A review of methods for the reconstruction and analysis of integrated genome-scale models of metabolism and regulation

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Abstract (89/250)

The current survey aims to describe the main methodologies for extending the reconstruction and analysis of genome-scale metabolic models and phenotype simulation with Flux Balance Analysis mathematical frameworks, via the integration of Transcriptional Regulatory Networks and/or gene expression data. Although the surveyed methods are aimed at improving phenotype simulations obtained from these models, the perspective of reconstructing integrated genome-scale models of metabolism and gene expression for diverse prokaryotes is still an open challenge.

Introduction

High-throughput large-scale omics experiments are nowadays disseminated in biochemical research, supporting the study of the genomics, transcriptomics, and metabolomics layers of the cellular's molecular machinery. Currently, the volume of studies in the different omics fields has provided means for systems biology to thrive (1). This interdisciplinary field proposes differentiated approaches, such as the reconstruction of *in silico* networks and models, to provide quantitative and qualitative descriptions of biological systems as a whole.

Reconstruction of GSMMs

Nowadays, the generation of Genome-Scale Metabolic Models (GSMMs) is a common practice in systems biology. The reconstruction of these comprehensive models, through modeling techniques and genomics data, allows predicting cells' metabolic behavior (2–4).

A GSMM is an *in silico* representation of the biochemical reactions taking place within a the metabolism of a given organism (5). A genome-wide functional annotation that provides the required metabolic information over the organism of interest should be performed to assemble this representation. This information is linked to existing metabolic knowledge retrieved essentially from biochemical databases and literature. These steps help to create the reaction set, upon which the metabolic network is assembled.

The link from metabolic genes to proteins (mainly enzymes or membrane transporter proteins), as well as from proteins to reactions, is established by Gene-Protein-Reaction (GPR) associations. GPR associations must be cautiously defined during the reconstruction, taking into account isoenzymes, protein complexes and cascade reactions (3), through the use of *AND* or *OR* Boolean rules.

In the next iteration, biomass and organism-specific constraints are formulated from the retrieved knowledge to assemble a final stoichiometric model. The final GSMM may then be exported in a standard format, such as the Systems Biology Markup Language (SBML) (6). Several platforms, such as *merlin* (7), ModelSEED (8), RAVEN (9) and CarveMe (10), have been developed specifically for performing or assisting the reconstruction of these models (11).

The classic principles of chemical engineering are used to infer the dynamic mass balances of all metabolites in the metabolic network. A single ordinary differential equation (ODE) is created for each metabolite, accounting for its stoichiometry in the whole reaction set. Due to the lack of kinetic rates for all reactions in the ODE set, a steady-state approximation is used to reduce the mass balances to a set of linear equations. In a pseudo-steady-state paradigm, the concentration of a metabolite is assumed to remain constant throughout time (4).

When used to determine flux values, the set of linear equations defines a linear system, typically underdetermined, as the number of fluxes is much higher than the number of mass balance constraints, also referred to as the null space of S (12). Additional mass balance constraints can be added to the system to limit the flux that each reaction can acomodate by the imposition of both lower and upper bounds.

The system can be solved mathematically transforming it into an optimization problem,using several constraint-based approaches to predict the phenotypic behavior of the organism on a wide variety of environmental and genetic conditions. One of the most popular approaches is the Flux Balance Analysis (FBA) framework (12). FBA can compute an optimal solution, out of the feasible space determined by both mass balance and flux constraints using linear programming (LP). FBA requires the definition of an objective function, which should be relevant to the undergoing problem, which is commonly defined as the maximization or minimization of a specific metabolic flux (e.g., biomass reaction), and quantitatively determines how much each reaction contributes to a phenotype (4).

Parsimonious Flux Balance Analysis (pFBA) (13) and Flux Variability Analysis (FVA) (14), are alternative mathematical frameworks that also employ LP to allow analyzing *in silico* flux distributions. This set of tools is extremely helpful for validating a reconstructed model using experimental data of the organisms of interest. COnstraint-Based Reconstruction and Analysis (COBRA) Toolbox (15), COBRApy (16), OptFlux (17), and ReFramed (https://github.com/cdanielmachado/reframed) are prominent computational tools that have implemented these methods.

Although GSMMs have proven to be valuable throughout the years (18–24), there are limitations. Indeed, they are not yet capable of accounting for biological regulatory phenomena, such as the control of gene expression (25). The lack of this additional layer of information in these models can lead to erroneous *in silico* phenotype simulations, due to the lack of constraints that allow reaching the most accurate flux distribution according to experimental data.

Several methods have been proposed to improve phenotype simulations obtained from GSMMs, which will be herein surveyed. Most of these new methodologies are aimed at combining additional layers of omics data, namely transcriptomics, to limit the cone of allowable flux distributions. Also, these methods often resort to the integration of gene expression data and/or regulatory information obtained from Transcriptional Regulatory Networks (TRN)s being, therefore, prominent efforts made towards the reconstruction of integrated genome-scale models of metabolism and gene expression. The utilization of these integrated models can be useful to improve phenotype simulations or extend the analysis of regular GSMMs.

