. Measured vs. predicted fitness

E. Cell morphology images

F. Cell length (μm)

G. Minimum negative curvature (μm, abs. value)

Cell length
Positive curvature
Negative curvature

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**Cellular Behavior during the Depletion of Essential Proteins**

To determine how rapidly terminal phenotypes are established, we performed time-lapse imaging on nine representative knockdown strains in the presence of inducer. We placed exponentially growing cells on agarose pads of LB+xylose to initiate depletion, and imaged them every 5 min to determine microcolony growth rates (Figures 6D and S5E). During the first 60-min post-induction, WT cells maintained a constant elongation rate. The mbi knockdown maintained the WT growth rate; murD had a ~50% reduction; and the rest had a 20%–30% reduction. Bulging (tagD) was the first prominent morphological phenotype observed (~40 min; 1–2 cell doublings on agarose pads) with filamentation (ppbB), bending (accD), and lysis (murD) occurring after ~60 min (2–3 doublings; Figure 6E). As depletion is primarily by dilution, these data indicate that reduction of at least 50%–75% below basal knockdown is required to substantially affect growth rate and morphology. Many factors prevent precise quantification, including differential protein stabilities and polar effects on transcription.

We examined growth of the entire library during dcas9 induction by diluting stationary-phase cultures into liquid LB+xylose. Approximately one-third of the strains never emerged from stationary phase (OD600 < 0.06 at 7 hr; Figure S6A). These strains covered the entire range of lags, and hence lack of growth could not be explained by the variable stationary-phase outgrowth exhibited under basal knockdown conditions (Figure S6B). Instead, these strains identify the subset of essential proteins present in limiting amounts such that additional depletion beyond basal knockdown results in growth cessation or lysis (Table S4).

Among strains that grew, we identified two distinct phenotypes (Figure S6C; Table S4; Supplemental Experimental Procedures). 13 strains showed nearly linear growth; 9/13 affected cofactor biosynthesis (e.g., heme biosynthesis) or electron transport (Figure S6D) and may reflect cofactor availability. 14 strains enriched in either cell-envelope synthesis (5/14), or DNA replication (7/14) (Table S4) stalled after initial growth and then exhibited a marked decrease in OD, indicative of lysis (Figure S6E). This common phenotype may reflect the close network and morphological connections observed between DNA replication and envelope synthesis (Figure 3A).

**Dissecting a Highly Redundant Gene Network with Multiplexed CRISPRi**

Genetic redundancy, prevalent in complex processes (e.g., construction of PG; Meeske et al., 2015), can mask the contributions of individual genes to essential processes. CRISPRi can simultaneously knockdown two genes (Qi et al., 2013); we show that CRISPRi can simultaneously, effectively knockdown eight unrelated, nonessential genes (Figures 7A and 7B). Using this multiplexing capability, we examined redundancy for the 16 penicillin binding protein (PBP)-encoding genes involved in PG synthesis. Simultaneous knockdown of pairwise ppb genes were viable at full dcas9 induction, except for ppbA/ppbH, a known synthetic lethal pair, and those including ppbB, the only essential PBP (Figures 7C and S7A–S7C; Table S6) (Wei et al., 2003). The observed fitness of double knockdowns was largely predicted by multiplying the fitness of single mutants, a formalism developed for null mutations in parallel pathways (Figure S7A; Supplemental Experimental Procedures).

We identified double knockdowns hypersensitive to PBP inhibitors (mecillinam, cefoxitin, and aztreonam; Figures S7D and S7E), as these suggest possible triple synthetic gene combinations. Fitness of the ppbA/ponA/ppbD triple knockdown was lower than predicted (Figure 7D), and we could not construct a ΔppbA/ΔponA/ΔppbD triple deletion, although we could introduce an innocuous deletion (ΔtrpC) or an unrelated ppb deletion (ΔppbC) into ΔppbA/ΔponA. Thus, the triple deletion is either extremely sick or synthetic lethal.

PBP4 (ppbD), PBP2a (ppbA), and PBP1a/b (ponA) have transpeptidase activity and are septally localized (Scheffers et al., 2004). Reduced septal PG transpeptidation during cell division may underlie the severe growth defect of the triple knockdown. Control and single- and double-knockdown cells exhibited the expected rod-like shape, albeit with reduced cell length (Figures 7E and 7F), potentially implicating these three genes in the regulation of cell division. By contrast, the triple mutant had distinct phenotypes, including filamentation, cell lysis (Figure 7E, white arrows), and bending along the cell contour (Figure 7G; p < 10^{-6}), whether constructed with complete knockdown of all three genes or ponA knockdown in the ΔppbA/ΔppbD double. This phenotype is consistent with lethality caused by reduced septal transpeptidation and showcases the ease of dissecting redundant pathways with CRISPRi.

**DISCUSSION**

Bacteria typically have several hundred essential genes that encode the core reactions central to viability, together constituting ~10% of their total genetic complement. Lacking a facile way to reduce their expression, we had little understanding of in vivo relationships among essential gene processes or how subtle imbalances in essential pathways impacted cellular homeostasis. This work is a major advance in the study of bacterial...
essential genes, providing a systematic, unbiased study of their phenotypes in vivo using CRISPRi (Figure 1) to obtain facile and precise downregulation. Using chemical genomics profiling and high-throughput microscopy, we identified complex phenotypes, including chemical vulnerabilities (Figure 3), growth and shape phenotypes (Figures 4 and 5), and terminal death phenotypes (Figure 6). Together, these revealed a complex web of connections among essential processes. Given that CRISPR systems are broadly active in bacteria, our approach can be readily extended to other bacterial species, including pathogenic and non-culturable species.

