HYBRID MODELS: RELATING GENOMIC DETAIL TO CELL PHYSIOLOGY AND EXTRACELLULAR ENVIRONMENT

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Abstract

While genomics (the set of experimental and computational tools that allows the blueprints of life to be read) opens the doors to a more rational approach to the design and use of living cells to bring about desirable chemical transformations, genomics is, by itself, insufficient. We need tools that allow us to relate genomic and molecular information to cellular physiology and then to the response of a population of cells. Chemical engineering tools applied to the design of chemical plants can be adapted relating system performance (i.e. the cell) to genomic information. We propose the development of hybrid cellular models. In such models genomics and chemical detail for a cellular subsystem (e.g. pathogenesis) is embedded in a coarse-grain cell model. Such a construct allows the quantitative and explicit linkage of genomic detail to cell physiology to the extracellular environment.

I. Overall Vision

Our vision is to develop a framework using computer and experimental models to quantitatively and explicitly link genomic/molecular level insights to the physiology of whole organisms. Our group works at two extremes: (1) a microbial model, and (2) a model of the human body. The microbial model can be done in much greater detail than the human model and is easier to verify and is the subject of this paper. The human model may have more impact and demonstrates the broad potential of hybrid models.

This vision addresses what we believe is the essential challenge to biology and medicine. Namely, how to make sensible use of genomic (and related "…omic" information). Biology is in transition from a data poor to data rich field thanks to advances in the underlying technology (e.g. high-throughput techniques). The output of such studies is information, but we lack a theoretical framework in which to organize, test, and use this basic information. We believe we can construct both experimental and computer models that will allow us to quantitatively and explicitly cross scales; through such models we can directly link genomic and molecular information to physiological response.

We are particularly interested in a new approach "hybrid" models. We believe this approach will be our best opportunity to build models of complex organisms that are both realistic, tractable, and useful. Hybrid models start with a genomically/molecularly detailed model of a subsystem of interest. We then insert this detailed submodel into a cellular model with pseudo-molecular detail (i.e. "coarse grain" models), and then the cellular model into a system model. For bacteria the system is a population of cells in a bioreactor or a national environment. For a human this would mean putting a genomically detailed model of a subcellular mechanism into a cell model (could be in multiple cell types) into corresponding tissue/organ model(s) which is embedded in a physiologically-based pharmacokinetic model (PBPK). PBPK models have been long used by the biomedical community, but often lack mechanistic and kinetic detail, which would now be provided by input sub-models. Such models could be computational, but can be made as physical devices using the techniques of microfabrication (eg. we have constructed crude devices with liver-fat-lung-other tissue compartment; see Sin *et al.* (2004) and Viravaidya & Shuler (2004a; 2004b)).

The benefits of applying such modeling approaches to either a population of bacteria or to the human body are two-fold. First, to "understand" a system you must be able to recreate it. A parts list (genome) is insufficient. Regulatory structure is critical. An isolated submodel removed from the context of the complete biological system is of limited utility. An effective model tests the level of understanding by making predictions that can be verified or denied. While no model can "prove" a proposed mechanism, it can be used to confirm its plausibility. A second benefit is the practical application of the model. For humans the type of models envisioned provides a rational basis to evaluate proposed therapeutic strategies (e.g. combination therapy for cancer or gene therapy) or evaluate molecular targets for their potential to offer cheaper, less invasive, and earlier diagnosis of disease. For a population of microbes we might better understand how molecular mechanisms relate to the biotechnological application of that organism.

II. Building Bacterial Cell Models: The "Minimal Cell" as a Foundation

While our overall aim is to build a model for real organisms using this hybrid approach, we believe a fully-detailed model of a hypothetical "minimal cell" is critical to both testing fundamental concepts about microbial physiology and to building the methodology necessary to construct hybrid models of real cells quickly and efficiently.

A minimal cell is a hypothetical cell defined by the essential functions required for life [Castellanos *et al.*, 2004]. The model seeks to identify a minimum number of genes necessary and sufficient for the cell to divide and grow continuously in a rich environment with preformed nutrients and relatively constant temperature and pH. The model, which contains kinetic and thermodynamic

constraints as well as stoichiometric constraints, can be used as a tool to identify the organizing principles which relate the dynamic non-linear functioning of the cell to the static linear sequence information of the genome.

