Automatic policing of biochemical annotations using genomic correlations

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With the increasing role of computational tools in the analysis of sequenced genomes, there is an urgent need to maintain high accuracy of functional annotations. Misannotations can be easily generated and propagated through databases by functional transfer based on sequence homology. We developed and optimized an automatic policing method to detect biochemical misannotations using context genomic correlations. The method works by finding genes with unusually weak genomic correlations in their assigned network positions. We demonstrate the accuracy of the method using a cross-validated approach. In addition, we show that the method identifies a significant number of potential misannotations in Bacillus subtilis, including metabolic assignments already shown to be incorrect experimentally. The experimental analysis of the mispredicted genes forming the leucine degradation pathway in B. subtilis demonstrates that computational policing tools can generate important biological hypotheses.

RESULTS
Strategy of the computational approach
The algorithm presented in this study identifies genes that have either unusually poor genomic correlations with their network neighbors, or alternative network locations with significantly better correlations. The problems of assigning the correct function and identification of misannotations have different objectives and require different algorithms. In many cases, it is possible to reject an existing annotation based on poor genomic correlations, while these correlations are not strong or unique enough to accurately predict the correct function.

Similar to our previous studies, we represent the metabolic network as a graph with nodes being metabolic genes and edges being connections established by shared metabolites (see Methods). Suppose two genes X and Y in different organisms are annotated to catalyze the metabolic activity specified by the EC number 1.2.3.4 (Fig. 1). The developed approach will suggest that the annotation of the gene X is likely to be correct due to strong context-based correlations with neighboring genes. On the other hand, the gene Y displays poor genomic correlations to its network neighbors, and its annotation is likely to be an error.

To predict potential misannotations, we integrated sequence and context correlations using the AdaBoost algorithm with alternating decision trees. AdaBoost has been successfully applied to several large-scale integration problems in biology, including prediction of gene regulatory response and identification of genes responsible for orphan metabolic activities. The AdaBoost algorithm was trained with a collection of context genomic descriptors: phylogenetic profiles, mRNA co-expression, chromosomal distance between genes, gene clustering across genomes and protein fusion. For each descriptor, we considered two different scores: the largest pair-wise correlation between the target gene and its direct network neighbors and the average fitness score in the assigned network location calculated as described in the Methods.

The average fitness score quantifies the overall context correlations of the target gene with all its network neighbors. To represent the relative fitness of the existing annotation, the AdaBoost score for
In the second method (TN2), to simulate misannotations due to a residual sequence homology to non-native metabolic activities, genes were only reassigned to incorrect activities for which they had >30% sequence identity. A random choice was made if several reassignments were possible for a gene. The classification algorithm was then independently trained using TN2 examples (Fig. 2a). The mean area under the ROC curve for the TN2 set, based on four independent reassignment experiments, was 0.93 (95% CI: 0.90–0.95). In spite of the large fraction (40%) of misannotations in the reassigned network, the algorithm identified about 90% of true misannotations, with only 20% of correct annotations misclassified as misannotations.

Finally, in the third method (TN3), the genes were reassigned only if they had similar (within 10%) or even higher sequence identities to the newly assigned (incorrect) activities. This test simulated misannotations that are especially difficult to detect using sequence homology. In total, 26% of the network genes were reassigned using the third method. The mean area under the ROC curve for the TN3 examples (Fig. 2a), based on four independent reassignment runs, was 0.87 (95% CI: 0.86–0.88). The algorithm identified about 80% of misannotations while misclassifying 20% of correct annotations. Because many metabolic assignments in existing databases have been made based primarily on sequence homology, it is likely that the errors simulated using the second and the third methods dominate real world misannotations.

To understand the transferability of our approach to other species, we repeated the analysis using the curated Escherichia coli metabolic model iJR904 (ref. 28). The negative examples TN2 and TN3 for the bacterial metabolic model were generated in the same way as for the yeast network. The classifiers optimized for the yeast network were directly applied to the bacterial model without further modification or optimization. The resulting performance for the E. coli network was similar to that for S. cerevisiae (Supplementary Fig. 1). Consequently, the optimized method is able to detect misannotations in different species. The policing approach should also be quite effective in non-model organisms because the context correlations, with the exception of co-expression, can be calculated directly from genomic sequences; the decrease in sensitivity without expression information was less than 3% (at 25% false positive rate). The accuracy of other context correlations will only improve as more genomes are sequenced.

