SUPPLEMENTARY METHODS

Statistical analysis of the Tn-seq output.

The output of the Tn-seq analysis pipeline for the chromosomal genes was used in the fitness classification of genes as follows. First, all genes with no observed insertions (i.e., no reads) were classified as essential. Although this step may result in small genes (that lack insertions by chance) being falsely annotated as essential, manually checking these genes showed many are expected to be truly essential (e.g. ribosomal proteins), supporting the inclusion of this step. Next, GEI scores were imported into R version 3.2.3 and log transformed. Initial clustering of the log transformed GEI scores into fitness categories was performed using the *Mclust* function of the *Mclust* package in R [1]. In short, this function attempted to explain the distribution of GEI values by fitting a series of overlapping Guassian distributions, with the number and shape of the distributions determined by *Mclust* (Bayesian Information Criteria was used to determine the optimal number of distributions [and hence clusters] that best explains the data). The data were then assigned to different categories based on the probability of the data point arising from each of the distributions. Sample output data from *Mclust* showing the distribution of the data and the clustering is provided as Figure S16. As high uncertainty in the classification of genes at the borders of groupings exists, the clusters were refined through the use of affinity propagation implemented by the *apcluster* function of the *apcluster* package of R [2], which groups data points in a method independent of *k-means* or Gaussian distributions. All genes belonging to the same group as determined by affinity propagation implemented in *apcluster* that contained an essential gene, as determined in any of the previous steps, were re-annotated as essential. Additionally, all genes belonging to the same group in the *apcluster* output that spanned the border of two groups as determined by *Mclust* were transferred to the same classification, based on which cluster the genes had a higher median probability of being derived from in the *Mclust* analysis. Finally, genes that were classified as 'essential' in one medium and 'large growth impairment' in the second medium, but that were identified as having no medium specificity based on clustering of the fold changes between media, were considered as essential in both media. Few genes were impacted by this final step, and these were those that straddle the threshold between these two fitness categories. The reclassification was performed based on the logic that a gene cannot i) contribute to growth equally in both media but ii) be essential in only one of the two media.

Genes with GEI scores significantly different between conditions were determined as follows. The GEI fold changes between the conditions of interest were imported into R, log transformed, and the following clustering performed independently for each fold change comparison. The log transformed fold changes were clustered using *Mclust* in R as described above for the GEI scores. In the case of the media comparisons, three clusters were produced by *Mclust*: 'Little to no difference', 'Moderate difference', and 'Large difference'; only genes with a fold change classified as 'Large difference' were considered to display growth medium specificity. In the case of comparing between strains, only two clusters were produced by *Mclust*: 'Little to no difference' and 'Difference between strains'. Sample output data from *Mclust* showing the distribution of the fold change data and the clustering is provided in Figure S16, which helps to illustrate why there is a difference in the number of categories for these comparisons. These clusters were then refined using *apcluster* in R as described above for the GEI scores.

Gene functional enrichments.

Assignment of chromosomal genes into specific functional categories was performed largely based on the annotations provided in the *S. meliloti* Rm1021 online genome database (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi). This website pulls annotations from several databases including PubMed, Swissprot, trEMBL, and Interpro. Additionally, it provides enzyme codes, PubMed IDs, functional classifications, and suggested Gene Ontology (GO) terms for most genes. The numerous classifications were simplified to 18 functional categories, designed to adequately cover all core cellular processes. Each category was based on a generic GO term, and the categories (with related GO terms in parentheses) were: signal transduction (GO:0007165), lipid metabolism (GO:0006629), amino acid metabolism (GO:0006520), translation (GO:0006412), electron transport (GO:0009055), transport (GO:0006810), carbohydrate metabolism (GO:0005975), nucleic acid metabolism (GO:0090304), cell division (GO:0051301), motility and attachment (GO:0048870 and GO:0022610), oxidoreductase activity (GO:0016491), cofactor metabolism (GO:0051186), DNA metabolism (GO:0006259), transcription (GO:0097659 plus GO:1903506), cell wall (GO:0042546), hypothetical (no GO term), and miscellaneous (no GO term).