Reconstruction of TRNs

A TRN can be represented as a bipartite graph that comprehends vertices and edges. Vertices are usually regulators and target genes, whereas edges determine how these regulatory elements are connected and interact with each other, often under a causal relationship.

Inferring TRNs is fundamentally an underdetermined problem associated with a large search space where many solutions explain the data equally well (25,26). High-quality transcriptional information is scarce, in databases or literature and focused on a few well-studied organisms. Thus, the number of potential regulatory interactions between a Transcription Factor (TF) and target genes is considerably larger than the actual true biological interactions.

Although methods for reconstructing TRNs have been extensively reviewed in the literature (25–31), there are a panoply of classification systems and procedures. Moreover, as new methods are released each year, the complexity increases. Hence, assigning classes to these new approaches can be a complicated task. More importantly, this reveals that standard platforms and methodologies to assemble TRNs using diverse

sources of regulatory information, such as gene expression data (32,33) or transcriptional information (34–36), are still missing.

Nevertheless, an integrative workflow for reconstructing bacterial TRNs has been proposed by Faria *et al.* 2014 (25). The authors suggested that comparative-genomics approaches, namely the inference of TRNs using template curated networks and the prediction of cis-regulatory elements, can be integrated with the output of *de novo* reverse engineering tools. This workflow addresses the possibility of reconstructing TRNs for less described prokaryotic organisms using a variety of sources of regulatory information.

Template-network methodologies are based on the conservation of prokaryotic TRNs across evolution (37–39). As described by Faria *et al.* 2014 (25), template-network-based methods usually perform a search for orthologous genes in the genome of the organism of interest to propagate TRNs to strains of a well-characterized model organisms or closely related ones.

Cis-regulatory elements detection rely on the assumption that a regulatory interaction between a given TF and target gene can be inferred from the detection of the Transcription Factor Binding Sites (TFBS). The prediction of these *cis*-regulatory elements is a problem in which computational methods can assist (27,40). Although these computational tools are unable to infer a complete TRN from TFBS data, they can be integrated in the following workflow towards such a goal. The principles of this methodology were implemented by Alkema *et al.* 2004 (41) in Regulogger and the RegPredict web-based platform (42).

De novo reverse engineering tools are widely used for inferring TRNs from gene expression data. Indeed, a vast repertoire of computational tools based on the *de novo* reverse engineering approach can be found in the literature, and consequently numerous ways to classify these tools (28–30). Nevertheless, *de novo* reverse engineering methods are usually classified by mathematical formulation. Hence, to highlight the most used mathematical formulations, data-driven methods are usually based on the following:

- Correlation (e.g. COREGNET (43)),
- Information-theoretic (e.g. (44)),
- Boolean algebra (making use of the widely known binary operators *AND*, *OR,* and *NOT* to describe regulatory interactions (45), e.g. ModEnt (46)),
- Regression-based (e.g. GENIE3 (47)),
- ODEs (e.g. Inferelator (48)),
- Bayesian models (e.g. Gat-Viks *et al* 2007 (49)),

Available state-of-the-art TRNs' reconstructions for prokaryotic organisms include well-known prokaryotic organisms such as *Escherichia coli* (50,51), *Bacillus subtilis* (52,53) and *Mycobacterium tuberculosis* (54), having hundreds of regulators and thousands of target genes. These TRNs can, therefore, be used as gold-standards in the template-network-based approach or supervised methods. Interestingly, other TRNs for

prokaryotic organisms less described in the literature or having less amount of gene expression data (44,55–61), are also available, though in less number.

The TRNs available in the literature are usually the result of a specific gene expression data-driven analysis or the collection of regulatory information from literature and databases. Although these TRNs can be used in the comparative genomics or data-driven approaches, not all of them can be easily integrated in GSMMs, as only genome-scale TRNs are actually useful for the integration and simulation of integrated models.

Integrated models

Combining regulatory elements with information on metabolic stoichiometry is a complex task. There are many ways for controlling metabolism (62), which are well represented in the large diversity of methods proposed to quantify such influence (25,63–75). Nevertheless, the common denominator is that most methods start with GSMMs.

In detail, several of these methods integrate complete functional TRNs (76–84) or gene expression data (85–95) into GSMMs, whereas others impose additional constraints using information on allosteric and post-translational modifications (66,67,73). A different strategy is the combination of multiple layers of regulation (63,65,72,74). For higher eukaryotes such as humans, the control of gene expression also plays an essential role in the differentiation between different tissues or cell-types. Thus, algorithms for tailoring a GSMM according to a specific cell-line or tissue, commonly referred to as context-specific models, have been proposed (75,96–104). These principles and their main implementations are depicted in the Figure 1.

