Optimal flux spaces of genome-scale stoichiometric models are determined by a few subnetworks

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The metabolism of organisms can be studied with comprehensive stoichiometric models of their metabolic networks. Flux balance analysis (FBA) calculates optimal metabolic performance of stoichiometric models. However, detailed biological interpretation of FBA is limited because, in general, a huge number of flux patterns give rise to the same optimal performance. The complete description of the resulting optimal solution spaces was thus far a computationally intractable problem. Here we present CoPE-FBA: Comprehensive Polyhedra Enumeration Flux Balance Analysis, a computational method that solves this problem. CoPE-FBA indicates that the thousands to millions of optimal flux patterns result from a combinatorial explosion of flux patterns in just a few metabolic sub-networks. The entire optimal solution space can now be compactly described in terms of the topology of these sub-networks. CoPE-FBA simplifies the biological interpretation of stoichiometric models of metabolism, and provides a profound understanding of metabolic flexibility in optimal states.

A comprehensive view of the metabolic capacities of organisms can be obtained with genome-scale stoichiometric models of their metabolic networks21-26. The development of these models has been greatly facilitated by the availability of annotated genome sequences and semi-automated computational pipelines for reconstruction13,14,21. Models currently exist for various unicellular organisms, including various pathogens, industrially relevant microorganisms and man11, and their number continues to grow. They typically incorporate hundreds to thousands of reactions and metabolites.

In the last decade, a large number of computational methods have been developed for studying the systemic properties of genome-scale metabolic networks for applications in biotechnology and medicine24,26. Flux balance analysis (FBA)24,30 is arguably the most frequently used method for analysis of stoichiometric models. FBA predicts maximal yields of metabolic products (e.g. biomass) and the associated optimal flux distributions of genome-scale stoichiometric models and, therefore, FBA sets bounds for metabolic engineering studies24,26. The predictions of FBA often come close to the outcome of laboratory evolution studies15,43 and can be used to identify metabolic constraints and objectives at the level of the entire metabolic network36. Generally, the constraints defined by the stoichiometric model are insufficient in number to guarantee a unique optimal flux route through the metabolic network. A whole solution space of flux distributions is then consistent with the prediction of the maximal yield. The set of all optimal flux distribution solutions of a FBA problem defines a so-called polyhedron35.

In this work, we report a computational method, Comprehensive Polyhedra Enumeration Flux Balance Analysis (CoPE-FBA), that gives directly a network-topological understanding of the solution spaces resulting from FBA, including flux ranges (in the literature also referred to as flux spans) and flux coupling20, in terms of a compact set of subnetworks that display alternative flux distributions in the optimal state calculated by FBA. The software for the computations of CoPE-FBA is described in the Methods section.

Methods proposed in the past for the full characterization of polyhedra of metabolic networks67,19,44-46,57,60, e.g. elementary flux modes (EFMs) and extreme pathways (ExPas), have their limitations for two reasons: excessive running times and output (millions of flux vectors) that is too large for any sensible analytical biological inspection. Here we present how those limitations can be overcome for FBA polyhedra, which are smaller than
those associated with EFMs and ExPas. However, they are still considered intractable. Previous studies have obtained various partial characterizations of FBA polyhedra. Mixed-integer linear programming has been used to partially characterize the optimal flux space. Flux variability analysis was introduced to quantify the range of flux values that a single reaction can take in a polyhedron. Flux coupling analysis allows for the identification of reaction pairs with a fixed flux ratio or flux direction across all optimal solutions within the polyhedron. Monte Carlo sampling of optimal solutions has also been used to probe the geometric properties of a polyhedron. However, these studies do not give any insight into how the topology of the entire metabolic network gives rise to a polyhedron of a specific FBA problem.