Potential misannotations in B. subtilis metabolic network

To test our algorithm on a less-studied network, we applied it to the model Gram-positive bacterium B. subtilis. We investigated the B. subtilis metabolic annotations available in KEGG29 (655 genes), Swiss-Prot30 (528 genes) and MetaCyc30 (369 genes). The different number of annotated genes in these databases is a consequence of different annotation strategies. While some databases strive for maximum coverage, others focus on the most accurate annotations. There are 277 B. subtilis annotations shared by all three databases and an additional 122, 10 and 20 unique annotations in KEGG, MetaCyc and Swiss-Prot, respectively. We applied the developed algorithm to all B. subtilis metabolic assignments in the three databases using the parameters optimized for the TN3 yeast examples. The cumulative distributions of the AdaBoost classification scores for B. subtilis annotations (Fig. 2b) show that the metabolic assignments shared by all databases (red curve) are on average more accurate compared to annotations present exclusively in a single database (black curve, Kolmogorov-Smirnov test \( P = 2 \times 10^{-18} \)). Nevertheless, the database-unique annotations display, on average, significantly better scores compared to the scores of misannotated genes (TN3 yeast examples, blue curve, \( P = 2 \times 10^{-4} \)). This demonstrates that it is not possible to detect potential misannotations simply by identifying database-unique functional assignments.
Based on the ROC characteristics (Fig. 2a), the most efficient part of the TN3 curve allows identification of 70% of misannotations, while classifying only about 5% of correct assignments as misannotations. Considering the total number of analyzed B. subtilis metabolic assignments (679) and assuming that about 10% of the database assignments are misannotations4,5, the red point in Figure 2a corresponds to the analysis of 80 genes with the worst classification scores; about half of these genes should represent true misannotations. Indeed, we manually analyzed the list of 80 genes with the worst classification scores, and for 34 cases we either found counter evidence or could not identify any experimental study supporting the annotations (Table 1). Although the potential misannotations usually have weak sequence homology (usually <40% identity) to known enzymes, the classifier is not simply relying on homology to identify misannotations. For about 35% of the annotations with good classification scores, sequence identity was also weak (<40%), but these metabolic assignments are supported by good context-based correlations.

For each potential misannotation, we show in Table 1 the gene name, annotation source, the highest sequence identity to enzymes responsible for the annotated activity in other species, the relative strength of various context-based correlations and the existence of good alternative network locations (see Supplementary Methods). In the table the context correlation values are represented by their relative percentile ranks based on the average fitness scores (see Methods). For example, the “expression profile” rank of 10% indicates that the target gene has better co-expression scores in 10% of all possible network locations compared to the location assigned in the database. Overall, the results in Table 1 suggest that Swiss-Prot and MetaCyc are more conservative in their functional assignments compared to KEGG, which has the largest number of annotations and potential misannotations. We want to emphasize that the majority of KEGG-unique annotations displayed good confidence scores, indicating that only a fraction of them are likely to be incorrect.

The B. subtilis gene dgkA is a typical example of a potential misannotation. The gene is annotated in all considered databases as “diacylglycerol kinase” (DagK, EC 2.7.1.107), possibly based on weak sequence homology. However, dgkA has poor context-based correlations with the network neighbors of the EC 2.7.1.107 activity (Table 1). In a recent study6, the authors confirmed that dgkA is not a diacylglycerol kinase but rather an undecaprenol kinase. Another example is the B. subtilis gene ywrD, which is annotated in KEGG as an ortholog of the γ-glutamyltransferase (EC 2.3.2.2). Weak context-based correlations (Table 1) with neighboring network genes suggest that ywrD is unlikely to catalyze the EC 2.3.2.2 function. The γ-glutamyltransferase activity (EC 2.3.2.2) is required for growth on extracellular glutamyl compounds, such as glutathione (GSH, 1), as the source of sulfur. However, a ywrD-null mutant was demonstrated12 to grow well on minimal medium with GSH as the sole sulfur source. In addition, histidine tag–purified ywrD could not hydrolyze GSH. These findings strongly suggest that ywrD does not encode a γ-glutamyltransferase. Further analysis of each case in Table 1 is presented in Supplementary Table 1.