Our essential gene network (Figure 3) reveals numerous inter-process connections not previously annotated and is highly enriched in novel connections among distant processes. Process inter-connectivity may provide the cell with mechanisms for restoring cellular homeostasis in response to transient imbalances. Some connections make intuitive sense. For example, folate is likely linked to replication via its necessity for dTTP synthesis. Such correlations readily suggest specific hypotheses that can be directly tested experimentally; e.g., a particular DNA polymerase subunit senses dTTP levels. Other connections have no facile explanation and underscore that connections between core processes are abundant and understudied. Our network likely significantly underestimates connections because it is based only on the highest confidence interactions to avoid false positives. Comparable datasets in other organisms will provide the basis for evolutionary studies to investigate the extent to which the logic of these functional circuits is conserved across organisms.

Our studies suggest that the levels of essential B. subtilis proteins are higher than necessary to maintain optimal growth, as the vast majority of basal knockdown strains grew indistinguishably from WT in exponential phase (Figure 4B). Thus, either protein levels are set high enough to be robust to small (1.5–3-fold) decreases in expression, or their levels are maintained by an elaborate posttranscriptional regulatory system. Moreover, there is a sufficient excess of essential proteins that most strains (70%) emerge from stationary phase even when their gene products are depleted during regrowth. The 30% of strains unable to exit stationary phase (Figure S6A) are those whose protein levels are closest to the levels necessary for normal growth.

In stark contrast, almost all basal knockdowns strains exhibited increased cell inviability during the exit from the stationary phase. Thus, even a small decrease in protein product increases the vulnerability of cell regrowth (Figure 4A). Importantly, there is little overlap between strains ceasing growth soon after depletion and those most vulnerable to inhibition of outgrowth from stationary phase (Figure S6B). This discordance suggests that the set of essential genes whose expression level is close to that necessary for rapid cell growth in ideal conditions is distinct from the set necessary for survival during stationary phase. Speculatively, protein levels may reflect the ecological niche of B. subtilis in soil, where cells spend much more time in a non-growing state and must survive long enough to enter the sporulation program with high efficiency.

Classically, genetic perturbations resulting in morphological variation, such as homeotic mutants and variegated maize, provided critical insights into fundamental cellular circuits. However, the essentiality or redundancy of most cell-wall synthesis proteins has made it difficult to uncover the molecular mechanisms underlying bacterial cell-shape and size determination. Our CRISPRi knockdown approach allowed us to probe this relationship both under conditions of partial knockdown (1.5–3-fold) and during complete depletion. Partial knockdown identifies outliers exceptionally sensitive to depletion. As a consequence, we identified the critical envelope gene actuators of the response, showed that cell width, as well as length, is controlled (Figure 5), and determined that cell width monotonically varies with the extent of depletion of mur genes (Figures 5D and 5E). The few outliers involved in other processes identify putative regulators of cell-wall synthesis. The translation and DNA-replication-related outliers may tie the rate of protein synthesis and DNA replication to cell-wall growth, possibly using previously unrecognized moonlighting functions of these proteins. Moreover, since maximal growth rate is not substantially affected across the basal knockdown strains, our findings indicate that cell size and growth rate can be, at least, partially decoupled. Additionally, a graded morphological response to the levels of essential proteins may drive physiological heterogeneity, enabling population adaptation to dynamic environments.

In contrast, complete depletion probes the intimate relationship between growth and morphology. Here, cells exhibit a wide array of terminal phenotypes with alterations in both growth and morphology (Figure 6A). By comparison, only a single nonessential E. coli gene deletion (rodZ) has a morphological phenotype (Shiomi et al., 2008). Some phenotypes are the result of gradual depletion of function (Figure 6C), which may mimic imbalances that transiently occur due to stochasticity in gene expression. Tracing changes at different rates of depletion may provide clues to the cellular program for morphology. Moreover, the dynamics of removing an essential function may be as important to growth and viability as the presence or absence of such a protein.

The evolution of essential processes has remained largely mysterious. Long-term evolution experiments have detected many mutational events in the RNA polymerase complex, cell-wall synthesis, and cell-shape determination (Tenaillon et al., 2012), suggesting a great diversity of molecular adaptations, including to essential processes. The apparent excess abundance of most essential proteins (Figures 6D and 6E) suggests that new functions could rapidly develop from the existing repertoire of proteins without compromising growth, possibly explaining the prevalence of moonlighting proteins (Huberts and van der Klei, 2010). For example, actin and tubulin homologs have diverse roles in bacteria and eukaryotes (Busiek and Margolin, 2015). Phenotypic heterogeneity and variable survival during the stationary phase, as observed here (Figure 4), may indicate that stressful environments have shaped the evolutionary history of essential genes. The complexity of the network (Figure 3A) suggests context dependence that may be critical for the evolution of essential genes and their interactions; perhaps, certain essential genes can be rendered non-essential in environments such as biofilms. Expansion of our study to varied environments may provide a more nuanced view of the instances in which a particular process is limiting for growth.
EXPERIMENTAL PROCEDURES

CRISPR Library Design, Cloning, Chemical Screens, and Growth Analysis

sgRNAs targeted all putative essential genes (subtiwiki.uni-goettingen.de) and those recently identified in our B. subtilis gene knockout library (B.M.K., J.M.P., and C.A.G., unpublished data). sgRNAs were designed to target within the gene body near the 5′ end of the gene on the non-template strand. sgRNA libraries were cloned via inverse PCR as previously described (Larson et al., 2013) and strains were constructed using natural competence transformation.

