

1 Cellular determinants of metabolite concentration ranges

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11 **Abstract:** Cellular functions are shaped by reaction networks whose dynamics are determined
12 by the concentrations of underlying components. However, cellular mechanisms ensuring that
13 a component's concentration resides in a given range remain elusive. We present network
14 properties which suffice to identify components whose concentration ranges can be efficiently
15 computed in mass-action metabolic networks. We show that the derived ranges are in excellent
16 agreement with simulations from a detailed kinetic metabolic model of *Escherichia coli*. We
17 demonstrate that the approach can be used with genome-scale metabolic models to arrive at
18 predictions concordant with measurements from *Escherichia coli* under different growth
19 scenarios. By application to 14 genome-scale metabolic models from diverse species, our
20 approach specifies the cellular determinants of concentration ranges that can be effectively
21 employed to make predictions for a variety of biotechnological and medical applications.

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26 **Author Summary**

27 We present a computational approach for inferring concentration ranges from genome-scale
28 metabolic models. The approach specifies a determinant and molecular mechanism underling
29 facile control of concentration ranges for components in large-scale cellular networks. Most
30 importantly, the predictions about concentration ranges do not require knowledge of kinetic
31 parameters (which are difficult to specify at a genome scale), provided measurements of
32 concentrations in a reference state. The approach assumes that reaction rates follow the mass
33 action law used in the derivations of other types of kinetics. We apply the approach with large-
34 scale kinetic and stoichiometric metabolic models of organisms from different kingdoms of life
35 to show that we can identify a proportion of metabolites to which our approach is applicable.
36 By challenging the predictions of concentration ranges in the genome-scale metabolic network
37 of *E. coli* with real-world data sets, we further demonstrate the prediction power and limitations
38 of the approach.

39

40 **Introduction**

41 Advances in systems biology studies have been propelled by the availability of high-quality
42 genome-scale metabolic reconstructions for many organisms across all kingdoms of life [1].
43 Metabolic network reconstructions contain information about metabolites and reactions
44 through which they are transformed to support different cellular processes [2, 3]. Alongside
45 enzyme concentrations and phenomenological constants, reaction rates and metabolite
46 concentrations—as two faces of the metabolic phenotype—characterize key aspects of the
47 metabolic capabilities of an organism. Since metabolic concentrations are important
48 determinants of reaction rates [4], understanding what controls their physiological ranges can
49 point to cellular mechanisms that control phenotypic plasticity to ensure viability of organisms
50 under changing conditions [5].

51 A naïve approach to determine a concentration range for a given component is to
52 assume that it is present with a single molecule or that the entire cell dry weight under an
53 investigated scenario is composed solely of that component. This derivation requires only
54 knowledge of the component’s molecular weight, which is readily available. However, the
55 derived ranges are vast and largely invariant under different scenarios; therefore, they are not
56 informative. Here we ask whether large-scale metabolic models can be used for accurate
57 prediction of concentration ranges. Resolving this question is tantamount to identifying a
58 cellular mechanism underlying the control of concentration range for given cellular component.

59 The change in concentration of metabolites can be described by a system of coupled
60 ordinary differential equations (ODEs), $\frac{dx(t)}{dt} = Nv(t)$, where $v(t) = (v_1(t), \dots, v_n(t))^T$
61 denotes reaction rates and $x(t) = (x_1(t), \dots, x_m(t))$ the metabolite concentrations at time t ,
62 and N represents the stoichiometric matrix. The rows of the stoichiometric matrix correspond
63 to metabolites, columns stand for reactions, and its entries denote the stoichiometric
64 coefficients with which metabolites participate in reactions as substrates or products [6].

65 Reaction rates are modeled according to a kinetic law, $v(t) = f(x(t), \theta)$, which often leads to
66 nonlinearities and involves multiple parameters, denoted by θ [7]. As a result, the coupled
67 nonlinear ODEs are often not analytically tractable and their simulations are challenging. These
68 issues arise since parameters remain poorly specified at a genome scale for the majority of
69 model organisms [8, 9] and the nonlinear ODEs may lead to numerical issues [10]. In addition,
70 determining the steady-state concentration ranges by characterizing the solutions to the system
71 of non-linear equations $Nf(x(t), \theta) = 0$ is intractable for large-scale networks even when the
72 equations have a simplified mass action form often used in metabolic modeling [11].

73 Feasible steady-state reaction rates, v , for which $Nv = 0$, can be predicted based solely
74 on the structure of the network with computational approaches from the constraint-based
75 modeling framework [12]. However, since intracellular reaction rates cannot be measured
76 directly, the validation of these predictions requires laborious labeling experiments and model
77 fitting procedures [13]. By neglecting the effect of concentrations on reaction rates, constraint-
78 based approaches do not facilitate the usage of metabolic network reconstructions to predict
79 concentrations of metabolites, which are becoming more accessible by quantitative
80 metabolomics technologies [14].

81 The existing constraint-based approaches that can make predictions of metabolite
82 concentrations and their ranges are based on consideration of thermodynamics constraints.
83 Thermodynamics-based metabolic flux analysis (TMFA) produces flux distributions that do
84 not contain any thermodynamically infeasible reactions or pathways, and it provides
85 information about the free energy change of reactions and the range of metabolite
86 concentrations in addition to reaction fluxes [15]. However, due to uncertainty in the estimation
87 of the standard Gibbs free energies, TMFA usually predicts unconstrained ranges for
88 metabolite concentrations (see Discussion in Henry et al. [15]). Metabolic Tug-of-War
89 (mTOW) extends TMFA but is based on a non-convex optimization approach which comes at

90 a cost of local optima and lack of robustness of predictions (validated by correlation [16]). A
91 method to predict metabolite concentration ranges with limited knowledge about the
92 underlying kinetic laws and parameter values would allow direct integration and validation of
93 genome-scale models with experimental data from metabolomics technologies, enabling
94 systems biology applications, from engineering of intervention strategies to design of new
95 drugs [17-19].

96 Here we provide an approach which relies on the structure of the network, encoded in
97 the stoichiometric matrix, to provide simulation-free prediction of steady-state concentration
98 ranges by employing mass action kinetics. We focus on mass action kinetics since it underlies
99 the derivation of more involved types of kinetics for different reaction mechanisms [20], allows
100 for consideration of enzyme concentration if enzymes appear as reaction substrates, and
101 provides a simple mathematical form that may be amenable to analytical treatment. The usage
102 of mass action was here also favored due to lack of information about reaction mechanisms at
103 a genome-scale level. The approach expands on the well-established concept of full coupling
104 of reactions [21] to consider pairs of reactions whose ratio of mass-action-compatible fluxes
105 depends only on the respective rate constants. Thus, this flux ratio is invariant at any, not
106 necessarily steady, state of the system. The approach is also refined to predict concentration
107 ranges for unseen cellular scenarios provided concentration data from a reference experiment.
108 Our method complements the constraint-based modeling framework, focused on analysis of
109 steady-state reaction rates, and thus enables a comprehensive characterization of feasible
110 concentrations in genome-scale metabolic networks under specified conditions.

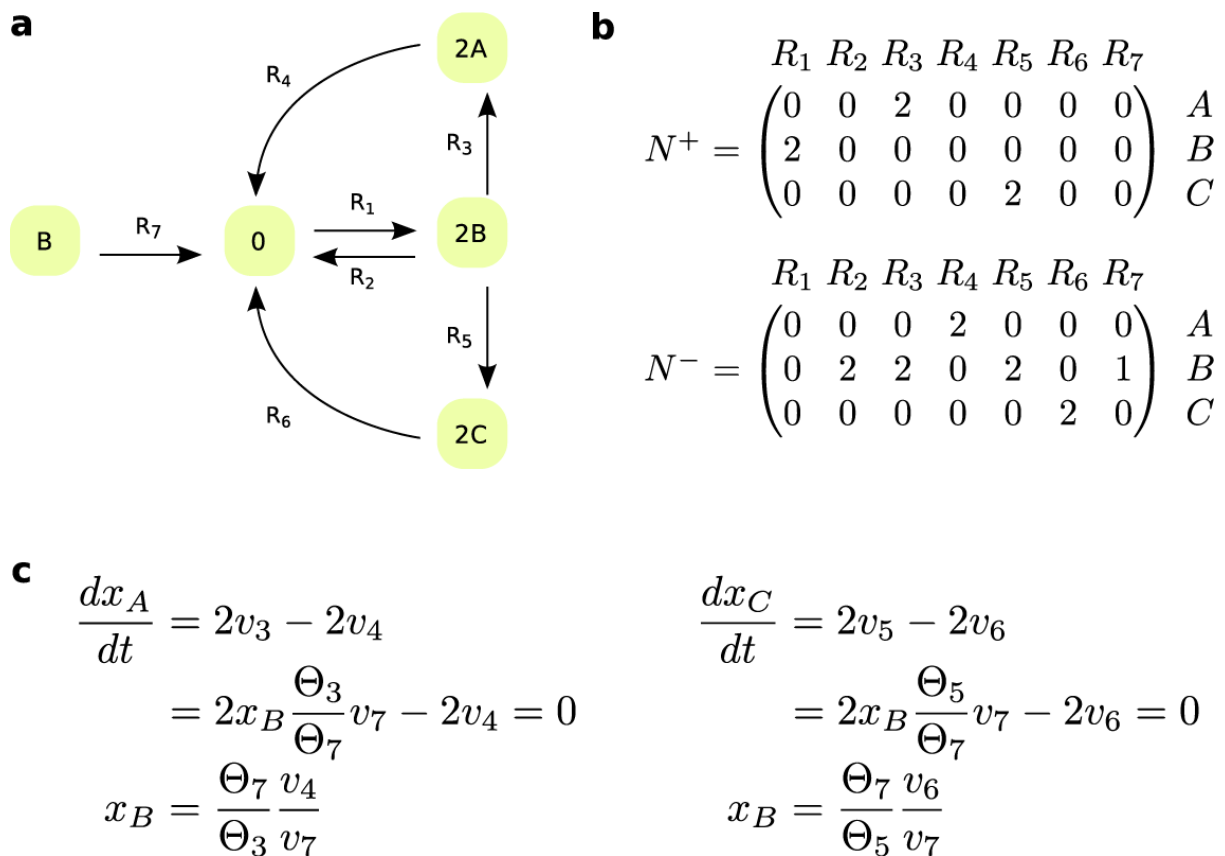
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112 **Results**

113 **Metabolites with structurally constrained concentrations (SCC)**

114 Consider a metabolic network composed of m metabolites that participate in n reactions. The
 115 $(m \times n)$ stoichiometric matrix, N , can be written as a difference of two non-negative matrices,
 116 $N = N^+ - N^-$, where N^+ includes the stoichiometry of the products and N^- comprises the
 117 stoichiometry of the substrates of each reaction. For instance, the stoichiometry of substrates
 118 and products given in Fig. 1b describes the metabolic network on Fig. 1a. We assume that the
 119 rate of reaction R_i is modeled according to mass action kinetic, whereby $v_i = \theta_i \prod_j x_j^{N_{ji}^-}$, where
 120 $\theta_i > 0$ is the reaction constant and the concentration of each substrate molecule appears in v_i
 121 as a multiplicative factor.

122



123

124 **Fig. 1. Network with a component exhibiting structurally constrained concentration. (a)**

125 Reaction diagram that includes seven reactions, $R_1 - R_7$, and three components, $A - C$. (b)

126 stoichiometric matrices associated with substrates, N^- , and products, N^+ , for the network in
127 (a). Reaction R_7 lacks one substrate molecule of B in comparison to R_2 , since $N_{22}^- - N_{27}^- = 1$
128 and $N_{i2}^- - N_{i7}^- = 0$ for every $i \neq 2$. Reactions R_3 and R_5 share the same substrate components
129 with same stoichiometry, and hence their fluxes are fully coupled under the assumption of mass
130 action kinetic. Reaction R_3 is fully coupled to reaction R_4 , as are reactions R_5 and R_6 . (c)
131 Component B exhibits structurally constrained concentration from the ODEs of components A
132 and C . The network exhibits different positive steady states with changing rate of reaction R_1 .

133 To state our main result, we require some concepts and terminology which we next
134 introduce and illustrate. We will say that a reaction R_k lacks one substrate molecule of X_i in
135 comparison to reaction R_l , if $N_{il}^- - N_{ik}^- = 1$ and for every $i' \neq i$, $N_{i'l}^- - N_{i'k}^- = 0$. For the
136 network in Fig. 1a, reaction R_7 lacks one substrate molecule of component B in comparison to
137 reaction R_2 . Under the assumption of mass action kinetic, if a reaction lacks one substrate
138 molecule in comparison to another, the reactions differ in their orders by one. As a result, the
139 ratio of fluxes for such reactions at any state of the system depends only on the rate constants
140 and the concentration of the substrate in which the reactions differ.