### Leucine degradation pathway in B. subtilis

The developed method can be used to identify suspicious functional assignments for several genes in a pathway. An example is the yngI/HGFE gene cluster in B. subtilis (Fig. 3a). The yngI gene is listed in KEGG as a hypothetical protein, yngI is listed as acyl-CoA synthetase (EC 2.3.1.86) (until recently it was listed as long-chain fatty acid-CoA ligase, EC 6.2.1.3), yngH is listed as the acetyl-CoA carboxylase biotin carboxylase subunit (EC 6.4.1.2/6.3.4.14), yngG is listed as hydroxymethylglutaryl-CoA lyase (EC 4.1.3.4), yngF is listed as enoyl-CoA hydratase (EC 4.2.1.17) and yngE is listed as propionyl-CoA carboxylase β chain (EC 6.4.1.3). In MetaCyc, yngE is listed as similar to propionyl-CoA carboxylase and yngF is listed as enoyl-CoA hydratase (EC 4.2.1.17). In Swiss-Prot, yngI is listed as probable acyl-CoA dehydrogenase (EC 1.3.99), yngH is listed as biotin carboxylase 2 (EC 6.3.1.4/6.4.1.2), yngG is listed as hydroxymethylglutaryl-CoA lyase (EC 4.1.3.4) and yngF is listed as putative enoyl-CoA hydratase/isomerase.

Our algorithm predicted as potential misannotations the assignments of the EC 6.4.1.3 function to yngE, EC 4.2.1.17 to yngF and
Table 1 | Potential misannotations in the B. subtilis metabolic network

| Gene name | Annotated function (EC number) | Homology score | Phylogenetic profile rank (%) | Expression profile rank (%) | Gene distance rank (%) | Protein fusion? | Significantly better alternative location? |
|-----------|-------------------------------|----------------|-----------------------------|---------------------------|------------------------|---------------|---------------------------------|
| adhB      | 1.1.2.284 (K)                 |                | 90                          | 90                        | 83                     | N             | Y                              |
| alaT      | 2.6.1.17 (K)                  |                | 58                          | 23                        | 28                     | N             | Y                              |
| bcsA      | 2.3.1.74 (K, S, M)            |                | 29.7/10-04                  | 74                        | 79                     | S             | Y                              |
| bsaA      | 1.11.3.9 (K, S, M)            |                | 55.2E-51                    | 47                        | 57                     | M             | Y                              |
| Cad       | 4.1.11.8 (M)                  |                | 22.8/2E-14                  | 45                        | 80                     | S             | Y                              |
| dgkA      | 2.71.107 (K, S, M)            |                | 32.3/2E-11                  | 64                        | 21                     | M             | Y                              |
| hipO      | 3.5.1.32 (K, M)               |                | 35.9/6E-59                  | 45                        | 64                     | K             | Y                              |
| Pps       | 2.79.2 (K, M)                 |                | 43.5/0.002                  | 44                        | 38                     | 71            | Y                              |
| xpt       | 2.4.2.7 (M)                   |                | 29.2/5E-07                  | 4                         | 1                      | 7             | Y                              |
| ybbD      | 3.2.1.52 (K)                  |                | 34.2/1E-27                  | 49                        | 1                      | 54            | Y                              |
| ycgT      | 1.8.19 (K)                    |                | 29.8/2E-25                  | 50                        | 21                     | 33            | Y                              |
| yhcV      | 1.1.205 (K)                   |                | 37/0.002                    | 22                        | 68                     | 44            | Y                              |
| yhdR      | 2.6.1.1 (K)                   |                | 30.1/3E-30                  | 2                         | 1                      | 9             | Y                              |
| yhfR      | 5.4.2.21 (K)                  |                | 38.3/1E-12                  | 22                        | 65                     | 22            | Y                              |
| yisP      | 2.5.1.32 (K)                  |                | 27.8/8E-24                  | 87                        | 49                     | 60            | Y                              |
| yitC      | 3.1.3.71 (K, S)               |                | 38.7/4E-18                  | 87                        | 42                     | 72            | Y                              |
| yjmC      | 1.1.3.7 (K)                   |                | 39.8/2E-60                  | 68                        | 30                     | 48            | Y                              |
| yktC      | 3.1.3.25 (K, S)               |                | 38.1/2E-28                  | 73                        | 49                     | 49            | Y                              |
| ykuR      | 3.5.1.47 (K)                  |                | 35.6/3E-43                  | 75                        | 70                     | 50            | Y                              |
| yngF      | 6.4.1.3 (K)                   |                | 40.1/8E-92                  | 1                         | 4                      | 2             | Y                              |
| yngF      | 4.2.1.17 (K, M)               |                | 38.9/5E-39                  | 1                         | 2                      | 2             | Y                              |
| yngI      | 6.2.1.3 (K)                   |                | 31.6E-63                    | 1                         | 10                     | 56            | Y                              |
| yoaD      | 1.1.95 (K)                    |                | 33.8/1E-39                  | 1                         | 1                      | 24            | Y                              |
| yogA      | 1.1.11 (K)                    |                | 29.7/2E-21                  | 39                        | 81                     | 71            | Y                              |
| yqhT      | 3.4.11.9 (K)                  |                | 34.9/4E-22                  | 50                        | 54                     | 11            | Y                              |
| yrhE      | 1.2.1.2 (K)                   |                | 37.5/1E-129                 | 2                         | 60                     | 58            | Y                              |
| ysfC      | 1.1.3.15 (K)                  |                | 27.3/4E-10                  | 55                        | 66                     | 76            | Y                              |
| yumB      | 1.6.99.3 (K)                  |                | 26.3E-25                    | 1                         | 18                     | 26            | Y                              |
| yumC      | 1.8.19 (K)                    |                | 29.3/1E-21                  | 81                        | 32                     | 35            | Y                              |
| yvcN      | 2.3.1.5 (K)                   |                | 28.6/6E-13                  | 78                        | 36                     | 77            | Y                              |
| yvcT      | 1.11.215 (K, S)               |                | 47.3/8E-79                  | 53                        | 59                     | 47            | Y                              |
| ywD       | 2.3.2.2 (K)                   |                | 31.4/9E-55                  | 25                        | 85                     | 43            | Y                              |

