Cell Geometry and Membrane Protein Crowding Constrain Growth Rate, Overflow Metabolism, Respiration, and Maintenance Energy

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Abstract

The physical dimensions and membrane properties of a cell constrain its phenotype. A metabolic theory is presented for interpreting maximum growth rate, overflow metabolism, respiration efficiency, and maintenance energy flux based on the intersection of cell dimensions, membrane protein crowding, and metabolism. The presented theory employs biophysical properties and metabolic systems analysis to successfully predict phenotypes of *Escherichia coli* K-12 strains MG1655 and NCM3722. The strains are genetically similar but differ in surface area to volume (SA:V) ratios by up to 30%, maximum growth rates on glucose salts media by 40%, and overflow-inducing growth rates by 80%. Six *in silico* predictions were tested against experimental evidence including phenomics data, membrane proteomics data, and SA:V mutants. The predictions were remarkably consistent with experimental data and provided a cell membrane-centric explanation for maximum growth rate, maintenance energy generation, respiration chain efficiency (P/O number), and optimal biomass yield of a strain. These analyses did not consider cytosolic macromolecular crowding, highlighting the distinct properties of the presented theory and gaps in current cell biology literature. Cell geometry and membrane protein crowding are significant biophysical constraints, and consideration of both provide a more complete theoretical framework for improved understanding and control of cell biology.
Keywords: systems biology, cell biology, metabolism, cell geometry, surface area to volume ratio

Introduction

Bacterial cell geometry, including cell shape and size, is highly regulated and constrains the surface area available for acquiring nutrients and the volume available for synthesizing proteins (1-4). Cell geometry also impacts molecular crowding by fixing the two- and three-dimensional spaces available for macromolecules (5-9). Three-dimensional cytosolic molecular crowding has been analyzed both explicitly and implicitly using computational biology to predict catabolite repression and phenotype shifts including overflow metabolism or the production of fermentation products in the presence of electron acceptors (5-7, 10, 11). A quantitative and predictive understanding of the intersection of cellular geometry, two-dimensional membrane protein crowding, and phenotype, however, is not well studied (12-17).

Cell membranes provide a barrier against environmental stressors, retain macromolecules, provide a platform for the selective transport of molecules, and play a crucial role in energy metabolism including respiration (18, 19) (Fig. 1A). The lipid bilayer is a major building block of the membrane and has a finite capacity to host embedded and adsorbed proteins due to a combination of steric protein crowding effects and the loss of membrane integrity at high protein loading (18, 20-23).

Cellular surface area to volume ratios (SA:V) and membrane macromolecular crowding are the two foci of the presented work. Systems biology studies have applied enzyme metrics, such as total protein mass, to approximate crowding on a two-dimensional membrane surface, but these approaches generally miss the significant difference between a two- and three-dimensional crowding constraint (10, 19, 24, 25) (Fig. 1B, 1C). The current study makes a distinction between two- and three-dimensional protein crowding on the two-dimensional membrane surface by accounting for biophysical properties of embedded and associated enzymes (10, 24). Enzyme requirements for surface area are not readily predictable from the enzyme mass or volume

