

A Genomically/Chemically Complete Module for Synthesis of Lipid Membrane in a Minimal Cell

Mariajose Castellanos,¹ Keiichiro Kushiro,² Samuel K. Lai,² Michael L. Shuler²

¹University of Maryland Baltimore County, 1000 Hilltop Circle, MD 21250; telephone: 410-455-8151; fax: 410-455-1049; e-mail: mariajose@umbc.edu

²School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY 14853-5201

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ABSTRACT: A minimal cell is a hypothetical cell defined by the essential functions required for life. We have developed a module for the synthesis of membrane precursors for a mathematical minimal cell model. This module describes, with chemical and genomic detail the production of the constituents required to build a cell membrane and identifies the corresponding essential genes. Membranes allow selective nutrient passage, harmful substance exclusion, and energy generation. Bacterial membrane components range from lipids to fatty acids with embedded proteins and are structurally similar to eukaryotic cell membranes. Membranes are dynamic structures and experimental analyses show great variations in bacterial membrane composition. The flexibility of the model is such that different membrane compositions could be obtained in response to simulated changes in culture conditions. The model's predictions are in close agreement with the observed biological trends. The model's predictions correspond well with the experimental values of total lipid content in cells grown in chemostat culture, but less well with data from batch growth. Cell shape and size results agree especially well for data for growth rate relative to maximum growth rate larger than 0.5; and DNA, RNA, and protein predictions are consistent with experimental observations. A better understanding of the simplest bacterial membrane should lead to insights on the more complex behavior of membranes of higher species as well as identification of potential targets for antimicrobials.

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Introduction

Overview

A new framework is needed to link genomic data into an understanding of cell function and explicitly link genomic/molecular level insights to physiology. Our approach is to ask how would we design a cell to achieve the essential functions of life and from that design how would we write the minimum number of genomic instructions. To accomplish this goal we are constructing a chemically detailed minimal cell computer model (Browning and Shuler, 2001; Browning et al., 2004; Castellanos et al., 2004). By modeling the regulatory structure and functions to maintain a living cell, we expect to better understand the relationship of genomic instructions to cell function and regulation. We define a minimal cell as a chemoheterotrophic bacterium culturable *in silico* in a maximally supportive environment (rich environment with preformed nutrients and constant T and pH) with the minimum number of genes. The minimal cell possesses only the essential functions required for sustained growth and reproduction.

We have provided a general framework for a functionally complete whole cell computer model with dimensionless parameters (Browning and Shuler, 2001) and developed deterministic and statistical descriptions for control of initiation of DNA replication (Browning et al., 2004). Further, we have demonstrated that the coarse-grain description can be described in genomic/chemical detail by treating pseudochemicals aggregates as “modules.” Modularity was demonstrated by integrating genomic descriptions for pyrimidine and purine metabolism in our minimal cell model (Castellanos et al., 2004). In this work we develop a lipid and fatty acids module; this new

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component describes the regulated synthesis of precursors to form a chemically defined membrane for a minimal cell.

Any living organism needs a cell membrane as a barrier between the inside of the cell and the external environment, thus allowing a cell to function as a unit of life. If the membrane is broken, the integrity of the cell is destroyed and cell death occurs. The bacterial membrane has several functions: (a) acting as an osmotic barrier that is a selectively semi-permeable layer, (b) containing transport systems for the entry of nutrients and excretion of waste products, and (c) playing a major role in cell wall biosynthesis. The cell membrane is a specialized structure with lipids and proteins as its main components. Lipids form the basic structure of the membrane arranging themselves in a sheet like configuration. The major proteins in the membrane have large hydrophobic regions and become embedded in the lipid matrix.

The lipid bilayer is fluid-like, permitting movement of the imbedded lipids and proteins by lateral diffusion within the membrane. Temperature affects the cell's membrane fluidity. Lipid bilayers do not allow larger ions and polar molecules to be transported across the membrane unaided. Movement of these substances across the membrane involves imbedded membrane proteins, which form either "channels" or molecule specific transport systems (i.e., facilitated or active transport). Small ions, carbon dioxide and oxygen can easily pass through the channels in the membrane. Larger molecules, such as those making up simple sugars, may pass through the membrane by facilitated diffusion or an active transport mechanism. Non-polar molecules can diffuse across the membrane at metabolically useful rates, while charged species such as organic acids, amino acids, and inorganic salts require specific carrier proteins.

There are three major kinds of membrane lipids: phospholipids, glycolipids, and sterols. Both phospholipids and glycolipids readily associate spontaneously to form a lipid bilayer. Phospholipids are mixed glycerol esters of fatty acids and a phosphate ester of a non-fatty acid unit.

All cell membranes are characterized by a different composition and must be fluid at the temperature at which the cell is grown. Despite the variable compositions of biological membranes, the basic structural unit of all biomembranes is the phospholipid bi-layer which is essential for normal cell growth and reproduction. In natural membranes, thermal motion permits phospholipid and glycolipid molecules to rotate freely. Bacterial cells adapt to a decrease in growth temperature by increasing the proportion of unsaturated to saturated fatty acids in the membrane, thus maintaining appropriate fluidity of their membranes.

The diversity of lipids in both gram negative and positive bacteria is immense. The two bacteria most relevant to construction of a minimal cell model are *Escherichia coli* and *Mycoplasma genitalium*. The well understood physiology and regulation in *E. coli* provides useful guidance. *M. genitalium* is instructional as it is the microbe the fewest

number of genes (470). Most of the lipid research (distribution and transport) has focused on phospholipids (Huijbregts et al., 2000). Therefore, the first step to define lipids in a generalized minimal cell is focused on this type of lipid.

Phospholipids

Most bacterial membranes contain the anionic phospholipids: phosphatidylglycerol (PG), cardiolipin (CL), and zwitterionic phosphatidylethanolamine (PE) as the major membrane phospholipids. Many other phospholipids including phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC) can be found in smaller amounts or in specific bacteria.

Phospholipids participate in several different functions; foremost among their function is the ability to act as a permeability barrier for cells by forming phospholipid bilayers. The phospholipid bilayer acts as a matrix, supporting functions such as protein targeting, DNA replication, solute transport and signal transduction.

Phospholipids in *E. coli*

The lipid composition of *E. coli* is one of the simplest in biology. This organism contains: PE, PG, and CL. PE constitutes approximately 75% of the total phospholipids in most gram-negative bacteria. *E. coli* cells with inhibited phospholipid synthesis grow normally for one generation before they cease to divide (Rock and Jackowski, 1982). *E. coli* carefully regulates the composition of its membrane glycerolphospholipids. Tight mutations in phospholipid biosynthesis enzymes have resulted in cell death or arrest of cell growth. Significant changes in phospholipid composition cause marked changes in the physiology of the cell (Coleman, 1990). *E. coli* mutants with limited ability to synthesize phosphatidic acid become restricted in their capacity to grow and synthesize macromolecules. Reduction in lipid synthesis correlates well with reduction in macromolecule synthesis and growth rate, suggesting a correlation between these processes (Pizer et al., 1974). Defects in lipid synthesis in *E. coli* mutants, that is, glycerol phosphate production, glycerol phosphate acyltransferase reaction, phosphatidyl synthesis; result in dramatic inhibition of cell growth and macromolecular synthesis. The balance between synthesis and degradation (turnover rate) of lipid components maintains a relatively constant lipid composition (Pizer et al., 1974).