CoPE-FBA of various genome-scale metabolic networks shows that a few relatively small subnetworks (involving typically about 5–10% of all the reactions) shape the geometry of the polyhedron of optimal FBA solutions. We tested whether our results apply to genome-scale stoichiometric models in general. We compared eight such models and nine different growth conditions and found in all cases that the solution space could be understood in terms of the topology of a few small subnetworks. The computational pipeline that we have developed for the calculation of polyhedra of FBA problems is described in the Methods section. We start by explaining the compact mathematical representation of the optimal flux space using a toy metabolic network. Subsequently, we present the results we obtained by applying CoPE-FBA to real-life genome scale metabolic networks. The main body of the paper ends with a discussion. The paper is completed by a section on methodology with information on the design and implementation of the associated computational pipeline.

Results

Network topological interpretation of optimal solution spaces. Any FBA polyhedron can be described in two ways. One by linear equalities and inequalities, which is given by the specification of the FBA problem as a linear program (see Methods). The other, which is more relevant for biological investigations, involves the geometric description of a polyhedron in terms of its extremities, which are expressed as flux vectors. All these flux vectors have a topological interpretation in terms of cycles and paths in metabolic networks. However, existing methodologies have so far been incapable of determining these polyhedral properties of genome-scale stoichiometric models, even when restricted to optimal FBA spaces.

We start with analyzing a toy metabolic network (figure 1A) to introduce the mathematical description of optimal solution spaces (polyhedra) that arise in FBA. The network consists of 26 reactions and contains reversible as well as irreversible reactions. To facilitate the exposition we assume that each reaction, apart from R25, transforms one molecule of substrate into one molecule of product. We consider the network at steady state, i.e. for every intermediate metabolite the net production and synthesis rates balance. The FBA objective will be to maximize flux through reaction R26 under a restriction on the network input flux, i.e. R1 = 1. Formulating this

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Figure 1 | Topological characterization of the optimal solution space of an artificial metabolic network in terms of vertices, rays, and linealities. A. A metabolic network with 23 metabolites and 26 reactions. The source and sink metabolites, X, Y, T and U, are underlined to indicate that their concentrations are considered fixed in order to ensure a steady state, which we assume to be stable. Reversible reactions are depicted by two-way arrows, irreversible reactions by one-way arrows. A reaction carries a positive flux when running from lower alphabetic to higher alphabetic order (e.g. for R19 and R21 production of O and L correspond to positive fluxes). FBA was applied to maximize the flux through reaction R26 under the constraint that the flux of reaction R1 is smaller than or equal to 1. B. Overview of the linealities (green subnetworks) and the single ray (blue) that exist for this FBA program. The linealities correspond to reversible cycles whereas rays resemble irreversible cycles. These cycles are elementally balanced, such that no net conversions take place. Irreversible cycles (rays) are thermodynamically infeasible. The reactions in these cycles that are dashed in the figure show a choice of reactions included in vertices. C. The four vertices of this FBA solution space are displayed. They each represent a route from source to sink metabolites that have the same maximal yield. Reaction R25 is not used among the optimal vertices because it would give rise a lower yield than any of the other vertices. Any optimal flux distributions can be reconstructed from the vertex, rays, and linealities.
problem as a linear programming (LP) problem and solving by any
LP-solver (or in this case by inspection of figure 1A) it can be verified
that the solution value to this FBA program is a maximal flux of
reaction R26 equal to 1. For additional introductory expositions of
flux balance analysis we refer the reader to three papers24,25,26.

Clearly any route in the toy network from X to Y that avoids R25
corresponds to an optimal flux vector. Each route has value 1 for each
of the reactions on the route and 0 otherwise. Inspection of the
network indicates that multiple such routes exist. One such a route
is composed out of the reactions, \{R1, R2, R5, R6, R7, R8, R12, R13,
R14, R15, R22, R23, R26\}. Let us denote the corresponding flux vector
by \( f_2 \). Another one is obtained by replacing reactions R6, R7, and R8
by reactions R9, R10, and R11. Let \( f_3 \) be the corresponding flux vector.
Any convex combination of these two fluxes, i.e., for any positive \( \lambda \),
sending a flux of \( \lambda \) over the first route and 1 - \( \lambda \) over the second,
constitutes an optimal flux vector \( f = \lambda f_2 + (1 - \lambda) f_3 \). In
addition, various cycles exist in the network that, when run at differ-
ent rates, do not influence the yield of Y on X. For instance,
reaction R2, R3 and R4 can run at any rate as long as metabolites
A, B, and C are at steady state and this will not enhance nor reduce the
yield of Y on X. This analysis indicates that alternative optimal flux
distributions exist in the network and that each of those agrees with
the FBA optimum.