The success of whole organism genome sequencing and high-throughput measurements provides an opportunity for system-level analysis of whole organisms or what has been termed "systems biology" eg. [Kitano, 2002]. Systems biology investigates the "behavior and relationships of all of the elements in a particular biological system while it is functioning". The emphasis in our project is on modeling the complete functionality of a cell and its explicit response to perturbations in its environment [Browning & Shuler, 2001] and to build hybrid models starting with a genomically/molecularly detailed model of a subsystem of interest, inserting that submodel into a cellular model with pseudo-molecular detail (i.e. "coarse grain" models), and then the cellular model into a system model. Our attempt to generate "complete" and hybrid models that predict time-dependent responses of a cell differentiates this project from others.

Many investigators have made significant contributions to our understanding of bacterial metabolism, particularly central carbon metabolism. The studies have taken advantage of detailed genomic information and some models are based primarily on stoichiometry and techniques involving flux balance analysis, metabolic control theory, and mathematical techniques for optimization eg. [Burgard *et al.*, 2001; Edwards & Palsson, 2000]. Since these models are intrinsically static, they have limited ability to predict aspects of cell regulation and dynamic response (although by the addition of constraints, such as uptake rates of a nutrient, these models provide some insight into the dynamic state that can be achieved). Others have proposed methodology to incorporate more directly dynamic (kinetic) information into models of central metabolism eg. [Chassagnole *et al.*, 2002]. Others have attempted to model whole cells [Tomita, 2001], but those models, while attempting to be whole cell models neglect important, non-metabolic aspects of cell growth (eg. control of chromosome replication or spatial issues associated with position of septa). These studies, and many other similar ones, have contributed towards a systems biology perspective. However, all of these approaches are "incomplete" descriptions.

Incomplete descriptions may lead to conclusions that are inaccurate as there is an implicit assumption in such studies. The assumption is most easily illustrated by considering the metabolic flux analysis of an isolated pathway. As shown by Schlosser and Bailey [1990], such analysis is correct only if the output of the pathway cannot influence any input into the pathway. Any "cell" model that is "incomplete" assumes that no output of the model either directly or indirectly can influence any input or state within the model within the timescale of interest.

A "complete" model of a real organism is a daunting task [McAdams & Shapiro, 2003; Bailey, 2001], but we believe our goal of a hypothetical minimal cell model is both achievable and will provide insights into biological questions of immediate importance. McAdams & Shapiro [2003] write "… to develop "whole-cell" models… major, perhaps insurmountable, difficulties must be overcome… Problems include lack of quantitative data on molecular concentrations and kinetic parameters as well as only piecemeal characterization of the cell's regulatory circuitry". While we agree with the problems they identify, we are optimistic and we agree with Alon [2003] that a "reverse engineering" approach that takes advantage of the natural characteristics of biological systems: modularity, robustness, and use of recurring circuits elements can succeed. This is the basis of the approach we will describe. Just as an engineer will design an airplane based on functional constrains and make use of prior designs we will design a cell (using guidance from existing cells) to perform the essential tasks necessary to survive indefinitely and translate that design into a hypothetical genome, just as the airplane design is translated into blueprints and construction documents. We describe in this paper methodology to rapidly estimate a credible set of kinetic parameters overcoming one of the key limitations suggested by McAdams & Shapiro [2003].

Perhaps the question of "What is essential for life?" is one of the most fundamental questions facing humanity and a minimal cell model begins to answer this question. By constructing a minimal cell *in silico* we can seek to understand and identify underlying regulatory and organizational concepts central to life. This exercise is very important since our understanding of things is never fully complete until we can reconstruct them.

Although a minimal cell is hypothetical, the applicability of such a detailed model is enormous. The proposal model can lead to a better understanding of the behavior of chemoheterotrophic bacteria. While a minimal cell model will suggest the essential components of regulation, a deeper insight into the logic of cell regulation can be achieved in future studies by perturbing the environment with large changes until the model cell fails ("dies") and then finding regulatory approaches that allow survival. In essence, we wish to understand how selective pressure relates to microbial evolution. A more complete understanding of essential cellular structure and regulation is important for bioprocess engineers to metabolically engineer cells for production of desirable metabolites and/or to design improved operating strategies for bioprocesses.

Additionally, we can use the minimal cell as a basis to learn to build hybrid models of real cells. The key requirements for such hybrid modules is "modularity" and the ability to construct species specific coarse grain models rapidly. Using the minimal cell we demonstrate modularity and also techniques to evaluate kinetic parameters rapidly.