Analysis of nucleotide pools shows a reduction of ATP when *E. coli* cells were deprived of glycerol phosphate, indicating an effect on cell function due to reduced lipid synthesis. Additionally experimental evidence has shown that ppGpp is involved in the regulation of phospholipid synthesis when cells experience amino acid deprivation. Studies show inhibition of the synthesis of phosphatidic acid

and PG by ppGpp (Pizer et al., 1974), which indicates that cell growth and phospholipid synthesis are tightly coupled.

Phospholipids in Mycoplasma

Mycoplasmas lack a bacterial cell wall, periplasmic space and an outer membrane. Membrane protein patterns are little affected by small changes in growth conditions. Membranes consist of 50–60% protein and 30–40% lipids.

Most of the lipids in Mycoplasma are synthesized de novo and phospholipids are dominant. Neither biochemical studies nor sequence analysis have identified genes involved in fatty acids or cholesterol de novo synthesis in Mycoplasma species. Kinetic studies of exogenous fatty acids incorporation into membrane phospholipids reveal that the process is protein mediated, exhibiting saturation, energy dependence, and inhibition (Dahl, 1988; Dahl et al., 1981). Fatty acids transport appears to be tightly coupled with phospholipid biosynthesis since there is a very small accumulation of free fatty acids or acyl CoA inside the cell. Many details concerning the regulatory biochemical mechanisms for the uptake of exogenous fatty acids and membrane phospholipid biosynthesis in Mycoplasma are unknown. We infer a possible mechanism from a combination of what is observed in *E. coli* and what is seen in Mycoplasma species for a minimal cell.

PG and diphosphatidylglycerol (DPG) occur in most Mycoplasma species. The amount of lipids varies with species and the age of the culture (Smith and Koostra, 1967). The fatty acids distribution in the phospholipids mimics the distribution of the fatty acids in the culture medium. Mycoplasma, Ureaplasma, and Spiroplasma cannot elongate exogenous fatty acids. Fatty acids chains are needed for the synthesis of membrane phospholipids. Presumably the phospholipid biosynthesis pathway in Mycoplasma is thought to be very similar to *E. coli*; however, it has not been biochemically determined. There are several observations that support this idea, as the finding of lysophosphatidic and phosphatidic acids in Mycoplasma and the presence of phosphatidyl glycerol phosphate (PGP) in *M. gallinarum* (Romano et al., 1972). The composition of the cell membrane of *M. hominis* is relatively simple; composed primarily of PG. *M. hominis* contains mostly palmitic acid as the fatty acid (Rottem and Greenberg, 1975).

Lipids in a Minimal Cell

The percentage of the genes (or reactions) involved in fatty acids and phospholipid metabolism in the minimal gene sets described by Hutchinson's (Hutchison et al., 1999), Mushegian and Koonin (Koonin et al., 1996) and Costas D. Maranas (Burgard et al., 2001) are: 8.4%, 18.2%, and 37%, respectively. The previous studies were based on different organisms, distinct experimental techniques and in silico analyses, which explains the percentage difference. Most of the enzymes required for lipid synthesis and

degradation are identified by sequence comparison. However, no homologs of the *fadL* or *fadR* proteins have been identified, indicating that fatty acids may be transported across the envelope of the gram-positive bacteria by a different mechanism than the gram-negative bacteria. *M. genitalium* and *M. pneumoniae* have a very limited set of lipid genes in comparison to *E. coli* or even *Haemophilus influenzae*. The sequence analysis suggests that Mycoplasma have the capacity to synthesize phospholipids, but neither has fatty acids synthase genes with significant homologies. Either all fatty acids are derived from the medium or the genes are very divergent and cannot be identified by sequence analysis. Among the limited lipid genes identified, there are lipases in both organisms. These enzymes might serve to scavenge fatty acids and other lipid precursors from the environment.

By combining the experimental studies on genes of several bacterial species (outlined in the preceding paragraphs), previously published minimal gene sets (experimental and theoretical, for a brief description see (Castellanos et al., 2004)), and the essential macromolecules needed by the cell, we propose a lipid and fatty acids module. The integration of the new module is facilitated by the fact that the original coarse grain model (Domach et al., 2000) included cell envelope precursors, a cell envelope and the molecules involved in directing cross wall formation and cell envelope synthesis. The lipid and fatty acids module contains pathways for conversion of all precursors into the cell membrane, but also how these pathways are regulated. In some cases, kinetic parameters and regulatory effectors such as ppGpp have been determined experimentally by different studies and calculations as described in the kinetic parameters section.

Materials and Methods

The *E. coli* single cell model has fully been described (Ataai and Shuler, 1986; Browning and Shuler, 2001; Domach et al., 2000; Shuler and Domach, 1983; Shu and Shuler, 1991; Shuler et al., 1979). The coarse grain minimal cell model (Browning and Shuler, 2001) consists of sixty-one non-linear equations, which compute the interactions, synthesis, and degradation of the model components as well as algebraic expressions monitoring cycle parameters.

Kinetic Equations

We incorporated eight non-linear differential equations that describe lipid and fatty acids metabolism (Tables I–III). The equations were formulated by writing first pseudochemical reactions that represented the stoichiometric relationship for the lipids and fatty acids and their precursors and products. Then these eight equations express our best estimate of the kinetic relationships corresponding to these

Table I. Lipid metabolism enzymes.

| Enzyme | Gene name and identification |
|--|-------------------------------|
| 1 Glycerol 3-phosphate acyltransferase | <i>plsB</i> , MYP1140, MSC577 |
| 2 1-Acylglycerol-3-phosphate acyltransferase | <i>plsC</i> , MG212 |
| 3 CDP-diglyceride synthetase | <i>cdsA</i> , MG437 |
| 4 Phosphatidylglycerophosphate synthase | <i>pgsA</i> , MG114 |
| 5 Phosphatidylglycerophosphatase | <i>pgP</i> * |

The numbers in the first column correspond to the numbers used in Figure 1.

*There is no gene identified with this enzyme yet. The subunit structure is unknown.

metabolic pathways and include metabolic control through the concentration of the chemical components acting as signals. These kinetic relationships are typically semi-empirical in form and reflect the known factors modulating activity. Table I shows the enzymes that form part of the minimal cell model lipid module. Tables II–III describe the nomenclature for the symbols representing the macromolecules in lipid and fatty acids metabolism, and all the equations currently used in the lipid and fatty acid module, respectively.

Kinetic Parameters

The eight non-linear differential equations include 45 new parameters, as equilibrium rate constants, stoichiometric values, maximum rates, and saturation/inhibition constants. The exact numerical value of the kinetic parameters is not critical to a hypothetical minimal cell (see Browning and Shuler, 2001) although the ratios of kinetic parameters are important. Further, such parameters need to be physiologically plausible (allow the cell to divide for essentially an infinite number of generations). We used experimental evidence and enzyme studies from well-characterized organisms to estimate kinetic parameters, while we relied heavily on the well-studied gram-negative cell, *E. coli*; we used *B. subtilis*, *Bacillus cereus*, and *H. influenzae* data as available. The enzyme characterizations for these organisms

Table II. Names of the species that form part of the lipid minimal cell module (shown in Fig. 1), the abbreviations used in the code comments, and the nomenclature used in the model equations.

| Species | |
|-------------|-----------------|
| G3P | P ₂₆ |
| AsnG | P ₂₇ |
| PA | P ₂₈ |
| CDPd | P ₂₉ |
| PGP | P ₃₀ |
| PG | P ₃₁ |
| Fatty acids | P ₃₂ |
| acp | P ₃₃ |

Table III shows the model equations for the lipid module and Table IV shows the values for the kinetic parameters and sources or justification.

yielded at least three or more kinetic parameters for orthologous genes in the different species. The parameter values vary significantly from one species to another and we used values from gram-positive organism where possible.

