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A review of genome-scale metabolic flux modeling of anaerobiosis in biotechnology

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The genome-scale metabolic flux modeling of anaerobic metabolism relevant to biotechnology has recently expanded in focus. In particular, there is interest in modeling facultative anaerobes (including yeast) to learn how to effectively eliminate microaerobic environments in favor of anaerobiosis. This is advantageous to bioprocessing and maximizes product formation from metabolic pathways that require substantial reducing power. Recent modeling efforts have also focused on CO/CO_2 and lignocellulosic sugar utilization for the production of advanced biofuels and chemicals. Several genome-scale models (GEMs), representing diverse metabolic traits, now exist for the non-pathogenic clostridia, methanogen, and *Geobacter* spp. obligate anaerobes, and microbial consortia interactions are now being modeled. Several new modeling tools to automate GEM construction, incorporate −omics datasets, and derive metabolic engineering strategies can now apply to anaerobiosis.

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Introduction

Anaerobic fermentation processes in biotechnology

A few years ago, a single review article captured the brief history of genome-scale metabolic flux modeling for both industrial and biomedical applications [1]. Since this time, the number of genome-scale models (GEMs) and their applications has continued to grow on an accelerated exponential trajectory due to the utility of new methods of automated GEM construction and increasing interest in commercializing bioprocesses. This review focuses on recent advances in genome-scale metabolic flux modeling related to anaerobiosis in biotechnology applications and new tools with potential uses for studying the systems biology of anaerobic metabolism. A summary of GEMs reviewed is provided in Table 1. As detailed in this review, genome-scale metabolic flux modeling of anaerobic metabolism has recently focused on the following topics: first, driving product formation from metabolic pathways that require significant reducing power [2], second, assimilating CO/CO_2 as a sole carbon source, third, producing H_2 through the anoxic ferredoxin-driven hydrogenase, fourth, fermenting pentose sugars to ethanol, and fifth, bioremediation. Several facultative anaerobes have been characterized by GEMs under anaerobic conditions in order to take advantage of $NAD(P)^+$ regeneration with a non-O_2 terminal electron acceptor to produce biofuels and chemicals. *Clostridium* spp., *Geobacter* spp., and methanogens remain the dominant focus for genome-scale modeling of obligate anaerobes. Finally, novel developments have been made in genome-scale modeling tools that are significant to studying anaerobiosis by first, automating GEM construction, second, allowing the incorporation of high-throughput −omics datasets, third, the generation of regulatory network models, and fourth, the derivation of effective metabolic engineering strategies.

The basics of genome-scale metabolic flux modeling and the influence of redox potential

Constraint-based metabolic modeling of biological systems has been employed for approximately 20 years (built upon long-standing mathematical principles) and has been the subject of numerous reviews. The modeling approach is built upon assumptions that first, the stoichiometries for all biochemical reactions within a given system are known, second, collectively, laws of mass conservation apply to the biological system, and third, metabolism can be approximated on short time-scales by pseudo-steady states. With these assumptions, system-wide metabolism is modeled as $\boldsymbol{S}\cdot\boldsymbol{v} = \boldsymbol{0}$, where $\boldsymbol{S}$ is the stoichiometric coefficient matrix and $\boldsymbol{v}$ is the vector of optimized reaction fluxes. Largely due to the underdetermined nature of genome-scale systems, there have been continuous improvements to the modeling paradigm, but the baseline modeling approach has much value in modeling anaerobiosis. Constraint-based models are inherently good at accounting for connectivity and balances on a large-scale. This attribute is particularly useful for anaerobic systems that typically have large carbon substrate fluxes, limited ATP generation, and overflow metabolism where metabolic byproducts need