141 Furthermore, two reactions R_k and R_l are fully coupled if there exists $\lambda > 0$, such that
142 $v_l = \lambda v_k$ for any positive steady-state reaction rate v , *i.e.*, $Nv = 0$ [21]. Therefore, fully
143 coupled reactions have an invariant ratio over all positive steady states that the network admits,
144 and full coupling is a transitive relation. For the network in Fig. 1a, reaction R_3 is fully coupled
145 to R_4 and R_5 is fully coupled to R_6 . Such reactions, which are fully coupled irrespective of the
146 kinetic law, can be efficiently determined based on the stoichiometry of large-scale networks
147 by linear programming [21, 22] (see Materials and Methods).

148 Under the assumption of mass action kinetic, two reactions that share the same
149 substrates of same stoichiometry are also fully coupled [23]. In this case, the coupling holds
150 for any, not necessarily steady, state of the system. Therefore, the consideration of mass action

151 kinetic expands the set of fully coupled reactions. For instance, this is the case for reactions R_3
 152 and R_5 that have the substrate components of same stoichiometry in Fig. 1a, whereby $\frac{v_3}{v_5} = \frac{\theta_3}{\theta_5}$.

153 Consider now a metabolite X_j with an ODE given by $\frac{dx_j}{dt} = \sum_{k \in P_j} N_{jk}^+ v_k - \sum_{l \in S_j} N_{jl}^- v_l$,
 154 where P_j is the set of reactions with X_j as one of their products and S_j is the set of reactions
 155 which have metabolite X_j as one of their substrates. A metabolite X_i , not necessarily different
 156 from X_j , has structurally constrained concentration (SCC), if the following conditions hold: (i)
 157 for each reaction R_l in S_j , there exists a non-empty subset Q_l^{-i} of reactions lacking one substrate
 158 molecule of X_i in comparison to R_l ; the union of all Q_l^{-i} yields the set of reactions S_j^{-i} ; (ii) all
 159 reactions in S_j^{-i} are mutually fully coupled; and (iii) all reactions in P_j are mutually fully
 160 coupled. A similar argument can be made with respect to condition (i) in terms of reactions in
 161 the set P_j (Materials and Methods). A metabolite X_i that satisfies the conditions above will be
 162 referred to as a SCC metabolite.

163 In the following, we use the ODE for metabolite X_j to derive the concentration bounds
 164 for a metabolite X_i with SCC. Let Q be a subset of S_j^{-i} that contains one and only one reaction
 165 from each of Q_l^{-i} . Under mass action, for the flux of every reaction $R_l \in S_j$, it then holds that
 166 $v_l = x_i \frac{\theta_l}{\theta_l^{-i}} v_l^{-i}$ (see Materials and Methods), where θ_l^{-i} is the reaction constant and v_l^{-i} the
 167 flux of a reaction $R_l^{-i} \in Q$. The expression for $\frac{dx_j}{dt}$ above then becomes $\sum_{k \in P_j} N_{jk}^+ v_k -$
 168 $x_i \sum_{l \in S_j} N_{jl}^- \frac{\theta_l}{\theta_l^{-i}} v_l^{-i}$.

169 At any positive steady state, it then holds that $\frac{dx_j}{dt} = v_p \sum_{k \in P_j} N_{jk}^+ \frac{v_k}{v_p} -$
 170 $x_i v_s^{-i} \sum_{l \in S_j} N_{jl}^- \frac{\theta_l}{\theta_l^{-i}} \frac{v_l^{-i}}{v_s^{-i}} = 0$, for any flux v_p of reaction $R_p \in P_j$ and flux v_s^{-i} of reaction $R_s^{-i} \in$
 171 Q . Due to the conditions (iii), above, the sum $\sigma_p = \sum_{k \in P_j} N_{jk}^+ \frac{v_k}{v_p}$ is a constant which, in the

172 simplest case, when all reactions in P_j are fully coupled irrespective of the kinetic rate law,
 173 depends only on the network structure. In addition, due to condition (ii), above, the value of
 174 $\sigma_s^{-i} = \sum_{l \in S_j} N_{jl}^- \frac{\theta_l v_l^{-i}}{\theta_l^{-i} v_s^{-i}}$ is also a constant which depends on both the network structure and a
 175 subset of rate constants. The rate constants which appear in the expression for σ_s^{-i} and σ_p for
 176 any $Q \subseteq S_j^{-i}$ will be referred to as *relevant rate constants*, while the flux ratio $\frac{v_p}{v_s^{-i}}$ will be called
 177 *relevant flux ratio*.

178 Therefore, given a steady-state flux distribution, v , a set $Q \subseteq S_j^{-i}$, and two reactions
 179 $R_p \in P_j$ and $R_s^{-i} \in Q$, we have that $x_i = \frac{\sigma_p}{\sigma_s^{-i}} \frac{v_p}{v_s^{-i}}$. This derivation establishes a direct relation
 180 between a flux distribution, under specified inputs from the environment, and the concentration
 181 of a SCC metabolite. We can also use the derived expression to obtain the concentration bounds
 182 for x_i over any set, F , of steady-state flux distributions and subset Q (per definition above),
 183 yielding the following:

$$184 \quad \min_{\{Q,F\}} \frac{\sigma_p}{\sigma_s^{-i}} \frac{v_p}{v_s^{-i}} \leq x_i \leq \max_{\{Q,F\}} \frac{\sigma_p}{\sigma_s^{-i}} \frac{v_p}{v_s^{-i}}. \quad (1)$$

185 For instance, component B in Fig. 1a is SCC, derived from the ODE of component A , whereby
 186 the relevant flux ratio is $\frac{v_4}{v_7}$ and the relevant rate constants are θ_3 and θ_7 (Fig. 1c). Similarly,
 187 one can show that component B is SCC from the ODE of component C .

188 Let the lower and upper bounds for the concentration of metabolite X_i derived from the
 189 ODE of metabolite X_j in Eq. (1) be denoted by $L_i^j = \min_{\{Q,F\}} \frac{\sigma_p}{\sigma_s^{-i}} \frac{v_p}{v_s^{-i}}$ and $U_i^j = \max_{\{Q,F\}} \frac{\sigma_p}{\sigma_s^{-i}} \frac{v_p}{v_s^{-i}}$,
 190 respectively. If there are r metabolites X_d , $1 \leq d \leq r$ for which Eq. (1) applies, then the lower
 191 and upper bounds for the concentration of X_i are given by the intersection of the ranges derived
 192 from the ODEs of X_d , i.e.,

$$193 \quad \max_d L_i^d \leq x_i \leq \min_d U_i^d. \quad (2)$$

194 Therefore, the lower bound is the minimum of the maxima, while the upper bound is the
195 maximum of the minima derived from the individual ODEs. In case that the SCC of a
196 metabolite can be derived from multiple ODEs, Eq. (2) provides more constrained predictions
197 about metabolite concentration ranges than Eq. (1) alone. For instance, component B in Fig. 1a
198 is SCC not only from the ODE of component A but also from that of C , whereby the relevant
199 flux ratio is $\frac{v_6}{v_7}$ and the relevant rate constants are θ_5 and θ_7 (Fig. 1c). In case that the upper
200 bound is smaller than the lower bound in Eq. (2) then the system of ODEs does not have a
201 positive solution for X_i , which implies that the network does not allow a positive steady state.
202 Therefore, the approach can also be used to check for existence of positive steady state with
203 respect to a SCC metabolite under mass action kinetics.

204

205 **Validation of the approach with a large-scale kinetic model of *E. coli***

206 The proposed approach can be employed to determine metabolite concentration ranges by
207 using information about full coupling of reactions, fluxes entering relevant flux ratios, and the
208 relevant reaction rate constants. To validate the predictions, we employ a detailed kinetic model
209 of elementary metabolic reactions of *E. coli* [8] from which these inputs are readily available.
210 Of the 830 metabolites interconverted by 1,474 elementary reactions in the model, our
211 approach determines that 23 metabolites exhibit SCC. The ranges for these SCC metabolites
212 are fully determined by 67 relevant rate constants (4.6% of all rate constants) and fluxes of 67
213 reactions (4.6% of all reactions) which enter in the relevant flux ratios. We use the kinetic
214 model to simulate 100 steady states from different initial conditions (Supplementary Table S1).

215 We determined the Euclidean distance between the predicted and simulated lower and
216 upper bounds to demonstrate their quantitative agreement. Since metabolite concentrations
217 vary over several orders of magnitude, the results based on Euclidean distance will be biased
218 by the presence of very large metabolic pools; therefore, we also considered two variants of

219 relative Euclidean distance (see Materials and Methods). Our results from the quantitative
220 comparison demonstrate a very good agreement between the predicted and simulated bounds
221 (Supplementary Table S2, Supplementary Fig. S1). We also employ the Pearson correlation to
222 assess if the predicted and simulated bounds agree qualitatively across the metabolites with
223 SCC. We determine that there is a perfect qualitative match between the predicted and
224 simulated lower (1, p-value $< 10^{-6}$) and upper bounds (1, p-value $< 10^{-6}$) of the SCC
225 metabolites (Supplementary Table S2).

226 It has been recently proposed that the shadow prices of metabolites can be used to
227 quantify the ranges of metabolite concentrations, under the assumption that the cellular system
228 optimizes an objective [24]. To compare the performance of shadow prices as a measure of
229 metabolite concentration ranges, we employ the stoichiometric matrix of the analyzed kinetic
230 model by using the maximization of metabolic exchange fluxes as cellular objective, shown to
231 outperform yield as a predictor of growth rate [25]. We did not use optimization of yield, most
232 widely used in flux balance analysis, since the model has been parameterized without
233 consideration of a biomass reaction. We observe that for the analyzed model and the
234 physiologically relevant objective, the calculated shadow prices for the 23 SCC metabolites
235 cannot be used as indicators of concentration variability due to the weak negative correlation
236 with the concentration ranges as well as with the coefficients of variation of the SCC
237 metabolites (Supplementary Table S2). These findings point out that our approach, in absence
238 of a cellular objective but with knowledge about a few rate constants and selected flux ratios,
239 outperforms the existing contender for quantifying concentration ranges in large-scale
240 metabolic networks.

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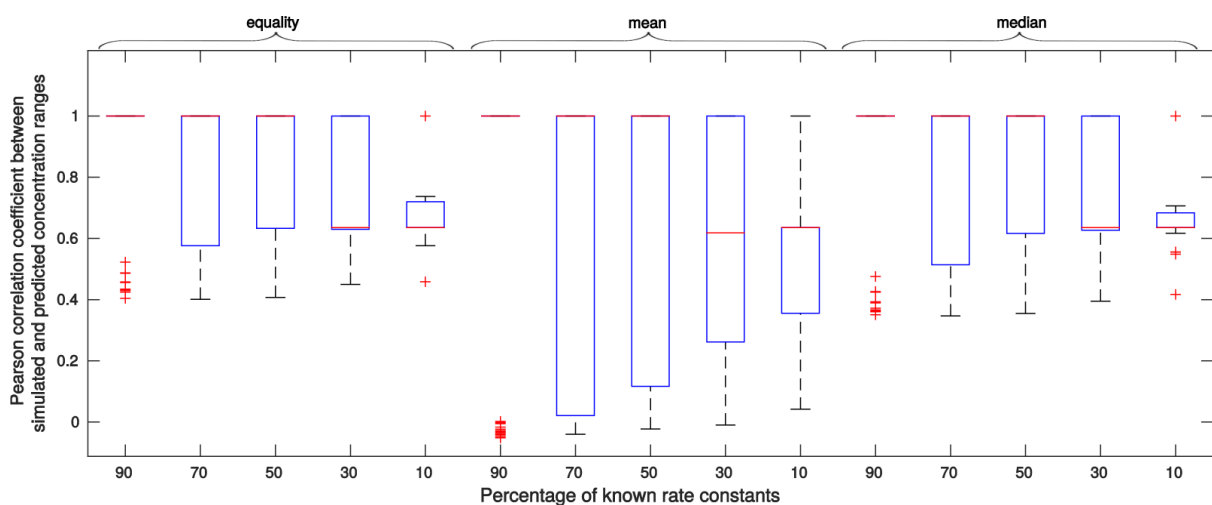
243 **Effects of missing information about rate constants**

244 While the full reaction couplings considered by our approach can be readily obtained given the
245 structure of the network and flux ratios are increasingly available from labeling approaches
246 [26], the resulting predictions can be affected by missing information about rate constants. To
247 assess the effect of missing information on the accuracy of predictions, we consider the cases
248 that 10 – 90% of rate constants used in the derivation of the ranges for the metabolites with
249 SCC are known (see Methods). We consider three scenarios whereby the missing ratios of rate
250 constants, appearing in Eq. (1), are substituted by: (i) a value of one, simulating a scenario in
251 which all relevant rate constants are of the same value, (ii) the mean, or (iii) the median of the
252 ratios of relevant rate constants that are present (*i.e.*, known) in the model equation from which
253 the conditions for SCC are established. We note that the units of the rate constants are not
254 relevant since rate constants enter Eq. (1), above, as ratios.