Figure 1: Overview of several methods for integrating additional constraints into GSMMs based on the regulation of metabolism. Whereas some methods integrate complete functional TRNs or gene expression data into GSMMs, others impose further constraints based on allosteric and post-translational modifications. Additionally, other methods integrate multiple omics layers of regulation of metabolism. For higher eukaryotes such as humans, context-specific models have also been based on tailoring the flux cone of solutions.

Surveying all approaches is out of the scope of this review. The following sections will cover the integration of TRNs or gene expression data into GSMMs, focusing on the control of gene expression at the transcriptional level. Figure 2 highlights both approaches, namely the integration of TRNs (Figure 2 A) and gene expression data (Figure 2 B) into GSMMs.

The differences between the integration of TRNs and gene expression data into GSMMs are associated with the type and amount of data that these sources can offer to the metabolic landscape of GSMMs.

Methods capable of integrating TRNs into GSMMs provide comprehensive knowledge regarding the metabolic and regulatory events occurring inside the cell at the genome-scale. As a result, both regulatory and metabolic networks can be analyzed together at the genome-scale, extending the range of applications of a regular GSMM.

On the other hand, gene expression data comprehend a set of snapshots of the transcriptome for several experimental conditions. Thus, a gene expression dataset can solely offer gene expression levels at a given experimental condition.

The group of methods aimed at integrating gene expression data with GSMM's comprises methods using only transcriptomics data for tailoring the flux distributions, so no structure or rules describing the regulatory interactions are observed in this class of methods. Thus, the integration of gene expression data focuses on improving the prediction of flux distributions, rather than the study and analysis of an additional biochemical network at the genome-scale.

Methods have also been classified according to the main formulations, as previously suggested by Machado *et al.* 2014 (70). Organizing methods into containers, according to their main formulations and features, facilitates the decision process when selecting an adequate method for the existing constraints and data sources.

Hence methods were classified into discrete (Figure 2 C) or continuous (Figure 2 D), according to whether phenotype simulations were performed with discrete, namely Boolean logic ("ON/OFF"), or continuous constraints.

Accordingly, a method is systematically classified as discrete if the result of the integration is a Boolean value (e.g., 1 for "ON" and 0 for "OFF"), imposing additional constraints on the system. These methods are also referred to as *switch,* since TRN or gene expression data switch reactions on or off. The state of a given metabolic gene is determined by evaluating either the Boolean regulatory rule or thresholding the gene expression level. Then, metabolic reactions mapped to metabolic genes are accessed according to the GPR rules to determine the resulting states. Thus, reactions having a one-to-one direct GPR rule are active/inactive according to the state of the metabolic gene. Reactions catalyzed by enzyme complexes, encoded by multiple yet mandatory genes, are considered inactive if at least one metabolic gene is not available. In contrast, reactions catalyzed by isoenzymes, namely multiple enzymes catalyzing the same reaction, are considered active if at least one metabolic gene is active.

Alternatively, there are methods aimed at circumventing the rigid Boolean logic, called *valve* methods, which impose continuous constraints to adjust a given flux distribution gradually and according to penalties, expression scores, or normalized expression levels obtained from the gene expression data. Typically, continuous integration is performed through the implementation of slack variables in the constraints' formulations, altering the reactions' bounds. The slack variable represents penalties, expression scores, or normalized expression levels retrieved from gene expression data for the metabolic genes associated with a given reaction. As before, the state of the metabolic reactions mapped to metabolic genes is assessed through GPR

rules, through selecting the best penalty, expression score, or normalized expression level for the slack variable.

The methodology for assigning a value to the slack variable, when a set of isozymes catalyzes a given reaction, comprises several distinct approaches. These include: methods in which the slack variable assumes the maximum expression score of the associated genes; methods where the slack variable takes the sum of expression scores of all genes encoding the isozymes catalyzing a single reaction; and, methods in which the reaction is replicated, according to the number of isozymes, and each new reaction is associated with one, and one only, gene.

Regarding reactions catalyzed by an enzyme complex, a group of methods establishes that the minimum expression score of all encoding genes is assigned to the slack variable. In contrast, other methods define the utilization of either the geometric or arithmetic mean of the expression score of all genes associated with an enzyme complex or isozymes, respectively.

Furthermore, methods capable of integrating gene expression data into GSMMs were also divided into single-condition (Figure 2 A) or multi-condition (Figure 2 B). Notice that this classification was not used to classify those methods aimed at integrating TRNs into GSM models, as it will be explained next.

Methods were classified as single-condition (Figure 1 A) or multi-condition (Figure 1 B) according to whether phenotype simulations were performed for one or more conditions/states in the gene expression dataset, respectively. For instance, a given method is considered multi-condition if it adjusts the flux cone of solutions by considering all conditions in the gene expression dataset or the gene differential expression between two conditions. Otherwise, the methods are classified as single-condition.