Chemical screening was performed and chemical-gene scores were calculated as previously described (Nichols et al., 2011). B. subtilis and S. aureus UppS proteins were purified using nickel-affinity chromatography, and assayed using a Kinetic EnzChek pyrophosphate assay (Life Technologies). The B. subtilis essential gene network was constructed by calculating all pairwise Pearson correlations between sgRNA knockdown strains, randomly permuting gene identity relative to chemical-gene scores to generate a background distribution, then applying a significance cutoff to the correlations based on the 95% confidence interval of the background distribution. Population growth curves were obtained from a microplate reader, and growth information was extracted by fitting the curves to a Gompertz equation (Zwietering et al., 1990).

High-Throughput Microscopy

Images of single cells were acquired after transferring cells from a 96-well plate onto a large-format agarose pad. Analysis of cellular morphologies was performed using custom MATLAB code. A minimum of 100 cells were analyzed for quantitative descriptions of single-cell phenotypes. Terminal phenotypes were examined by spotting cultures outgrown from stationary phase for two hours in liquid LB medium, transferred to agarose pads containing LB + 1% xylose, then imaged after overnight incubation. Time-lapse images were taken in an active-control environmental chamber at 37 °C (Haisontech). Further details of methods are in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the RNA-seq and NET-seq data reported in this paper is GEO: GSE74926.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.05.003.

AUTHOR CONTRIBUTIONS


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REFERENCES


Supplemental Figures

Figure S1. Further Characterization of the *B. subtilis* CRISPRi System, Related to Figure 1

(A) Flow cytometry of cells constitutively expressing rfp, with or without *P*<sub>xyl</sub>-dcas9 and an rfp-targeting sgRNA. RFP levels are relative to the sgRNA- control.

(B) Flow cytometry of cells constitutively expressing rfp, with or without *P*<sub>iptg</sub>-dcas9 and an rfp-targeting sgRNA. RFP levels are relative to the sgRNA- control. rfp knockdown without IPTG induction is < 15%.

(C) Flow cytometry of cells in which dcas9 was induced by adding the specified concentration of xylose that constitutively express rfp and an rfp-targeting sgRNA shown as distributions of RFP fluorescence for selected xylose concentrations. RFP levels per cell are relative to the sgRNA- control.

(D) NET-seq of cells maximally induced for dcas9 (1% xylose) and constitutively expressing rfp and an rfp-targeted sgRNA versus cells without an sgRNA. Bars show the total number of normalized RNA 3' ends downstream of the sgRNA-dCas9 binding site.

(E) Flow cytometry of cells constitutively expressing an rfp-gfp operon and various sgRNAs targeting gfp at maximal induction of dcas9 (1% xylose). RFP and GFP levels are relative to the sgRNA- control. The dashed line is y = x. Note that the sgRNAs with low efficacy target the template strand (violet).

(F) Growth curves of *P*<sub>xyl</sub>-dcas9 sgRNA- (red), *P*<sub>xyl</sub>-dcas9 sgRNA* (green), and *B. subtilis* 168 cells (blue), with 1% xylose supplemented. Shaded areas are mean ± std for each strain.
Figure S2. Essential Gene Knockdown Screen Reproducibility and Chemical-Gene Phenotypes, Related to Figures 2 and 3

(A) Reproducibility between replicate colonies for all chemical conditions (R: Pearson’s correlation).
(B) Hierarchical clustering of chemical-gene scores (S-scores; Collins et al., 2006) for all chemical conditions.

(legend continued on next page)
(C) A tetrahydrofolate biosynthesis and utilization cluster defined by knockdown strain sensitivity to the DfrA inhibitor trimethoprim and the Sul inhibitor sulfamonomethoxine.

(D) A cell division/RNA polymerase cluster defined primarily by sensitivities to cell wall-acting antibiotics (aztreonam, cefoxitin, D-cycloserine, and fosfomycin) and DNA intercalators (ethidium bromide, and acriflavine).

(E) Concentration-dependent inhibition of purified S. aureus UppS by MAC-0170636. Each point is an individual measurement.

(F) A cluster containing ylaN, isoprenoid biosynthesis, and iron-sulfur cluster biosynthesis genes defined by resistance to cell wall-targeting antibiotics, especially fosfomycin.
Figure S3. An Essential Gene Network Reveals Numerous Intra- and Inter-process Connections, Related to Figure 3
The *B. subtilis* essential gene network as depicted in Figure 3A, except showing sub-networks with only one or two connections and gene labels.
Figure S4. Further Characterization of the Essential Gene Network and Basal Knockdown Levels, Related to Figures 3 and 4
(A) ROC curves depicting true positive rates and false positive rates for either intra- or inter-process connections between essential operons.
(B) Absolute number of connections between essential operons in the STRING database (blue) or our essential network (red).
(C) Previously uncharacterized connections between cell division and transcription (red lines); these connections are largely due to shared sensitivities of ftsL/divIC and rpoB/C knockdowns to cell wall-acting antibiotics and DNA intercalators (see also Figure S2D). Black lines are connections between genes in different operons and gray lines are connections within operons. The dashed line is $y = x$. The rpoBC transcript is reduced $\sim$40% by the rpoB sgRNA. Outliers and genes of interest are colored as indicated. The operon containing manP/A-yjdF is abbreviated as “man.”
(D) RNA-seq of cells with basal expression of dCas9 and an sgRNA targeting rpoB versus cells without an sgRNA. Reads per gene were normalized by RPKM. (E) Previously uncharacterized connections among iron-sulfur cluster biosynthesis, isoprenoid biosynthesis, and the gene of unknown function ylaN (red lines). Black lines are connections between genes in different operons and gray lines are connections within operons. (F) ylaN is no longer essential in the presence of additional iron. B. subtilis 168 cells were transformed with PCR products that replaced either trpC (B. subtilis 168 is already mutant for trpC, so the replacement is neutral), or ylaN with a kanamycin-resistance marker. (G) Basal CRISPRi knockdown of rfp modestly increases during stationary phase. (H) Culture optical densities (ODs) determine the instantaneous growth rates for all strains. Instantaneous growth rates were calculated from OD measurements in Figure 4A. Shaded area is mean ± std for sgRNA- control.
Figure S5. Further Characterization of Cellular Dimensions and Terminal Depletion Phenotypes, Related to Figures 5 and 6