Table 1

Summary of GEMs reviewed

Organism	Model	# Genes	# Reactions	# Metabolites	Use	Reference
Clostridium acetobutylicum ATCC 824	iCAC490	490	794	707	Model acetone, butanol, and ethanol (ABE) selectivity	[27••]
Clostridium beijerinckii NCIMB 8052	iCM925	925	938	881	Study butanol production and the influence of hydrogenase activity	[30]
Clostridium cellulolyticum H10	iFS431	431	621	603	Study cellulose utilization, inhibition by cellobiose, and discover growth bottlenecks	[34]
Clostridium ljungdahlii	iHN637	637	785	698	Characterize autotrophic and heterotrophic metabolic programs	[35•]
Clostridium thermocellum ATCC 27405	iSR432	432	577	525	Study cellulose metabolism and the relationship between redox and ethanol productivity	[32]
Dehalococcoides pan-genome	iAI549	549	518	549	Gain insights into organohalide respiration	[46]
Escherichia coli MG1655	iJO1366	1366	2251	1136	Updated *E. coli* model for systems biology and metabolic engineering	[16]
Geobacter metallireducens	iAF987	987	1284	1109	Broadly applicable to understanding *Geobacter* metabolism	[37•]
Klebsiella oxytoca	iYZ1315	1315	2260	1666	Predict metabolic flux changes with altered redox	[5••]
Klebsiella oxytoca	KoxGSC1457	1047	1457	1099	Improve 2,3-butanediol production with genetic and environmental perturbations	[8]
Lactococcus lactis MG1363	–	518	754	650	Discover metabolic characteristics required to produce different flavor compounds	[11]
Methanosarcina acetivorans C2A	iMB745	745	756	715	Study methane production from CO	[41]
Methanosarcina barkeri Fusaro	iMG746	746	816	718	Discover metabolic influences on methane production	[40]
Methanococcus maripaludis S2	iMM518	518	570	556	Determine the impact of C and N sources on methane production and growth	[42••]
Neurospora crassa	iJDZ836	836	1374	737	Serve as a model eukaryotic GEM and simulate anaerobic ethanol production from xylose	[12]
Pichia pastoris	iLC915	915	1423	899	Produce recombinant proteins from methanol and glycerol or sorbitol	[22]
Saccharomyces cerevisiae	iTO977	977	1566	1353	Simulate yeast metabolism under C-limited and N-limited and aerobic/anaerobic conditions	[21]
Saccharomyces cerevisiae	Yeast 5	918	2110	1655	Model yeast metabolism and ethanol fermentation	[19]
Saccharomyces cerevisiae	Yeast 6	900	1888	1458	Improved anaerobic capabilities compared to Yeast 5	[20]
Shewanella denitrificans OS217	iOS217_672	672	865	638	Investigations into *Shewanella* metabolism	[14]
Scheffersomyces stipitis	iBB814	814	1371	971	Determine xylose metabolism and anaerobic growth requirements	[23]
Scheffersomyces stipitis	iSS884	884	1332	920	Predict ethanol production from xylose at different oxygen uptake rates	[22]
Shewanella spp.	Core	552	673	565	Composite of 21 sequenced *Shewanella* species	[14]
Shewanella sp. M4-4	iMR4_812	812	986	655	Investigations into *Shewanella* metabolism	[14]
Shewanella sp. W3-18-1	iW3181_789	789	918	643	Investigations into *Shewanella* metabolism	[14]

to be balanced in terms of redox potential. Thus, charge balancing of reactions, in particular, is very important under anaerobic conditions.

Anaerobic fermentation is of interest to biotechnology due to the large amount of metabolic byproducts produced. Typically, there is a broad range of byproducts

that are produced due to the requirement to maintain an intracellular redox potential. The relative redox potential (or O/R value) of different metabolic byproducts can be gauged relative to a reference carbohydrate (i.e. $(CH_2O)_X$) by considering excesses or shortages of hydrogen and oxygen atoms [3]. The O/R values for several metabolic byproducts are given in Table 2 along with an explanation of the calculations. The approach is significantly different from the degree of reduction approach found in many biochemical engineering texts. The O/R values of metabolic byproducts can be applied to calculate redox balancing and should be able to serve in a predictive manner when metabolically engineering anaerobic metabolism with mixed acid/alcohol products.