Notice that the latter classification was not used to classify those methods aimed at integrating TRNs into GSMMs. Methods capable of integrating TRNs into GSMMs do not require a gene expression dataset, thus classifying them into single- or multi-condition would be meaningless. Other methods capable of assembling and integrating TRNs into GSM models GSMMs often use the whole dataset and can then perform condition-specific phenotype simulations. Hence, classifying these methods as single-condition would be misleading.

Figure 2: Two examples of the integration of TRNs (A and C) or gene expression data (B and D) into GSMMs. The integration of TRNs (A) does not require gene expression data, while methods that integrate gene expression data (B) are capable of tailoring the flux cone of solutions by accounting for one (single-condition) or more (multi-condition) conditions in the gene expression dataset. Both types of integration can be mediated by discrete (C) or continuous (D) variables.

An analysis of these methods, encompassing the year of publication, availability of a tool with a user-friendly interface (namely a Graphical User Interface (GUI) without the requirement of coding skills), type of reaction constraint formulation, as well as the organism used for proof of concept has also been conducted. This information is available at the supplementary material 1. Figure 3 provides, on the other hand, a complete understanding of the methods described next, as well as their categorization according to the classification axes described above.

Integrating TRNs

For simulation purposes, the first attempts to integrate TRNs within GSMMs, namely Regulatory Flux Balance Analysis (rFBA) (76,105–107), Steady-state Regulatory Flux Balance (SR-FBA) (77) and the method proposed by Herrgård *et al.* 2006 (79), are based on the *switch* approach, to complement the metabolic system with additional constrains outlining which genes are activated or silenced in the network for specific *stimuli*.

As proof of concept, rFBA was successfully used to create the very first integrated genome-scale model of metabolism and gene expression for *E. coli* (106,107). In this reconstruction, as well as in the integrated network of *S. cerevisiae* provided by Herrgård *et al.* 2006 (79), a Boolean network collected from literature was integrated through a set of GPR rules with the GSMM imposing regulatory events as additional time-dependent constraints. On the other hand, SR-FBA performs steady-state simulations by including all valid metabolic and regulatory constraints in the system in a single step, through a Mixed-Integer Linear Programming (MILP) formulation. For that, nested Boolean expressions are formulated as a set of linear constraints, by recursively iterating over the structure of the regulatory layer and GPR rules, to add auxiliary variables representing intermediate Boolean terms (77). As shown in Figure 3, these methods have been classified as discrete, and none provides a user-friendly interface without the requirement of coding skills.

Two platforms, namely Toolbox for Integrating Genome-scale Metabolism (TIGER) (84) and FlexFlux (83), have been developed for integrating Boolean-based TRNs into GSMMs. TIGER can convert a series of logic Boolean rules, which can be thought of as a Boolean TRN, into a set of mixed-integer inequalities. Then, several algorithms for integrating gene expression data into the metabolic model and simulating phenotypic behavior can be implemented in the toolbox. Other implementations already available in this toolbox, such as Metabolic Adjustment by Differential Expression (MADE) (95), can be used for simulations.

FlexFlux differs from TIGER insofar as it is the only tool that provides a user-friendly interface for the integration of TRNs into GSMMs. This computational tool developed in Java® allows the input of Systems Biology Markup Language (SBML) (6) with the SBML Qualitative (SBML-qual) extension. SBML-qual is the standard file format extension for storing and sharing qualitative multi-state TRNs (108). In this way, a regular SBML file can hold a computer representation of qualitative models of biological networks.

Qualitative multi-state regulatory networks can then be used to determine multi-state qualitative constraints for metabolic flux analyses using FBA. Furthermore, FlexFlux allows the translation of the discrete qualitative states into continuous intervals, thereby constraining a reaction flux continuously or discretely (83).

Probabilistic Regulation of Metabolism (PROM) (78), PROM2.0 (109), and Integrated Deduced REgulation And Metabolism (IDREAM) (82) are all based on a probabilistic model for TRNs, which are integrated with a constraint-based model using a continuous method. PROM and PROM2.0 were the first attempts to circumvent the previous rigid discrete constraints added to a GSMM by setting the reactions' flux bounds proportional to the probabilities of their associated metabolic genes. In turn, the probability of a metabolic gene being activated in the whole set of conditions is defined together from the TRN and gene expression dataset provided as input. In short, PROM approaches can determine the probability of a given gene being or not activated, when the set of regulating TFs is either activated or silenced. The probability is calculated according to the frequency that each gene is active in the dataset (of either perturbed or over/under-expressed TFs). Likewise, the effect of perturbations on the regulatory network can also be robustly predicted.

Although PROM-based approaches are probably the best examples for integrating both TRNs and gene expression data into a GSMM, the gene expression dataset must have a large number of measurements per condition. PROM and PROM2.0 have been validated with *E. coli* and *M. tuberculosis* experimental gene expression data and the respective TRNs.