(A) Median cell lengths for a subset of strains measured from both phase contrast images and FM4-64 staining. The two measurements are highly correlated. We note that our strains did not show as strong a propensity for chaining as other *B. subtilis* strains, and that it was possible to identify the positions of septa from phase contrast images.

(B) Cells from a subset of strains with representative terminal phenotypes stained with DAPI and FM4-64 to reveal nucleoid structure and the cell membrane, respectively.

(C) Distribution of cell lengths of the valS knockdown strain in its terminal phenotype in LB versus LB + 0.005% xylose. Xylose-treated cells were significantly shorter and exhibited the overdivision phenotype.

(D) Distribution of cell lengths of wild-type cells in its terminal phenotype in LB versus LB + 0.6 mg/mL serine hydroxamate. Cells treated with serine hydroxamate were significantly shorter and exhibited the overdivision phenotype.

(E) Post-induction instantaneous growth rates for selected strains with different terminal phenotypes. Most strains maintained constant growth rates in the first hour, except for murD, which decreased in growth rate after 40 min.
Figure S6. Essential Gene Depletion Growth Curves, Related to Figure 6

(A–E) Microplate reader growth curves of essential gene knockdown library strains at full induction of dCas9. Set of strains that displayed noteworthy growth patterns are plotted in each subfigure. (A) The set of strains that never emerged from stationary phase with dCas9 induction. (B) Strains in (A) displayed a range of apparent lags without dCas9 induction, indicating that cell death during depletion is not correlated to vulnerability in stationary phase. (C) Strains that continued growing after dCas9 induction. (D) Strains that exhibited slower, almost linear growth curves. (E) Strains that exhibited biphasic behavior, involving initial growth followed by stalling and a marked decrease in OD.
Figure S7. *pbp* Knockdown-Specific Examples, Chemical Screens, and Reproducibility, Related to Figure 7

(A) Relationship between double knockdown fitness predicted by the multiplicative model and fitness measured by colony size. The gray, dashed y = x line represents the ideal relationship between predicted and measured fitness according the multiplicative model; the red line is a linear fit to the data. Strains with two sgRNAs targeting the same gene were removed for clarity.

(B) Relative fitness defect of the *pbpB* single knockdown strain based on normalized colony size.

(C) Agreement between normalized colony sizes for reciprocal double knockdown sgRNA pairs (e.g., *amyE::sgRNA*~pbpA~ and *thrC::sgRNA*~pbpH~ versus *amyE::sgRNA*~pbpH~ and *thrC::sgRNA*~pbpA~) at maximal induction of *dcas9* (1% xylose).

(D) The difference between measured and predicted fitness ($\epsilon$ values) for each *pbp* double knockdown based on normalized colony size at maximal induction of *dcas9* (1% xylose) and in the presence of the indicated PBP inhibitor.

(E) Agreement between normalized colony sizes for reciprocal double knockdown sgRNA pairs at maximal induction of *dcas9* (1% xylose) and in the presence of the indicated PBP inhibitor.
Supplemental Information

A Comprehensive, CRISPR-based Functional Analysis of Essential Genes in Bacteria

EXTENDED EXPERIMENTAL PROCEDURES

Plasmid Construction

Plasmids are available from the Bacillus Genetic Stock Center (http://www.bgsc.org/). dcas9 was PCR-amplified from pdCas9-bacteria (Addgene #44249) using primers containing BamHI-compatible Bsal sites, digested with Bsal, then ligated into plasmid pAX01 digested with BamHI to generate pJMP1 (P\textsubscript{xyr}-dcas9, Erm\textsuperscript{R}). The rfp-targeting sgRNA\textsuperscript{RRI} was PCR-amplified from pgRNA-bacteria (Addgene #44251) using primers containing the veg promoter and EcoRI sites, digested with EcoRI, then ligated into either pDG1662 digested with EcoRI to generate pJMP2 (P\textsubscript{veg}-sgRNA\textsuperscript{RRI}, Cm\textsuperscript{R}), or pDG1731 digested with EcoRI to generate pJMP3 (P\textsubscript{veg}-sgRNA\textsuperscript{NTI}, Spc\textsuperscript{R}). Essential gene and \textit{pbp} library plasmids were generated from either pJMP2 or pJMP3 using inverse PCR as previously described (Hawkins et al., 2015; Larson et al., 2013). \textit{uppS\textsuperscript{Ss}} was PCR-amplified from \textit{B. subtilis} 168 genomic DNA using primers containing Sall and Nhel sites, digested with Sall and Nhel, then ligated into pDR110 digested with BamHI to generate pDR-\textit{uppS\textsuperscript{Ss}} (P\textsubscript{spank}-\textit{uppS\textsuperscript{Ss}}, Spc\textsuperscript{R}). \textit{uppS\textsuperscript{Ss}} was PCR-amplified from \textit{S. aureus} N315 genomic DNA using primers containing HindIII and SphI sites, digested with HindIII and SphI, then ligated into pDR110 digested with HindIII and SphI to generate pJMP11 (P\textsubscript{spank-\textit{uppS\textsuperscript{Ss}}}, Spc\textsuperscript{R}). Multiple sgRNA plasmids were constructed using Bsal-mediated cloning as previously described (Hawkins et al., 2015), except for double \textit{pbp} sgRNA plasmids, which were constructed by Gibson assembly (Gibson et al., 2009) using the Gibson Assembly master mix kit (New England Biolabs).