255 We find that the substitutions for the missing ratios of rate constants according to the
256 three scenarios, as expected, decrease the Pearson correlation between predicted and simulated
257 ranges over 100 instances of models in which relevant rate constants were removed at random
258 (Fig. 2). Nevertheless, even when only 30% of the relevant rate constants are known for the
259 cases (i) and (iii), we obtain a median Pearson correlation coefficient between the predicted
260 and simulated ranges of at least 0.6 (Fig. 2). Substituting the missing ratio of rate constants
261 with the mean of the ratios shows the largest variability over the 100 instances of models with
262 partial knowledge of rate constants. The reason for this finding is that the distribution of rate
263 constants and their ratios are highly left-skewed (Supplementary Fig. S2). Therefore, we
264 conclude that even in the absence of information about rate constants that matches the current
265 state-of-the-art of knowledge about *E. coli* (Supplementary Table S3), our approach provides
266 qualitatively reliable estimates of concentration ranges in large-scale models. The ordering of
267 lower and upper bounds between metabolites can be predicted well (median significant

268 Spearman correlation above 0.75 at significance level of 0.05 for all scenarios). However, we
269 observe that the median over relative and log-transformed Euclidean distances between
270 predicted and simulated lower as well as upper bounds over the 23 SCC metabolites are small
271 (<0.71 and <0.08 , respectively) when more than 50% of the relevant rate constants are known
272 (Supplementary Fig. S3-S6). Therefore, the approach can be used for the frequently employed
273 comparison of metabolite concentration ranges within and between conditions.

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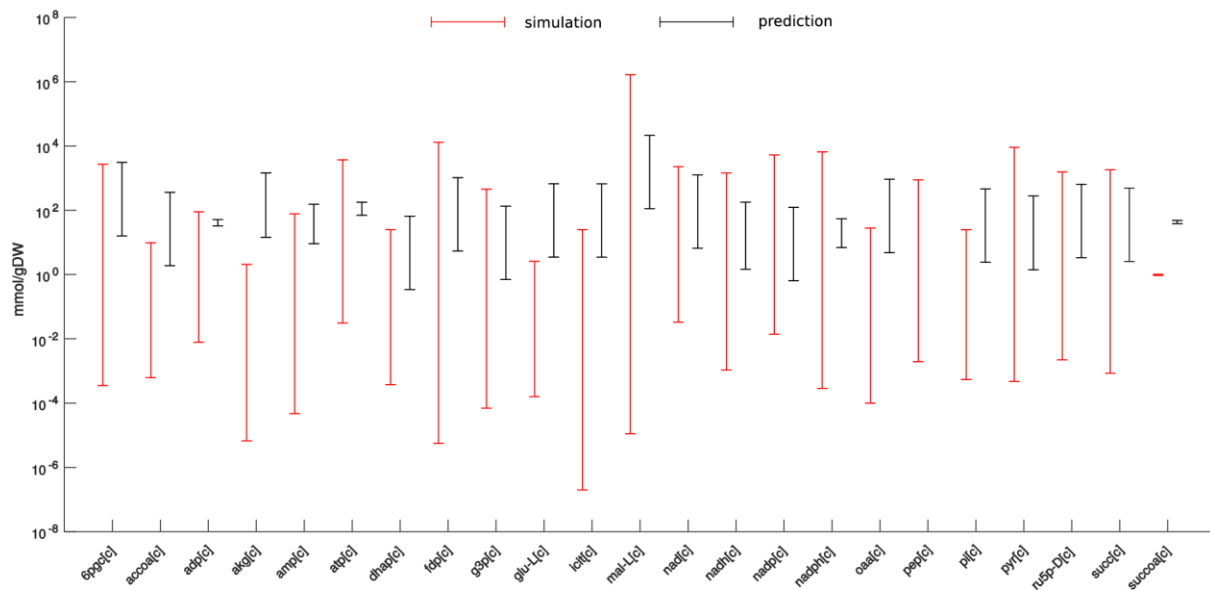
276 **Fig. 2. Effect of missing information about relevant rate constants on the accuracy of**
277 **concentration range predictions for a large-scale kinetic model of *E. coli*.** We consider 10
278 – 90% of the relevant rate constants to be unknown by random removal. We consider three
279 scenarios for the substitution of missing ratios of rate constants: (i) equality (i.e., kinetic rate
280 constants are assumed to be the same), (ii) the mean, or (iii) the median of the ratios of relevant
281 rate constants that are still present in the model. Shown are the boxplots (red lines inside each
282 box denote the corresponding medians) of the resulting Pearson correlation coefficients
283 between the predicted and simulated ranges over the SCC metabolites in the kinetic model of
284 *E. coli*.

285

286 **Effect of missing information about flux ratios**

287 We also investigate the accuracy of the predictions of concentration ranges when full
288 information about relevant rate constants is available and relevant flux ratios are obtained from
289 constraint-based modeling approaches. To obtain physiologically relevant predictions, we
290 constrain the model with the simulated exchange fluxes (Supplementary Table S1), since they
291 can be readily obtained from experiments (e.g. by following substrate depletion). As the
292 employed kinetic model does not specify a biomass reaction, we optimize a weighted average
293 of ATP production and total flux, known to lead to predictions in line with flux estimates from
294 labeling experiments [2]. To this end, we determine the range for the relevant flux ratios at the
295 optimal value for the objective and used them together with Eq. (2) to obtain concentration
296 ranges for the 23 SCC metabolites (Materials and Methods). We find that for 13 out of 23 SCC
297 metabolites the predicted concentration range reside inside the respective simulated range. For
298 additional 6 metabolites the ranges overlap, while the remaining metabolites show no overlap
299 in the predicted and simulated range using the objective of optimized ATP production and total
300 flux (Fig. 3). Since the approach provided accurate quantitative and qualitative predictions
301 with perfect information in the case of kinetic modeling, the discrepancy is due to the objective
302 used to constrain the physiologically reasonable fluxes.

303



304

305 **Fig. 3. Effect of missing information about relevant flux ratios on the accuracy of**

306 **concentration range predictions for a large-scale kinetic model of *E. coli*.** Relevant flux

307 ratios are obtained by constraint-based modeling in which the objective of weighted ATP

308 production and total flux is maximized. Red bars denote simulated ranges resulting from 100

309 different initial conditions of the large-scale kinetic model of *E. coli*. Black bars denote the

310 predicted ranges following Eq. (2). Concentration ranges are predicted for 23 SCC metabolites

311 in the employed metabolic model.

312

313 **Concentration ranges in a genome-scale metabolic model of *E. coli***

314 Arguably the most interesting scenario for application of our approach is with genome-scale

315 metabolic networks. We find 199 SCC metabolites in the cytosol and 168 in the periplasm and

316 extracellular space of the most recent genome-scale metabolic network of *E. coli* [27]

317 (Supplementary Table S8). However, for this model, we observe that there are data available

318 for only 28% of relevant rate constants (Supplementary Table S3), and we have no estimates

319 of the relevant flux ratios available from labeling experiments [28-30]. Therefore, the approach

320 cannot be used without extensions. Given a steady-state flux distribution, v , the concentration

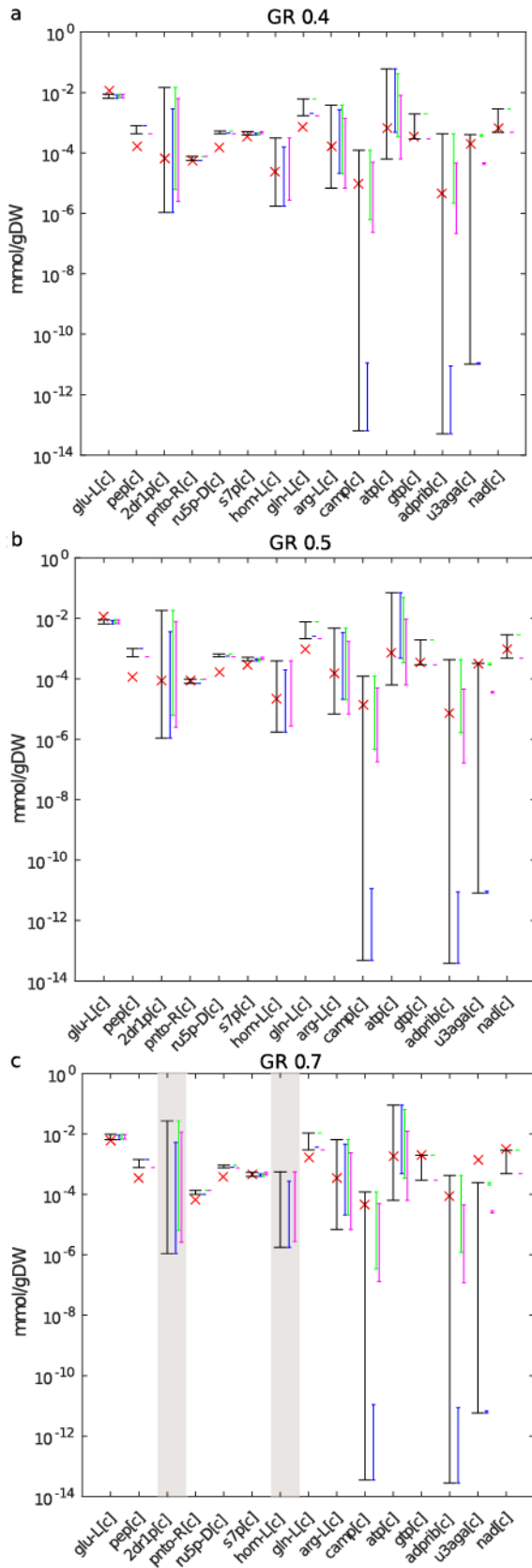
321 of a SCC metabolite X_i is given by $x_i = \frac{\sigma_p}{\sigma_s} \frac{v_p}{v_s}$. If we have data on concentration of SCC
322 metabolites and flux predictions from the constraint-based modeling framework, we can
323 readily obtain estimates for the ratio $\frac{\sigma_p}{\sigma_s}$. By definition, this ratio is invariant over the conditions
324 where all steady-state fluxes appearing in relevant flux ratios are non-zero. Therefore, we can
325 use the estimates for $\frac{\sigma_p}{\sigma_s}$ together with flux predictions to make predictions about concentration
326 ranges following Eq. (2) for another scenario. We note that the prediction about concentration
327 ranges inherit the uncertainty in the estimation of $\frac{\sigma_p}{\sigma_s}$ as well as the flux ratios from flux balance
328 analysis, which may contribute to the size of the predicted ranges.

329 **Metabolite concentration data set of Ishii et al. [28].** We use the measurements of steady-state
330 concentrations of 182 metabolites from *E. coli* under different growth scenarios [28]. This data
331 set includes 15 of the 199 cytosolic SCC metabolites found in the genome-scale model. We
332 also have access to rates of glucose and oxygen uptakes, carbon dioxide release as well as
333 growth from the same experiments [28], which we use as constraints to a genome-scale
334 metabolic network of *E. coli*. It has been shown that *E. coli* does not optimize a single objective
335 (e.g., growth), but its steady-state flux distributions result from the trade-off between tasks of
336 optimizing growth, ATP synthesis, and total flux [2]. Since growth rate is fixed from
337 measurements, we optimize the weighted average of ATP synthesis and total flux, with a
338 weighting factor of 0.1 on ATP synthesis to reduce the effect of the order difference in the
339 respective optimum observed when ATP production and total flux are optimized individually.
340 Here, too, at the obtained optimum we can efficiently estimate ranges for the relevant flux
341 ratios (Materials and Methods). In addition, we compare obtained concentration ranges with
342 those predicted when maximization of ATP is used as the only objective. To obtain estimates
343 for $\frac{\sigma_p}{\sigma_s}$, we use three replicates for the concentration data and predictions of ranges for relevant
344 flux ratios at growth rate of $0.2h^{-1}$ (Supplementary Table S4). Eq. (2) can then be applied to

345 determine concentration ranges based on $\frac{\sigma_p}{\sigma_s^i}$ for a combination of replicates, to investigate the
346 effect of outliers. We predict in turn the concentration ranges for three other growth rates (i.e.,
347 0.4, 0.5, and $0.7h^{-1}$).