Cofactors (e.g. NAD(P)H) are sources of reducing power (i.e. redox) for biosynthetic reactions that produce valuable chemicals and biofuels. A recent review [4•] details the progress in manipulating cofactor availability and specificity. This is done through enzyme and metabolic engineering to produce effective cofactor circuits that provide adequate reducing power. Recent research has shown that the extracellular redox potential plays a significant role in anaerobic metabolism. For example, the facultative anaerobe *Klebsiella oxytoca* can natively convert glycerol to 1,3-propanediol under microaerobic conditions. Decreasing the extracellular redox from −150 to −240 mV increased 1,3-propanediol production while slowing cell growth. Flux balance analysis (FBA) modeling results with GEM iYZ1315 revealed a significant metabolic shift to reductive glycerol metabolism at low redox [5••]. Recently, the sensing of redox by *Escherichia coli* was elucidated by a quantitative model [6••]. The model describes how two global transcription factors, ArcA and Fnr, sense intracellular redox and ultimately regulate genes connected to 80% of the total metabolic flux and 95% of differentially expressed genes between fermentive and respiratory conditions. This shift between fermentive and respiratory conditions is also governed by a trade-off between two objectives as determined from several sets of ^{13}C-labeled metabolic flux analysis (^{13}C-MFA) data: first, optimality under one condition and second, minimal adjustment between conditions [7].

Table 2

Relative oxidation-reduction values (O/R value) per mole of compound for typical fermentation products

Compound	Formula	2H [b]	O [c]	O/R value [d]
X = 0 Reference[a]	Null	0	0	0
Hydrogen	H_2	−1	0	−1
X = 1 Reference[a]	CH_2O	0	0	0
Carbon dioxide	CO_2	+1	+1	+2
Formic acid	CH_2O_2	0	+1	+1
X = 2 Reference[a]	$C_2H_4O_2$	0	0	0
Acetic acid	$C_2H_4O_2$	0	0	0
Ethanol	C_2H_6O	−1	−1	−2
X = 3 Reference[a]	$C_3H_6O_3$	0	0	0
Acetone	C_3H_6O	0	−2	−2
Lactic acid	$C_3H_6O_3$	0	0	0
Propanol	C_3H_8O	−1	−2	−3
Propionic acid	$C_3H_6O_2$	0	−1	−1
X = 4 Reference[a]	$C_4H_8O_4$	0	0	0
2,3-Butanediol	$C_4H_{10}O_2$	−1	−2	−3
Butanol	$C_4H_{10}O$	−1	−3	−4
Butyric acid	$C_4H_8O_2$	0	−2	−2
Succinic acid	$C_4H_6O_4$	+1	0	+1

[a] Reference compounds correspond to the general formula of a carbohydrate $(CH_2O)_X$.
[b] Each 2H excess (relative to the reference) is given a value of −1, and each 2H shortage is given a value of +1.
[c] Each O excess (relative to the reference) is given a value of +1, and each O shortage is given a value of −1.
[d] The overall O/R value is the sum of the 2H and O values [3].

Gems and their applications

Facultative anaerobes

A GEM (KoxGSC1457) for *K. oxytoca* was developed to study 2,3-butanediol production from glycerol and improve production through single gene knockouts to increase the pyruvate pool and by optimizing oxygen levels. In this case, production was achieved in anaerobic conditions, but a microaerobic environment (5% O_2) maximized production [8]. However, an emphasis of this review is to identify conditions where anaerobiosis is beneficial. The reducing power provided in anaerobiosis was used to produce 1,4-butanediol from engineered *E. coli* [9] and 4-hydroxybutyric acid (for chemical conversion to γ-butyrolactone) from the rumen bacterium *Mannheimia succiniciproducens* LPK7 [10••]. Both approaches used genome-scale modeling to effectively derive metabolic engineering strategies for high productivity by comparing flux maps that first, maximized the cell growth rate and second, maximized desired product formation. The GEM for *Lactococcus lactis* MG1363 was used to study the flavor-forming pathways of industrial dairy fermentations [11]. A GEM (iJDZ836) for the eukaryotic model organism and filamentous fungus *Neurospora crassa* was created and validated by existing experimental data gene essentiality, nutrient rescue, and synthetic lethal interactions. The model was used to study xylose fermentation to ethanol under anaerobic conditions [12]. Significant research has also been performed with anaerobic growth of *Shewanella oneidensis* MR-1, a Gram-negative bacterium with uses in bioremediation (e.g. carbon cycling and metal reduction) and microbial fuel cell applications. A dynamic FBA (dFBA) study of *S. oneidensis* MR-1 was performed, and ^{13}C-labeling of amino acids was used to check dFBA results. In this case, the dFBA framework predicted the dynamic metabolic shifts that occurred from early consumption of lactate to re-uptake of acetate and pyruvate metabolic byproducts late in the culture [13]. Ultimately, a new GEM of *S. oneidensis* MR-1 was created along with new GEMs for *Shewanella* sp. MR-4, sp. W3-18-1, *S. denitrificans* OS217, and a core *Shewanella* model consisting of all 21 sequenced strains. Model