These alternative optimal flux distributions are each related to
three topological features of the solution space: vertices, rays, and
linealities. Vertices represent paths in the metabolic network (fig-
ure 1) and they correspond to corner points of (a suitably chosen
projection) of the polyhedron describing the solution space. A ray
is generally an irreversible cycle in the network (figure 1). In linear
algebraic terms, a ray is a direction (flux vector) \( v \) such that given
any point \( v' \) in the polyhedron the point \( v' + \mu v \) is also in the
polyhedron, for all values of \( \mu \). These directions together form a
cone. Linealities are reversible cycles in the network (figure 1). In
linear algebraic terms, they are defined as directions (flux vectors)
\( v \) such that given any point \( v' \) in the polyhedron the point \( v' + \nu v \) is also in the polyhedron, for all values of \( \nu \). The latter directions
together span a linear subspace, the lineality space, which can be
fully characterized by a relatively small number of basis vectors,
which we call linealities in this paper. We emphasize that the rays
and the linealities do not belong to the optimal solution space them-
selves; they do not contribute to optimization of the metabolic
objective. They merely give directions in which the solution space
is unbounded. Every flux vector in the optimal FBA polyhedron can
be expressed in terms of these three sets of vectors. For a precise
mathematical exclamation of these concepts we refer to the
Supplementary Information. In figure 1B,C we display the vertices,
rays, and linealities for the toy network.

Rays and linealities correspond to, respectively, irreversible and
reversible cycles, in which no net conversion takes place, see
Figure 1B. For instance, the conversion by the lineality composed
of R2, R3, and R4 involves no net conversion, only the recycling of B.
The same holds for the single ray solution and the other linealities.
We notice that rays correspond to thermodynamically infeasible
loops (see Discussion). Four vertices exist for this FBA problem
(Figure 1C). They differ in the routes taken through the reactions
R6 to R11 and R13 to R18. They each give rise to the maximal yield of
1 unit of Y per 1 unit of X. For incompletely defined networks, rays
and linealities can in principle also occur as paths rather than cycles.

Several computational pipelines have been proposed to compute
these extremities of polyhedra. We refer especially to Polco26 de-
veloped for determining EFMs in metabolic networks. The size of
the problems usually prevents these methods to find a complete enu-
meration of the extremities. We emphasize here two aspects of our
method that allows us to overcome these problems. Firstly, we cor-
rect the common practice in FBA to model fluxes without bounds by
bounds with artificially high numbers. Such bounds are entirely
superfluous and cause the disappearance of the rays and the line-
alities at the expense of an explosion of the number of vertices.
Secondly, we perform a preprocessing step. We noticed that in
optimal FBA solution space, usually many fluxes have a fixed value
throughout the space. We detect these fixed fluxes first by flux vari-
ability analysis. Fixing them at these values reduces the search space
so much that e.g. Polco can be used for enumeration. Moreover,
fixing these fluxes shows that the variability in the optimal solution
space is captured by relatively small subnetworks constituted by
reactions with variable fluxes. We find these subnetworks by per-
forming a correlation analysis on the vertices found. This allows for
a compact and insightful description of the optimal solution space in
terms of subnetworks that can be studied independently by visual
inspection. We illustrate this at the hand of the toy model. All details
of the method are described in the Methods section.