348 For the objective of optimizing ATP synthesis and total flux, our results demonstrate
349 that measurements for 9, 10, and 6 of the 15 SCC metabolites fall in the predicted concentration
350 range for the three growth rates, respectively (Fig. 4). Nevertheless, the Spearman correlation
351 between the measured values and the predicted lower and upper bounds is significant and larger
352 than 0.57 and 0.56, respectively (Supplementary Table S5). Therefore, the approach can be
353 used to compare the ordering of lower or upper bounds between different experimental
354 scenarios (Supplementary Fig. S7). In addition, this analysis highlights the effect of the
355 replicates of metabolite concentrations used in calculating the values of $\frac{\sigma_p}{\sigma_s^i}$, since estimates for
356 some of the replicates may be outliers (Fig. 4). In contrast, we find that 4, 5 and 2 of the 15
357 SCC metabolites fall in the measured range for the three growth rates when maximization of
358 ATP is used as objective (Supplementary Fig. S9). Moreover, we cannot predict concentrations
359 for 8 out of the 15 SCC metabolites due to numerical instabilities arising when using this
360 objective under the additionally imposed constraints on growth. The reasons for the
361 discrepancy between the predicted and measured values under both objectives include the
362 combination of at least three factors: the inability to distinguish the concentrations of free
363 metabolites from those bound to macromolecules experimentally [31], model (and objective)
364 inaccuracies, and the simplifying assumption of mass action kinetic. Nevertheless, the
365 approach can be extended to consider networks with kinetic laws derived from mass action
366 which involve enzyme forms (e.g., Michaelis-Menten, see Discussion) at cost of increased data
367 requirements for application.

368



369

370 **Fig. 4. Comparison of predicted ranges with measured metabolite concentrations under**

371 **the objective of optimizing ATP synthesis and sum of total flux. Comparison of the**

372 predicted concentration ranges for 15 intracellular metabolites in *E. coli* with absolute
373 concentrations measured at growth rates (GR) of (a) 0.4, (b) 0.5 and (c) $0.7h^{-1}$. For metabolites
374 with grey background, there is no access to measurements. The colored bars denote the
375 predicted ranges from each of the three different replicates, while the black bar represents the
376 prediction over all replicates. The red cross denotes the measured value at the respective GR.
377 For some metabolites there is no overlap between the colored bars, indicating poor
378 reproducibility over the replicates in the reference scenario. The nomenclature of the
379 metabolites is provided in Supplementary Table S5.

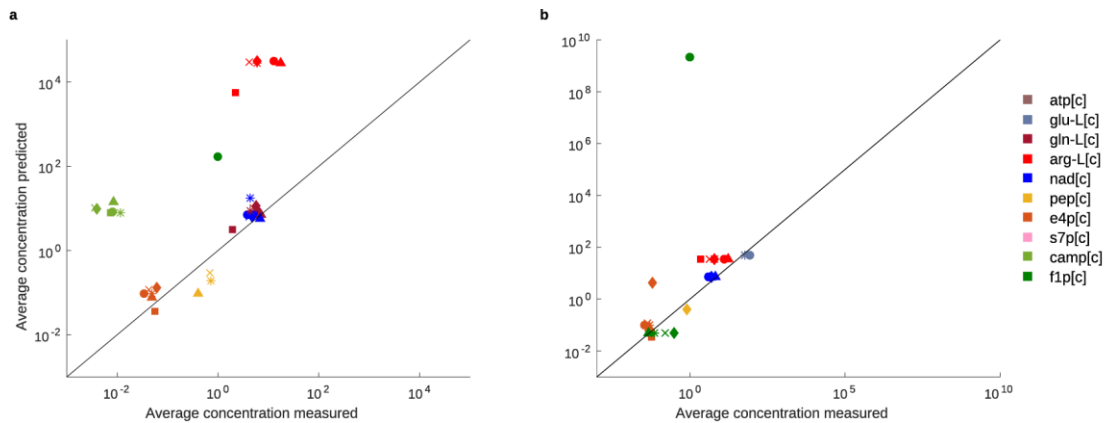
380

381 **Metabolite concentration data set of Gerosa et al. [32].** We use the measurements of steady-
382 state concentrations of 43 metabolites from *E. coli* grown in eight different carbon sources [32].
383 This data set includes ten of the 199 cytosolic SCC metabolites found in the genome-scale
384 model. We also have access to rates of carbon uptake, some secretion rates, as well as growth
385 from the same experiments (see Supplementary Table S10), which we use as constraints to a
386 genome-scale metabolic network of *E. coli*. Since growth rate is fixed from measurements, as
387 above, we optimize the weighted average of ATP synthesis and total flux, with weighting
388 factors 0.001 for ATP synthesis and 1000 for total flux to reduce the effect of the order
389 difference and make the comparison to optimization of ATP synthesis. Different weighting
390 factors are used in comparison to the analysis of the data set from Ishii et al., above, since
391 different constraints are used that affect the optimal values of the individual objectives. Here,
392 too, at the obtained optimum we can efficiently estimate ranges for the relevant flux ratios
393 (Materials and Methods). To obtain estimates for $\frac{\sigma_p}{\sigma_s}$, we use the metabolite concentrations
394 from growth on acetate (Supplementary Table S10). We then predict the concentration ranges
395 for the ten SCC metabolites for the seven other carbon sources (Supplementary Fig. S10, S11).

396 In Supplementary Figures S10 and S11 measured concentration ranges are denoted by red bars
397 and predicted concentration ranges are shown in black. In case of succinate as only carbon
398 source we obtain a model with no feasible solution, so no concentrations could be predicted for
399 that case without further model adaptations. In the remaining growth conditions, depending on
400 the objective and growth condition analyzed, three to five predictions of concentrations resulted
401 in minimum values larger than the respective maximum (missing black bars). This observation
402 is a result of numerical instabilities occurring if flux values v_p and v_s^{-i} in Eq. (1) differ by
403 several orders of magnitude. The Spearman correlation between the average measured and
404 predicted concentrations (Fig. 5) when optimizing ATP synthesis is 0.63 (p-value $3 \cdot 10^{-4}$),
405 while it is only 0.33 (p-value 0.03) when ATP synthesis and total flux are optimized. In
406 addition, the Spearman correlation between the measured and predicted upper and lower
407 bounds when maximization of ATP is used results in higher correlation values (upper bounds
408 0.61 (p-value $4.3 \cdot 10^{-4}$), lower bounds 0.85 (p-value $5.9 \cdot 10^{-9}$)) than those when optimization
409 of ATP synthesis and total flux are employed (upper bounds 0.21 (p-value 0.17), lower bounds
410 0.54 (p-value $1.6 \cdot 10^{-4}$)). These findings imply that the usage of different objectives to estimate
411 flux ratios and through them concentrations of metabolites can also be used to discern
412 importance of optimized objectives in a particular experiment.

413

414



415

416 **Fig. 5. Average measured and predicted concentration of SCC metabolites under**

417 **different carbon sources.** Each data point represents a SCC metabolite (different colors, see

418 legend) under one carbon source (● fructose, ■ galactose, ◆ glucose, * glycerol, × gluconate,

419 ▲ pyruvate). Note that due to numerical instabilities a concentration could not be calculated

420 for all (SCC metabolite, carbon source) combinations, see also Supplementary Fig. S10, S11;

421 (a) concentration prediction using optimization of ATP synthesis and total flux (Spearman

422 correlation 0.33) (b) concentration prediction using optimization of ATP synthesis (Spearman

423 correlation 0.63).

424

425 **Changes in metabolite concentrations in knock-out mutants**

426 The fully parameterized kinetic model of *E. coli* can be used to test the applicability of the

427 approach to predict changes in metabolite concentrations in metabolic engineering scenarios.

428 Here, we test the performance of the approach with knock-out mutants based on the following

429 procedure: We make use of the model parameterization to simulate a steady-state concentration

430 and flux distribution from initial physiologically reasonable values for metabolite

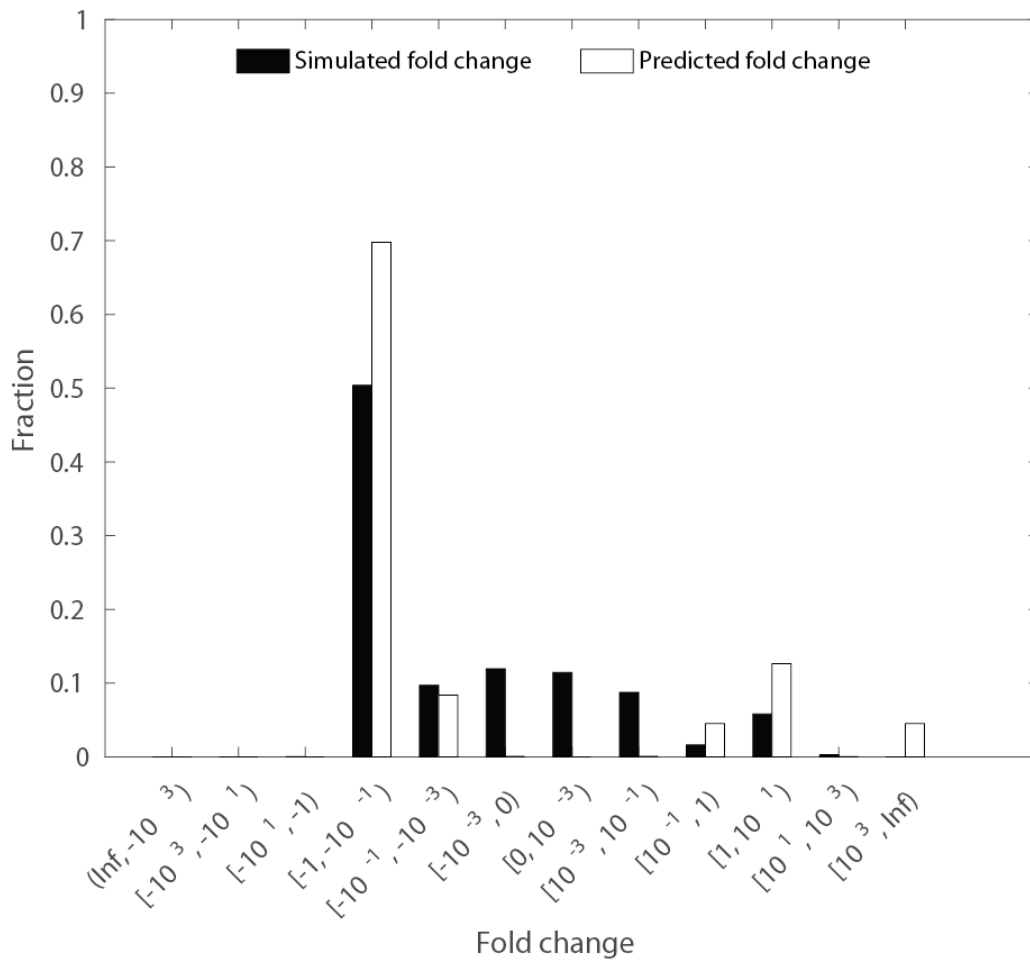
431 concentrations. The resulting steady-state concentrations and fluxes yield a wild type reference.