Evaluation of Parameters

Values for the kinetic parameters and sources or justification are given in Table IV. Most of the maximum rate constants can be determined from basic measurements on appropriate enzymes or by using mass balances to determine the maximum net synthesis rate. Some saturation constants were determined from references to the literature, while others are estimated based on a prior heuristic rule (Browning and Shuler, 2001) as 1/25 of the normal intracellular concentration (“normal” is defined by an average value over a range of bacteria in exponential growth). The stoichiometries of the identified chemical reactions are used for the stoichiometric coefficients. Parameters were not adjusted to fit experimental data. In some cases, parameters in the literature were reported with several significant figures, in practice the parameter values were rounded to two significant figures.

Model Construction

Transport

Glycerol 3 Phosphate Transport

The initial substrate in most phospholipid de novo synthesis pathways is glycerol. Glycerol is a three-carbon substance that forms the backbone of fatty acids. Some *Mycoplasma* species use glycerol, but not all of them, suggesting that glycerol-3 phosphate (G3P) and not free glycerol may be the substrate for acylation. Isolated membranes from *M. hominis* utilize G3P (Rottem and Greenberg, 1975). The protein coded by the *glpT* gene has been biochemically characterized in *H. influenzae* for G3P transport (Song et al., 1998). Wild-type *B. subtilis* transports G3P efficiently (Lindgren, 1978). A gene found in *Myxococcus xanthus* revealed 40% functional similarity to the permease for G3P of *B. subtilis* and *V. cholerae* (Moraleda-Munoz et al., 2001). Sequence comparison analysis identified the gene MG188 in *M. genitalium* as an ABC transporter permease protein and probable G3P transport system.

Experiments provide evidence to believe that exogenously provided G3P is the primary source for de novo phospholipid synthesis in *E. coli* (Merlie and Pizer, 1973). G3P potentially could serve as a precursor for phospholipid biosynthesis for a minimal cell. Based on the above observations, we include a single gene product for G3P transport in the minimal cell using Equation (1) (Table III). G3P is maintained at low intracellular level by feedback

Table III. Lipid minimal cell equations.

$$\begin{aligned}
R_{P26} &= v_{P26} \left(\frac{C_{P26^*}}{K_{P26^*} + C_{P26^*}} \right) \left(\frac{P_{21T}}{V} \right) \left(\frac{K_{P26}}{K_{P26} + \frac{P_{26}}{V}} \right) \\
\frac{dP_{27}}{dt} &= k_{27} \left(\frac{K_{P27}}{K_{P27} + \frac{P_{27}}{V}} \right) \left(\frac{P_{26}}{V} \right) \left(\frac{P_{21T}}{V} \right) \left(\frac{K_{P32i}}{K_{P32i} + \frac{P_{32}}{V}} \right) \left(\frac{K_{P17}}{K_{P17} + \frac{P_{17}}{V}} \right) V - \varepsilon_{27} \left[\frac{P_{28}}{dt} \right] \\
\frac{dP_{28}}{dt} &= k_{28} \left(\frac{K_{P28}}{K_{P28} + \frac{P_{28}}{V}} \right) \left(\frac{P_{27}}{V} \right) \left(\frac{P_{21T}}{V} \right) \left(\frac{P_{32}}{V} \right) \left(\frac{K_{P17i}}{K_{P17i} + \frac{P_{17}}{V}} \right) V - \varepsilon_{28} \left[\frac{P_{29}}{dt} \right] \\
\frac{dP_{29}}{dt} &= k_{29} \left(\frac{K_{P29}}{K_{P29} + \frac{P_{29}}{V}} \right) \left(\frac{P_{28}}{V} \right) \left(\frac{P_{22T}}{V} \right) \left(\frac{P_{21T}}{V} \right) \left(\frac{K_{P22Ti}}{K_{P22Ti} + \frac{P_{22T}}{V}} \right) \left(\frac{K_{P22dT}}{K_{P22dT} + \frac{P_{22dT}}{V}} \right) \left(\frac{P_{22T}}{K_{P29/P22T} + \frac{P_{22T}}{V}} \right) V - \varepsilon_{29} \left[\frac{P_{30}}{dt} \right] \\
\frac{dP_{30}}{dt} &= k_{30} \left(\frac{K_{P30}}{K_{P30} + \frac{P_{30}}{V}} \right) \left(\frac{P_{29}}{V} \right) \left(\frac{P_{27}}{V} \right) \left(\frac{P_{21T}}{V} \right) \left(\frac{K_{P30/P17}}{K_{P30/P17} + \frac{P_{17}}{V}} \right) V - \gamma_{P30} \left(\frac{M_{31}}{dt} \right) \\
\frac{dM_{31}}{dt} &= k_{31} \left(\frac{K_{M31}}{K_{M31} + \frac{P_{31}}{V}} \right) \left(\frac{P_{30}}{V} \right) \left(\frac{P_{21T}}{V} \right) \left(\frac{K_{P17}}{K_{P17} + \frac{P_{17}}{V}} \right) V \\
R_{P32} &= v_{P32} \left(\frac{C_{P32^*}}{K_{P32^*} + C_{P32^*}} \right) \left(\frac{P_{21T}}{V} \right) \left(\frac{P_{28}}{V} \right) \left(\frac{P_{27}}{V} \right) \left(\frac{P_{27}}{K_{P32/P27} + \frac{P_{27}}{V}} \right) v_{P33} \\
\frac{dP_{33}}{dt} &= k_{P33} \left(\frac{C_{P33^*}}{K_{P33} + C_{P33^*}} \right)
\end{aligned}$$

inhibition (McIntyre et al., 1977; Shopsis et al., 1974) (Table III fourth term, Eq. 1).

Fatty Acids Composition and Transport

After entering the cell, fatty acids are catabolized or directly incorporated into complex lipids (Nunn, 1986; Schaffer, 2002). Analysis of biological membranes derived from different organisms has shown that the fatty acid membrane composition is highly specific under standardized conditions. Even changes in culture conditions (temperature, age, and environment) bring few changes in fatty acid composition (Van Deenen, 1965). It is believed that the membrane structure and function are not critically dependent on a limited and highly specific composition of the lipid hydrocarbon tails, as any kind of severe modifications in the fatty acid composition of the medium for *M. laidawaii* B does not appear to affect the viability of the culture (McElhane and Tourtell, 1969).

The transport systems for long and medium fatty acids in *E. coli* are saturable, while each transported substance differs in affinity (Maloy et al., 1981). *E. coli* K12 transport systems for long and medium chain fatty acids were shown to be dependent on the *fadD* gene product, acyl-CoA synthetase. However, further studies (Rock and Jackowski, 1985) concluded that the *fadD* gene product is not required for exogenous fatty acids transport and the incorporation of fatty acids may follow an alternate route. Non-orthologous genes corresponding to *fadD* have not been found in Mycoplasma.