The data in the table are based on annotations available in February 2009. Annotation source: K, KEGG; M, MetaCyc; S, Swiss-Prot. Homology score is the highest protein-protein sequence identity to another Swiss-Prot protein with the target activity; the corresponding BLAST E-value is also shown. The context genomic correlations are represented as the relative percentile ranks. For example, the “expression profile” rank of 20% indicates that the target gene has better co-expression values in 20% of all other possible network locations compared to the location assigned in the database. Lower percentile ranks indicate better consistencies with genomic context correlations. For the protein fusion, “Y” (“N”) indicates that fusion events between an ortholog of the candidate gene and a network neighbor were detected (not detected). The presence of a significantly better alternative location (“Y”/”N”) was determined by the ALR ratio as described in Supplementary Methods.

EC 6.2.1.3 to yngI. These genes have considerably better genomic correlations in different network locations (functions): yngF in EC 6.4.1.4, yngF in EC 4.2.1.18 and yngl in EC 6.2.1.16. Overall, the yng genes form the consecutive reactions in the leucine (2) degradation pathway. Based on the predicted functional assignments, we can also suggest the likely functions for yngl (EC 1.3.99.10) and yngH (EC 6.4.1.4 subunit, forming the enzyme complex with yngE). Consequently, the yng cluster is likely to form a complete degradation pathway from 3-methylbutanoyl-CoA (3) to acetoacetyl-CoA (4), which can be further catalyzed by the bacterial citric acid cycle.

What is the biological role of the leucine degradation pathway in B. subtilis? In early stages of sporulation, B. subtilis cells divide into two unequal compartments. The smaller compartment develops into a bacterial spore, and the larger compartment forms the mother cell, which protects and nurtures the spore until the spore is fully developed. Notably, the yng genes are under transcriptional control of the σE factor and are primarily expressed early in the mother cell during sporulation—that is, when extracellular nutrients are limited. The expression of the gene yngA, which is responsible for the last step of leucine catabolism—acetoacetyl-CoA to acetyl-CoA (5) conversion (EC 2.3.1.9; see Fig. 3a)—is also controlled by the σE factor.

Owing to the structure of its citric acid cycle, B. subtilis cannot grow on leucine as the sole carbon source. Nevertheless, the catabolism of the leucine and fatty acids through the citric acid cycle can provide additional energy during early sporulation stages. The selection of the energy source becomes logical if one considers the membrane and amino acid composition of B. subtilis. Leucine is one of the most abundant amino acids in logarithmically growing B. subtilis cells; it is responsible for about 8–10% of all protein residues (see also Supplementary Fig. 2). In addition, B. subtilis lipids are
predominantly (>90%) composed of branched chain fatty acids, odd-iso fatty acids can be oxidized to 3-methylbutanoyl-CoA. Sequence identity of yngf to EC 6.2.1.3 (31%) and yngf to EC 1.3.99.2 (48%) suggests that these genes may also directly participate in the fatty acid degradation pathway. It is likely that during sporulation, branched chain fatty acids and amino acids are present in the extracellular media due to the bacterial cannibalism process, which allows a fraction of B. subtilis cells to kill their nonsporulating siblings and feed on the released nutrients.