The IDREAM method resulted from the combination of Environment and Gene Regulatory Influence Network (EGRIN) (55,110) and PROM frameworks to create an enhanced genome-scale model of metabolism and gene expression for *Saccharomyces cerevisiae* (82). Contrariwise to the previous approaches, this methodology has used a *de novo* reverse engineering method called EGRIN to complement the yeast TRN collected from the database YEAst Search for Transcriptional Regulators And Consensus Tracking (YEASTRACT) (111). Then, the phenotype simulations are conducted in a similar way as in the PROM-based approaches.

Transcriptional Regulated Flux Balance Analysis (TRFBA) (81) and CoRegFlux (80) also provide a framework for the integration of gene expression data and TRNs in a continuous manner. Whereas the former requires a TRN for the organism of interest, the latter provides tools for inferring the regulatory network from gene expression data using CoRegNet (43). Nevertheless, CoRegFlux allows us to use a curated TRN rather than using the provided data-driven method.

Regarding the TRFBA methodology, this FBA-based approach considers gene expression levels as two additional types of continuous constraints. The first is represented by a constant parameter that converts the gene expression levels to the upper bounds of the reactions. The second type of linear constraints to be added to the

system can be thought of as the linear regression of each target gene from the regulating TFs.

CoRegFlux differs from TRFBA in that it uses a statistical reverse engineering method to infer targets of a given set of regulators at the genome-scale. Then, the influence score (similar to correlation scores for activation or repression) of each regulator in the set of target genes is calculated with CoRegNet from a large gene expression training dataset. Influence scores are used to train a linear model capable of predicting the gene expression of metabolic genes using a new gene expression dataset. These predicted levels of expression are then translated into flux bounds for the phenotype simulations using FBA or Dynamic Flux Balance Analysis (dFBA) (112).

All methods surveyed here are listed in the supplementary material 1.

Integrating gene expression data

The method proposed by Åkesson *et al.* 2004 (87), followed by MADE (95), were the earliest approaches for tailoring the flux cone of solutions using discrete variables obtained solely from gene expression data. In the case of the method developed by Åkesson *et al.* 2004 (87), a reaction is simply switched "off" with a zero flux bound if the associated genes are found to be under-expressed in the corresponding condition (single-condition method). MADE, on the other hand, tries to surpass the problem of arbitrary thresholding under-expression by considering multiple conditions (multi-condition method). Statistical significance between changes in gene expression levels across sequential conditions is calculated to infer whether a gene is activated (95).

E-Flux (113) and the method proposed by Lee *et al.* 2012 (94) have introduced several novelties when compared with the previous methodologies. These methods were the first attempts to constraint an FBA-based model using continuous variables. Nevertheless, these approaches are radically different. E-Flux directly maps gene expression levels into flux bound constraints, assuming the maximum flux of a given reaction to be a linear function of the expression of the associated genes in the same condition (single-condition method). Lee and coworkers (94) do not introduce or alter flux bound constraints directly into the GSMM. An alternative objective function that minimizes the distance between flux distributions and gene expression data is applied for each phenotype simulation (single-condition method).

The Transcriptional-controlled Flux Balance Analysis (TFBA) method, proposed by van Berlo *et al.* 2011 (93), is aimed at overcoming the problem of setting an arbitrary threshold to determine whether a gene is activated or not. The TFBA assumption is that differential gene expression between two conditions should also be reflected in the flux of the reactions associated with this gene. For that, the authors formulated constraints defining upper and lower limits for fluxes according to the gene expression, though assuming their transgression to be possible. The optimization problem (MILP

formulation) consists of finding the flux distribution that minimizes the number of transgressions.

Likewise, the method developed by Fang *et al.* 2012 (92) is based on the differential gene expression between two conditions, namely reference and perturbed conditions. This method assumes that the flux distribution of a reference condition can be determined using the FBA or FVA frameworks, while the differential gene expression between the reference and perturbed conditions is used for tailoring the flux distribution of the perturbed one. Also, this method considers the variation of the biomass composition between reference and perturbed conditions.

Similarly to TFBA and the method proposed by Fang *et al.* 2012 (92), the Gene Expression Flux Balance Analysis (GX-FBA) method (91) also determines the flux distribution for the reference condition using FBA. Then, GX-FBA employs a new objective function and new constraints derived from the difference between reference and perturbed states to perform the *in-silico* phenotype simulation of the latter state. A wide range of phenomena associated with temperature and known to induce virulence in the gram-negative bacterium *Yersinia pestis* was used as proof of concept.