validations were performed by predicting growth/no-growth phenotypes for wild-type and knockout strains given different carbon sources and electron acceptors. Cluster analysis revealed similarities/differences among models [14]. This methodology represents a good approach to discover shared metabolic traits and broader capabilities among phylogenetically related organisms. There is also much interest in how anaerobic processes of *Enterobacteriaceae* evolved and diversified in an approach termed 'paleo' systems biology [15]. With this understanding, a more complete picture of anaerobic capabilities will be generated. A recent trend in genome-scale modeling of anaerobiosis is the generation of high-quality GEMs for the purpose of metabolic engineering, but a common theme is that this approach is aiding biological discovery and improving the understanding of anaerobic metabolism. This is particularly clear with the integration of −omics datasets in genome-scale modeling. One study of anaerobiosis in *E. coli* MG1655 used an updated GEM (iJO1366) [16] and metabolomics data of aerobic and anaerobic growth to calculate *in vivo* reaction free energies, which enabled a more accurate representation of anaerobic metabolism. This approach generated new understandings of dNTP synthesis and function of the beta-oxidation pathway for fatty acids synthesis during anaerobiosis [17].

Yeast

The modeling progress with yeast is discussed separately from the other facultative anaerobes because of the strong historical development of yeast GEMs and recent concerted efforts to use genome-scale modeling to study and engineer productive anaerobic phenotypes. A recent study identified a single gene knockout strain (Δ*dic1*) of *Saccharomyces cerevisiae* capable of over-producing succinic acid. Genome-scale modeling using an existing GEM revealed production was tied directly to mitochondrial redox balancing and reductive TCA cycle activity [18]. Several new versions of the *S. cerevisiae* GEM have been published recently. Both the Yeast 5 [19] and Yeast 6 [20] GEMs were produced with emphasis on improving metabolic flux characterizations of anaerobic ethanol formation, which requires sterol supplemented growth medium. Here, genome-scale modeling provides a platform to understand the limitations to anaerobiosis in yeast, such as sterol biosynthesis, and allows metabolic simulations under supplementation or microaerobic conditions to understand how these impact growth and the use of product formation pathways. Significant global changes in metabolic flux distributions under aerobic and anaerobic conditions with carbon and nitrogen limitations were quantified using genome-scale modeling with integrated transcriptomics data using another new GEM (iTO977) of *S. cerevisiae* [21]. However, xylose utilization remains a high priority with yeast metabolic engineering and fermentation, and multiple investigators have focused on the native xylose utilizing *Scheffersomyces stipitis* (formerly *Pichia stipitis*). The first GEM (iSS884) of *P. stipitis* (*S. stipitis*) was produced along with an updated GEM for *P. pastoris* (iLC915), which together have the ability to utilize all lignocellulosic sugars [22]. Another GEM (iBB814) for *S. stipitis* used high-throughput growth phenotyping data for model validation and flux variability analysis to identify potential metabolic bottlenecks in xylose utilization and cofactor recycling. While *S. stipitis* cannot be grown anaerobically, a computational reaction insertion analysis identified 28 reactions necessary to simulate anaerobiosis with this model [23]. These may be translated into metabolic engineering strategies to produce an anaerobic strain. This represents another example of how genome-scale modeling is being used to drive biological discoveries, which will eventually be used to engineer anaerobiosis in yeast. *S. stipitis* (iBB814) has been simulated in co-culture with a respiratory deficient *S. cerevisiae* using dFBA. Both models were adapted by adding furfural and 5-hydroxymethyl furfural (HMF) degradation pathways. Results found that inoculums that reduced acetate production by *S. cerevisiae* yielded the highest ethanol productivity [24].