Fatty acids are essential components for the formation of the lipid bilayer and cell viability; *E. coli* mutants defective in fatty acids synthesis continue to grow and synthesize phospholipids until metabolite leaking occurs and the cell lyses. A simpler route for a bacterial cell is to scavenge the metabolites from the medium instead of synthesizing them. This is shown by experiments where an exogenous supply of fatty cells is added to the medium with growing *E. coli* cells; which in turn reduces the content of acyl moieties derived from de novo synthesis in cell membrane phospholipids (Rock and Jackowski, 1982). Also, *E. coli* fatty acids auxotrophs can grow on a wide variety of unsaturated and saturated fatty acids, although, the precise growth conditions may vary (Cronan, 2003). The update of exogenous sources of fatty acids would be an attractive strategy for a minimal cell.

Fatty acids uptake in Mycoplasmas consists of entry from the environment, activation and esterification. Specific enzymes have not yet been identified (Rock and Jackowski, 1982). Uptake of fatty acids by *M. capricolum* has been characterized (Dahl, 1988), showing protein-mediated transport. *Streptomyces coelicolor*, the best genetically characterized gram-positive organism, utilizes exogenous fatty acids of different chain lengths. Palmitate transport conformed to Michaelis-Menten kinetics with a concentration above 100 μ M in the medium (Banchio and Gramajo, 1997). Studies with related organisms demonstrate saturable uptake (Black and DiRusso, 2003; Calmes and Deal, 1976).

Based on the above observations, the lipid minimal cell module includes saturable, carrier protein mediated fatty acids uptake (Table III, Eq. 7).

Table IV. Lipid module kinetic parameters.

| Parameter | Variable name in code | Value | Units | Reference |
|-----------------|----------------------------|----------|--------|--|
| ν_{P26} | Glycerol_3P.v | 13.1E-6 | g/h•ml | a, c, (Huijbregts et al., 2000; Raetz, 1978) |
| K_{P26^*} | Glycerol_3P.Ksat [0] | 1E-4 | g/ml | (Huijbregts et al., 2000; Raetz, 1978) |
| K_{P26} | Glycerol_3P.Ksat[1] | 1E-5 | g/ml | (Huijbregts et al., 2000; Raetz, 1978) |
| $K_{P26/P21T}$ | Glycerol_3P.Ksat[4] | 1.E-3 | g/ml | (Huijbregts et al., 2000; Raetz, 1978) |
| k_{27} | AsnG.k[0] | 4.48E-2 | g/h•ml | a, c, (Green et al., 1981; Ray and Cronan, 1987; Rock et al., 1981) (Raetz, 1978; Cronan and Bell, 1974) |
| $K_{P27/P26}$ | AsnG.Ksat[0] | 1.0E-4 | g/ml | (Pizer et al., 1974) (Green et al., 1981; Ray and Cronan, 1987; Rock et al., 1981) (Raetz, 1978; Cronan and Bell, 1974) |
| K_{P27} | AsnG.Ksat[1] | 1.0E-5 | g/ml | (Pizer et al., 1974) (Green et al., 1981; Ray and Cronan, 1987; Rock et al., 1981) (Raetz, 1978; Cronan and Bell, 1974) |
| $K_{P27/P21T}$ | AsnG.Ksat[2] | 1.0E-4 | g/ml | b, (Pizer et al., 1974) (Green et al., 1981; Ray and Cronan, 1987; Rock et al., 1981) (Raetz, 1978; Cronan and Bell, 1974) |
| ϵ_{27} | Coef.e[3] | 0.017E-1 | | a, c, (Raetz, 1978) |
| K_{P32i} | AsnG.Ksat[3] | 1.0E-4 | g/ml | (Pizer et al., 1974; Raetz, 1978) |
| K_{P17} | AsnG.Ksat[4] | 0.98E-5 | g/ml | (Merlie and Pizer, 1973); (Raetz, 1978) |
| k_{28} | Phosphatidicacid.k [0] | 8.32E-8 | g/h•ml | a, c, (Raetz, 1978); (Silber et al., 1980) |
| K_{P28} | Phosphatidicacid.Ksat[0] | 1.2E-8 | g/ml | (Raetz, 1978); (Silber et al., 1980) |
| $K_{P28/P27}$ | Phosphatidicacid.Ksat[1] | 1.5E-5 | g/ml | (Raetz, 1978); (Silber et al., 1980) |
| $K_{P28/P21T}$ | Phosphatidicacid.Ksat[2] | 1.1E-6 | g/ml | b, (Raetz, 1978); (Silber et al., 1980) |
| $K_{P28/P17i}$ | Phosphatidicacid.Ksat[3] | 1.0E-5 | g/ml | b, (Merlie and Pizer, 1973), (Raetz, 1978), (Silber et al., 1980) |
| $K_{P28/P32}$ | Phosphatidicacid.Ksat[4] | 0.56E-6 | g/ml | b, (Raetz, 1978), (Silber et al., 1980) |
| ϵ_{28} | Coef.e[4] | 0.27E-0 | | a, c, (Raetz, 1978), (Silber et al., 1980) |
| k_{29} | CDP_diacylglycerol.k[0] | 2.39E-8 | g/h•ml | a, c, (Raetz, 1978), (Silber et al., 1980) |
| $K_{P29/P28}$ | CDP_diacylglycerol.Ksat[0] | 1.2E-6 | g/ml | (Coleman, 1990), (Raetz, 1978), (Silber et al., 1980) |
| K_{P29} | CDP_diacylglycerol.Ksat[1] | 1.0E-8 | g/ml | (Coleman, 1990), (Raetz, 1978), (Silber et al., 1980) |
| $K_{P29/P21T}$ | CDP_diacylglycerol.Ksat[2] | 1.7E-4 | g/ml | b, (Coleman, 1990), (Raetz, 1978), (Silber et al., 1980) |
| $K_{P29/P17}$ | CDP_diacylglycerol.Ksat[3] | 1.2E-5 | g/ml | (Merlie and Pizer, 1973), (Raetz, 1978), (Silber et al., 1980) |
| K_{22Ti} | CDP_diacylglycerol.Ksat[4] | 6.3E-3 | g/ml | (Coleman, 1990), (Raetz, 1978), (Silber et al., 1980) |
| K_{P22dT} | CDP_diacylglycerol.Ksat[5] | 4.5E-4 | g/ml | (Coleman, 1990), (Raetz, 1978), (Silber et al., 1980) |
| $K_{P29/P22T}$ | CDP_diacylglycerol.Ksat[6] | 1.5E-3 | g/ml | b, (Coleman, 1990), (Raetz, 1978), (Silber et al., 1980) |
| ϵ_{29} | Coef.e[5] | 0.12E-1 | | a, c, (Raetz, 1978) |
| k_{30} | PGP.k[0] | 8.36E-7 | g/h•ml | a, c, (Raetz, 1978), (Silber et al., 1980) |
| $K_{P30/P29}$ | PGP.Ksat[0] | 1.2E-8 | g/ml | (Raetz, 1978), (Silber et al., 1980) |
| K_{P30} | PGP.Ksat[1] | 1.8E-8 | g/ml | (Raetz, 1978), (Silber et al., 1980) |
| $K_{P30/P21T}$ | PGP.Ksat[2] | 1.7E-9 | g/ml | b, (Raetz, 1978), (Silber et al., 1980) |
| $K_{P30/P17}$ | PGP.Ksat[3] | 2.4E-7 | g/ml | b, (Raetz, 1978), (Silber et al., 1980) |
| γ_{P30} | Coef.g[6] | 0.02E-0 | | a, c, (Raetz, 1978) |
| k_{M31} | PG.k[0] | 8.9E-10 | g/h•ml | a, c, (Raetz, 1978) |
| $K_{M31/P30}$ | PG.Ksat[0] | 9.9E-4 | g/ml | (Raetz, 1978), (Silber et al., 1980) |
| K_{M31} | PG.Ksat[1] | 1.2E-4 | g/ml | (Raetz, 1978), (Silber et al., 1980) |
| $K_{M31/P21T}$ | PG.Ksat[2] | 5.1E-3 | g/ml | b, (Raetz, 1978), (Silber et al., 1980) |
| $K_{M31/P17}$ | PG.Ksat[3] | 1.6E-5 | g/ml | b, [24,(Raetz, 1978), (Silber et al., 1980) |
| ν_{32} | Fatty.v | 0.62E-2 | g/h•ml | a, c, (Banchio and Gramajo, 1997), (Raetz, 1978), (Dahl, 1988) |
| K_{P32^*} | Fatty.Ksat[0] | 0.55E-3 | g/ml | (Banchio and Gramajo, 1997), (Raetz, 1978), (Calmes and Deal, 1976), (Dahl 1988) |
| $K_{P32/P21T}$ | Fatty.Ksat[1] | 0.27E-8 | g/ml | b, (Banchio and Gramajo, 1997), (Raetz, 1978), (Calmes and Deal, 1976; Dahl, 1988) |
| $K_{P32/P27}$ | Fatty.Ksat[2] | 1.0E-5 | g/ml | b, (Banchio and Gramajo, 1997), (Raetz, 1978), (Calmes and Deal, 1976; Dahl, 1988) |
| $K_{P32/P28}$ | Fatty.Ksat[3] | 0.62E-2 | g/ml | (Banchio and Gramajo, 1997), (Raetz, 1978), (Calmes and Deal, 1976), (Dahl, 1988) |
| k_{P33} | Acp.k[0] | 7.38E-06 | g/h•ml | a, c |
| K_{P33} | Acp.Ksat[0] | 0.83E-2 | g/ml | (Nakanishi et al., 2001; Rock et al., 1981; Rock and Jackowski, 1982) |