Temporal Expression-based Analysis of Metabolism (TEAM) (90) and Adaptation of Metabolism (AdaM) (89) are the only methods developed for integrating time-series gene expression data into constraint-based models. The former uses dFBA (112) to predict time-series flux distributions based on temporal gene expression profiles. Using a cost minimization scheme similar to the strategy proposed in the context-specific Gene Inactivity Moderated by Metabolism and Expression (GIMME) method (98), TEAM is capable of determining the flux distribution of a GSMM, constrained with gene expression levels of each time step in the dataset. TEAM was tested with time-series gene expression data from *Shewanella oneidensis*.

AdaM consists of a flux-based bilevel optimization problem that extracts minimal operating networks from a given GSMM (89). This algorithm infers the minimal operating networks in agreement with the differential gene expression pattern between time-steps. Then, Elementary Flux Modes (EFM)s (114) are computed with these minimal operating networks rather than computing the flux distributions at each time step. Reactions are weighted according to the number of EFMs in which these are present. The optimization problem consists of finding the minimal network having the largest weight.

Angione *et al.* 2015 & 2016 (86,88) formulated methods, for example, the Metabolic and Transcriptomics Adaptation Estimator (METRADE), aimed at measuring the adaptability to a changing environmental condition over time. These approaches have provided equally valid methodologies for integrating gene expression data in metabolic networks. In short, these methods have modeled both upper and lower bounds of each reaction as a continuous logarithmic function of the associated gene expression levels.

Reaction Inclusion by Parsimony and Transcript Distribution (RIPTiDe) (85) is aimed at circumventing the assumption that reaction fluxes are directly related to the gene

expression levels for a given condition. The authors have proposed an unsupervised method that assigns weights (continuous variable) to reactions according to the normalized expression levels of associated genes over the entire dataset. Then, a pFBA simulation considering these linear coefficients is performed. The novelty of this method consists of its validation with precise transcript abundance obtained with RNA-sequencing (RNA-seq).

The methods capable of integrating gene expression data into GSMMs addressed herein are available in the supplementary material 1.

Synopsis

The reconstruction of GSMMs is common practice in systems biology nowadays. The advent of the GSMM reconstruction for many organisms was facilitated by the adoption of standard protocols (3), as well as the existence of user-friendly computational tools (7,8), capable of assembling these models from different genomic, enzymatic and stoichiometric data. Nevertheless, the simulation of GSMMs still presents today false-positive phenotypes for several environmental conditions.

The reconstruction of TRNs is a well-known strategy in systems biology for understanding the regulatory machinery of a given organism (26,28,30). Although there are many methodologies for assembling a TRN, standard protocols and computational platforms are yet missing to support the reconstruction of TRNs for less described organisms using different data sources. The workflow suggested by Faria *et al.* 2014 (25) highlighted several methodologies that can be combined to extend the reconstruction of TRNs to more bacterial species. To the best of our knowledge little progress has been made to provide a user-friendly platform capable of achieving such a goal. More importantly, the reconstruction of genome-scale TRNs using such integrative workflow, would be pivotal for the reconstruction and simulation of integrated models.

The integration of the control of gene expression into GSMMs has been surveyed in this work. A systematic classification that grasps the difference between the several methodologies, capable of integrating and simulating regulatory events into GSMMs was proposed herein. Although part of the reviewed methods have already been surveyed before (25,64,68–71,115), TIGER, FlexFlux, METRADE, IDREAM, TRFBA, CoRegFlux, RIPTiDe and the method proposed by Angione *et al.* (2016) have never been addressed elsewhere in reviews, to the best of our knowledge. Moreover, a detailed categorization that highlights the methodologies used to perform the integration of the regulatory layer into GSMMs has not been provided. This systematic categorization can guide the decision process of selecting the most adequate method of integration and simulation.

As shown in Figure 3, there are several methods and toolboxes capable of integrating and simulating TRNs into GSMMs using a discrete approach (76,77,79,83,84). The TRNs used by these methods and toolboxes were mainly reconstructed from literature, which

might be a time-consuming approach. The remaining methods allow to assemble TRNs from gene expression data using *de novo* reverse engineering methods. The resulting TRNs can be integrated and simulated with a given GSMM. FlexFlux is the prominent exception as it can perform the integration of the TRN in the GSMM using either discrete or continuous variables.

To date, only two prokaryotic organisms, *E. coli* (76–78,81,83) and *M. tuberculosis* (78,109), and the yeast *S. cerevisiae* (79–82,84), have integrated genome-scale models as a result of the integration of complete TRNs into a metabolic network. Nevertheless, some of these reconstructions still require gene expression datasets, namely several methods in the continuous sub-group.

Regarding the methods for integrating gene expression data, most of these have provided means for integrating transcriptomics data as continuous constraints from one or more conditions (Figure 3). Only the method proposed by Åkesson *et al.* 2004 (87), as well as MADE (95), use discrete variables to simulate integrated models of metabolism and gene expression.

Besides *E. coli*, *M. tuberculosis,* and *S. cerevisiae*, methods for integrating gene expression data have also provided integrated models for *S. oneidensis* (90) and *Y. pestis* (91).