Strain Construction

Strains are available from the Bacillus Genetic Stock Center (http://www.bgsc.org/). All \textit{B. subtilis} strains were constructed using natural competence via either a standard or high-throughput method (Koo et al., \textit{in preparation}).

\textit{Standard Method}
3 ml of MC medium (10.7 g/L potassium phosphate dibasic, 5.2 g/L potassium phosphate monobasic, 20 g/L glucose, 0.88 g/L trisodium citrate dihydrate, 0.022 g/L ferric ammonium citrate, 1 g/L casein hydrolysate, 2.2 g/L potassium glutamate monohydrate, 20 mM magnesium sulfate, 150 nM manganese chloride, 20 mg/L tryptophan) were inoculated with a single colony of *B. subtilis* and incubated at 37 °C overnight (≥10 h). The overnight culture was diluted to an OD$_{600}$ of 0.1 in 10 mL BMK medium (10.7 g/L potassium phosphate dibasic, 5.2 g/L potassium phosphate monobasic, 20 g/L glucose, 0.88 g/L sodium citrate dihydrate, 0.022 g/L ferric ammonium citrate, 2.5 g/L potassium aspartate, 10 mM magnesium sulfate, 150 nM manganese chloride, 40 mg/L tryptophan, 0.05% yeast extract), then grown in a 125 mL flask at 37 °C with shaking (250 rpm) until cells reached OD$_{600}$~1.5. 150 µL of culture were then mixed with ≥100 ng of plasmid DNA in a deep 96-well plate, covered with a breathable film, and incubated at 37 °C without shaking for 10 min, then incubated at 37 °C with shaking (900 rpm) for 2 h. After 2 h, cells were plated on LB agar containing selective antibiotics (7.5 µg/mL kanamycin, a combination of 1 µg/mL erythromycin and 15 µg/mL lincomycin, 6 µg/mL chloramphenicol or 100 µg/mL spectinomycin [by activity]).

**High-throughput Method**

Double and triple *pbp* knockdown libraries were constructed using high-throughput transformation. Individual wells of a deep 96-well plate containing 300 µL of MC medium were inoculated with single colonies of strains containing P$_{xyr}$-dcas9 and an sgRNA targeting one of the 16 *pbp* genes; this plate was incubated at 37 °C with shaking (900 rpm) for at least 10 h. Cells were then diluted to OD$_{600}$~0.1 in BMK medium, and 25 µL of diluted cells were added to ≥100 ng of plasmid DNA in a shallow, v-bottom 96-well plate, covered with a breathable film, and incubated at 37 °C in a humidified incubator without shaking for 16 h. 50 µL of LB were added to each well and the well contents were plated on LB agar containing selective antibiotics.

**Flow Cytometry**
Strains were grown overnight in LB in deep 96-well plates, and then back-diluted 1:300 into fresh LB containing the appropriate concentration of xylose to induce expression of dcas9. After ~5 h of growth, cells were diluted 1:300 into phosphate buffered saline, and red fluorescence levels (B-A laser) were determined using an LSRII flow cytometer (BD Biosciences). Data for at least 10,000 cells were collected. Median red fluorescence signals were extracted from FCS files using FlowJo (FlowJo, LLC); error bars are from three biological replicates.

RNA-seq
Cells were grown in LB or LB + 1% xylose to OD$_{600}$~0.3. RNA extraction and RNA-seq was performed as previously described (Larson et al., 2014). RNA-seq data was deposited at the Gene Expression Omnibus (GSE74926).

NET-seq
Cells were grown in LB + 1% xylose to OD$_{600}$~0.3. NET-seq was performed as previously described (Larson et al., 2014). NET-seq data was deposited at the Gene Expression Omnibus (GSE74926).

sgRNA Design
The 20-nucleotide guide sequences for our sgRNA library were designed to be effective and specific. We first found all GG dinucleotides in the target genome, and extracted the 20 bases ending one base 5' of that GG as the potential target candidates for the organism. We then scored the specificity of each 20-nt guide by passing all the guides through Bowtie with the genome as the reference, then passing each 19-nt suffix through Bowtie, then each 18-nt suffix, etc. The score for a guide was the shortest suffix for which only a single alignment was found. Lower specificity score numbers were therefore better. If we never found a unique alignment at any length, the score was listed as -1, and the guide was avoided. Next, we annotated each
target with any coding regions overlapping that target. We noted the position of the target relative to the first coding base of the gene. For each gene we chose a target that was as close as possible to the 5’ end of the coding sequence for that gene and that had a low (good) specificity score.