In the toy model flux variability analysis finds that in every optimal
solution R5, R12 and R22 have value 1. After fixing these fluxes, we
most clear see that the number of vertices of a FBA problem arises
through a combinatorial phenomenon. The 22 vertices, corresponding
to 22 paths from R1 to R26, that are obtained by firstly choosing
between \{R6, R7, R8\} and \{R9, R10, R11\} (which together form the
first subnetwork) and then choosing between \{R13, R14, R15\} and
\{R16, R17, R18\}, which form the second subnetwork. Notice that
because R23 and R24 together form a cycle (corresponding to a ray)
they are not regarded as a third subnetwork. In each vertex we
must choose the flux in R23 equal to 1 and in R24 to 0. The same flux
vectors with flux 0 on R23 and 1 on R24 is not another vertex: it can
be expressed as a convex combination of two other solutions: 1/2
times the vector with flux in R23 equal to 1 and in R24 to 0 plus 1/2
times the vector with flux in R23 equal to -1 and in R24 to 2.

Flux path variability in such subnetworks is the crux of the com-
binatorial explosion of vertices, which we will report in the next
sections for genome-scale models. This combinatorial explosion
can arise because subnetworks may exist in metabolism with alterna-
tive internal flux distributions that can be independently chosen
without compromising the optimality requirement. These subnet-
works have a fixed net input-output stoichiometry, i.e. \( D \rightarrow I \) and \( J \rightarrow Q \) in Figure 1, regardless of their internal flux distribution. If there
were \( k \) such subnetworks each having 2 alternative routes, then
there would have been (at least) \( 2^k \) vertices, emphasizing that the total
number of vertices can in general be much larger than the number of
reactions in the system.

CoPE-FBA for Escherichia coli on glucose. Using CoPE-FBA, we
characterized the optimal solution space for an FBA program where
a genome-scale model of Escherichia coli version iJR904w was
optimized for growth in mineral medium on glucose. The uptake
glucose flux was set such that a maximal growth rate of 1 was ob-
tained. Through subsequent enumeration we found 17280 vertices, 8
rays, and 1 lineality (i.e. the lineality space has dimension 1). Across all
vertices, out of the 1066 reactions in the model: 733 carried no flux,
274 had a single value and 59 were variable. Thus, 59 variable reactions
gave rise to the 17280 vertex solutions; below we explain how. The
software pipeline for CoPE-FBA and the scripts for obtaining the
results described in this section can be found in the Methods section.

Of these 59 variable reactions, 44 reactions had 2 different flux
values, 3 reactions had 16 values, 2 reactions had 4 values, and 10
reactions had 3 values across all 17280 vertices. The identity and
distributions exist in the network and that each of those agrees with
the FBA optimum.

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three topological features of the solution space: vertices, rays, and
linealities. Vertices represent paths in the metabolic network (fig-
ure 1) and they correspond to corner points of (a suitably chosen
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together span a linear subspace, the lineality space, which can be
fully characterized by a relatively small number of basis vectors,
which we call linealities in this paper. We emphasize that the rays
and the linealities do not belong to the optimal solution space them-
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method that allows us to overcome these problems. Firstly, we cor-
rect the common practice in FBA to model fluxes without bounds by
bounds with artificially high numbers. Such bounds are entirely
To determine the origin of the 17280 vertices in terms of the metabolic network topology we determined the Pearson correlation coefficients between the 59 variable reactions across all vertex solutions (figure 2A). The resulting 59×59 correlation matrix could be block diagonalized into five blocks, indicating that the fluxes of those 5 sets of reactions vary independently across all vertices. The sets contain 29, 18, 5, 4, and 3 reactions (59 in total). Each of these sets contain reactions that together form a (connected) metabolic subnetwork (figure 2B). For each subnetwork, we determined the number of different flux distributions that specify the vertices. The subnetwork with 29 reactions had 24 different flux distributions, the set with 18 reactions had 90 and the other three sets each had 2 such flux distributions. Multiplying these numbers yields 17280, the number of vertices. In other words, the 17280 vertices are derived from five subnetworks that each can independently be described by a relatively small number of flux distributions within the FBA optimum (see Supplementary Information for details of the various subnetworks).

Each subnetwork is linked to the core 274 reactions that are fixed in the optimum network state, i.e. across all vertices. This suggests that the overall stoichiometry of the subnetworks should be fixed across all vertices. This is indeed the case, as we verified computationally. Thus, the subnetworks can achieve the same net reaction stoichiometry using different internal flux distributions while the entire flux distribution obeys the same optimal yield. The overall stoichiometries of the five subnetworks can be found in the Supplementary Information. For Escherichia coli iJR904 we found eight rays and a single lineality which matched irreversible and reversible cycles.