432 We then knock-out each reaction and predict positive steady state flux distribution closest to

433 the wild type reference, following the Minimization of Metabolic Adjustment (MOMA)

434 approach [33]. The resulting flux distribution is used to calculate the concentrations of the 23
435 SCC metabolites following our approach (Eq. (1)). In the last step, the predicted changes in
436 concentration of the SCC metabolites with respect to the reference are compared to the changes
437 from kinetic simulations of the knock-out with the wild-type reference specifying the initial
438 conditions. We observe similar ranges for the predicted and simulated fold-changes in SCC
439 concentration over all 23 SCC metabolites and knock-outs of 929 reactions for which we were
440 able to simulate a steady-state knock-out flux distribution (Figure 6, fold changes for individual
441 SCC metabolites are shown in Supplementary Figure 12). We grouped the fold-changes into
442 12 bins, given in the x-axis of Figure 6. For ten SCC metabolites, the predicted fold change of
443 at least 29% of the knock-outs is in the same bin as the simulated fold change. The highest
444 overlaps are observed for AMP (39%), phosphoenolpyruvate (38%) and isocitrate (37%). In
445 contrast, the fold changes in concentration for metabolites like succinyl-CoA, acetyl-CoA,
446 oxaloacetate, malate and pyruvate are in the same class as simulated for at most 1% of the
447 knock-outs. The lack of correspondence between simulated and predicted concentrations for
448 some SCC metabolites (Supplementary Fig. S12) indicates that principles others than those
449 used in MOMA shape the metabolic adjustment of knock-out mutants.

450



451

452 **Fig. 6. Fold change in concentration of SCC metabolites upon reaction knock-out.** The
453 distribution of predicted and simulated fold change in concentration of 23 SCC metabolites
454 over 929 single knock-out mutants for which a steady-state flux distribution could be
455 simulated.

456

457 **Metabolites with SCC across species**

458 We next apply Eq. (1) to 14 large-scale metabolic networks which differ in complexity due to
459 the number of considered metabolites and reactions as well as their organization in subcellular
460 compartments (Supplementary Table S6). The investigated metabolic networks are mass- and
461 charge-balanced and support positive steady-state reaction rates (see Methods). Since reliable
462 kinetic information and measurements of absolute concentration measurements are currently

463 missing across diverse species, we report only the number of the metabolites with SCC across
464 the analyzed large-scale networks.

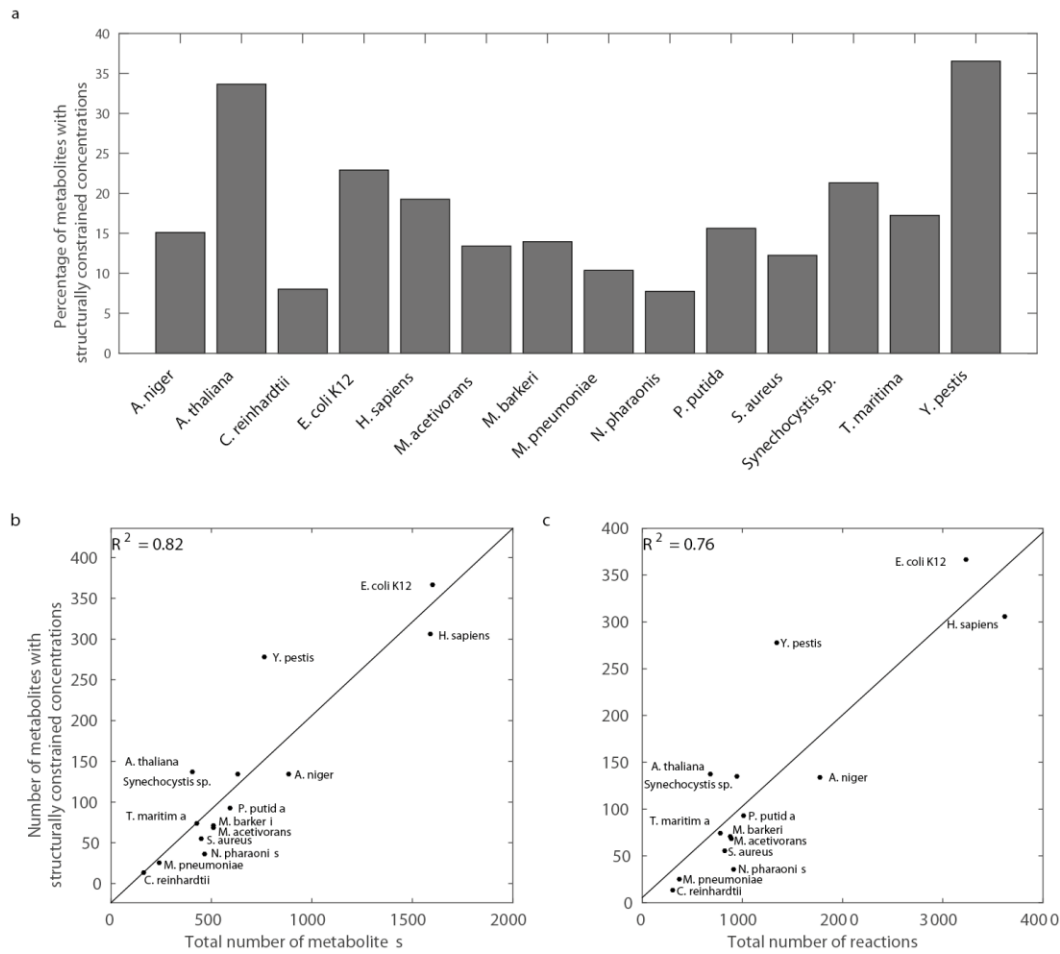
465 We find that the percentage of metabolites with SCC ranges from 7.74 % and 8.02% in
466 the models of *N. pharaonis* and *C. reinhardtii* to 33.66% and 36.53% in the models of *A.*
467 *thaliana* and *Y. pestis* (Fig. 7a). Interestingly, the number of metabolites with SCC scales
468 linearly with the total number of metabolites (Fig. 7b, $R^2 = 0.82$) and the number of reactions
469 in the examined networks (Fig. 7c, $R^2 = 0.76$). This finding indicates that the proposed
470 approach is not limited to networks of a particular size.

471

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475

476 **Fig. 7. Metabolites with structurally constrained concentration across species.** (a) The
 477 fraction of metabolites with structurally constrained concentrations in 14 large-scale metabolic
 478 networks from all kingdoms of life. The number of these metabolites scales linearly with (b)
 479 the total number of metabolites ($R^2 = 0.82$) and (c) the total number of reactions ($R^2 = 0.76$).

480

481 Different reasons can be used to explain the observation that larger networks contain
 482 more metabolites with SCC. For instance, larger networks may include more linear pathways,
 483 whereby the number of reactions which are fully coupled due to structure is expected to
 484 increase. Yet, in denser networks, which include more reactions on the same set of metabolites,
 485 it is more likely to identify reactions which share substrates of same stoichiometry, which then
 486 leads to full coupling due to mass action kinetics, as considered in our approach. To investigate

487 the reasons for the scaling of the number of metabolites with SCC, we determine the number
488 of: (i) metabolites which are synthesized and used by one reaction, respectively (in support of
489 the linear pathway explanation), (ii) fully coupled reactions only due to structure, (iii) coupled
490 reactions due to mass action (in support of the network density explanation), (iv) the
491 combination of (ii) and (iii), to assess the couplings due to both structure and kinetics
492 (Supplementary Table S7). We calculate the Pearson correlation coefficient between each of
493 these properties and the number of reactions over the analyzed networks, as a measure of
494 network size (Supplementary Table S7). Larger networks indeed contain a bigger number of
495 metabolites synthesized and used by a single reaction, respectively, and more reactions which
496 are fully coupled due to both structure and kinetics. Therefore, both the linear pathway and the
497 network density explanations contribute to the observed scaling in the analyzed networks.

498 Due to the derivation of Eq. (1), it may be expected that the approach is not applicable
499 to metabolites which participate in a large number of reactions, since they may be less likely
500 to be fully coupled. Nevertheless, our findings show that between 28.89% and 62.95% of the
501 SCC metabolites in the analyzed networks are involved in more than two reactions (see
502 Supplementary Table S6). One reason is that a SCC metabolite may also be determined by
503 applying Eq. (1) to the ODE of another metabolite (see Eq. (2) and Fig. 1c).

504 Since changes in relevant fluxes directly affect the concentration of a SCC metabolite,
505 they can be used to tightly control the concentration range. For essential metabolic processes
506 to be carried out efficiently, metabolites that serve as coenzymes and energy currency of
507 biological systems, namely, the oxidized and reduced version of NAD and NADP as well as
508 the adenosine phosphates (i.e. AMP, ADP, ATP), are maintained within certain concentration
509 ranges that can be readily controlled, as is the case for SCC metabolites. Despite the many
510 biochemical reactions in which these ubiquitous metabolites participate (Supplementary Table
511 S8), all of which must satisfy our conditions in order to invoke Eq. (1), we find that the

512 (sub)cellular concentrations of ATP and NAD are indeed structurally constrained in twelve and
513 ten of the analyzed networks, respectively. This implies that the network structure, alongside
514 the relevant rate constants and relevant flux ratio, imposes boundaries on and facilitates simple
515 control over their concentrations. In addition, we find that NADP shows SCC in four of the
516 investigated networks, including *A. thaliana* and *C. reinhardtii* (Table 1 and Supplementary
517 Table S8). In these photosynthetic organisms, NADPH is produced by ferredoxin-NADP+
518 reductase in the last step of the electron transport chain which constitutes the light reactions of
519 photosynthesis [34]. The produced NADPH provides reducing power for the biosynthetic
520 reactions in the Calvin cycle to fix carbon dioxide as well as in the reduction of nitrate into
521 ammonia for plant assimilation in the nitrogen cycle. Therefore, precise and simple control of
522 NADPH will provide uninterrupted functionality of these key metabolic pathways and
523 maintenance of carbon and nitrogen balance [35]. In addition, for ten models, we find that H+
524 is SCC, ensuring maintenance of the specific functions of individual organelles [36].
525 Altogether, our findings indicate that the concentration ranges for coenzymes and other
526 components essential for fueling metabolism can be established by controlling few ratios of
527 fluxes, despite their involvement in hundreds of reactions. Moreover, they imply that the
528 network architecture may be organized such that the concentrations of these metabolites are
529 intrinsically constrained and easy to control.

530

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535 **Table 1. Structurally constrained concentrations for metabolites serving as energy**
 536 **currency.** (h=chloroplast, c = cytosol, m = mitochondria, n = nucleus, p = periplasm, e =
 537 extracellular space). The table summarizes the networks in which Eq. (1) holds for NADH,
 538 NAD, NADP, NADPH, ATP, and H⁺. The table includes the respective compartments in
 539 which Eq. (1) can be applied for the investigated metabolites.

Network	NADH	NAD	NADP	NADPH	ATP	H ⁺
<i>A. niger</i>		c	c	c	c	
<i>A. thaliana</i>			h		h,c,m	
<i>C. reinhardtii</i>			h		h	h,c
<i>E. coli K12</i>		c			c	c,p
<i>H. sapiens</i>	c	c		c	c,n	c
<i>M. acetivorans</i>		c			c	c
<i>M. barkeri</i>		c			c	c
<i>M. pneumoniae</i>						
<i>N. pharaonic</i>		c			c	
<i>P. putida</i>					c	c,e
<i>T. maritima</i>		c	c		c	c,e
<i>S. aureus</i>		c				c,e
<i>Synechocystis sp.</i>		c			c	c,p
<i>Y. pestis</i>		c			c	c
Number of networks where Eq.(1) can be applied	1	10	4	2	12	10

540

541 Discussion

542 Genome-scale metabolic networks have propelled the understanding of the metabolic
 543 capabilities for a wide variety of organisms across all kingdoms of life. The existing large-scale
 544 modelling approaches examine the space of feasible fluxes, but cannot be used to infer the
 545 metabolite concentrations driving these fluxes without extensively relying on largely unknown
 546 kinetic parameters. Hence, the direct usage of large-scale metabolic networks to make

547 predictions about concentrations that are directly testable from high-throughput metabolomics
548 data is not possible with the existing modelling approaches.

549 Here we derive a condition that pinpoints that the structure of a metabolic network,
550 ratios of relevant rate constants, and ratios of relevant reaction fluxes constitute the determinant
551 of concentration ranges for selected metabolites. This link is based on the well-known concept
552 of full coupling of reactions [21, 23] which we expand under the assumption of mass action
553 kinetics to include reactions that share substrates of same stoichiometry. These concepts allow
554 us to efficiently determine the admissible concentration ranges in large-scale metabolic
555 networks endowed with mass action kinetics across all kingdoms of life. The derivation of Eq.
556 (1) can be generalized by considering reactions which differ in order larger than one with
557 respect to a single metabolite. For a given flux distribution this approach results in a polynomial
558 equation in a single variable which can be efficiently solved with the Newton's method.