Figure 3: Classification of methods aimed at the reconstruction of integrated genome-scale models of metabolism and gene expression. These methods have been divided according to the integration of TRNs (white boxes) or solely gene expression data into GSMMs. Discrete and continuous categories were used to classify these methods according to the usage of discrete, namely Boolean logic ("on/off"), or continuous constraints. Methods capable of integrating gene expression data into GSMMs have been further divided into single-condition (orange circles) and multi-condition (blue ellipses) whether phenotype simulations were performed for one or more conditions/states in the gene expression dataset, respectively. Each inner circle stands for a prokaryotic organism, while the outer circle stands for the baker's yeast *Saccharomyces cerevisiae*.

A vast diversity of methods for the integration of gene expression data in GSMMs has been found. Yet, most methods require large gene expression datasets to be robust, which might not be the case for all organisms. Other methods resort to mapping levels of gene expression directly with the reactions bounds, which again might not be the best approach (70,115,116).

Furthermore, the methods for integrating gene expression data with metabolic models previously evaluated by Machado and coworkers (70), namely E-Flux (113), MADE (95), GX-FBA (91) and the method developed by Lee *et al.* 2012 (94) have shown to perform poorly in the designed benchmark. None of the methods have outperformed

each other in the phenotype simulations nor pFBA, which indicates that the promising results reported by these methods seem to be mere artifacts related to rigid constraints created around the nature of the gene expression dataset.

The reconstruction of integrated models using TRNs is, in theory, more useful than merely integrating gene expression data into GSMMs. Integrated models that result from the integration of TRNs provide comprehensive knowledge regarding the metabolic and regulatory events happening inside the cell, thus leading to a broader range of applications when compared to a regular GSMM (117,118).

Moreover, the diversity of methods for reconstructing TRNs using different data sources, such as gene expression, transcription factor binding site, or comparative genomics analysis, eases the reconstruction of TRNs for most prokaryotic organisms having a sequenced genome (25). However, the absence of a user-friendly computational tool based on the ensemble of these different approaches is missing. In contrast, the same strategy has yielded results in the reconstruction of GSMMs (7–9,11,15,119).

In short, the existence of standardized protocols and easy to use computational tools for the generation of GSMMs has eased its practice in systems biology to study the metabolism of many organisms. In contrast, the absence of the computational tools that ease the reconstruction of TRNs from different sources of regulatory data hindered a similar approach.

The integration and analysis of regulatory events into GSMMs has been surveyed herein. A systematic classification has been created to grasp the difference between the several methodologies capable of integrating and simulating regulatory events into GSMMs. As a result, two primary approaches have been determined, namely the integration of TRNs and/or the integration of gene expression data.

The major obstacle when using the methods described in this survey to simulate integrated genome-scale models of metabolism and gene expression is not reproducing their results, but rather extending their implementations to other organisms and case studies. This hurdle poses a stiff challenge for using these methods out of the scope they were aimed at during development.

The requirement for large gene expression datasets with specific experimental conditions, the usage of TRNs reconstructed solely from literature, and the output of biased results strictly related to rigid constraints, are specific indicators of issues preventing the scaling-up of the reconstruction and analysis of integrated models. In short, there is a vast diversity of methods capable of integrating and simulating the effect of regulation into the metabolism, though few approaches that ease the reconstruction of these integrated models.

Hence, the perspective of reconstructing integrated genome-scale models of metabolism and gene expression for diverse prokaryotes is still a complex endeavor.

The implementation of a user-friendly computational framework that does not require coding skills, is capable of running a semi-automated pipeline for reconstructing TRNs or analyzing gene expression data, and performs its integration into standard GSMMs, would be a clear breakthrough towards the reconstruction and simulation of integrated genome-scale models of metabolism and gene expression. This hypothetical computational tool should be able to combine different sources of regulatory information which are seldom combined.

Perspectives

- The advent of high-throughput large-scale omics experiments has been supporting the study of the genomics, transcriptomics, and metabolomics layers of the cellular's molecular machinery. Systems biology can take advantage of the sheer volume of studies in these different omics fields by proposing differentiated approaches, such as the reconstruction of *in silico* networks and models
- The reconstruction and analysis of integrated models based on the integration of TRNs into GSMMs has not been conventional for non-model prokaryotic organisms. Usually, these lack large gene expression datasets, or have few sources of regulatory data. In addition to the absence of an established methodology and of easy to use tools and algorithms, the reconstruction and integration of TRNs into GSMMs is almost impracticable.
- Hence, a user-friendly computational framework that facilitates the reconstruction of TRNs and allows to integrate these into GSMMs would be a step towards facilitating the extension of integrated models to other prokaryotic organisms.