**sgRNA Efficacy Analysis**

To test the efficacy of our essential gene knockdown library, we pinned the library onto rectangular LB agar plates containing 1% xylose to fully induce expression of dcas9. Of the 299 strains in the library, 258 targeted bona fide essential genes as determined by a gold-standard gene deletion analysis that attempted to replace every open reading frame in the *B. subtilis* genome with an antibiotic resistance marker (Koo et al., in preparation). 244 of the 258 (95%) strains showed a colony size defect of at least 25% (Table S2). We then tested the 14 strains that failed to show a colony size defect by measuring growth in liquid LB + 1% xylose using a microplate reader. We found that 5 of the 14 strains showed 50% or less growth compared to the growth of the no-sgRNA control at mid-log phase (OD$_{600} \sim 0.2$; Table S2), bringing the percentage of effective sgRNAs to 97%. Of the nine strains that failed to show growth defects, five target phage genes; these genes may not be truly essential, as the antibiotic resistance marker used in the deletion analysis contains a strong promoter that may upregulate adjacent, toxic phage genes that are normally transcriptionally silent. Alternatively, dCas9 may be acting to repress phage transcription that is normally toxic (this appears likely for sgRNAs targeting the SKIN element repressor, *sknR/yqaE*). In the cases of *yezG* (a putative antitoxin) and *yhdL* (anti-SigM), CRISPRi repression of upstream genes on the same transcript appears to also silence expression of toxic proteins normally controlled by these gene products. We conclude that nearly all sgRNAs targeting essential genes showed repression activity.

**Quantitative PCR**
Overnight LB cultures of *B. subtilis* were diluted 1:1000 in 2 mL LB + 1% xylose. These liquid cultures were grown for 6 h at 37 °C. For RNA extraction, 600 µL of culture were mixed with 600 µL of -20 °C methanol and spun down at full speed for 5 min. Once decanted, the RNA was extracted with the Qiagen RNeasy RNA isolation kit protocol #7. Following RNA isolation, genomic DNA was eliminated with the Ambion DNA-Free kit per manufacturer’s instructions. cDNA was synthesized with the Invitrogen SuperScript III First-Strand Synthesis System. Random hexamers were annealed, and then cDNA was synthesized via SuperScript III RT. cDNA was diluted 10-fold to be used in conjunction with Agilent Brilliant II SYBR Green QPCR Master Mix and 0.6 µM final concentration primers. Primers were designed with Primer3 with the following parameters: 60 °C melting temperature, 200 base pair-amplified region, and amplified region within the middle of the gene. Control genes employed consisted of *sigA*, *gyrA*, and *rpoB*. The qPCR included RNA samples that had DNase treatment, as well as RNase treatment, to verify that no genomic DNA remained. qPCR experiments were conducted in a Stratagene Mx3005P qPCR System. Three biological replicates were used in each qPCR.

**CRISPRi pbp Double and Triple Knockdown Screening**

Double and triple knockdown strains containing two sgRNA plasmids that integrate at either *amyE* or *thrC* were constructed using the high-throughput transformation method described above. Single colony isolates were stored as glycerol stocks in 96-well format. To screen the library, cells were robotically pinned from glycerol stocks onto rectangular LB agar plates in 384-colony format using a ROTOR robot (Singer Instruments), then pinned once more to 1536-colony format. Cells in 1536-colony format were then pinned to LB + 1% xylose plates to fully induce *dcas9* expression. LB plates with PBP inhibitors also contained 1% xylose. Plates were imaged using a Powershot G10 camera (Canon) after ~7-9 h of growth at 37 °C, and colony size was extracted using the “opacity” setting in the Iris software package (Paradis-Bleau et al.,
Colony size was normalized within plates using internal controls that expressed dcas9, but did not contain sgRNAs.

The fitness \( W \) of double (or higher-order) deletion strains can be predicted by multiplying the fitness values of each of the single deletions (deletions of gene x and gene y) that comprise the double (predicted \( W_{xy} = W_x \times W_y \)); this is known as the multiplicative model (St Onge et al., 2007). Large values of the deviation between the measured fitness and the predicted fitness of double deletions (\( \epsilon = \) measured \( W_{xy} \) – predicted \( W_{xy} \)) indicate a genetic interaction. Because our PBP genetic interaction screen used CRISPRi knockdowns rather than gene deletions, it was unclear if the multiplicative rule would apply to our analysis. To test for agreement between the multiplicative model and our CRISPRi screen data, we plotted predicted fitness versus measured fitness for all double knockdown strains (Figure S7A). We found a strong, linear correlation (Pearson’s \( R = 0.95 \)) between predicted and measured fitness, suggesting that the multiplicative model can reliably be applied to our CRISPRi knockdown data.

The small deviation we observed from the ideal multiplicative model (ideal fit: \( y = x \); our fit: \( y = 0.81x + 0.13 \)) may be due to the relatively small sample size of our screen (\( n = 240 \) double knockdowns).