Comparison of the optimal solution space topologies across growth conditions and species. In order to address how different nutrients influence the geometry of the optimal solution space, we compared the polyhedra of Escherichia coli iJR904 growing on nine different carbon sources in mineral medium using CoPE-FBA. The number of vertices across these nine cases differ by a factor of about two and the number of subnetworks is always very small. This indicates that the polyhedron can be understood in terms of a small number of subnetworks each involving at most several tens of reactions. As
expected, the rays and linealities appeared to be independent of the growth medium.

To test how different network topologies affect the number and size of metabolic subnetworks we repeated our analysis on a number of genome-scale reconstructions representing different micro-organisms. Table 1 shows that network size is not the only determinant of the number of vertices of a polyhedron. For instance, *Mycobacterium tuberculosis* has only half the number of reactions of *Escherichia coli* iAF1260 and nearly an equal number of vertices. Comparison of the number of vertices of the two *Escherichia coli* metabolic network reconstructions, iJR904 and iAF1260, does indicate an effect of network size on the number of vertices. The number of subnetworks remained the same. In the Supplementary Information we report the subnetworks of iAF1260 and one of them involves a large segment of central metabolism. The increase in the number of vertices for growth on threonine (over 4000 fold) indicates that iAF1260 has greater flexibility in amino acid metabolism.

In Table 1, we present the polyhedral characterization of eight different microorganisms and find that a small fraction of the total number of reactions end up in the subnetworks that determine the number of vertices, i.e. the major topological feature of the polyhedron. Across all organisms, the number of subnetworks is always very small indicating that the optimal solution space (the polyhedron) can be quickly assessed by studying the individual subnetworks. By doing so this can greatly simplify the results of a FBA and can give direct insight into properties such as flux variability and coupling.

**Discussion**

Genome-scale stoichiometric models of metabolic networks allow for a comprehensive view of the metabolic capabilities of an organism. FBA is an indispensable tool for such studies. In this work, we presented Comprehensive Polyhedra Enumeration Flux Balance Analysis (CoPE-FBA), an approach to fully characterize the optimal solution space of genome-scale stoichiometric models (a polyhedron) corresponding to FBA. Using our methodology, the outcome of FBA can be quickly assessed in its entirety in terms of a few metabolic subnetworks, even though the models consist of thousands of metabolic reactions and reactants.

Through the development of our enumeration pipeline (CoPE-FBA) we developed several techniques to make the step from determining a single FBA optimum to the enumeration of all optima tractable at genome-scale. This entailed careful pre-processing of the genome-scale stoichiometric models. Redundant reactions, pairs of reactions with matching stoichiometry but that differed only in reversibility, were scanned for and in all cases the irreversible reaction was deleted. Explicitly-encoded infinity constraints i.e. bounds on reactions represented by a large number and reactions that carry a fixed flux at optimality (as determined by rational-arithmetic FVA) were removed. The technical details of these engineering techniques are discussed in the Supplementary Information.

We emphasize that enumerating all the elementary flux modes or the extreme pathways of a metabolic network is a computationally much more demanding task than determining all the rays and vertices of the FBA polyhedron. The reason is that there are in general a lot less of the latter than of the former; the vertices only consider reaction paths through the network that give rise to the maximization of an objective.