III. The Minimal Cell Concept

The minimal cell concept can be traced back to the 1950's when Harold Morowitz and colleagues began to seek the smallest, autonomous, self-replicating entity. They correctly identified *Mycoplasma* as the best living example of a minimal organism (both in terms of size and genome). Morowitz proposed that it should be possible to build a computer model of such a complete cell. He also wrote that with *Mycoplasma* "Their existence with all the properties of life says the 'logic of life' is finite, relatively simple, and subject to full exploration" [Morowitz, 1984].

By the mid-1990's the issue of a minimal gene set began to attract increased attention. In 1995, Itaya [1995] used random gene knockouts in *Bacillus subtilis* to estimate that 254 genes are essential. Mushegian and Koonin [1996] compared the full genome sequences of *Haemophilus influenzae* and *Mycoplasma genitalium* and proposed a set of about 250 genes as a minimal gene set. A large project was begun shortly afterwards to create a minimal cell. The ultimate goal was the experimental construction of an artificial minimal genome. Hutchinson *et al* [1999] used transposon knockouts of *M. genitalium* to predict that about 265 to 350 genes (about 100 with unknown function) were essential. The so named E-CELL model was created as a component of this minimal cell project [Tomita, 2001]. However, the project was abandoned [Peterson & Fraser, 2001]. A very similar project under Venter's leadership has been restarted to develop a synthetic chromosome as the first step toward making a self-replicating organism [Zimmer, 2003]. The technical difficulty of fitting the minimal genome with a working cell structure is acknowledged as a major challenge[Zimmer, 2003].

Peterson and Fraser [2001] reviewed key difficulties in a minimal cell project. They conclude, "We believe that the concept of the minimal genome is a useful tool in attempting to organize our thoughts about gene function - even though we may never, in practice, be able to reach a definition of a minimal gene set that is applicable to all types of organism". One specific issue is the existence of paralogous genes that "…greatly hampers genome-scale mutagenesis experiments". For example, an essential function can be accomplished by either gene product A or B; single gene knockout experiments will not reveal either A or B as essential as both must be eliminated simultaneously. As they point out one must "…take care to discriminate between dispensable genes and dispensable functions."

Koonin [2000] reviewed advances since their 1996 paper that demonstrate the complexity in using comparative genomics to establish a minimum gene set. For example, of the 256 genes identified as essential in 1996, 15% were found to be dispensable in knockout experiments. Further, when 21 genomes are compared only 80 genes (instead of 256) are universally present. These two results are ascribed, in part, to NODs (non-orthologous gene displacement - where the same function is performed by unrelated or very distantly related and nonorthologous proteins). Thus, comparative genomics is limited in its ability to reveal a true minimal set of genes suggesting the need for alternative approaches. Koonin differentiates between essential genes and essential functions.

A minimal gene set derived by comparative genomics approach is likely to be an underestimate (due to non-orthologous gene displacement). Additionally an experimental approach can overestimate the minimal set substantially (genome scale knockouts could identify genes as essential even when the deletion only slows growth [Koonin, 2003]). Computer simulations offer an alternative to comparative genomics and experiments to identify a minimal gene set.

A computer model approach should result in a set of minimal functions that will correspond to real genes, which exist in nature, and 200 genes is our projected number of essential genes. The model we are developing focuses on essential functions while finding examples of gene products that can perform those functions. While the set of minimal genes we postulate may change (eg. if a new multifunctional protein is found), we believe we can find a set of essential functions. Further, the technical difficulties associated with generating an experimental minimal cell and the ambiguities in interpretation of comparative genomic data argue for the establishment of a theoretical computer model of a minimal cell. This model must be explicit about minimal functions and include a realistic set of proteins to accomplish these functions. This is, we believe, the most practical route to a minimal cell.

IV. The Cornell Single Cell

We have previously developed a "complete" cell model of *E. coli* that contains all of the functional elements for the cell to grow, divide, and respond to a wide variety of environmental perturbations. All chemical species are included, but lumped into pseudochemical groups. This "coarse-grain" model serves as the basis for our efforts to build a minimal cell model. Basically, the *E.coli* model is a good summary of the functionality required for a minimal cell, but it does not capture explicitly the physical chemistry that supports those functions. We described our first mathematical model of a single *E. coli* cell in 1979 [Shuler *et al.*, 1979]; at that time, it was the only model of an individual cell that did not include artificially-imposed constraints on aspects such as mode of growth, timing of cell division (eg. growth rate), and