To experimentally investigate the role of the yng cluster during sporulation, we used $^{13}$C labeling experiments. First, we analyzed B. subtilis 168 cells in nonsporulating minimal medium supplemented with [U-$^{13}$C]leucine (see Methods). Because the degradation pathway leads from leucine to acetyl-CoA (Fig. 3a), we measured the fractional labeling of the acetyl-CoA m2 mass isotopomer using LC-MS/MS (see Methods) and calculated the fraction of acetyl-CoA originating directly from leucine. No $^{13}$C labeling above the natural abundance of the m2 isotopomer (8%) was detected in cells during vegetative growth. This result confirmed that the leucine degradation pathway is not active during favorable environmental conditions.

Next, we investigated the activity of the leucine pathway during sporulation. It was previously shown that 2.5 h after the start of sporulation the activity of $\sigma^t$-regulated genes is at the highest. We inoculated bacterial cells into sporulation medium supplemented with [U-$^{13}$C]leucine, and extracted metabolites after 2.5 h. In sporulating cells the fraction of acetyl-CoA derived from leucine was about 2.5–3 times higher than background, while all yng mutants displayed essentially background labeling levels (Fig. 3b). Consequently, the yng pathway is indeed active during sporulation.

Several genes from the yng cluster have been assigned in KEGG to the isoleucine (6) degradation pathway: yngE as an ortholog of EC 6.4.1.3, yngF as an ortholog of EC 4.2.1.17. To investigate the possibility that the yng genes also play a role in the degradation of isoleucine to acetyl-CoA, we tested the activity of the isoleucine degradation pathway during sporulation. Similar to the leucine experiments, we measured the labeling of acetyl-CoA in sporulation conditions supplemented with [U-$^{13}$C]-isoleucine. No labeling above background was detected (Supplementary Fig. 3). Consequently, the yng genes are unlikely to participate in the isoleucine degradation. Although B. subtilis can utilize isoleucine and valine (7) as the sole nitrogen source, our experiments demonstrate that either the isoleucine pathway is not active during sporulation or its products are not primarily degraded to acetyl-CoA.

**DISCUSSION**

The main idea of the presented approach is to use functional genomic correlations essentially in reverse. Instead of using them to assign protein function, we utilize the correlations to predict potential misannotations. The developed method, or similar approaches, can be automatically applied to many thousands of metabolic assignments in various functional databases. Based on this analysis the potential misannotations can be marked with corresponding confidence scores. As topologies of protein-protein interaction networks are discovered, similar methods can also be developed and optimized to identify misannotations in the context of molecular interaction networks. Importantly, the developed method was not conceived as a criticism of such valuable resources as Swiss-Prot, KEGG and MetaCyc. Our results clearly demonstrate that the majority of annotations in these databases are correct. Nevertheless, we think that the method can help the existing resources to improve the annotation quality and reduce the spread of misannotations.

**METHODS**

**Metabolic networks construction.** The metabolic networks were constructed using known enzymatic reactions for the considered organisms: the iLL672 model for S. cerevisiae, the iJR904 model for E. coli, and B. subtilis metabolic reactions from KEGG, MetaCyc and Swiss-Prot. Only genes with assigned EC numbers were considered; activities representing nonmetabolic reactions, such as EC 2.7.11.1 (nonspecific serine/threonine protein kinase) or EC 2.7.7.6 (RNA polymerase), were excluded. Each metabolic network was represented as a graph with nodes as metabolic genes and edges as functional connections established by metabolites shared between enzymes. The shortest path between a pair of nodes was used as the metabolic network distance between the corresponding genes. The 40 most connected co-factors and metabolites were not considered in calculating metabolic distances (Supplementary Table 2).