Clostridia

The concept of FBA was initiated in the 1980s with the acid-producing clostridia, and the first genome-scale models for this genus appeared in 2008 [25,26]. The mesophilic obligate anaerobe *Clostridium acetobutylicum* ATCC 824 has been of considerable research interest for its ability to produce acetone, butanol, and ethanol (ABE) fermentation products, degrade multiple complex substrates, and for studying its genetic programs of a metabolic shift (i.e. acids to solvents production) and sporulation. An updated GEM (iCAC490) of *C. acetobutylicum* ATCC 824 was produced containing thermodynamic reaction constraints and proton balancing based on metabolite pK_a values [27••]. The model was used along with a new tool of Flux Balance Analysis with Flux Ratios (FBrAtio) to show that knowledge of how metabolic flux is distributed at key critical metabolic branch points is critical for accurately predicting the selectivity of ABE and acid (i.e. acetate, butyrate, and lactate) fermentation products. It was also shown that merely adjusting the efflux of protons (consistent with the proton motive force), given a single set of five flux ratios, enabled an accurate portrayal of metabolism through the acidogenic exponential growth phase and solventogenic stationary phase of the culture [27••]. This approach has been used to predict ABE products in continuous fermentation with immobilized cells, and experimental results with varying substrates and dilution rates were used to validate model predictions [28]. The iCAC490 model also has a correct representation of the bifurcated TCA cycle (no carbon flux between α-ketoglutarate and fumarate) and an inactive oxidative pentose phosphate pathway as determined by ^{13}C-MFA [29•]. The original *C. acetobutylicum* GEMs published in 2008 [25,26] had differing versions of the

TCA cycle, and neither completely agreed with the findings of the fluxomics study. Thus, much remains unknown even in the central carbon metabolic pathways of anaerobes, and this should be considered in metabolic network reconstructions. This also points to ^{13}C-MFA as a complementary tool to genome-scale modeling for characterizing major metabolic pathways in the presence of incomplete genome annotation, which is also common among anaerobes.

A GEM (iCM925) for the butanol 'over-producer' *C. beijerinckii* NCIMB 8052 initially demonstrated the problem of acids/solvents selectivity, and identified the need for a new type of constraint. This model was also validated with experimental phenotype data and studied the role of the hydrogenase activity on global flux distribution and resulting products profile [30]. This model was also used to explore possible mechanisms of acetate and butyrate production when genes of their corresponding biosynthesis pathways (i.e. *pta* and *buk*) were knocked out [31].

Two cellulolytic clostridia have had GEMs constructed recently. First, a GEM (iSR432) for the thermophile *C. thermocellum* ATCC 27405 was constructed and verified with experimental phenotype data. The model revealed the relationship between redox state and ethanol productivity. Metabolic engineering targets were identified as single gene knockouts and supplemented carbon sources [32]. An additional study incorporated RNAseq transcriptomic data into the iSR432 GEM to identify the presence/absence of genes and identify pathways as on/off. The final result revealed significant deviations from traditional FBA with a maximized cell growth objective, and model results more closely matched experimental observations [33•]. Thus, the use of transcriptomic data in this manner can dramatically reduce the phenotypic solution space of the optimization problem. While it cannot provide information regarding individual flux levels, the knowledge of on/off states for particular pathways is highly significant. A GEM (iFS431) for the mesophile *C. cellulolyticum* H10 was produced and modeled in co-culture with *C. acetobutylicum* ATCC 824 in one of the first examples of dFBA using an interacting co-culture [34]. Results revealed that increased rate of cellulose consumption by the co-culture, relative to *C. cellulolyticum* alone, was not due to the removal of cellulose inhibition and that *C. cellulolyticum* is adapted to low carbon flow, suggesting that future metabolic engineering strategies must address this issue.