The table lists the variable names in equations, variable names in code, parameter values, parameters units, and the sources, with the following definitions: a, Mass balance; b, Heuristic rule; c, Stoichiometric coefficients; and reference number.

Sterols

Sterols incorporated into the membrane of all Mycoplasma species remain chemically unmodified and do not exhibit significant metabolic turnover. Sterols have a dual function in the cell membrane: act as a bulk component and facilitate unsaturated fatty acids uptake (Dahl et al., 1982). Studies of *M. capricolum* showed growth rate dependence on cholesterol presence in the medium. Cholesterol specifically stimulated the uptake of unsaturated fatty acids by lowering the K_m value for the uptake (Dahl and Dahl, 1983; Dahl et al., 1981). *M. pneumoniae* and *M. pulmonis* are capable of modifying exogenous PC but most other Mycoplasma species are not.

Some Mycoplasma species are unique among prokaryotes with a requirement for sterols for growth. However, experimental evidence (Rottem, 1975) of fatty acids incorporation into Mycoplasma cells without the presence of sterols shows the non-essentiality of this component. Based on this data, sterols are not included as components of the minimal cell.

Genes in Synthesis and Metabolism

Overview

Lipid synthesis in *E. coli* and *S. typhimurium* is divided in two parts: (1) synthesis of fatty acids (lipid hydrophobic part) and (2) attachment of fatty acids to G3P, followed by additions and modifications of the polar head group. The vast majority of Mycoplasma species only include genes that code for the attachment of fatty acids to G3P.

From the genome sequence of *M. genitalium*, 10 genes have been identified in the lipid related category. Hutchinson's (Hutchinson et al., 1999) minimal Mycoplasma genome gene set includes eight genes in this category (four of those are included already in the minimal cell model). Kobayashi et al. (2003) include 16 genes in the membrane lipid category. Mushegian and Koonin (Koonin et al., 1996) included six genes for lipid metabolism, these genes code for a biochemical pathway that includes G3P, however; the pathway is missing the reaction that produces 1-acyl glycerol 3 phosphate from G3P. Additionally, the genes proposed by Mushegian and Koonin code for enzymes that would produce PGP as the pathway's final product. Current lipid composition studies in Mycoplasma include PGP as an intermediate, not as a final component of the group of polar lipids that form the membrane; also the virtual absence of this later compound in most Mycoplasma species suggests the presence of an active phosphatase. This experimental evidence implies that this set of proposed genes is likely to be underestimated in number. By combining the genes in the different minimal gene sets proposed in the literature (experimental, theoretical or computational) with the basic cell needs, a lipid and fatty acids transport and metabolism pathway was designed for a minimal cell. Each of the pathways also includes regulatory effectors.

Figure 1 shows the lipid pathway containing the major lipids in several bacterial species. The dashed lines represent the proposed lipid pathway for the minimal cell. The following section describes important enzyme features to develop the kinetic models. The gene name is followed by enzyme characteristics and in some cases a reference to equation terms (right-hand side) that model the behavior of the enzyme. Table III contains all equations.

plsB

The G3P acyltransferase catalyzes the first step in membrane phospholipid formation and acylates the 1-position of G3P with either acyl-carrier protein (ACP) or acyl-coenzyme A (CoA) thioesters forming 1-acylglycerol P (Rock et al., 1981) (step 1 in Fig. 1). The *plsB* gene codes for this enzyme. Acylation of G3P activity has been observed in *M. hominis* and *A. laidwii* (Rottem and Greenberg, 1975). Phosphate (Green and Bell, 1984), acyl coenzyme A, and acyl carrier protein (Table III, fifth term, Eq. 2) (Green et al., 1981) act as inhibitors for G3P acyltransferase.

There is one sole pathway for de novo phospholipid biosynthesis in several bacterial species. This pathway involves the acylation of G3P with two fatty acids donated from the acyl carrier protein pool (Ray and Cronan, 1987). Experimental evidence from a study with two mutations that completely inhibited phospholipid biosynthesis, showed no alternate pathways in *E. coli* (Ray and Cronan, 1987). G3P acyltransferase is inhibited by ppGpp, (Table III, sixth term, Eq. 2) (Heath et al., 1994).

plsC

The gene *plsC* encodes the protein 1-acylglycerol-3-phosphate acyltransferase which catalyzes the transfer of a second fatty acid to Position 2 of the first intermediate forming phosphatidic acid (Rock et al., 1981; Shih et al., 1999) (Step 2 in Fig. 1). Sequence comparisons found a weak homolog in mycobacteria (Cronan, 2003).