559 Our approach is also applicable to networks with kinetic laws derived from mass action
560 which involve enzyme forms (e.g., Michaelis-Menten). This can be achieved by augmenting
561 the network to include reactions which model substrate-enzyme complex formation as well as
562 the synthesis and degradation of enzymes. However, these extensions come at a cost of
563 substantially larger data sets which are not yet readily available. In addition, our analyses
564 demonstrate that the casting of a kinetic rate law in terms of mass action mechanisms may
565 affect the findings regarding the SCC metabolites. For instance, we find that there are many
566 more SCC metabolites in comparison to other SCC components (i.e., enzymes and enzyme-
567 substrate complexes) in each of the analyzed models (Supplementary Table S9). With
568 exception of the network of *C. reinhardtii*, the usage of enzymatic forms explicitly in mass
569 action mechanisms leads to a decrease in the number of metabolites with SCC (Supplementary
570 Table S9), due to the decrease in the number of reaction pairs which differ in their order by

571 one. Applications of the approach to other forms of kinetics will be subject in future
572 investigations and extensions.

573 Our approach provides a links between metabolite concentrations, relevant rate
574 constants, and relevant flux ratios; therefore, information on two of these can be used to predict
575 the third. Our analyses demonstrate that there is a good quantitative agreement between
576 predicted and simulated concentration ranges based on full knowledge of rate constants from a
577 kinetic model of *E. coli*. Rate constants of elementary reactions are expected to become
578 increasingly available for model organisms, largely due to the development of computational
579 methods coupled with high-throughput data [8, 9]. In addition, by examining the scenario
580 where flux ratios are estimated from the constraint-based modeling framework, we observe
581 that the approach can be used to select which objective function (or a combination thereof) is
582 optimized by a biological system for which metabolite concentration measurements are
583 available.

584 Most importantly, we show that even in the absence of data on relevant rate constants
585 and relevant flux ratios, we can apply the approach to successfully predict concentration ranges
586 in *E. coli* under different growth conditions, provided measurements of concentrations for SCC
587 metabolites in one reference condition. Therefore, the proposed approach represents an
588 important step in complementing genome-scale metabolic networks with metabolite
589 concentrations, widening the applicability of large-scale models to a range of biotechnological
590 and medical applications.

591 **Materials and Methods**

593 **Components with structurally constrained concentrations**

594 A metabolic network can be represented by the stoichiometric matrix, $N = N^+ - N^-$, where
595 N^+ includes the stoichiometry of the products and N^- comprises the stoichiometry of the

596 substrates of each reaction. In the following, we derive the conditions for structurally
 597 constrained robustness of component X_i based on the ordinary differential equation (ODE) for
 598 the component X_j (not necessarily different from X_i) under the assumption that the reaction
 599 rates, $v(t)$, satisfy mass action kinetics, whereby $v_i(t) = \theta_i \prod_j x_j^{N_{ji}^-}$. Let the ODE be
 600 specified by $\frac{dx_j(t)}{dt} = \sum_{k \in P_j} N_{jk}^+ v_k(t) - \sum_{l \in S_j} N_{jl}^- v_l(t)$, where P_j is the set of reactions with X_j
 601 as one of their products and S_j is the set of reactions which have metabolite X_j as one of their
 602 substrates.

603 We consider the following two cases: (i) the concentration of X_i appears in every $v_k(t)$
 604 for which $N_{jk}^+ \neq 0$ and for every $v_k(t)$ there exist a set P_j^{-i} of reactions $R_k^{-i} \in P_j^{-i}$ such that
 605 $v_k(t) = x_i(t) \frac{\theta_k}{\theta_k^{-i}} v_k^{-i}(t)$ and (ii) the concentration of X_i appears in every $v_l(t)$ for which $N_{jl}^- \neq$
 606 0 and for every $v_l(t)$ there exist a set of reactions $R_l^{-i} \in S_j^{-i}$ such that $v_l(t) = x_i(t) \frac{\theta_l}{\theta_l^{-i}} v_l^{-i}(t)$.

607

608 **Case I:**

609 The rates of a reaction R_k and a reaction from the set R_k^{-i} are given by

$$610 \quad v_k(t) = \theta_k \prod_j x_j^{N_{jk}^-}(t) = \theta_k \prod_{j \neq i} x_j^{N_{jk}^-}(t) x_i^{N_{ik}^-}(t) = \theta_k x_i(t) \prod_{j \neq i} x_j^{N_{jk}^-}(t) x_i^{N_{ik}^- - 1}(t)$$

611 and

$$612 \quad v_k^{-i}(t) = \theta_k^{-i} \prod_j x_j^{N_{jk}^-}(t) = \theta_k^{-i} \prod_{j \neq i} x_j^{N_{jk}^-}(t) x_i^{N_{jk}^-}(t).$$

613 From rewriting the equation of $v_k^{-i}(t)$ above we have that $\prod_{j \neq i} x_j^{N_{jk}^-}(t) = \frac{v_k^{-i}(t)}{\theta_k^{-i} x_i^{N_{jk}^-}(t)}$. Since

614 $N_{jk}^- - N_{jk}^{-i} = 0$ for every $j \neq i$ and $N_{ik}^- - N_{ik}^{-i} = 1$ we can rewrite the equation of $v_k(t)$ such

615 that

$$616 \quad v_k(t) = \frac{\theta_k}{\theta_k^{-i}} x_i(t) v_k^{-i}(t) x_i^{N_{ik}^- - N_{ik}^{-i} - 1}(t) = \frac{\theta_k}{\theta_k^{-i}} x_i(t) v_k^{-i}(t).$$

617

618 The ODE for component X_j revealing structurally constrained concentration of
 619 component X_i is then given by:

$$620 \quad \frac{dx_j(t)}{dt} = \sum_{k \in P_j} N_{jk}^+ v_k(t) - \sum_{l \in S_j} N_{jl}^- v_l(t) = x_i(t) \sum_{k \in P_j} N_{jk}^+ \frac{\theta_k}{\theta_k^{-i}} v_k^{-i}(t) - \sum_{l \in S_j} N_{jl}^- v_l(t).$$

621 Let p and s bet two reaction indices such that $N_{jp}^+ \neq 0$ and $N_{js}^- \neq 0$. In any positive state $v(t)$,
 622 we have that

$$623 \quad \frac{dx_j(t)}{dt} = v_p^{-i}(t) x_i(t) \sum_{k \in P_j} N_{jk}^+ \frac{\theta_k v_k^{-i}(t)}{\theta_k^{-i} v_p^{-i}(t)} - v_s(t) \sum_{l \in S_j} N_{jl}^- \frac{v_l(t)}{v_s(t)}.$$

624 In a steady state then

$$625 \quad v_p^{-i} x_i \sum_{k \in P_j} N_{jk}^+ \frac{\theta_k v_k^{-i}}{\theta_k^{-i} v_p^{-i}} - v_s \sum_{l \in S_j} N_{jl}^- \frac{v_l}{v_s} = 0.$$

626 If for every $N_{jp}^+ \neq 0$, $\frac{v_k^{-i}}{v_p^{-i}}$ is constant because either reactions R_k^{-i} and R_p^{-i} are fully coupled or

627 share the same substrates, then $\sum_{k \in P_j} N_{jk}^+ \frac{\theta_k v_k^{-i}}{\theta_k^{-i} v_p^{-i}} = \sigma_p^{-i}$ is a constant that only depends on a

628 subset of rate constants and the network structure. Moreover, if for every $N_{js}^- \neq 0$, $\frac{v_l}{v_s}$ is constant

629 because either reactions R_l and R_s are fully coupled or share the same substrates, then

630 $\sum_{l \in S_j} N_{jl}^- \frac{v_l}{v_s} = \sigma_s$ is a constant, too, which in the simplest case when all reactions in S_j are fully

631 coupled irrespective of the kinetic rate law, only depends on the network structure. Therefore,

$$632 \quad v_p^{-i} x_i \sigma_p^{-i} - v_s \sigma_s = 0,$$

$$633 \quad \text{and } x_i = \frac{\sigma_s}{\sigma_p^{-i}} \frac{v_s}{v_p^{-i}}.$$

634 For each reaction R_k in S_j , there exists a non-empty subset Q_k^{-i} of reactions lacking

635 one substrate molecule of X_i in comparison to R_k ; the union of all Q_k^{-i} yields the set of reactions

636 S_j^{-i} . Let Q be a subset of P_j^{-i} that contains one and only one reaction from each of Q_k^{-i} . Since

637 the reaction indices p and s are arbitrarily chosen, the concentration range of metabolite X_i for
 638 a given subset Q over a given set of flux distributions, F , is given as

$$639 \quad \min_{\{Q,F\}} \frac{v_s}{v_p^{-i}} \frac{\sigma_s}{\sigma_p^{-i}} \leq x_i \leq \max_{\{Q,F\}} \frac{v_s}{v_p^{-i}} \frac{\sigma_s}{\sigma_p^{-i}}.$$

640 **Case II:**

641 The rates of a reaction R_l and a reaction from the set R_l^{-i} are given by

$$642 \quad v_l(t) = \theta_l \prod_j x_j^{N_{jl}^-}(t) = \theta_l \prod_{j \neq i} x_j^{N_{jl}^-}(t) x_i^{N_{il}^-}(t) = \theta_l x_i(t) \prod_{j \neq i} x_j^{N_{jl}^-}(t) x_i^{N_{il}^- - 1}(t)$$

643 and

$$644 \quad v_l^{-i}(t) = \theta_l^{-i} \prod_j x_j^{N_{jl}^-}(t) = \theta_l^{-i} \prod_{j \neq i} x_j^{N_{jl}^-}(t) x_i^{N_{il}^-}(t).$$

645 From rewriting the equation of $v_l^{-i}(t)$ above we have that $\prod_{j \neq i} x_j^{N_{jl}^-}(t) = \frac{v_l^{-i}(t)}{\theta_l^{-i} x_i^{N_{il}^-}(t)}$. Since

646 $N_{jl}^- - N_{jl}^{-i} = 0$ for every $j \neq i$ and $N_{il}^- - N_{il}^{-i} = 1$ we can rewrite the equation of $v_l(t)$ such

647 that

$$648 \quad v_l(t) = \frac{\theta_l}{\theta_l^{-i}} x_i(t) v_l^{-i}(t) x_i^{N_{il}^- - N_{il}^{-i} - 1}(t) = \frac{\theta_l}{\theta_l^{-i}} x_i(t) v_l^{-i}(t).$$

649

650 The ODE for component X_j revealing structurally constrained concentration of component X_i

651 is then given by:

$$652 \quad \frac{dx_j(t)}{dt} = \sum_{k \in P_j} N_{jk}^+ v_k(t) - \sum_{l \in S_j} N_{jl}^- v_l(t) = \sum_{k \in P_j} N_{jk}^+ v_k(t) - x_i(t) \sum_{l \in S_j} N_{jl}^- \frac{\theta_l}{\theta_l^{-i}} v_l^{-i}(t).$$

653 Let p and s bet two reaction indices such that $N_{jp}^+ \neq 0$ and $N_{js}^- \neq 0$. In any positive state $v(t)$,

654 we have that

$$655 \quad \frac{dx_j(t)}{dt} = v_p(t) \sum_{k \in P_j} N_{jk}^+ \frac{v_k(t)}{v_p(t)} - v_s^{-i}(t) x_i(t) \sum_{l \in S_j} N_{jl}^- \frac{\theta_l}{\theta_l^{-i}} \frac{v_l^{-i}(t)}{v_s^{-i}(t)}.$$

656 In a steady state then

$$657 \quad v_p \sum_{k \in P_j} N_{jk}^+ \frac{v_k}{v_p} - v_s^{-i} x_i \sum_{l \in S_j} N_{jl}^- \frac{\theta_l}{\theta_l^{-i}} \frac{v_l^{-i}}{v_s^{-i}} = 0.$$

658 If for every $N_{jp}^+ \neq 0$, $\frac{v_k}{v_p}$ is constant because either reactions R_k and R_p are fully coupled or
 659 share the same substrates, then $\sum_{k \in P_j} N_{jk}^+ \frac{v_k}{v_p} = \sigma_p$ is a constant that, in the simplest case when
 660 all reactions in P_j are fully coupled irrespective of the kinetic rate law, depends only on the
 661 network structure. Moreover, if for every $N_{jl}^- \neq 0$, $\frac{v_l^-}{v_s^-}$ is constant because either reactions R_l^-
 662 and R_s^- are fully coupled or share the same substrates, then $\sum_{l \in S_j} N_{jl}^- \frac{\theta_l v_l^-}{\theta_l^- v_s^-} = \sigma_s^-$. The
 663 constant σ_s^- then only depends on a subset of rate constants and the network structure.
 664 Therefore,

$$v_p \sigma_p - v_s^- x_i \sigma_s^- = 0,$$

665 and $x_i = \frac{\sigma_p v_p}{\sigma_s^- v_s^-}$.