Occasionally, ambiguous or conflicting annotations were observed. In these cases, protein BLASTp searches through the National Center for Biotechnology Information (NCBI) server were performed against the non-redundant protein database. If putative domains were detected within the amino acid sequence, a combination of the best hit (lowest E-value) and consensus among domain annotations were used to categorize the gene in question. If no putative domains were detected, the functional annotation was based on the best scoring protein hits in the database (E-value threshold of $1x10^{-25}$). The functional annotations of all chromosomal genes are provided in Data Set S6.

Development of iGD1575b.

For *in silico* analysis of redundancy in the *S. meliloti* genome, the previously published *S. meliloti* genome-scale metabolic model iGD1575 [3] was modified slightly. As indicated in Table S8, the biomass composition was updated to include 31 additional compounds at trace concentrations, including vitamins, coenzymes, and ions, in order to ensure the corresponding transport or biosynthetic pathways were essential. However, the original model iGD1575 was unable to produce vitamin B12 (calomide; cpd00166) and holo-carboxylate (cpd12847), as the addition of either compound into the biomass reaction prevented growth of the model. To rectify this, the reversibility of rxn00792_c0 was changed from 'false' to 'true', as this reaction must function in the reverse direction for production of holo-carboxylate, and the reactions rxn01609, rxn06864, and rxnBluB were added to the model. However, no new genes were included in the model. This updated model was termed iGD1575b and is available in SBML and MATLAB format in File S2.

Simulating the removal of pSymA and pSymB *in silico*.

Several modifications to iGD1575b were required in order to produce a viable model following the deletion of all pSymA and pSymB genes. As described previously [3], succinoglycan was removed from the biomass composition, 'gapfill' GPRs (gene-protein-reaction relationships) were added to the reactions 'rxn01675_c0', 'rxn01997_c0', 'rxn02000_c0', and 'rxn02003_c0' in order to allow the continued production of the full LPS molecule, as well as to 'rxn00416_c0' to allow asparagine biosynthesis. Additionally, 'gapfill'

GPRs were added to the reactions 'rxn03975_c0' and 'rxn03393_c0' so that removal of pSymA and pSymB did not prevent biosynthesis of vitamin B12 and ubiquinone-8, respectively. Finally, a glycerol export reaction via diffusion (rxnBLTPcpd00100b) was added to remove the glycerol build-up resulting from cardiolipin biosynthesis. The modified version of the model was termed iGD1575c, and is available in in SBML and MATLAB format in File S2. For simulating the removal of pSymA and pSymB in MATLAB, all pSymA and pSymB genes were deleted from the iGD1575b model using the *deleteModelGenes* function, followed by the removal of all constrained reactions using the *removeRxns* function.

Building the draft *R. leguminosarum* and *S. meliloti* metabolic models.

A draft, fully automated model containing no manual curation for *R. leguminosarum* bv. *viciae* 3841 was built using the KBase webserver (www.kbase.us). The Genbank file (GCA_000009265.1_ASM926v1_genomic.gbff) of the *R. leguminosarum* genome [4] was uploaded to KBase and re-annotated using the 'annotate microbial genome' function, maintaining the original locus tags. An automated metabolic model was then built using the 'build metabolic model' function, with gap-filling. This model included 1537 genes, 1647 reactions, and 1731 metabolites, and is available in in SBML and MATLAB format in File S2. The biomass composition was not modified from the default Gram negative biomass of KBase. All essential model genes were determined using the COBRA Toolbox in MATLAB with the *singleGeneDeletion* function and the MOMA protocol, with exchange reaction bounds set as provided in Table S7.

A draft, fully automated model containing no manual curation for *S. meliloti* Rm1021 was built as described above for *R. leguminosarum* but starting with the Genbank file (GCA_000006965.1_ASM696v1_genomic.gbff) for the *S. meliloti* genome [5]. The draft model included 1254 genes, 1698 reactions, and 1666 metabolites, and is available in in SBML and MATLAB format in File S2.

Building the *S. meliloti* core metabolic reconstruction, iGD726.