The *E. coli* enzyme can utilize acyl carrier protein or acyl-CoA as the fatty acyl donor (Table III, fifth term, Eq. 3). *E. coli* possesses one essential LPA acyltransferase (PlsC).

ppGpp regulation (by inhibition) of the synthesis of phosphatidic acid at the enzymatic level was observed in vitro for this protein (Table III, sixth term, Eq. 3) (Merlie and Pizer, 1973).

cdsA

The gene *cdsA* codes for the protein CDP-diglyceride synthetase. This protein catalyzes the synthesis of CDP-diacylglycerol (a product positioned at a branch point in the lipid biosynthetic pathway for most bacteria, one branch produces PE and the other PG and CL) (Step 3 in Fig. 1). Most sequenced bacterial genomes encode a protein with strong homology to CDP-diglyceride synthetase (Cronan, 2003). CDP-diacylglycerol is the source of the phosphatidyl

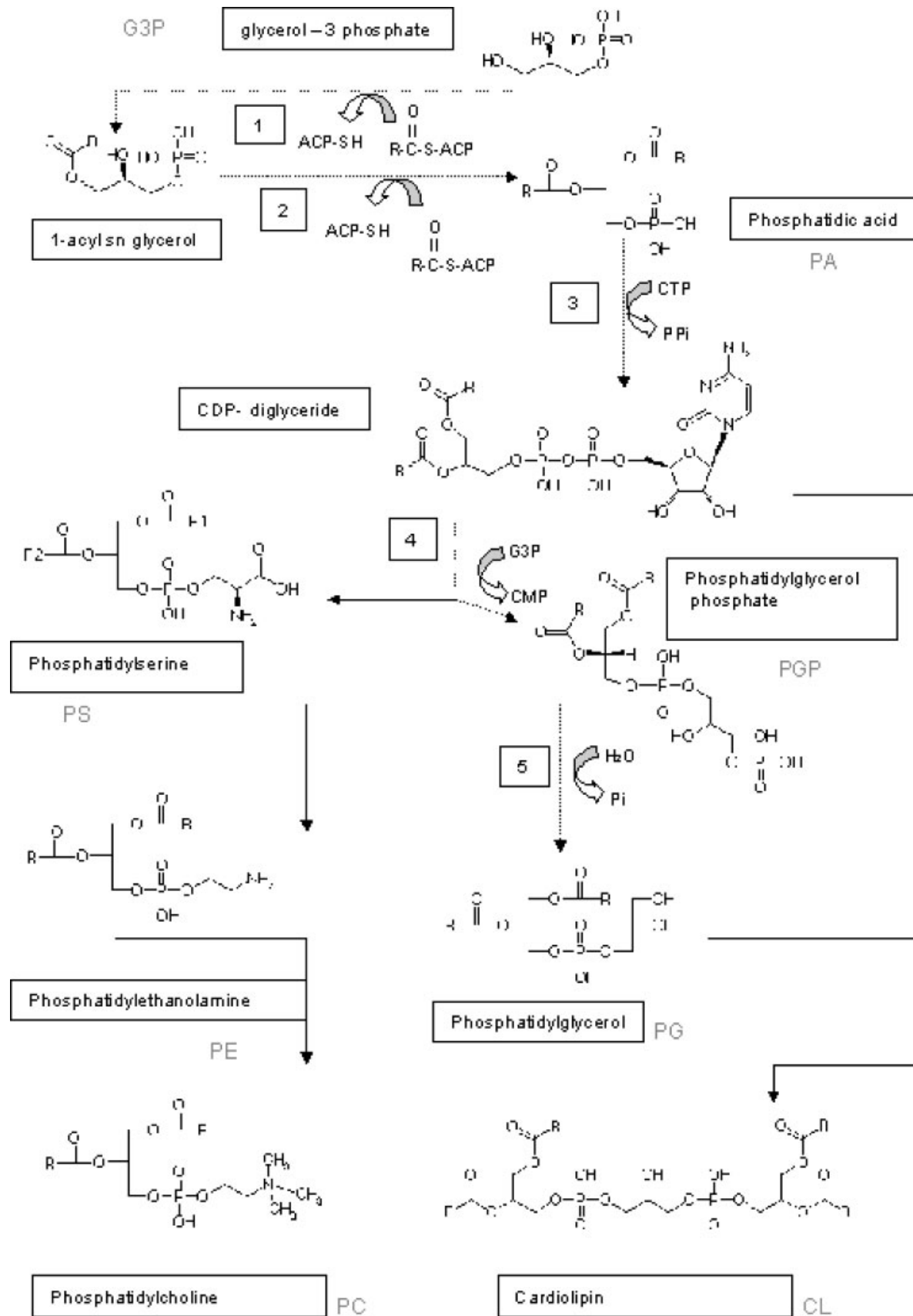


Figure 1. Biochemical pathways for the synthesis of the major membrane phospholipid for several bacterial species. Table I describes the enzymes catalyzing the various reactions. The dashed lines represent the steps included in the minimal cell model. The Metabolism section describes experimental evidence for the reactions (in different bacterial species) included in the model.

group for all the major phospholipids in *E. coli*. Inhibitors of this enzyme are CTP and dCTP (Table III, fifth and sixth terms, Eq. 4) (Langley and Kennedy, 1978). This gene was found essential in *B. subtilis* grown in LB media (Kobayashi et al., 2003). For simplicity, the equation approximating the kinetics of the formation of the gene product of CDP-diacylglycerol includes multiplicative terms.

pgsA

The gene *pgsA* encodes phosphatidylglycerophosphate synthase. This gene is widely conserved and has been previously shown to be essential in *E. coli* (Heacock and Dowhan, 1987).

Phosphatidylglycerophosphate synthase catalyzes the biosynthesis of PGP (Step 4 in Fig. 1). The major acidic phospholipids (synthesized from PGP) of *E. coli*, PG and CL are considered to play important roles in various cellular processes that are essential for viability. Mutation of *pgsA* or reduction in expression of the protein that controls the levels of PG plus CL is lethal (Suzuki et al., 2002). Regulation by inhibition of the synthesis of phospholipids at the enzymatic level by ppGpp was observed in vitro for this protein (Table III, fifth term, Eq. 5) (Merlie and Pizer, 1973).

pgp

The *pgp* gene codes for phosphatidylglycerophosphatase. This enzyme removes phosphate from PGP (Step 5 in Fig. 1). While, *E. coli* codes for at least three phosphatidylglycerophosphatases (PGP-tase): *pgpA*, *pgpB*, and *pgpC* they are not all essential. Cell with disruption of *pgpA* and *pgpB* genes (Funk et al., 1992; Icho and Raetz, 1983) showed normal growth properties, an increase in PGP levels (from 0.1% to 2%) and a 50% reduction of the phosphatidylglycerophosphate phosphatase activity, indicating that a different gene must also encode such an activity. The synthesis of PGP may be presumably carried out by *pgpC* activity, leaving the biosynthesis of PGP as the only step in *E. coli* phospholipid biosynthesis for which a gene locus has not been identified. Experimental evidence shows that PG is the most abundant polar lipid in *Achoeplasma*, *Mycoplasma*, *Spiroplasma*, and *Ureaplasma* species (Smith, 1992), which makes the dephosphorylation of PGP an essential activity. An analog of *pgpC* is postulated as necessary to code for the biosynthetic enzyme producing PG in our minimal cell pathway.

acp

The ACP synthase enzyme (encoded by *acp*) forms ACP, acyl carrier protein, which is the active form of the carrier in lipid synthesis (Elovson and Vagelos, 1968).

Most *Mycoplasma* sequenced genomes contain a gene that codes for the acyl carrier protein. Acyl-ACP rather than Acyl-CoA appears to be the most plausible in vivo acyl

donor for *E. coli* cells (Rock and Jackowski, 1982; Rock et al., 1981).