A recent GEM (iHN637) was published to describe the metabolic activity of the acetogen *C. ljungdahlii*. This organism is of considerable recent interest due its ability to use the Wood-Lungdahl pathway to grow autotrophically on CO_2/H_2 or on syngas ($CO_2/CO/H_2$). The organism also has use in microbial electrosynthesis, which uses electricity to reduce CO_2 to organic compounds. The iHN637 model was constructed with the use of transcriptomic data and proved capable of autotrophic growth and heterotrophic growth on several substrates including xylose, formate, and ethanol [35•].

Geobacter

Geobacter spp. are of interest for bioremediation due to their ability to utilize inorganic carbon and use metals or other microbes as terminal electron receptors. A high-profile review of genome-scale modeling in *Geobacter* was published a few years ago [36], so this review will summarize recent findings in the context of modeling anaerobic metabolism. An updated GEM of *Geobacter metallireducens* GS-15 (iAF987) was constructed and validated by growth experiments. The model was used to further understand the metabolism of carbon fixation as well as predict and evaluate terminal electron acceptors (e.g. Fe(III), nitrate, and fumarate) [37•]. Using a previously published GEM for *G. sulfurreducens*, genome-scale modeling was used to evaluate the organic-to-electricity metabolic potential of this organism in a microbial fuel cell given different mechanisms for the conveyance of an electron to the anode. Results showed that a maximum potential current of up to 3.710 A/gDCW is possible [38]. Recent research has also investigated the microbial interactions between U(IV) reducing *Geobacter* sp. and acetate-oxidizing sulfate reducing bacteria. Modeling results revealed that Fe(III) availability, rather than microbial competition, is the key factor in uranium bioremediation [39]. Thus, genome-scale modeling has enabled significant biological discoveries among the *Geobacter* spp. as well.

Methanogens

The methanogens are of considerable interest due to their ability to consume CO/CO_2 and produce methane as a metabolic byproduct. They are used heavily in anaerobic digestion and lend well for flue gas remediation. The existing GEM for *Methanosarcina barkeri* Fusaro has been updated (iMG746) and is more accurate at predicting gene knockout and batch growth phenotypes [40]. A GEM (iMB745) was produced for *M. acetivorans* C2A and was used to study the energetics of methane formation when grown on CO. This organism is of interest because it can also grow on methylated substrates and acetate [41]. Finally, a GEM (iMM518) was produced for *M. maripaludis* S2, a hydrogenotrophic mesophilic Gram-negative anaerobic archaebacterium that can use CO_2 as its sole carbon source and N_2 as its sole nitrogen source. Its genomic tools are well-established and its doubling time is about two hours, an order of magnitude greater than other well-studied methanogens. Simulation results suggested only slight differences when using N_2-fixation relative to ammonium supplementation in calculating growth and methane evolution rates. While CO_2/H_2 yields growth, supplementation with formate doubles