667 For each reaction R_l in P_j , there exists a non-empty subset Q_l^- of reactions lacking one
 668 substrate molecule of X_i in comparison to R_l ; the union of all Q_l^- yields the set of reactions
 669 P_j^- . Let Q be a subset of P_j^- that contains one and only one reaction from each of Q_l^- . Since
 670 the reaction indices p and s are arbitrarily chosen, the concentration range of metabolite X_i for
 671 a given subset Q over a given set of flux distributions, F , is given as

$$672 \quad \min_{\{Q,F\}} \frac{\sigma_p v_p}{\sigma_s^- v_s^-} \leq x_i \leq \max_{\{Q,F\}} \frac{\sigma_p v_p}{\sigma_s^- v_s^-}.$$

673 As a result, the ranges for steady-state concentration x_i can be expressed as a function of a set
 674 of given flux distributions, ratios of specific fluxes and constants that depend only on the
 675 structure of the network and values for a subset of rate constants. Since fluxes are the integrated
 676 outcome of transcription, translation, and post-translational modifications and their interplay
 677 with the environment and nutrient availability, our derivation provides a direct relation between
 678 concentration ranges, flux ratios, and rate constants.

679 Flux coupling

680 Let $C(N) = \{v \in \mathbb{R}^n | Nv = 0, v \geq 0\}$ be the steady-state flux cone for a given stoichiometric
681 matrix N with n reactions, under the assumption that every reaction is irreversible. Here, we
682 restrict our analysis to the subspace $F \subset C(N)$ by bounding the fluxes: $F = \{v \in \mathbb{R}^n | Nv =$
683 $0, 0 \leq lb \leq v \leq ub\}$, where lb and ub are lower and upper flux bounds. We will refer to $v \in F$
684 as the feasible flux distributions. A reaction R_i is called blocked if for every $v \in F$, $v_i = 0$. A
685 pair of reactions R_i and R_j is called fully coupled, if there exists $\lambda > 0$, such that for every $v \in$
686 F , $v_i = \lambda v_j$.

687 The minimum and maximum value for the ratio $\frac{v_i}{v_j}$ over the flux distributions in F can
688 be determined by the linear-fractional programming:

$$\begin{aligned} 689 \quad & \text{opt } \frac{v_i}{v_j} \\ 690 \quad & Nv = 0 \\ 691 \quad & lb \leq v \leq ub, \end{aligned}$$

692 which can be rewritten following the Charnes-Cooper transformation [37] to the following
693 linear program:

$$\begin{aligned} 694 \quad & \text{opt } v_i \\ 695 \quad & Nv = 0 \\ 696 \quad & v_j = 1 \\ 697 \quad & t \cdot lb \leq v \leq t \cdot ub \\ 698 \quad & t \geq 0. \end{aligned}$$

699 If the minimum and maximum values for the linear program are the same, then the reactions
700 R_i and R_j are fully coupled. Such reactions can be efficiently computed for large-scale
701 networks[4, 21].

702 In addition, under the mass action kinetics, two reactions are fully coupled in any state of the
703 system if they share the same substrates with the same stoichiometry. This leads to additional
704 full couplings due to the transitivity of the relations, as demonstrated in the main text.

705 **Metabolites with structurally constrained concentrations in mass action networks**

706 In the following, we present an algorithm determining SCC metabolites under the assumption
707 of mass action kinetics:

708 **Input:** metabolic network, list of fully coupled reactions

709 **Output:** metabolites with structurally constrained concentration

710 **for each** metabolite x_i in the network **do:**

711 $S_i \leftarrow$ set of reactions having x_i as substrate

712 $M_i^P \leftarrow$ set of all products of the reactions in S_i

713 **for each** metabolite $x_j \in M_i^P$ **do:**

714 $S_j \leftarrow$ set of reactions having x_j as substrate

715 $P_j \leftarrow$ set of reactions having x_j as product

716 $P_j^{-i} \leftarrow$ set of reactions lacking one substrate molecule of x_i in comparison to a
717 reaction $R_p \in P_j$

718 **if** for each reaction in P_j there is a reaction in P_j^{-i} **and** all reactions in P_j^{-i} are
719 fully coupled **and** all reactions in S_j are fully coupled:

720 x_i has SCC

721 **end if**

722 **end for**

723 $M_i^S \leftarrow$ set of all substrates of the reactions in S_i

724 **for each** metabolite $x_j \in M_i^S$ **do:**

725 $S_j \leftarrow$ set of reactions having x_j as substrate

726 $S_j^{-i} \leftarrow$ set of reactions lacking one substrate molecule of x_i in comparison to a
727 reaction $R_s \in S_j$

728 $P_j \leftarrow$ set of reactions having x_j as product

729 **if** for each reaction in S_j there is a reaction in S_j^{-i} **and** all reactions in S_j^{-i} are
730 fully coupled **and** all reactions in P_j are fully coupled:

731 x_i has SCC

732 **end if**

733 **end for**

734 **end for**

735

736

737 **Correlation analysis**

738 Using a large-scale kinetic model of *E. coli* we simulate 100 steady-state flux distribution and
739 steady-state concentrations from different initial concentrations. Initial concentrations were
740 obtained by perturbation of the original initial concentration of a metabolite by 1, 5, 10 or 20%.
741 We run the model until a steady-state was reached. Using the simulated steady-state flux
742 distributions we can predict concentration ranges for 23 metabolites using Eq. (2)
743 (Supplementary Table S1). The Pearson correlation was then calculated for (i) simulated and
744 predicted upper bounds, (ii) simulated and predicted lower bounds, and (iii) the absolute range
745 over simulated and predicted concentrations. In addition, we also determined the correlation
746 between shadow price for the respective metabolites and the simulated range, as well as, to the
747 coefficient of variation obtained over simulated concentrations (Supplementary Table S2).
748 Moreover, we calculated the Euclidean distance between upper and lower bound from
749 prediction and simulation, respectively. Due to the high difference in the order of magnitude
750 over the analyzed metabolites we also calculated Euclidean distance after normalizing the data.
751 We considered the Euclidean distance of log-transformed concentration vectors, and the
752 Euclidean distance between the concentration vectors normalized by the respective maximum
753 value.

754 **Effect of missing information on rate constants**

755 To assess the effect of missing information about rate constants on the accuracy of the predicted
756 concentration range, we simulated missing knowledge about parameters by removing 10, 30,
757 50, 70 or 90% of the relevant rate constants uniformly at random. We consider only removing
758 information about relevant rate constants to avoid bias due to removal of information in parts
759 of the network that have no effect on the predictions of the concentration ranges. We compare
760 the Pearson and Spearman correlation coefficient between predicted and simulated

761 concentration ranges as well as the two versions of Euclidean distance for each percentage
762 obtained over 100 random removals of rate constants.

763 **Effect of missing information on flux ratios**

764 To assess the effect of missing information about flux ratios on the accuracy of the predicted
765 concentration range, we obtained relevant flux ratios from constraint-based modeling.
766 Therefore, we solve the following linear program optimizing a weighted average of ATP
767 production and total flux:

$$768 \quad \max z^* = v_{atp} - 0.01 \sum_i^{n-1} v_i$$

$$769 \quad Nv = 0$$

$$770 \quad v_{sim_min} \leq v_{exchange} \leq v_{sim_max}$$

$$771 \quad v_{min} \leq v \leq v_{max}$$

$$772 \quad v_{min} \geq \epsilon = 10^{-7}$$

773 In addition, the flux through exchange reactions is constrained by the respective minimum,
774 v_{sim_min} , and maximum value, v_{sim_max} , obtained over 100 simulations (Supplementary Table
775 S1) to obtain a physiologically reasonable flux distribution. The weighting factor of 0.01 was
776 chosen to reduce the effect of three orders of magnitude difference in the respective optimum
777 observed when ATP production and total flux are optimized individually.

778 Next, we determine the range for the relevant flux ratios $\frac{v_p}{v_s^i}$ at the optimum z^* using a

779 transformed linear-fractional program:

780 opt v_p

$$781 \quad Nv = 0$$

$$782 \quad v_{atp} - 0.01 \sum_i^{n-1} v_i = z^*$$

783 $v_s^{-i} = 1$

784 $t \cdot v_{sim_min} \leq v_{exchange} \leq t \cdot v_{sim_max}$

785 $t \cdot v_{min} \leq v \leq t \cdot v_{max}$

786 $t \geq \epsilon$

787 $v_{min} \geq \epsilon = 10^{-7}$.

788 We then used the obtained ranges for $\frac{v_p}{v_s^{-i}}$ together with Eq. (2) to calculate concentration ranges
789 for SCC metabolite X_i .

790 **Extension of the approach based on available concentration measurements**

791 Using the most recent genome-scale metabolic network of *E. coli* [27] together with
792 measurements of steady-state concentrations from *E. coli* under different growth scenarios [28]
793 we predict concentration ranges for 15 SCC metabolites using the following procedure. We
794 first use the concentration measurements from three replicates at a growth rate of $0.2h^{-1}$
795 (reference state) together with flux ratios obtained from constraint-based modelling to estimate
796 the ratio $\frac{\sigma_p}{\sigma_s^{-i}}$ given that $x_i = \frac{\sigma_p}{\sigma_s^{-i}} \frac{v_p}{v_s^{-i}}$.

797 For each replicate we solve the following linear programs in order to obtain ranges for the
798 relevant flux ratios $\frac{v_p}{v_s^{-i}}$.

799 $\max z^* = 0.1v_{atp} - \sum_i^{n-1} v_i$

800 $Nv = 0$

801 $v_{bio} = 0.2$

802 $v_{O2\ uptake} = \beta_{1,j}, 1 \leq j \leq 3$

803 $v_{Glc\ uptake} = \beta_{2,j}$

804 $v_{CO2\ release} = \beta_{3,j}$

805 $v_{min} \leq v \leq v_{max}$

806 $v_{min} \geq \epsilon = 10^{-7}$.

807 The linear program above constrains rates of glucose and oxygen uptakes, carbon dioxide
808 release as well as growth by values $\beta_{i,j}$ (which differ between replicates j , $1 \leq j \leq 3$) available
809 from measurements [28]. We optimize the weighted average of ATP synthesis and total flux.
810 The weighting factor of 0.1 and 0.001 for ATP synthesis, for the data set of Ishii et al. [28] and
811 Gerosa et al. [32], respectively, is chosen to reduce the effect of the order difference in the
812 respective optimum observed when ATP production and total flux are optimized individually.
813 In addition, we use weighting factors of 1 and 1000 for optimization of total flux in the case of
814 Ishii et al. [28] and Gerosa et al. [32], respectively. To obtain ranges for the relevant flux ratios
815 $\frac{v_p}{v_s^{-i}}$, which are employed to calculate ranges for ratios $\frac{\sigma_p}{\sigma_s^{-i}}$, we solve the following linear program
816 at the optimum z^* :

817 opt v_p

818 $Nv = 0$

819 $v_{bio} = 0.2$

820 $v_{O_2 \text{ uptake}} = \beta_1$

821 $v_{Glc \text{ uptake}} = \beta_2$

822 $v_{CO_2 \text{ release}} = \beta_3$

823 $0.1v_{atp} - \sum_i^{n-1} v_i = z^*$

824 $v_s^{-i} = 1$

825 $t \cdot v_{min} \leq v \leq t \cdot v_{max}$

826 $v_{min} \geq \epsilon = 10^{-7}$

827 $t \geq 0$.

828

829 From Eq. (2) we predict concentration values for *E. coli* cells with growth rates of 0.4, 0.5, and
830 $0.7h^{-1}$ using the previously obtained estimates for ranges of $\frac{\sigma_p}{\sigma_s^{-i}}$ together with ranges of $\frac{v_p}{v_s^{-i}}$. The
831 latter can be obtained following the same procedure as described above using rates of glucose
832 and oxygen uptakes, carbon dioxide release as well as growth for *E. coli* cells grown at rates
833 of 0.4, 0.5, and $0.7h^{-1}$.