The physiological role of the gene *acpD* (one of the genes that form part of the minimal gene set by (Mushegian and Koonin, 1996)) is not clear. *acpD* was believed to code for the acyl carrier protein phosphodiesterase that converts holo-ACP to apo-ACP by hydrolytic cleavage of the phosphopantetheine prosthetic group from ACP. Nakanishi et al. (2001) identified that the *acpD* gene product could not perform the expected function under in vitro and in vivo conditions, but identified the *acpD* gene as an *azoR*, an azoreductase. Thus the proposed minimal cell model includes explicitly a gene for ACP synthase, but not *acpD* as suggested elsewhere (Table III, Eq. 8).

cls

There are two pathways for PC synthesis, a methylation pathway and a PC synthetase pathway. Many bacteria are able to use both pathways (Lillich and White, 1971). The condensation of two molecules of PG to produce CL and glycerol is catalyzed in *E. coli* by the *cls* gene (widely conserved) product, CL synthase (Cronan, 2003). *E. coli* cells lacking CL (*cls* mutants) were found to have a minor growth disadvantage compared to the wild type. Nevertheless, they did survive poorly under stationary phase growth (Cronan, 2003). In most *Mycoplasma* species, CL does not form part of the phospholipid components and the *cls* gene has been determined non-essential by Gerdes et al. (2003). The minimal cell does not include CL.

Model Simulations

The simulation results can be evaluated by comparison to typical data from chemoheterotrophic bacteria. Available data over a range of dimensionless growth rates can be used to determine whether the overall predicted model response is physiologic in nature. In Figure 2 model predictions of total lipid content in the cell grown in chemostat culture are compared to actual data over a range of growth rates. The model's predictions are in close agreement with the observed trends, for cells in continuous culture, while there is greater deviation for batch growth cultures from the "u" shaped response predicted. The discrepancy in response between batch and continuous culture suggest that altering growth rate by changing medium composition changes multiple aspects of cell physiology. Additionally, studies of *Mycobacterium* cultures have shown that lipid content can potentially increase and decrease during cell growth (Chandramouli and Venkitasubramanian, 1982; Ratledge, 1982). It has also been shown that the lipid content of *Mycobacterium bovis* decreased with growth rate (Beste et al., 2005); when the organism was grown in a carbon limited chemostat at two different growth rates (low and high).

A positive correlation between growth rate and cell size for bacteria grown on different media has been previously

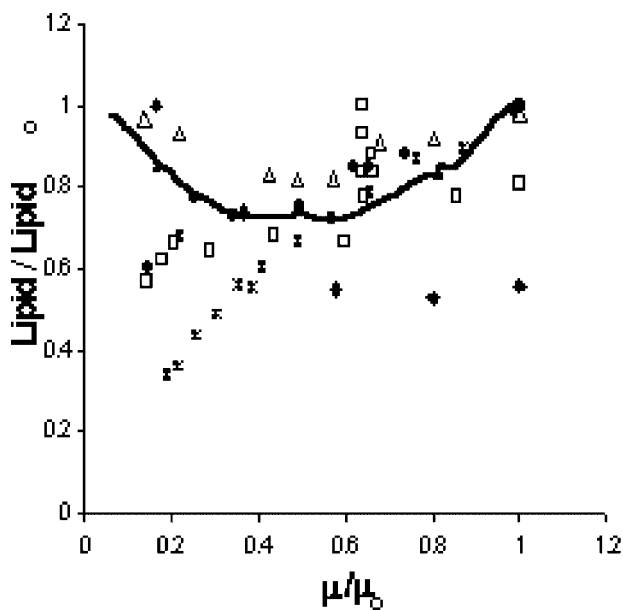


Figure 2. Lipid content relative to maximum lipid content as a function of growth rate relative to maximum growth rate. Asterisks (*) represent *E. coli* K12 in a glycerol phosphate supplemented/glycerol phosphate deprived batch cultures (McIntyre et al., 1977). Open squares (□) represent a *B. subtilis* glycerol auxotroph (Lillich and White, 1971). Experimental data was obtained from batch experiments where the medium was altered (to vary the growth rate), making the comparison of the model predictions and the experiments difficult. Additionally, open triangles (Δ) represent the macromolecular lipid composition of *Cellulomonas* sp. LC-10 grown in glucose limited continuous culture at 30°C (Summers and Srinivasan, 1979). Full circles (●) represent the changes in the lipid content of *Acinetobacter calcoaceticus* grown in a chemostat ethanol limited cultures at various growth rates (Abbott et al., 1974). Full diamonds (◆) represent the phospholipid composition of *Pseudomonas aeruginosa* under glucose limitation in continuous culture at 37°C (Gilbert and Brown, 1978). The solid line represents the minimal cell model results.

demonstrated (Akerlund et al., 1995; Bremer and Dennis, 1987). Additionally, Mongold and Lenski (1996) measured the average cell size of ancestral and derived genotypes of *E. coli* cells over a range of growth rates in chemostat cultures. Both cell genotypes showed higher mean cell size at higher growth rates, although the derived genotype led to larger cells than its ancestor. In Figure 3 model predictions of cell volume are compared to actual data. The amount of lipids is used as a part of the computer code that calculates the shape and size of the cell which allows calculation of cell shape (volume). The model predictions are in close agreement with the trends in terms of the shape and size from the observed data (esp. for $\mu/\mu_0 < 0.5$) which suggest that the model correctly generates the lipid content of the cell.

Figure 4 compares the model predictions for the relative amount of DNA, RNA and protein with experimental data of *E. coli* cultures that were grown on a medium with glucose as the main primary carbon source, casamino acids and supplemented/deprived with/of glycerol phosphate (McIntyre et al., 1977). These comparisons allow additional testing of the model. Unfortunately, volume data were not available from these experiments and similar experiments with *M. genitalium* would have been useful.

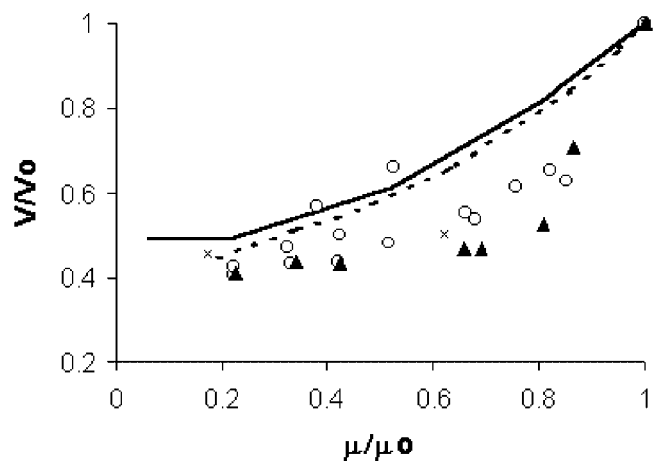


Figure 3. Cell volume relative to maximum cell volume as a function of growth rate relative to maximum growth rate for *E. coli* in continuous culture. Open circles (○) represent measurements from the Coulter Counter (Domach et al., 2000); crosses (×) represent measurement from (Helmstetter, 1974); closed triangles (▲) represent volumes calculated from electron microscopy measurements of cell dimension with glutaraldehyde-fixed cells; the solid line represents the minimal cell model results, and the dashed line represents the coarse grain model results.

The model clearly predicts a trend similar to the experimental data. The predictions for DNA content at lower growth rates are higher than the experimental measurements at growth rates of less than 1/2 the maximum growth rate. However, in the absence of error information on experimental data, one cannot tell whether predicted values fall within the experimental range of error. Further, since we are comparing results with *E. coli* to a hypothetical model based on *M. genitalium* the results should be viewed in regard to whether appropriate trends are predicted.