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public access to these results of federally sponsored research in accordance with the DOE Public Access Plan.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

Contribution

FC and OD conceived and designed the study. FR assessed the methods and drafted the manuscript. OD managed its coordination and helped to draft the manuscript. JPF, MR and IR participated in the coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

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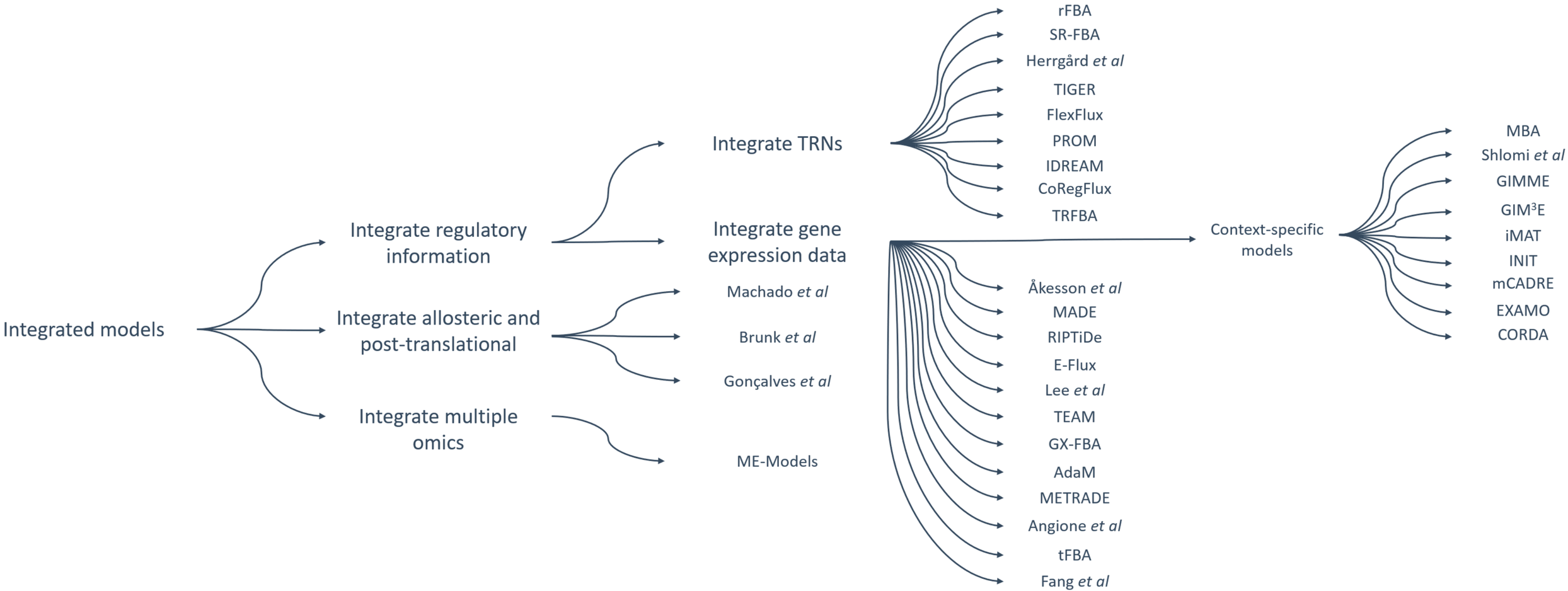
Integrated models
Integrate regulatory information
Integrate allosteric and post-translational
Integrate multiple omics
Integrate TRNs
Integrate gene expression data
Machado et al
Brunk et al
Gonçalves et al
ME-Models
rFBA
SR-FBA
Herrgård et al
TIGER
FlexFlux
PROM
IDREAM
CoRegFlux
TRFBA
Åkesson et al
MADE
RIPTiDe
E-Flux
Lee et al
TEAM
GX-FBA
AdaM
METRADE
Angione et al
tFBA
Fang et al
Context-specific models
MBA
Shlomi et al
GIMME
GIM3E
iMAT
INIT
mCADRE
EXAMO
CORDA

Regulatory Network

(A)

TF1

TF2

G1

G2

G3

Absolute

G1 and G2

G3

Discrete

Metabolic Network

Gene Expression Data

(B)

single-condition

multi-condition

G1

G2

G3

G1 and G2

G3

Continuous

Metabolic Network

Regulator
Under expressed

Regulator
Over expressed

Gene
Over expressed

Gene
Under expressed

Limited flux

Maximum flux

Discrete TRN

Continuous TRN

S. cerevisiae

E. coli

M. tuberculosis

S. oneidensis

Y. pestis

Herrgård *et al*

FlexFlux

CoRegFlux

rFBA

TRFBA

TIGER

IDREAM

SR-FBA

PROM

GX-FBA

RIPTiDe

E-Flux

TEAM

Lee *et al*

Åkesson *et al*

AdaM

Fang *et al*

METRADE

Angione *et al*

tFBA

MADE

Discrete gene expression data

Continuous gene expression data

Single-Condition

Multi-condition