834 **Fold changes in SCC metabolite concentrations in knock-out mutants**

835 We use a large-scale kinetic model of *E. coli* [8] to simulate a steady-state concentration and
836 flux distribution from initial physiologically reasonable values for metabolite concentrations
837 provided in the original publication. The simulated steady-state concentrations and fluxes yield
838 a wild type reference. Next, we simulate single reaction knock-outs and predict positive steady
839 state flux distribution closest to the wild type reference, following the Minimization of
840 Metabolic Adjustment (MOMA) approach [33] for each mutant. The resulting flux distribution
841 is used to calculate the concentrations of the 23 SCC metabolites following Eq. (1). In addition,
842 we simulate steady-state flux distributions and concentrations for knock-out mutants from the
843 kinetic model using the wild type reference as initial concentrations. For 929 out of 1474
844 reaction knock-outs we could simulate steady-state values. Based on these knock-out mutants
845 we then compare fold changes in concentration of the SCC metabolites with respect to the
846 reference obtained from kinetic model simulations and predictions using MOMA.

847

848

849

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854

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974

975

976 **Author contributions:**

977 Conceptualization, Z.N.; Methodology, J.M.O.E.-M., Z.N.; Software, A.K.; Formal Analysis,
978 A.K, Z.N.; Investigation, A.K., G.B.; Writing – Original Draft, G.B., Z.N.; Writing – Review
979 and Editing, A.K., J.M.O.E.-M., G.B., and Z.N.

980

981 **Competing interests**

982 The authors declare no competing interests.

983

984 **Data availability**

985 The code and data to reproduce the results are available on GitHub
986 <https://github.com/ankueken/SCC>.

987

988 **Supplementary Figure legends:**

989

990 **Fig. S1. Agreement between simulated and predicted bounds from a kinetic metabolic**
991 **model of *E. coli*.** The simulated and predicted (a) lower and (b) upper concentration bounds
992 for 23 SCC metabolites in the large-scale kinetic model of *E. coli*. The very small discrepancies
993 are due to numerical instabilities.

994

995 **Fig. S2. Distribution of rate constants used in calculation of concentration ranges for SCC**
996 **metabolites in a genome-scale metabolic model of *E. coli*.** Distribution of (a) the relevant
997 rate constants and (b) their ratios for reactions coupled due to mass action kinetics; log-log
998 distribution of (c) the relevant rate constants and (d) their ratios for reactions coupled due to
999 mass action kinetics.

1000

1001 **Fig. S3. Effect of missing information about relevant rate constants on the accuracy of**
1002 **concentration range predictions for a large-scale kinetic model of *E. coli*.** We consider 10
1003 – 90% of the relevant rate constants to be unknown by random removal. We consider three
1004 scenarios for the substitution of missing ratios of rate constants: (i) equality (i.e., kinetic rate
1005 constants are assumed to be the same), (ii) the mean, or (iii) the median of the ratios of relevant
1006 rate constants that are still present in the model. Shown are the boxplots (red lines inside each
1007 box denote the corresponding medians) of the resulting Spearman correlation coefficients
1008 between the predicted and simulated (a) lower bound vectors and (b) upper bound vectors of
1009 concentrations over the SCC metabolites in the kinetic model of *E. coli*.

1010

1011 **Fig. S4. Effect of missing information about relevant rate constants on the accuracy of**
1012 **concentration range predictions for a large-scale kinetic model of *E. coli*.** We consider 10

1013 – 90% of the relevant rate constants to be unknown by random removal. We consider three
1014 scenarios for the substitution of missing ratios of rate constants: (i) equality (i.e., kinetic rate
1015 constants are assumed to be the same), (ii) the mean, or (iii) the median of the ratios of relevant
1016 rate constants that are still present in the model. Shown are the boxplots (red lines inside each
1017 box denote the corresponding medians) of the average Euclidean distance between the
1018 predicted and simulated (a) lower bound vectors and (b) upper bound vectors of concentrations
1019 over the SCC metabolites in the kinetic model of *E. coli*.

1020

1021 **Fig. S5. Effect of missing information about relevant rate constants on the accuracy of**
1022 **concentration range predictions for a large-scale kinetic model of *E. coli*.** We consider 10
1023 – 90% of the relevant rate constants to be unknown by random removal. We consider three
1024 scenarios for the substitution of missing ratios of rate constants: (i) equality (i.e., kinetic rate
1025 constants are assumed to be the same), (ii) the mean, or (iii) the median of the ratios of relevant
1026 rate constants that are still present in the model. Shown are the boxplots (red lines inside each
1027 box denote the corresponding medians) of the Euclidean distance between the log-transformed
1028 predicted and log-transformed simulated (a) lower bound vectors and (b) upper bound vectors
1029 of concentrations over the SCC metabolites in the kinetic model of *E. coli*.

1030

1031 **Fig. S6. Effect of missing information about relevant rate constants on the accuracy of**
1032 **concentration range predictions for a large-scale kinetic model of *E. coli*.** We consider 10
1033 – 90% of the relevant rate constants to be unknown by random removal. We consider three
1034 scenarios for the substitution of missing ratios of rate constants: (i) equality (i.e., kinetic rate
1035 constants are assumed to be the same), (ii) the mean, or (iii) the median of the ratios of relevant
1036 rate constants that are still present in the model. Shown are the boxplots (red lines inside each
1037 box denote the corresponding medians) of the Euclidean distance between the predicted and

1038 simulated **(a)** lower bound vectors of concentrations normalized by the respective maximum
1039 value and **(b)** upper bound vectors of concentrations normalized by the respective maximum
1040 value over the SCC metabolites in the kinetic model of *E. coli*.

1041

1042 **Fig. S7. Predicted concentration ranges for 15 intracellular metabolites in *E. coli* at**
1043 **growth rates (GR) of 0.4, 0.5 and $0.7h^{-1}$ under the objective of optimizing ATP synthesis**
1044 **and sum of total flux.** The bars denote the predicted ranges from each of the three different
1045 scenarios **(a)** over all three replicates and **(b)** over replicates with not more than one magnitude
1046 difference in estimated range for the ratio of $\frac{\sigma_p}{\sigma_s}$. The marked points denote the measured
1047 concentrations in the employed data set.

1048

1049 **Fig. S8. Distribution of average Euclidean distance between simulated and predicted**
1050 **concentration.** From each of the 100 simulated steady-state flux distributions we predict
1051 concentrations for the SCC metabolites and calculate the average Euclidean distance between
1052 the simulated and predicted concentrations.

1053

1054 **Fig. S9. Comparison of predicted ranges with measured metabolite concentrations under**
1055 **the objective of optimizing ATP synthesis for the data set of Ishii et al.** Comparison of the
1056 predicted concentration ranges for 15 intracellular metabolites in *E. coli* with absolute
1057 concentrations measured at growth rates (GR) of **(a)** 0.4, **(b)** 0.5 and **(c)** $0.7h^{-1}$. The colored
1058 bars denote the predicted ranges from each of the three different replicates, while the black bar
1059 represents the prediction over all replicates. For some metabolites no value could be predicted
1060 due to numerical instabilities. The red cross denotes the measured value at the respective GR.
1061 For metabolites with missing red cross, there is no access to measurements. The nomenclature
1062 of the metabolites is provided in Supplementary Table S5.

1063

1064 **Fig. S10. Comparison of predicted ranges with measured metabolite concentrations**

1065 **under the objective of optimizing ATP synthesis and total flux for the data set of Gerosa**

1066 **et al.** Comparison of the predicted concentration ranges for 10 intracellular metabolites in *E.*

1067 *coli* with absolute concentrations measured at seven different carbon sources. The red bars

1068 denote the measured ranges over three different replicates, while the black bar represents the

1069 predicted concentration. For some metabolites no value could be predicted due to numerical

1070 instabilities. For the model simulating growth on succinate no steady-state solution could be

1071 obtained without further model adaptation, therefore, no SCC concentration could be predicted.

1072

1073 **Fig. S11. Comparison of predicted ranges with measured metabolite concentrations**

1074 **under the objective of optimizing ATP synthesis for the data set of Gerosa et al.**

1075 Comparison of the predicted concentration ranges for 10 intracellular metabolites in *E. coli*

1076 with absolute concentrations measured at seven different carbon sources. The red bars denote

1077 the measured ranges over three different replicates, while the black bar represents the predicted

1078 concentration. For some metabolites no value could be predicted due to numerical instabilities.

1079 For the model simulating growth on succinate no steady-state solution could be obtained

1080 without further model adaptation, therefore, no SCC concentration could be predicted.

1081

1082 **Fig. S12. Fold change in concentration of SCC metabolites upon reaction knock-out.**

1083 Distributions of predicted and simulated fold change in concentration for the 23 SCC

1084 metabolites over 929 single knock-out mutants, for which a steady-state flux distribution could

1085 be simulated.

1086

1087

1088 **Supplementary Table captions**

1089

1090 **Table S1.** (A) Initial conditions sampled for simulations of the large-scale kinetic model of *E.*
1091 *coli*. The initial concentration is given in units mmol/gDW. (B) Steady-state concentrations
1092 obtained from simulations of the large-scale kinetic model of *E. coli* starting from the
1093 respective initial conditions presented in Table S1A. The first two columns show the respective
1094 minimum and maximum steady-state concentration over all 100 simulations. The concentration
1095 is given in units mmol/gDW. (C) Steady-state flux distributions obtained from simulations of
1096 the large-scale kinetic model of *E. coli* starting from the respective initial conditions presented
1097 in Table S1A. The flux is given in units mmol/gDW/hr. (D) Simulated and predicted
1098 concentration ranges for 23 SCC metabolites in a kinetic metabolic model of *E. coli*.

1099

1100 **Table S2.** (A) Correlation between predicted concentration range and shadow price for 23
1101 structurally constrained metabolites to the corresponding metabolic concentrations obtained
1102 from 100 simulations of a kinetic model of *E. coli* core metabolism. (B) Euclidean distance
1103 between simulated and predicted concentration bounds for 23 SCC metabolites in large-scale
1104 kinetic model of *E. coli*. In addition the table provides simulated and predicted concentration
1105 bounds in mmol/gDW.

1106

1107 **Table S3.** List of rate constants for reactions in the genome-scale model iJO1366 of *E. coli*. In
1108 addition to the used rate constants and the related organism in BRENDA, the table reports the
1109 reaction abbreviation used in the model and the enzyme EC number related to each reaction.
1110 In case more than one rate constant is known per reaction we consider the average value.

1111

1112 **Table S4.** (A) Measured concentrations of SCC metabolites in *E. coli* under different growth
1113 scenarios. The three replicates at growth rate 0.2h^{-1} are used as reference state. Measured

1114 volumetric concentrations¹ were converted to mmol/gDW by using a ratio of aqueous *E. coli*
1115 cell volume to dry weight of 0.0023L/g². **(B)** Specific flux rates for *E. coli* grown under
1116 different scenarios.

1117

1118 **Table S5.** **(A)** Predicted concentration ranges for the 15 SCC metabolites in a genome-scale
1119 metabolic model of *E. coli* with available data on concentration. **(B)** In addition correlation
1120 values between predicted and simulated bounds are provided.

1121

1122 **Table S6.** Number of metabolites with structurally constrained concentrations for each of the
1123 metabolic networks analyzed. The numbers of reactions and metabolites correspond to the
1124 number after reaction splitting into irreversible reactions and removal of blocked reactions. The
1125 latter is needed to satisfy the prerequisite for a positive steady state.

1126

1127 **Table S7.** Fraction of fully coupled reactions and reactions coupled due to mass action kinetics
1128 in 14 analyzed genome-scale metabolic networks.

1129

1130 **Table S8.** Structurally constrained metabolites across the 14 analyzed metabolic networks. In
1131 addition, the in- and out-degree for these metabolites are provided. Metabolites marked in red
1132 correspond to energy metabolism (see Table 1 in the main text) and metabolites marked in
1133 green exhibit absolute concentration robustness. Metabolite names and their abbreviations are
1134 used as provided in the original models.

1135

1136 **Table S9.** Number of metabolites with structurally constrained concentrations metabolic
1137 networks analyzed including enzyme information. The numbers of reactions and metabolites
1138 correspond to the number after rewriting in Michaelis-Menten format, reaction splitting into

1139 irreversible reactions and removal of blocked reactions. Model components correspond to
1140 metabolites, enzymes and enzyme-substrate-complexes.

1141

1142 **Table S10.** (A) Measured concentrations of SCC metabolites in *E. coli* under growth on
1143 different carbon sources. Replicates for growth on acetate are used as reference state. (B)
1144 Specific flux rates for *E. coli* under growth on different carbon sources.

1145