**Context genomic correlations.** We used the following context correlations: phylogenetic profiles, mRNA co-expression, chromosomal distance, gene clustering (chromosomal co-localization across a set of genomes) and fusion of protein domains. The phylogenetic profile correlations were constructed using BLASTP searches, using E-value cutoff $10^{-7}$, against a collection of 70 evolutionarily distinct genomes; pair-wise phylogenetic profiles were calculated using Pearson’s correlation coefficient. The co-expression values were calculated using Spearman’s rank correlation between expression profiles obtained from the Rosetta Compendium dataset for S. cerevisiae, Stanford Microarray Database (SMD) for E. coli and the GEO database for B. subtilis. The physical distance between genes from target genomes was used as a chromosomal distance. To calculate the chromosomal clustering of genes across genomes, orthology mapping was established using the KEGG SSDB database; the chromosomal clustering values were calculated based on a collection of 105 diverse genomes. A pair of genes was considered fused if at least 70% of each protein could be aligned to.
nonoverlapping regions of a third protein in the US National Center for Biotechnology Information NR database (using BLAST E-value cutoff 10⁻²). Detailed descriptions of the data sources and the methods used to calculate the context-based correlations are given in our previous publications²²,²³.

**Context-based fitness functions.** We calculated the “fitness” of every gene in its assigned network position using the following equation:

\[
F(x) = \frac{1}{N} \sum_{i=1}^{R} \sum_{y \in \text{Layer}_y} w_i \cdot c(x,y)^\beta
\]

where \(x\) is the gene to be tested at the target network position, \(y\) is a neighboring gene from the \(i\)th network layer, \(c(x,y)\) is a context-based correlation between genes \(x\) and \(y\), \(w_i\) is the weight factor for Layer \(i\), and \(\beta\) is the optimized power factor for the context-based correlation. The summation in equation (1) is, first, over all genes in a given Layer; around the network position of the tested gene and, second, over all layers up to the layer \(R\) (\(R = 3\) in our calculation). \(N\) is the total number of genes in all considered layers. The parameters for each context-based method were optimized using a simulated annealing (SA) algorithm⁴ so that the log sums of the ranks of the correct functions for all known metabolic functions were minimized.

**Sequence homology information.** The sequence homology descriptor of protein function was represented as the highest sequence identity to a Swiss-Prot protein (using BLAST E-values cutoff 5 × 10⁻³) annotated to carry out the target function excluding genes that are (i) from the query genome or (ii) likely annotated based on computational methods—that is, genes with keywords “probable,” “like,” by similarity,” “hypothetical” or “putative” in their annotations.

**Combining sequence-based and context-based descriptors.** All context and sequence homology descriptors were combined using the AdaBoost algorithm with two additional AdaBoost scores: (i) the total score for the network neighbors. For each target gene, we also supplied the classification to all other network positions. For each context descriptor, we consider two context correlation ranks of the target gene at the annotated location compared with the target gene. The performance of the method was benchmarked using the performance.

**Cross-validation.** The performance of the method was benchmarked using the 5/50 cross-validation in which all samples were randomly divided into two sets with approximately equal numbers of TN and TP cases. Results from the two sets were pooled to estimate the overall performance. We also applied multivariable logistic regression to combine the different descriptors and predict misannotations. Although the AdaBoost algorithm tends to slightly outperform logistic regression, a comparable performance was observed for the two methods (Supplementary Fig. 4). All results reported in the paper are based on the AdaBoost algorithm.

**Labeling experiments.** *B. subtilis* 168 mutants (yngE-null, yngF-null, yngG-null, yngH-null, yngL-null and yngN-null mutants) were obtained from the Medicago Main Collection. Growth of these strains was tested using the minimal medium M9 supplemented with various carbon sources. The strains were grown on sporulation agar medium (DSM) and incubated overnight at 37 °C. On the following day, cells were inoculated into sporulation medium supplemented with 5 mM of [U-¹³C]-leucine or [U-¹³C]-isoleucine (Cambridge Isotope Laboratories) at the beginning of the growth curve. The cells were harvested 2.5 h after the onset of the sporulation.

Cellular metabolites were extracted using EtOH:H₂O (60:40) and 10 mM ammonium acetate solution at 70 °C. Cell debris was removed from the extract by centrifugation and the supernatant was completely dried. Samples were injected in an LC-MS/MS (Agilent) with a C18 column (Waters Atlantis T3 150x2.1x3). The identity of the peaks was established by verifying the peak retention time and mass spectrum for each mass isotope of acetyl-CoA. The natural (background) abundance of the m₂ isotope of acetyl-CoA (8%) was calculated by Analyst software (Agilent).

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T.-L.H., L.C. and D.V. performed computational research and data analysis. D.V. conceived and directed computational research. O.R. performed experimental research and analysis. U.S. conceived and directed experimental research. L.C., T.-L.H. and D.V. cowrote the paper. All authors read and edited the manuscript.

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