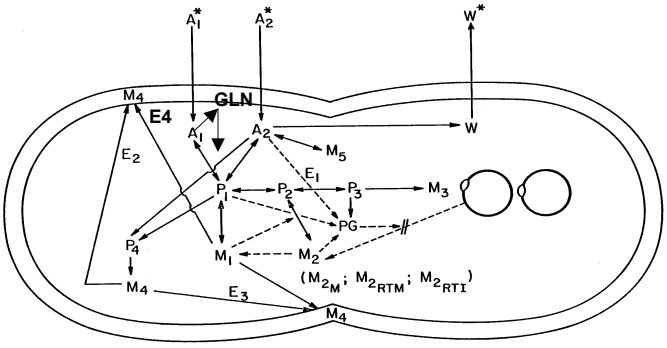

Figure 1. An idealized sketch of the model of E. coli B/rA growing in a glucose-ammonium salts medium with glucose or ammonia as the limiting nutrient. At the time shown, the cell has just completed a round of DNA replication and initiated cross-wall formation and a new round of DNA replication. Solid lines indicate the flow of material, while dashed lines indicate flow of information.

A_1= ammonium ion
A_2= glucose (and associated compounds)
W= waste products (CO_2, H_2O, and acetate)
P_1 = amino acids
PG= *ppGpp*
P_2 = ribonucleotides
P_3= deoxyribonucleotides
P_4= cell envelope precursors
M_1 = protein (both cytoplasmic and envelope)
$M2_{RTI}$= immature "stable" RNA
$M2_{RTM}$=mature *r*-RNA and *t*-RNA
$M2_M$=messenger RNA
M_3=DNA
M_4=non-protein part of cell envelope
M_5=glycogen
E_2, E_3=molecules involved in cross- wall formation and cell envelope synthesis
GLN=glutamine
E_1=enzyme in the conversion of P2 to P3
E4 = glutamine synthetase
*— the material is present in the external environment

cell size. Also, it was unique in its ability to respond explicitly to concentrations of nutrients in the environment [Bailey, 1998]. This base model [Domach *et al.*, 1984] has been embellished with additional biological details to allow prediction of a wide-range of responses to environmental and genetic manipulations. The initial model included only 18 pseudochemical species that represented large groups of related chemical species; Fig. 1 lists the components and graphically depicts the relationships between components. The mathematical description of cellular function, which is the core of the model, is based on time-variant mass balances for each component. Each mass balance takes into account the component's synthesis (as a function of availability of precursors and energy, relevant enzymes), utilization, and degradation. Stoichiometric coefficients for relating components through mass balances were derived primarily from published research, and in some cases, from our own experiments. It is important to note that the model was **NOT** developed by using adjustable parameters to fit model predictions to experimental results, nor did the stoichiometric mass balances assume a steady state (i.e. the amount of each component was allowed to vary with time). Despite the simplifications that were made in describing cell composition and relationships, the model can accurately predict changes in cell composition, size, and shape, and the timing of chromosome synthesis as a function of changes in external glucose and ammonium concentration [Domach *et al.*, 1984].

The dynamic mass balances are solved by forward integration from an initial condition (both extracellular concentrations of nutrients and intracellular estimates of all pseudochemical species) using a Runge-Kutta type technique. A variable step size is used as the equations become very stiff when chromosome replication is initiated (i.e. large flux through a small pool P_3). Conditional statements are included for fork position, gene dosage, potential for initiation of chromosome replication, cell shape and position and completeness of cross-wall formation.

Other biochemical details have been added in subsequent studies that allow the study of the effects of amino acid supplementation [Shu & Shuler, 1991] and of competition between recombinant mRNA and ribosomal mRNA in the context of high translational activity [Laffend & Shuler, 1994a]. The model has also been utilized extensively to improve the use of plasmids for recombinant protein production; (eg.[Kim & Shuler, 1990, 1991; Laffend & Shuler, 1994b]). The calculations have proven to be quite robust and results are reproducible.

We have used the structure of the *E.coli* model to build a coarse-grain minimal cell model [Browning & Shuler, 2001] that is a generalized model of chemoheterotropic bacteria. By using dimensionless concentrations and growth rates, we demonstrate that it is the relative value of parameters, rather than absolute values, that are key to physiological response and thus a generalized chemoheterotroph can be construct that is consistent with a wide range of experimental data (Browning & Shuler, 2001). The coarse-grain minimal cell model is "complete" in terms of function and is "modular". By modular we mean that we can "delump" a pseudochemical species into individual components while still maintaining the essential connectivity to other functions in the cell [Castellanos *et al.*, 2004]. This allows us to add detail in parallel efforts on different "modules" and then have confidence that they can be recombined into a functional and functioning whole. This strategy is our basic approach to constructing a genomically and chemically detailed minimal cell model. It depends on our ability to treat pseudochemical compounds as modules.