The phospholipids and fatty acids composition remains practically constant, in the cell membrane of *M. hominis* (Rottem, 1975). In Figure 5 the intracellular lipid and fatty acids content as predicted by the minimal cell model over 250 generations is shown. The range of values seen in both graphs are maintained for as many generations as the program is run and presumably would be maintained for infinity. However, the ability of the model to self correct is evident in the first five cell generations. Additionally, the same equilibrium conditions are obtained with different values for the initial external fatty acids and lipid external composition in the in silico medium.

Conclusion

A new module describing the lipid and fatty acids transport and metabolism has been presented. As shown, the model's formulation is an attempt to insert genomic details into a whole cell model (coarse grain model) while maintaining the "connectivity" of all model elements. The cell's in silico medium has two new elements, G3P and fatty acids, and the

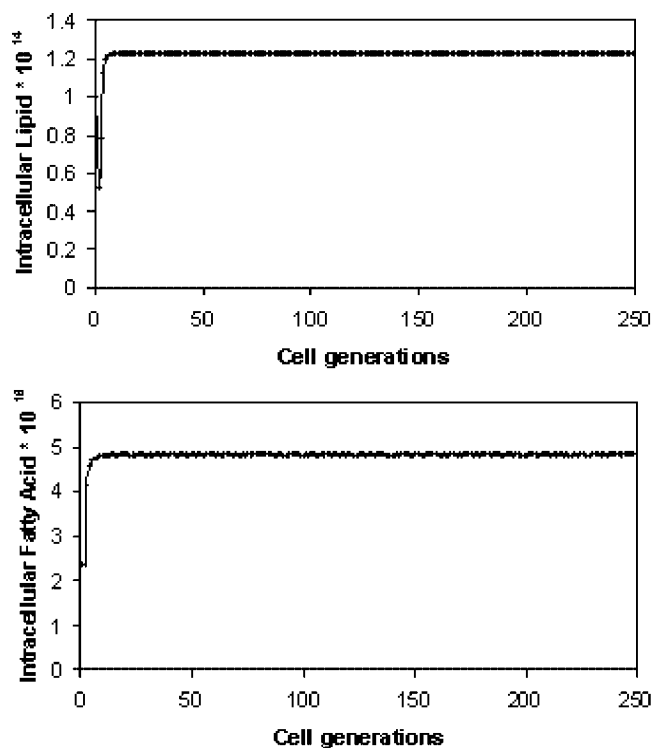
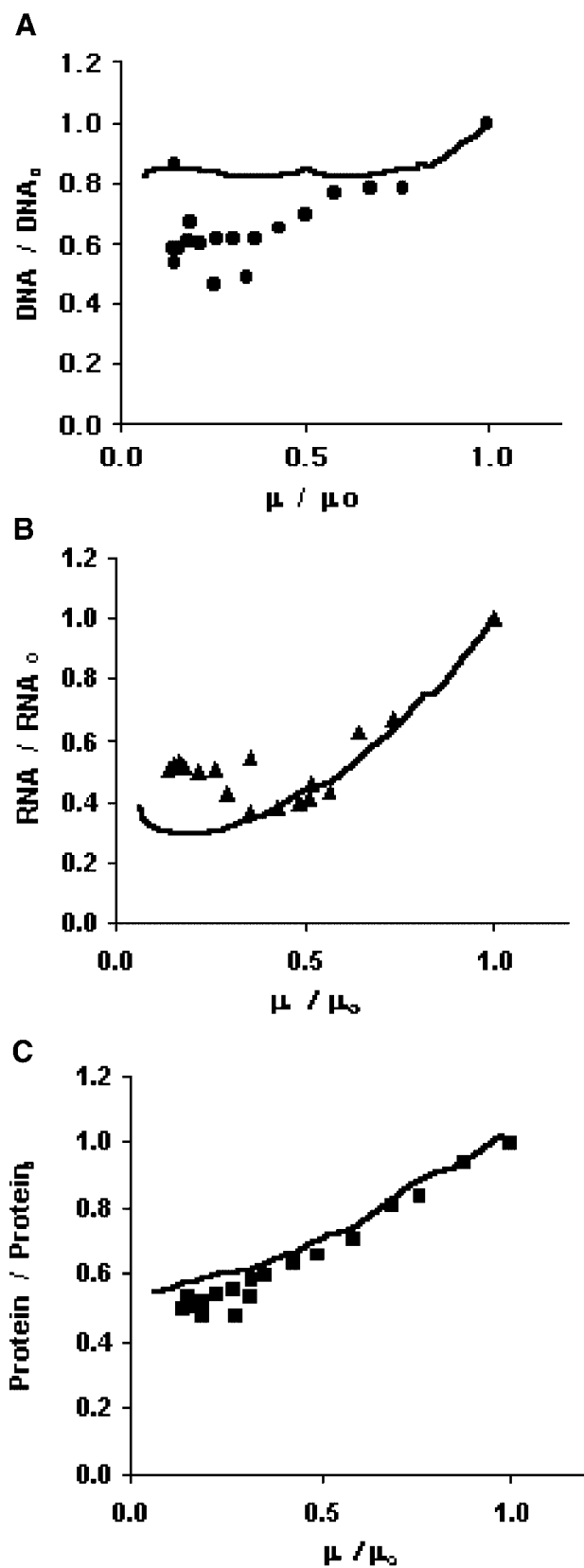


Figure 5. Simulation results of model. The mass of lipids inside the cell starts at an estimated initial value and equilibrates after five generations. The mass of fatty acids inside the cell starts at an estimated initial value and equilibrates after three generations.

internal cell's composition has eight new chemical species. The new module insertion did not affect the ability of the minimal cell model to predict interactions between the environment and the cellular physiological state with molecular level changes.

In our minimal cell lipid module, we defined a pathway with essential function and have found examples of genes that can accomplish this function, with the exception of *pgp* (a gene not yet determined in *E. coli*-best studied organism). Our lipid and fatty acids transport and metabolic pathways include eight functions (eight genes). The possibility that a smaller set of genes might be found exists, but at this point, this set of functions is minimal with the current experimental data available.

Figure 4. A: DNA content relative to maximum DNA content as a function of growth rate relative to maximum growth rate. Closed circles (●) represent glycerol phosphate supplemented/deprived *E. coli* cultures (McIntyre et al., 1977) and the line represents the minimal cell model. B: RNA content relative to maximum RNA content as a function of growth rate relative to maximum growth rate. Closed triangles (▲) represent glycerol phosphate supplemented/deprived *E. coli* cultures (McIntyre et al., 1977) and the line represents the minimal cell model. C: Protein content relative to maximum protein content as a function of growth rate relative to maximum growth rate. Closed squares (■) represent glycerol phosphate supplemented/deprived *E. coli* cultures (McIntyre et al., 1977) and the line represents the minimal cell model.

The nature of the lipid/fatty acids module could be expanded to include the effect of temperature on cell membranes. Many organisms including bacteria regulate their lipid composition with growth temperature. These mechanisms involve increasing the fraction of unsaturated phospholipid acyl chain as the growth temperature decreases (Cronan, 2003). The addition of a complementary mathematical expression including temperature effects on membrane composition could be developed; this would also be accompanied by integrating the variation of enzymatic reaction rates with temperature.

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