**MAC-0170636 Screening and UppS Activity Assay**

**Cloning, Overexpression, and Purification of B. subtilis UppS**

The gene uppS (GeneBank sequence NC_000964.3) was cloned into the pET-19b vector (Novagene) modified to encode an engineered Tobacco Etch Virus (TEV) protease cleavage site using primers uppS_FWD: CTAGCATATG CTCAACATACTCAAAAATTG and uppS_REV: CTAGCTACGAG CTAAATTCCGCCAAA. Primers used to generate the construct are listed below. UppS was over-expressed in *E. coli* BL21 (Rosetta) using auto-induction (Studier, 2005). Cells were harvested by centrifugation at 6000 × g for 25 min at 4 °C. Cells were suspended in lysis buffer containing 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, 250 KU
rLysozyme (Novagen) and an EDTA-free protease inhibitor tablet (Roche Diagnostics). Cells were lysed using a cell disrupter (Constant Systems Limited, Daventry, UK). Lysates were cleared by centrifugation at 30,000 × g for 30 min at 4 °C. Nickel-affinity chromatography was performed on the lysates using a 30 mL free-flow gravity column and 5 mL of Ni-NTA agarose (Sigma). Once loaded, the column was washed with buffer (10 column volumes) containing 50 mM NaH$_2$PO$_4$ (pH 8.0), 300 mM NaCl, 20 mM imidazole. His-tagged UppS was then eluted in buffer containing 50 mM NaH$_2$PO$_4$ (pH 8.0), 300 mM NaCl, 250 mM imidazole. Elution fractions were dialyzed overnight against 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 1 mM DTT. Once dialyzed, the elution fraction was concentrated using Amicon Ultra centrifugation filters (10kDa cut-off) and quantified using a NanoDrop (Thermo Scientific).

**Assay for UppS Inhibition by MAC-0170636**

A Kinetic EnzCheck pyrophosphate assay (Life Technologies) was used to assess inhibition of MAC-0170636 in vitro in accordance with the manufacturers guidelines. The IC$_{50}$ value was determined in 100 μL reaction volumes using a flat bottom 96-well plate (Costar 3370) in duplicate. Reactions with varying concentrations of MAC-0170636 were conducted with a final concentration of 1% DMSO in the presence of 0.2 mM 2-amino-6-mercapto-7-methyl-purine ribonucleoside (MESG), 0.625 U of purine ribonucleoside phosphorylase (PNP), 0.2 U of inorganic pyrophosphatase (PyroP), 0.125 μg of purified UppS$^{Ba}$ enzyme, 0.82 μM farnesyl pyrophosphate (FPP) (1xKM) and 65 μM isopentenyl pyrophosphate (IPP) (5x KM). For UppS$^{Sa}$, 0.5 μg of purified UppS enzyme, 0.7 μM farnesyl pyrophosphate (FPP) (1xKM), and 80 μM isopentenyl pyrophosphate (IPP) (5x KM) were used in the reaction. Data were fit using GraFit V5 (Erithacus Software).

**CRISPRi Essential Gene Knockdown Screening and Network Construction**

*Essential Gene Knockdown Screening*
sgRNA plasmids were transformed into a strain containing dcas9 (CAG74209) using the standard method described above. Single colony isolates of each transformation were grown overnight in LB + 6 µg/mL chloramphenicol, and stored as glycerol stocks at -80 °C in 96-well plates. Prior to screening, cells were robotically pinned onto rectangular LB agar plates in 384-colony format, and then pinned once more to 1536-colony format using a ROTOR robot (Singer Instruments). To screen the essential library, we pinned from rectangular LB agar plates in 1536-colony format to plates containing the indicated concentration of antibiotic or chemical stress. Each plate contained four technical replicates for each sgRNA strain and each chemical concentration was replicated at least twice; most concentrations had four replicate plates. Chemical concentrations were empirically determined by streaking wild-type B. subtilis 168 onto round agar plates containing chemicals and then visually inspecting the plates for colony size defects. We used concentrations in our screen that inhibited growth by 50% or less; chemical concentrations that inhibited growth such that the median colony size after 14 h was less than the median colony size on LB after 7 h were discarded. Importantly, the effective concentration of chemical in our screening plates is likely to be lower than the concentration added to the plate because of chemical breakdown during the two-day period in which plates were dried at room temperature to reduce problems with colony smearing due to wet plates. Pinned cells were grown on rectangular plates for 7-14 h at 37 °C, and then imaged using a custom light box with a Canon Powershot G10 (Canon).

Data Analysis and Network Construction

Colony sizes were extracted from plate images using the Iris software package (Paradis-Bleau et al., 2014). Spatial effects were normalized using a quadratic function, median and variance of colony sizes were normalized between plates, and S-scores were computed using previously developed software (Collins et al., 2006). False discovery rates were computed from S-scores as previously described (Nichols et al., 2011).
To construct an essential gene network from gene-chemical scores (i.e., S-scores), these scores were correlated (Pearson correlation) in a pairwise manner for all sgRNA knockdown strains, resulting in ~90,000 gene-gene correlations. To establish a cutoff for correlation significance, we estimated the background distribution of correlations by randomly permuting gene identity relative to the chemical-gene scores 5,000 times, and then used the 95% confidence interval of this background distribution (after discarding self correlations that equaled 1) to establish a correlation significance threshold of 0.572 (method adapted from Nichols et al., 2011); this procedure resulted in 412 significant connections between distinct sgRNA knockdown strains. Network data was visualized using Cytoscape v3.2.1 (Cytoscape Consortium; Shannon et al., 2003).

Network Comparison to the STRING Database

Essential operons were defined based on manual curation of global expression data (Nicolas et al., 2012) and functional annotations from the SubtWiki database (http://subtiwiki.uni-goettingen.de/). For any essential operon with genes in different annotations, the operon was split into subsets of genes with the same annotation; correlations between genes in the same operon were eliminated from the analysis. Pairs of essential operons were defined as interacting if any pair of genes spanning both operons had a correlation above the threshold value 0.572. The connectivity between two functional groups was defined as the number of essential operon interactions between the two groups. If two genes within the same essential operon fell into distinct functional groups, the interaction between them did not contribute to the functional group connectivity. Similarly, the interaction between operons that have been classified as single operons in other studies did not contribute to the functional group connectivity.