Table 3

New tools and databases useful to the genome-scale modeling of anaerobiosis

Tool	Use(s)	Acknowledged limitations	Classification(s)	Reference
BioMet Toolbox 2.0	Online tools for GEM analysis and simulation as well as −omics dataset analysis	Flux analysis limited to FBA and random sampling	Flux balance analysis −Omics integration Network visualization GEM database Fluxomics	[50]
BioMog	*De novo* biomass equation generator	Excludes flux and −omics data in generating biomass equations	Model building	[45]
COBRA Toolbox v2.0	MATLAB toolbox containing functions related to FBA, fluxomics, network gap filling, metabolic engineering, and visualization	Available for MATLAB and Python only. Programming knowledge is necessary	Flux balance analysis −Omics integration Metabolic engineering Model building Network visualization Fluxomics	[51]
CONGA	Used to find biomass, metabolic, and genetic differences between GEMs	Only an optimal subset of differences is identified	Model building	[14]
eQuilibrator	Calculates Gibbs free energies of reactions given metabolite concentrations, ionic strength, and pH	Repetitive parameterization may be necessary. Available only in Python	Model building	[52]
FBA-Gap	Aids in metabolic network completion and minimal medium formulation	Manual validations required to ensure accuracy	Model building	[53]
FBrAtio	Applies flux ratio constraints in a GEM to accurately model wild-type metabolism or design metabolic engineering strategies	Target identification is not automated. Available only in MATLAB	Metabolic engineering	[27••,54]
GEMINI	Construction of regulatory networks	Restricted to microbes with a large library of gene knockout phenotype data. Large−omics data input requirement	−Omics integration Model building	[55]
MEMOSys 2.0	GEM database and analysis tool	Only serves to develop and manage GEMS	Model building GEM database	[56]
MetaNetX	Web application for manipulating and analyzing GEMs by FBA, FVA, and gene essentiality	Only single gene/reaction knockouts are possible, but this is specific to determine essentiality	Flux balance analysis GEM database Model building Metabolic engineering	[57]
MILP	A method to utilize −omics data to reduce the solution space of a GEM	Multiple optima exist	−Omics integration	[58]
Model SEED	Automated high-throughput generator of GEMs	Non-essential pathways may contain gaps and manual curation may be necessary	Model building GEM database	[59]
OptCom	Enables the study of various types of interactions among multi-species microbial systems	Environmental and microbial signaling factors may be uncharacterized	Metabolic engineering	[49•]
ORCA	A COBRA Toolbox extension enabling multi-objective optimization, futile loop identification/elimination, and dFBA with incorporated kinetic constants	Available only in MATLAB	Flux balance analysis	[60]
Path2Models	Automated model generator based on data from available biochemical databases	The models are frameworks that may require additional building	Model building GEM database	[61]
ReacKnock	An updated version of the revolutionary program OptKnock	Solutions may not give the optimum product yield in some cases	Metabolic engineering	[62]
Redirector	Designs metabolic engineering strategies by altering cellular objectives	The number of targets returned is large, but these are ordered by relevance	Flux balance analysis Metabolic engineering	[63]
SUMEX	Microbial growth rate prediction algorithm	A simple first-generation algorithm that will likely incorporate additional inputs in future generations	Metabolic engineering Model building	[64]
TUX GA	A framework for optimizing the biomass equation of a GEM	May not find the optimal solution as an inherency of the genetic algorithm	Model building	[44]

Table 4

Applications of high-throughput datasets and redox

Dataset type	Impact on modeling	Tools to implement	Reference(s)
Transcriptomics	Eliminates un-utilized metabolic pathways	Boolean logic 'on/off' constraints	[33•]
Proteomics	Eliminates un-utilized metabolic pathways. Potential extrapolation to pathway capacities and kinetics	Boolean logic 'on/off' constraints and dFBA	[58]
Metabolomics	Enables calculation of *in vivo* reaction free energies for correct assignment of thermodynamic constraints	Thermodynamics-based metabolic flux analysis (TMFA)	[17,65]
Fluxomics	Characterization of central carbon metabolism. Especially useful in cases of incomplete genome annotation	Boolean logic for 'on/off' pathway constraints. FBrAtio for defining flux distribution at metabolic nodes (branch points)	[29•]
Phenotyping	Generation of realistic biomass equations. Results in global flux re-distribution.	Laboratory measurements incorporated directly as stoichiometry. BioMog and TUX GA for updates *in silico*	[44,45]
Fermentation product redox balancing	Re-distribution of metabolic flux based on allowable product secretion profiles	FBrAtio (although results have not been published to date)	[4•,54]

the growth rate [42••]. Recent advances have created significant diversity among the available GEMs for the methanogens.

Tools, databases, and −omics datasets

Along with individual models, several new genome-scale modeling tools and related databases have been published recently that assist considerably in: first, the construction of GEMs, second, the analysis of anaerobic metabolic processes, and third, the derivation of metabolic engineering strategies. These tools are listed and described briefly in Table 3. In addition, high-throughput −omics datasets have aided biological discovery and modeling accuracy of anaerobiosis. The uses and methods for implementing −omics datasets are summarized in Table 4. It is emphasized that tools for model building and metabolic engineering must be used iteratively as part of a design, build, and test strategy. It is recognized that experimental validation is an important part of genome-scale modeling, and this is especially true of anaerobiosis, for which significantly less biochemical data is available in the literature.