V. Demonstration of Modularity of Basic Minimal Cell Model

We have tested the hypothesis that it is not the exact values of parameters in the model that determine function, but that the values relative to one and another is critical. We tested this hypothesis by varying all kinetic rates by a scaling factor (or kinetic ratio). The growth rate scales directly with the kinetic ratio over about two orders of magnitude. At low values of growth rate, membrane energization becomes important and linearity is lost. Cell composition (eg. protein/cell, RNA/cell, etc.) remains constant for a wide range of kinetic ratios. Further, relative growth rate

changes for models with different kinetic ratios is essentially the same for a wide variety of perturbations to cell function (which also confirms the computational robustness of the model). Also the general physiological behavior of a variety of common bacteria (based on experiment) scales with a dimensionless growth rate, suggesting that the lessons from a hypothetical cell model will be broadly applicable to chemoheterotrophic bacteria.

A key aspect of the original Cornell *E. coli* model was a model that mechanistically coupled cell growth, chromosome replication, and cell division. We have updated the model for control of chromosome replication based on new experimental evidence since 1984. While the revised model is significantly different in terms of biological mechanism (positive vs. negative control), the mathematical characteristics are quite similar. Indeed, it may be that any functional mechanism for control of replication must satisfy similar mathematical constraints.

A manuscript on the revised model for chromosome replication is in press. While this model shares similarities with the initiator-titration model of Hansen *et al* [Hansen *et al.*, 1991], it includes ATP-bound DnaA as the active species. We have developed both deterministic and stochastic versions of control of initiation of chromosome replication in the model (to determine robustness to intracellular fluctuations in concentrations). The model also takes advantage of the genomic data on *E. coli* to locate all three types of DnaA-binding sites on the chromosome. The model examines the effects of various binding strengths on the robustness of the system. The model allows an evaluation of potential "costs" of strategies for number and placement of such binding boxes.

Our minimal cell model for nucleotide metabolism [Castellanos *et al.*, 2004] confirms the concept of modularity by testing a functional nucleotide subsystem model with significantly fewer gene-encoded functions (12) than estimated previously. In the *M. genitalium* genome sequence [Fraser, 1995] 25 genes can be associated with nucleotide transport and metabolism. Mushegian and Koonin [1996] estimated that the minimal gene set includes 23 genes for nucleotide metabolism. Hutchinson *et al* [1999] concluded that only 18 genes were essential for transport and metabolism of ribonucleosides. Kobayashi *et al* [2003] include 10 genes in the nucleotide category but they point out that due to single gene inactivation, the number of genes in their minimal gene set is likely to be underestimated. Their list of essential genes appears incomplete, based on the diagramed pathways (supplemented information to Kobayashi *et al.*, 2003. Our minimal cell pathway with 11 functions (12 genes) permitting growth from preformed ribonucleosides precursors is the most efficient (fewest genes) of any study with a complete pathway. Our minimal cell pyrimidine nucleotide biosynthesis pathway includes: uracil phosphoribosyltransferase, cytidylate kinase, ribonucleotide reductase, thymidylate synthetase, deoxyuridine triphosphatase, adenylate kinase, and thymidylate kinase. Our minimal cell purine nucleotide biosynthesis pathway includes: adenine phosphoribosyltransferase, guanine phosphoribosyltransferase, adenylate kinase, ribonucleotide reductase, and guanylate kinase.

An example of the equation used in the nucleotide model [Castellanos *et al.*, 2004] describing the reduction of dUMP to synthesize dTMP by thymidylate synthase is shown below:

$$\frac{dP_{24dM}}{dt} = k_{12}\left(\frac{K_{P_{24dM}}}{K_{P_{24dM}} + \frac{P_{24dM}}{V}}\right)\left(\frac{\frac{P_{25dM}}{V}}{K_{P24dM/P25dM} + \frac{P_{25dM}}{V}}\right)\left(\frac{\frac{P_{21T}}{V}}{K_{P24dM/P21T} + \frac{P_{21T}}{V}}\right)\cdot V - \varepsilon_9\left[\frac{P_{24dD}}{dt}\right] \quad 5$$

k12, is the maximum rate of synthesis for dCMP synthesis respectively; K_{P24dM}, $K_{P24dM/P25dM}$, $K_{P24dM/P21T}$ are saturation or equilibrium constants; P_{24dM}, P_{25dM}, P_{21T}, P_{24dD} are the amount per cell of dCMP, dCMP, ATP, and dCDP respectively, and V is for the cell volume. All parameters were estimated from experiments reported in the literature.