Rays and linealities of a polyhedron generally represent irreversible and reversible cycles that catalyze no net conversions; hence, they only achieve the recycling of components. From a thermodynamic point of view, this means that they are not driven by any Gibbs free energy potential at steady state. As a consequence, linealities represent subnetworks that are only thermodynamically feasible at steady states if all their reactions carry zero flux, i.e. they operate in thermodynamic equilibrium. Rays are thermodynamic inconsistencies in the network. For instance, consider the ray network composed out of the following reactions: \( A \leftrightarrow B, B \leftrightarrow C \) and \( A \rightarrow C \). Clearly, the third reaction should be reversible as the first two reactions together form a reversible path. All the rays we found for the genome-scale stoichiometric models were of this kind. If rays exist then the model contains thermodynamical inconsistencies. CoPE-FBA therefore detects such inconsistencies and can be used as a tool to improve the description of metabolic networks (cf.11–18). For instance, networks with many rays (such as *Lactococcus bulgaricus*, Table 1) suffer from a significant number of thermodynamic inconsistencies. Even though mass-conserving reversible cycles (linealities) are not thermodynamically infeasible, a great number of them in a metabolic network does warrant further investigation into their physiological role (e.g. *M. tuberculosis* and *L. lactis* in table 1).

From a biological perspective, CoPE-FBA greatly simplifies the communication of FBA simulation results to the experimental biologist as it can be done completely in terms of network structures.

| Table 1 | Overview of optimal solution space characterizations with CoPE-FBA for eight different genome-scale metabolic network models for growth on various carbon sources. The calculations in this table were obtained from our CoPE-FBA method described in the Supplementary Information. Unpublished *L. lactis*, *S. thermophilus*, *L. bulgaricus* reconstructions were provided by Prof. B. Teusink (VU University, Amsterdam). |
|---|---|---|---|---|---|---|---|---|
| reactions | C-source | vertices | rays | lin. sp. columns | modules | reactions in modules | zero fluxes | fixed nonzero fluxes | variable fluxes |
| M. barkeri | 688 | methanol | 512 | 5 | 5 | 7 | 34 | 294 | 338 | 56 |
| E. coli iJR904 | 1066 | glucose | 17280 | 8 | 1 | 5 | 59 | 716 | 271 | 79 |
| E. coli iJR904 | 1066 | threonine | 1152 | 8 | 1 | 7 | 34 | 728 | 283 | 55 |
| E. coli iJR904 | 1066 | arginine | 2304 | 8 | 1 | 7 | 39 | 750 | 277 | 59 |
| E. coli iJR904 | 1066 | citrate | 1920 | 8 | 1 | 8 | 42 | 749 | 275 | 62 |
| E. coli iJR904 | 1066 | fumarate | 640 | 8 | 1 | 8 | 37 | 752 | 277 | 57 |
| E. coli iJR904 | 1066 | glutamine | 1920 | 8 | 1 | 7 | 40 | 750 | 276 | 60 |
| E. coli iJR904 | 1066 | lactate | 17280 | 8 | 1 | 5 | 59 | 728 | 279 | 79 |
| E. coli iJR904 | 1066 | malate | 320 | 8 | 1 | 7 | 34 | 753 | 279 | 54 |
| E. coli iJR904 | 1066 | tryptophane | 1728 | 8 | 1 | 4 | 48 | 743 | 275 | 68 |
| M. tuberculosis | 1020 | glycerol | 1327104 | 7 | 11 | 12 | 96 | 542 | 347 | 131 |
| L. bulgaricus | 474 | lactose | 2240 | 35 | 1 | 1 | 122 | 160 | 179 | 135 |
| L. lactis | 735 | glucose | 192 | 1 | 1 | 7 | 33 | 354 | 337 | 53 |
| S. thermophilus | 555 | lactose | 96 | 2 | 1 | 5 | 50 | 239 | 259 | 57 |
| S. PC6803 | 331 | glycerol | 384 | 4 | 2 | 4 | 32 | 123 | 162 | 46 |
| E. coli iAF1260 | 2374 | glucose | 1679616 | 25 | 1 | 4 | 118 | 1900 | 324 | 150 |
| E. coli iAF1260 | 2374 | threonine | 5038848 | 25 | 1 | 7 | 76 | 1899 | 364 | 111 |
are not regarded as bounds, whence constraints of the type SCIENTIFIC
to experimental biologists. E. coli physiologists would immediately observe that subnetwork 3 (figure 2B) involves the respiratory chain of E. coli and that the flux variability partially derives from the usage of alternative electron carriers, i.e. ubiquinone-8, menaquinone-8, and demethylmenaquinone-8. But the levels of these quinones are dependent on the oxygen availability and this knowledge further reduces the solution space when the aerobicity of the environment is specified. In addition, consideration of secondary objectives to reduce the optimal solution space (e.g. minimal pathway length or protein costs) is greatly simplified by CoPE-FBA; reduction of the solution space will only concern reactions in the subnetworks (only 10 s of reactions; table 1). Another advantage of CoPE-FBA is that it gives a network topological explanation of flux coupling, flux correlation and flux variability analysis. Only fluxes within the same subnetwork will correlate or be coupled in the optimal solution space (cf. Supplementary Information).