Comparison to the STRING database was achieved by downloading the *Bacillus subtilis* 168-specific interactions scores from http://string-db.org/ (Szklarczyk et al., 2015). Scores were culled to the “experimental” or “database” categories. Genetic interactions in the STRING
database were combined into essential-operon interactions in an equivalent manner to our chemical genomics data.

Annotation distance analysis between essential gene operons was performed using annotations from the SubtiWiki online database (http://subtiwiki.uni-goettingen.de/). SubtiWiki annotations are broken down into four levels: the first level is very general (e.g., metabolism) and the fourth level is specific (e.g., RNA polymerase). In cases in which there were only three levels of annotation, the annotation from the third level was also used for the fourth level (Table S3). Operons with the same annotation at the fourth level (most specific) were given an annotation distance of 0, while operons with no overlapping annotations were assigned a 4. As the annotation distance was subject to bias based on the extent of annotation (e.g., whether or not strains were annotated at the fourth level), we suggest that the distance be interpreted as a qualitative metric for annotation differences.

High-resolution Liquid Growth Curve Acquisition and Analysis

All strains were grown in deep 96-well plates for 18 h, then back-diluted 1:200 into 200 µL fresh LB (for induced growth curves, LB was supplemented with 1% xylose, and the overnight cultures were grown for 26 h) and grown with shaking at 37 °C in an Infinite M200 plate reader (Tecan) for 7 h. The absorbance at wavelength 600 nm (OD$_{600}$) was measured at 7.5-min intervals.

For uninduced growth curves, the natural logarithm of the optical density was fit to the Gompertz equation (Zwietering et al., 1990) to determine apparent lag time, maximum specific growth rate, and from that, the doubling time. Each growth curve was fit individually.

For induced growth curves, several criteria were used to classify the curves into different categories: 1) no growth: OD$_{600}$<0.06 throughout the measurement; 2) linear growth: final OD$_{600}$>0.2, Pearson correlation coefficient >0.98 between time and OD$_{600}$ after removal of the lag and saturation regions of the curve, and the slope at the end of the growth phase is >90% of
the slope at the beginning of growth; 3) growth and death: maximum OD$_{600}$ is $>1.2 \times$ the final OD$_{600}$, with final OD$_{600}>0.06$; 4) others: strains that do not fall into any of the previous three categories.

**Viable Counts**

Strains were grown in deep 96-well plates for 18 h. The OD of each overnight culture was measured using a Genesys 20 spectrophotometer (Thermal Scientific). Overnight cultures were serially diluted in LB, and at each dilution, 100 µL of culture were plated on LB. Plates were incubated overnight at 37 °C, and the dilutions with ~100 colonies were used to count the number of colony forming units (CFUs).

Each overnight culture used for CFU counting was also diluted 1:100 in fresh LB, and 1 µL of the diluted culture was spotted onto a pad of 1.5% agarose in fresh LB. The pads were incubated at 37 °C for 2 h and then imaged. Dead or non-growing cells remained small and isolated from other cells, and live cells grew into collections of larger cells. The number of dead/non-growing cells and live cells were manually counted for each strain; a total of at least 200 cells were counted for each strain.

**High-throughput Imaging and Analysis**

For phase-contrast and fluorescence imaging, cells were imaged on a Nikon Eclipse TE inverted fluorescence microscope with a 100X (NA 1.40) oil-immersion objective. Images were collected using an Andor DC152Q sCMOS camera (Andor Technology, South Windsor, CT, USA) and µManager v1.3 software (Edelstein et al., 2010). For time-lapse imaging, cells were maintained at 37 °C during imaging with an active-control environmental chamber (HaisonTech).

Custom MATLAB (MathWorks) image processing code was used to segment cells and identify active cell contours from phase microscopy (Monds et al., 2014; Ursell et al., 2014). Cell
widths and lengths were calculated using the MicrobeTracker meshing algorithm (Sliusarenko et al., 2011). For all single-cell quantification, at least 100 cells were analyzed for each strain. Enrichment of morphological defects in each functional group was identified by conducting a two-sample KS test between the average length trend line deviations of strains corresponding to a functional group, and the entire collection background. For \( pbp \) strains, cell lengths were approximated by half of the contour lengths, and cell bending was defined as the minimum negative curvature of the cell contour.

**Terminal Phenotypes**

*Microscopy*

Cells were back-diluted 1:200 from overnight culture into fresh LB and grown at 37 °C for 2 h in a plate shaker, and then 1 \( \mu \)L of cells was spotted onto a pad of 1.5% agarose in fresh LB supplemented with 1% xylose. Cells were incubated on the pads overnight at room temperature, and then imaged. For imaging under antibiotic treatment and xylose titration, the appropriate concentrations of antibiotics or xylose were added to the agarose pads. For staining, 1 \( \mu \)g/ML DAPI (Invitrogen) and/or 2 \( \mu \)g/mL FM4-64 (Invitrogen) were added to the pads.

*Analysis*

Terminal phenotype for each essential gene knockdown was manually classified using an eight-dimensional phenotype vector encompassing features of lysis, bulging, uniform shape loss, bending, filamentation, over-division (shorter cells), and level of post-induction growth. Severe bulging, filamentation, or lysing phenotypes were classified as dominant such that secondary phenotypes were ignored. A given functional group of essential operons was described by its fractional composition of terminal phenotypes from essential operons in that group. The cosine similarity of terminal phenotypes between functional groups was calculated between the normalized fractional composition vectors.
SUPPLEMENTAL REFERENCES