It may be noted that nucleoside diphosphate kinase (NDK) is missing from the list of essential proteins; most would anticipate its inclusion. However, *M. genitalium* does not encode a homolog of this enzyme; Mushegian and Koonin [1996] detected a novel gene *ndk* (MG268) to

perform this essential activity among genes of unknown function. Though, disruption of this gene in Hutchinson *et al*, [1999] experiments demonstrated that the gene was not essential. However Lu *et al* [1996] found that adenylate kinase possesses NDK activity and is unique among all the NDK's in this regard. The experimental evidence allows us to include adenylate kinase as the gene that catalyzes NDK reactions. A key achievement is the demonstration that we can delump a module, insert genomic and chemical details, and maintain a fully functional complete cell model. In essence, we have a hybrid "coarse-grain" whole cell model in which a genomically detailed model is embedded within the coarse grain minimal cell model. Thus, we have established the concepts of "modularity" and "connectivity" discussed earlier.

VI. Demonstration of Approach to Rapid Estimation of Kinetic Parameters

The construction of the coarse-train model with detailed nucleotide modules required a tedious search through the literature to estimate parameters and requires considerable biological insight by the modeler. To build such hybrid modules for real cells, it would be helpful to have a methodology to take a general coarse-grain structure and estimate parameters using growth data that can be obtained quickly.

Sethna's research group has developed a generalizable approach to extract falsifiable predictions from biological models using statistical mechanical type models [Brown & Sethna, 2003; Brown *et al.*, 2002]. The method involves using a specific cost function using all experimental data along with error values, and a corresponding model output evaluated with a parameter set *p*. For a minimal cell a "data" set is the required "design performance" of the minimal cell model and is obtained from the generalized behavior of chemoheterotrophic bacteria. This cost function is then related to the energy of a statistical mechanical system, and the function optimized to find a "*best*" parameter fit with the lowest cost. This parameter set becomes the starting point to generate an ensemble of parameters using the Monte Carlo method.

We have validated this approach with our base coarse grain model (*E. coli* model), for which we have significant experimental data. The optimization routine has provided a parameter set that allows an excellent match of model predictions to the experimentally observed behavior of *E.coli* in glucose-limited chemostats. Additionally we have found that most of the parameters previously estimated from mass balances, stoichiometry and the literature and from applying the Sethna approach to a series of chemostat data gave nearly the same value ($\pm$ 10 %) for almost all the parameters. Only 6 parameters (68 parameters were studied) differed more than 10% from original parameter estimate with a maximum difference of 25%. We have preliminary results of the parameter sensitivity study that follows the optimization. Future experiments will include studying the model responses to perturbations of these parameters.

VII. Application to Microbes

Consider how we might approach modeling a microbial pathogen. The first step requires construction of the coarse-grain model. For microbes that can be cultured we believe we can rapidly estimate all of the essential parameters. Relatively high throughput chemostat systems using mini reactors are available. Using a variety of steady state flow rates, nutrient levels and types, and flow perturbations as inputs and measuring cell composition, size, residual nutrient levels, and by-product levels it is possible to form a significant database. Microarray data from perturbation experiments would be useful but not essential. We can then apply the approach described in Section VI to estimate the basic parameters. Here we assume that the general chemoheterotrophic behavior applies to the microbe of interest.

The second step is to abstract a proposed mechanism, say of pathogensis, into a detailed model incorporating all suspected genes and known regulatory features. Of particular importance will be the connection of this mechanism to extracellular cues and to the physiologic state of the cell.

The third stage is to place the cell in the context of environments of interest. For validation purposes experiments with low density cultures can be used to compare to predictions of response to predetermined perturbations. A more sophisticated set of experiments would be to examine microbe to microbe interaction (e.g. quorum factors) and microbe-tissue interactions. Proposed mechanisms of interaction would have to be placed in the model to make experimental to model prediction comparisons.

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