Methods

The stoichiometry of a metabolic network with m metabolites and r reactions is described by a \( m \times r \) stoichiometry matrix \( N \). The \((i,j)\)th entry of \( N \), \( n_{ij} \), is the stoichiometric coefficient of the \( i \)-th metabolite in the \( j \)-th reaction, which denotes the amount of metabolite \( i \) consumed \( (n_{ij}<0) \) or produced \( (n_{ij}>0) \) per unit reaction rate. Any reaction rate \( \text{flux vector} \ v \) that satisfies

\[
Nv = 0
\]

contains reaction fluxes such that the system is in steady state. Typically, \( v = 0 \) is not the only steady state flux vector. In Flux Balance Analysis (FBA) some objective is optimised over the steady state flux vectors.

In FBA, the steady-state conditions (eq. 1) are augmented with capacity bounds on reaction fluxes. In addition, a linear objective is postulated, by which we obtain a linear programming problem. A typical FBA linear program has the form:

\[
\begin{align*}
\text{maximize} & \quad f(v) = cv \\
\text{subject to} & \quad \begin{cases}
Nv = 0 \\
\nu^{\min} \leq v \leq \nu^{\max}
\end{cases}
\]

Here \( c \) is a vector of objective coefficients and \( cv \) is the way we write the inner product of \( c \) and \( v \). \( \nu^{\min} \) and \( \nu^{\max} \) are column vectors representing lower and upper bounds (respectively) on each of the \( r \) fluxes. Irreversibility constraints on reactions can be expressed by setting \( \nu^{\min} \) or \( \nu^{\max} \) to 0. Reversible reactions without lower (or upper) bound get \( -\nu \) (or \( +\nu \)).

Typically, a few fluxes will be fixed to some experimentally determined value or one of their bounds correspond to a measured value. All predictions are relative to a few fixed fluxes and therefore FBA predicts yields (ratios of flux). FBA typically involves maximizing a growth rate given a fixed uptake rate of a given nutrient. In fact in our computations we have minimized the uptake rate under a fixed growth rate. A little thought should make it clear that this does not effect the space of optimal solutions (it only scales every value involved by the same constant multiplicative factor).

Therefore, we keep the presentation of the method as if we maximize growth rate.

Minimization of uptake rate is modeled by the objective coefficient corresponding to the uptake reaction equal to 1 and all other objective coefficients equal to 0. Fixing growth rate is simply a matter of setting the flux vector corresponding to the reaction representing growth to the fixed value (making the upper and lower bound on the variable equal to this value). As is common practice in LP \( +\infty \) and \( -\infty \) are not regarded as bounds, whence constraints of the type \( v_j \leq -\infty \) or \( v_j \geq \infty \) are omitted in the LP. As we will explain later, it is essential not to replace the \( \nu \)'s by arbitrarily large constants. Although this does not influence the optimal solution value it causes the polyhedral structure to change in a significant and undesirable way.

To facilitate the exposition we express the feasible set in (2) as a set of inequalities only. This is easily obtained by rewriting (2) as

\[
\begin{align*}
\text{maximize} & \quad f(v) = cv \\
\text{subject to} & \quad 
\begin{cases}
Nv = 0 \\
\nu^{\min} \leq v \leq \nu^{\max} \\
\nu_j = \nu^{\max}, \quad \text{for all } j \text{ with } v_j \neq -\infty \\
\nu_j = \nu^{\min}, \quad \text{for all } j \text{ with } v_j \neq \infty
\end{cases}
\end{align*}
\]

We write then the set of all these constraints shortly as \( Ax \geq b \).