Conclusions

The role of genome-scale modeling in characterizing anaerobiosis has increased significantly in the past few years. However, several basic developments are still needed for creating effective GEMs of anaerobes. For example, poor genomic annotation still exists for many anaerobes, and several metabolic pathways function in non-obvious ways, such as the bifurcated TCA cycle and non-functional oxidative pentose phosphate pathway of *C. acetobutylicum* [29•]. It is likely that automated gap-filling algorithms would incorrectly complete the TCA cycle of a *C. acetobutylicum* GEM (although this has not been tested). Thus, a knowledgebase of the possible metabolic flux distributions in central carbon metabolism, possibly constructed by a broad ^{13}C-MFA study, would be considerably helpful in building new and more accurate GEMs of anaerobes. Effective bioinformatics approaches exist to update genome annotation, but it is suspected that biological discovery is the key bottleneck in anaerobiosis currently. The generation and incorporation of −omics datasets has proven beneficial, but the number of available datasets for anaerobiosis is limited currently. In addition, improvements in biomass equations are necessary. An initial GEM of *C. acetobutylicum* [26] proved highly sensitive to perturbations of the biomass equation [43], and it is reasonable to conclude that well characterized cell compositions of Gram-negative microbes such as *E. coli* should not apply to the Gram-positive anaerobes, such as the clostridia. However, cell composition is difficult to measure in the laboratory and changes with environmental conditions and genetic perturbations. Thus, methods of determining a biomass equation accurately and easily are needed. Algorithmic approaches are now appearing [44,45], but it is anticipated that an approach that incorporates simple (and preferably real-time) laboratory measurements will be most effective at generating dynamic biomass equations that are needed for accurate genome-scale modeling.

Following these basic needs for genome-scale modeling anaerobiosis, the next obvious steps are to develop tools that incorporate the redox potential and predict its associated global metabolic shifts. This is needed to more accurately model time-course fermentations without kinetic inhibition constants in dFBA. Global regulatory networks are also expected to provide significant opportunities, such as modeling metabolic shifts that occur with programmed cell differentiation like sporulation. The FBrAtio method provides one possible method for implementing regulatory rules that redirect metabolic flux by other means than a simple Boolean 'on/off' logic gate. These additions may improve model predictions of anaerobiosis and help attain better agreement with *in vivo*

metabolic flux states determined by ^{13}C-MFA. Using genome-scale modeling to design metabolic engineering strategies that optimize expression of synthetic *de novo* metabolic pathways is now also possible. While successes are few currently, this is expected to be an area of intense progress soon as several *de novo* metabolic pathways have led to production of novel biofuels and non-native value-added chemicals. In addition, a transition from agricultural feedstocks is underway as syngas, CO_2, and methane are under serious consideration as substrates, and recent developments have also incorporated renewable electricity (e.g. electrochemical platforms) as an input into engineered metabolism. Genome-scale modeling provides the necessary platform to study, identify bottlenecks, and design new metabolic capabilities that will be required to optimize these scenarios.

In another application, genome-scale modeling efforts should be more focused on extremophiles so that their unique characteristics can be engineered into microbial cell factories. As examples of potentially useful metabolic traits, modeling efforts are underway to characterize organohalide respiration by *Dehalococcoides* [46,47], and the haloalkaliphilic anaerobe *Chitinivibrio alkaliphilus* is capable of growing on insoluble chitin, at pH 10, and at salinities up to 3.5 M [48]. Only a genome-wide understanding will allow these metabolic traits to be harnessed and transplanted into new hosts at some point in the future. In addition, many of these anaerobes are found in microbial consortia, including as both pathogens and non-pathogens in the human microbiome. While successes are noted in modeling these complex systems [49•], a continued dedication to the systems-level understanding of the interplay between internal metabolism and the external environment remains important.

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