The iGD726 model was built from the ground-up using the existing genome-scale model as a reaction and GPR database, and with the Tn-seq data as a guide. The iGD726 model began as an empty SBML formatted file, and the first reaction that was added was a biomass reaction involving a single substrate (e.g., protein). The pathways (including central carbon metabolism) required to produce this biomass substrate were added reaction-by-reaction to the new reconstruction file. These reactions were taken from the original model, or were taken from other sources, primarily the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [6], if an appropriate reaction was missing in the original model. Pathway reconstruction was guided by the metabolic maps of KEGG and the Tn-seq data; if multiple pathways could be used to generate the same compound, the essentiality data of the Tn-seq screen were used to inform the decision of which pathway to include. When the necessary pathways were built, the model was converted to COBRA format, and FBA simulations were run to test if biomass could be produced. If not, we attempted to identify gaps both manually, and with the *detectDeadEnds* function of the COBRA Toolbox. In cases where we remained unable to identify the missing reaction(s), all reactions from the original model were transferred to the new reconstruction, following which they were cumulatively deleted until only the essential reaction(s) remained. When it was understood why these reactions were essential, they were either retained in the new reconstruction, or replaced with an alternative pathway, guided by the Tn-seq data. When the

model was capable of producing biomass (based on FBA simulations), the next biomass component was added to the biomass reaction, and the process repeated. This iterative process continued until the model could produce all biomass reactions. As the original model is a full genome-scale metabolic reconstruction, encompassing core and accessory metabolism, not all reactions were transferred to iGD726; only those essential for biomass production or to accurately capture the Tn-seq data were included in iGD726.

Throughout the above process, the Tn-seq data were used to refine the gene-reaction associations. Each time a new reaction was added to the core reconstruction, the genes associated with the reaction were checked against the Tn-seq data, and a literature search for each associated gene was performed. The gene associations were then modified as necessary to ensure the model accurately captured the experimental data. For example, if a gene was experimentally determined to be essential (in the Tn-seq data or the literature), but the corresponding reaction for the gene was associated with multiple alternative genes, all genes but the essential gene were removed from the reaction. Similarly, for essential reactions associated with non-essential genes, either i) all genes associated with the reaction in the original model were added; ii) if only one gene was associated with the reaction in the original model, an Unknown was added to reflect the apparent redundancy in the genome; or iii) an alternative, redundant pathway was added. Where possible, Unknowns in the gene associations were replaced with genes whose gene product may catalyze the reaction; this was only done if there was some level of experimental support for doing so.

Core model reconstruction was performed providing sucrose as a carbon source during the FBA simulations. Once a functional model was prepared, it was expanded to include the necessary metabolic and transport reactions to also allow growth with glucose or succinate as the carbon source. Finally, when biomass could be produced using any of the three carbon sources, the list of model genes was compared with the list of 489 core growth promoting genes to identify genes not included in the model but experimentally determined to contribute to growth. When possible, missing genes and their corresponding reactions were added to the core model.

During the construction of the core model, we additionally updated the biomass composition. This included modifying the membrane lipid composition to include lipids with different sized fatty acids based on the ratio experimentally determined [7]; the original genome-scale model contained only one representative per each membrane lipid class. Additionally, essential vitamins, cofactors, and ions were added to the biomass composition at trace concentrations to ensure that their biosynthesis or transport was essential. The complete biomass composition is provided in Table S3.

The final model contained 726 genes, 681 reactions, and 703 metabolites, and is provided in SBML and MATLAB format in File S2, and as an Excel file in Data Set S5. The Excel file contains all necessary information for use as a *S. meliloti* metabolic resource, including the reaction name, the reaction equation using the real metabolite names, the associated genes/proteins, and references. Additionally, for each reaction, the putative orthologs of the associated genes in 10 related Rhizobiales species are included, allowing the model to provide useful information for each of these organisms.

SUPPLEMENTARY RESULTS

Validation of the between-strain differences in the Tn-seq data.