For genome scale model analysis we use PySCeS-CRM (an unpublished but online available extension of the PySCeS software) for reading, editing, translating and writing genome-scale models. However, other constraint based modelling tools e.g. the COBRA Toolkit could also be used.

In general optimal solutions of FBA programs are hardly ever unique. Suppose that the optimal value of (3) is \( Z^* \) then we are interested in describing the polyhedron

\[
\begin{align*}
\text{cv} & \geq Z^* \\
A & \geq b
\end{align*}
\]

in terms of its extremities: vertices, rays and linealities (see the Results section and the Supplementary Information).

Mathematical software exists for conversion between the two descriptions. Most popular are methods based on either the Double Description Method or for specific polytopes Reverse Search enumeration e.g. implemented in the software CDD and LRS. However, a theoretically efficient method for enumerating the vertices of polytopes has yet to be found. Indeed it is a major open question in computational geometry if such a method exists. This, together with the enormous number of vertices that we usually encounter in the high-dimensional polyhedra involved in modelling metabolism, implies that there is no guarantee that existing software will be able to cope with our problems. Indeed, initial attempts to do so in the literature have failed. We have reported vast numbers of vertices for small, reduced metabolic systems (hundreds of thousands of vertices is not atypical) or intractability. While these studies focussed on enumeration of entire metabolic networks we consider an analogous problem i.e. the enumeration of an optimal FBA space. We do this for complete genome scale metabolic networks by reducing the complexity of the problem, not by finding a better conversion method, but by smart preprocessing.

Our approach can be thought of as working in several steps. We work with rational (i.e. exact) arithmetic.

1. Compute the FBA optimum. We formulate the FBA program as the LP (3) described in the main text. We solve the LP using QSOPT_EX version 2.5.01, a rational LP-solver. Let \( Z^* \) be the optimal FBA value.

2. Formulate the optimal FBA set. This is done simply by replacing the objective in the LP by the optimality restriction \( f(v) \geq Z^* \). We write this constraint together with the set \( Ax \geq b \) of all constraints, as expressed in (4), shortly as \( Dv \geq d \).

3. Define a graph with vertices the row indices of \( D \) and an edge between \( (i,j) \) if \( D_{ij} > 0 \). For each flux \( v_{ij} \) for which \( D_{ij} > 0 \), remove \( D \) from the corresponding column \( D_i \) and subtract \( D_i \) from \( D \). The set of fluxes that have now become all \( 0 \)-rows. Let the new system be \( D'v \geq d' \). Define the basis for this linear subspace using a linear algebra library (such as LINALGO).

4. Compute rays and vertices of the system \( D'v \geq d' \). For genomescale systems we use the enumeration program POLCO (version 4.2.0) for this. Note that POLCO automatically detects whether the system has a linearity space, but it does not report a basis for it, it only returns rays and vertices.

5. Reintroduce the fixed fluxes that were removed earlier. In each of the vertices reintroduce the fluxes that are fixed across all optima and were removed. Note the latter fluxes have value 0 in rays and linealities.

To detect the subnetworks resulting from the vertices found, a complete metabolic subnetwork/module analysis was performed in three steps (details of these steps are found in the Supplementary Information):

1. The vertices are translated in an array \( K \), which is scanned for fixed and variable fluxes in order to now generate a sub-matrix \( K' \) by removing the fixed fluxes from \( K \).

2. Using \( K' \) the correlation coefficients are calculated, which are then stored as the correlation coefficient matrix, \( P \).

3. Define a graph with vertices the row indices of \( P \) and an edge between \( m \) and \( n \) if and only if \( P_{mn} \neq 0 \). Each connected component of this graph corresponds to a metabolic module/subnetwork. For each metabolic module/subnetwork a pattern matching algorithm is used to determine the number of unique flux dis tributions that occur within a particular module, across all vertices.

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Additional information
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