In an attempt to independently validate the results of the Tn-seq experiment, 16 genes showing strain-specific phenotypes (14 genes whose disruption appeared to result in a larger growth defect in the ΔpSymAB strain, and 2 genes whose disruption appeared to result in a larger growth defect in the wild-type strain) were mutated through single cross-over plasmid integration. As described in the Results section of this manuscript, the between-strain differences of ~ 75% of these genes were confirmed by the validation experiment. Even if only 75% of the between-strain differences in the Tn-seq data are real, this is still a remarkable number of between-strain differences. However, we expect that many of the differences between the Tn-seq and validation experiments are not false positives, but instead a result of differences in growth conditions. More specifically, the validation experiments involve isogenic cultures, whereas the Tn-seq experiment is based on competitive growth in which less than 0.01% of the initial cell population would contain the mutation of interest.

A possible example of the growth condition differences influencing the results is the data for *feuQ*. The *feuN*, *feuP*, and *feuQ* genes encode a system resulting expression of *ndvA* and *ndvB*, whose products are involved in periplasmic β-glucan biosynthesis [8,9]. In the Tn-seq data, all five of these related genes contained many fewer insertions in the ΔpSymAB background than in the wild-type background, and this was observed in both of the media. However, in the validation experiment, mutation of *feuQ* did not result in the expected phenotype (none of the other four genes were tested). Given that all five of these genes (encoded as three operons in two different chromosomal loci) are involved in a common process, and that all five have the same phenotype in the Tn-seq data (independent of medium), it is unlikely that these represent false positives. Instead, we interpret the difference between the Tn-seq and validation experiments as reflective of the difference in growth conditions of these two experiments, highlighting how a failure to validate a phenotype does not necessarily mean that the Tn-seq observed phenotype is a false positive.

In summary, it is expected that some of the genes identified as having between-strain differences in the Tn-seq data are false positives. However, many of those that could not be independently confirmed in the validation experiment many not represent false positives and instead reflect differences in experimental settings.

Comparison of pipelines for classification of gene fitness phenotypes.

The analyses presented in the manuscript are based on gene classifications performed using the clustering based approach described in the Materials and Methods. Subsequently, the TSAS pipeline [10] was run to verify that the clustering based approach did not result in the inclusion of excessive false positives. Here, these approaches are compared.

The main step of the clustering based approach involved assuming that the data are explained by multiple overlapping Guassian distributions (i.e., non-essential genes form one distribution, essential genes form an independent distribution, etc.). The number and shape of distributions that best explains the data were then found, and genes classified based on the probability that they belonged to each of the fitted distributions.

In the case of TSAS, the reads are reduced to the number of unique insertions; i.e., all reads mapping to the same location are reduced to a single insertion. The data are then assumed to belong to a single Binomial distribution, and the probability of finding the detected number of

insertions by chance is calculated. Different p-value corrections are then applied to correct for multiple testing. The key outputs of this approach are p-values indicating the probability that genes have fewer than expected insertions; it does not distinguish between essential genes and genes whose mutation result in a non-lethal growth defect.

To ensure that the various steps of clustering based approach did not result in the identification of an excessively large number of false positive essential genes, the outputs of these methods were compared. Of the 307 genes classified as essential in all four samples, 269 (88%) were considered to have fewer than expected insertions with the TSAS pipeline and a strict significance threshold (field wide error rate Bonferroni correction < 0.01). Most of the 38 genes not considered to have fewer than expected insertions were small genes with no insertions. However, inspection of their annotations revealed that most are expected to be essential, highlighted by the fact that this list includes 24 ribosomal proteins, and therefore their annotation as essential by the clustering pipeline is probably accurate. Additionally, if a somewhat less strict significance threshold is used (Benjamini-Hochberg adjusted p-value < 0.01), 300 (98%) of the 307 core essential genes are classified as having fewer than expected insertions. Given these results, we conclude that the clustering based approach did not result in an artificially high number of false positives, and that the global systems-level analyses reported in this study are unlikely to be significantly impacted by the choice of statistical analysis.

Results from both the clustering approach and the TSAS pipeline are provided in Data Set S1.

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