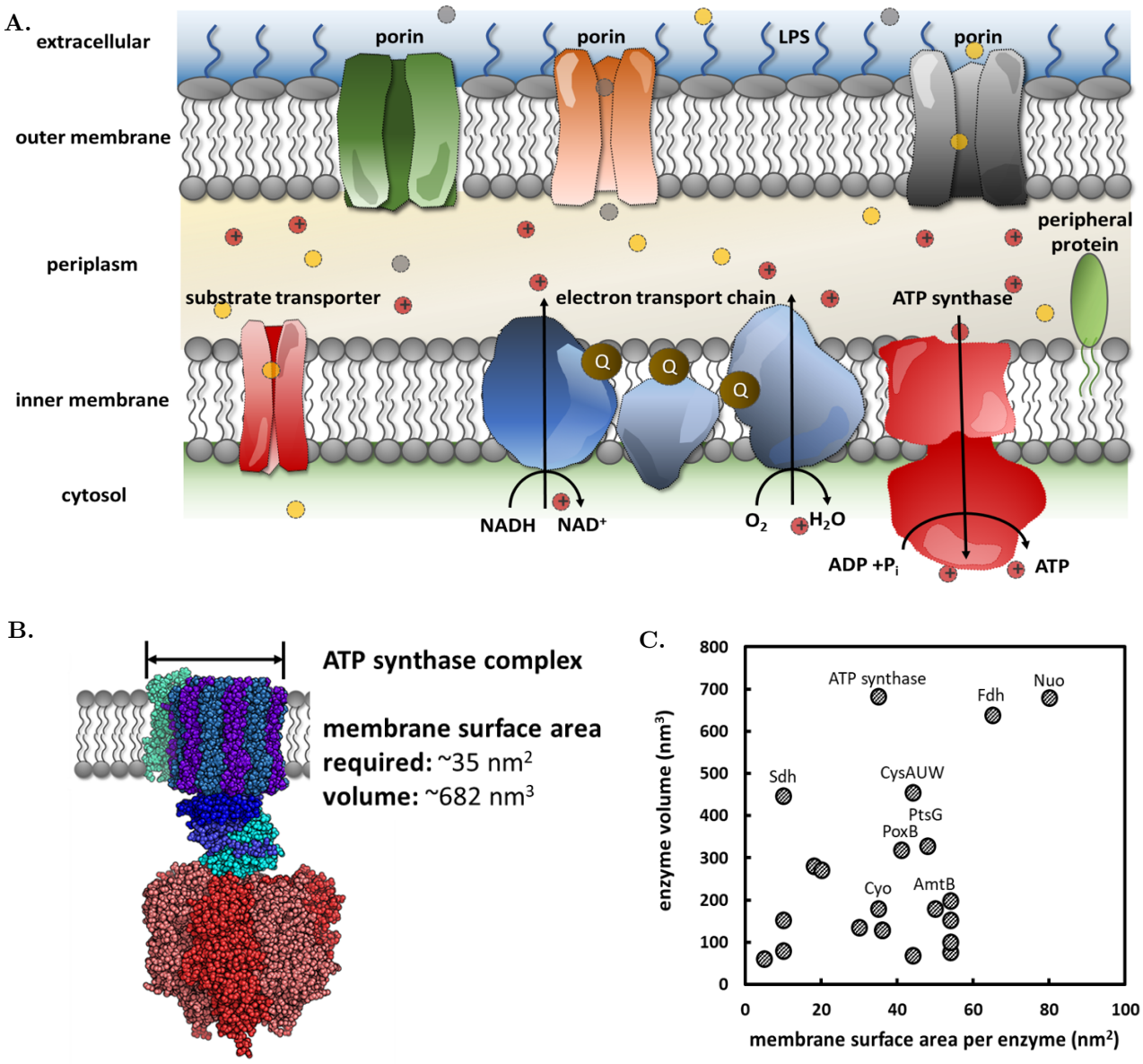


Figure 1: *E. coli* membrane and enzyme properties. A) The inner membrane enables critical cellular functions including substrate transport and oxidative phosphorylation. Membrane surface area is finite. **B)** The physical dimensions of membrane-associated enzymes influence both two- and three-dimensional molecular crowding. ATP synthase image modified from Wiki Commons. **C)** Membrane surface area requirements and enzyme volume for 22 central metabolism enzymes do not correlate well. Data available in supplementary data S1 and S4.

(Fig. 1B, 1C, supplemental S1), highlighting the need to study surface properties separately from intracellular crowding.

Escherichia coli is a facultative anaerobe and a convenient host for studying tradeoffs between cell geometry, membrane protein crowding, and phenotype. Two well studied and genetically similar *E. coli* K-12 strains, MG1655 and NCM3722, have distinct phenotypes (Fig. 2, supplemental S1, S2). These cells display exquisite control over their geometry including cell length and width, and we propose that this contributes to the phenotypic differences. The SA:V ratios influence the balance of nutrient and energy fluxes between surface area- and volume-associated processes (1-3). While theoretical studies have addressed the role of surface area on metabolism including Zhuang *et al.* 2011 and Szenk *et al.* 2017 (15, 16), no study has developed a quantitative and predictive molecular level theory that accounts for strain-specific differences in SA:V ratios, growth rate dependent changes in SA:V ratios, and growth rate dependent changes in membrane protein crowding (26). Additionally, maintenance energy calculations, which can account for a significant fraction (30 to nearly 100%) of substrate fluxes (27-30), have not previously considered the impact of biophysical constraints like finite membrane surface area and membrane protein crowding. The presented systems biology study demonstrates the remarkable theoretical impact of cell geometry and membrane protein crowding on foundational cellular processes including maximum growth rate, onset of overflow metabolism, respiration efficiency, and ultimately the cellular capacity for energy conservation

via maintenance energy generation. These predictions are all accomplished without consideration of cytosolic macromolecular crowding or absolute cellular volume, highlighting the incomplete nature of current cell biology theory. We propose that membrane-associated constraints are complementary to previously described cytosolic-based constraints.

Results

E. coli K-12 strains MG1655 and NCM3722 have different SA:V ratios and phenotypes

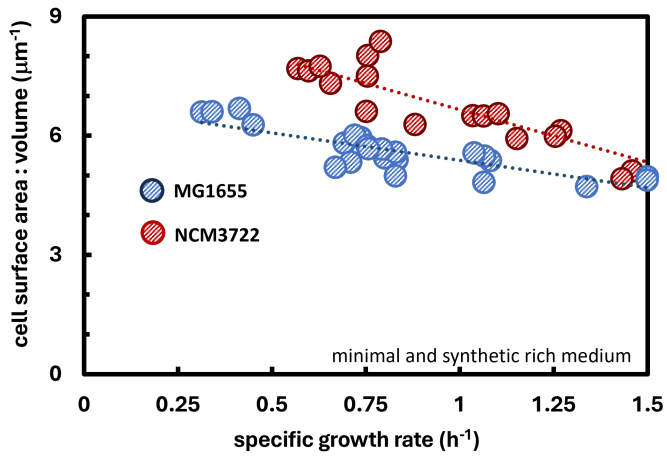
We hypothesize that the biophysical properties of the bacterial inner membrane constrain phenotype and developed a systems biology methodology to test this hypothesis. The methodology was developed using *E. coli* strain MG1655 data, and the predictions were then tested using data from *E. coli* strain NCM3722. These two strains are genetically similar but have distinct phenotypes including different maximum growth rates on minimal salts medium, different acetate overflow metabolisms, and different surface area to volume (SA:V) ratios (Fig. 2A, 2B, 2C). Bacterial cells follow "growth laws" and modulate their cell size and growth rate as a function of environment (31, 32). Si *et al.* measured wild type MG1655 and NCM3722 cellular dimensions and growth rates as a function of different nutrient conditions (2). Cell size measurements are foundational but are technically challenging and can differ substantially between research groups (2, 33, 34). Having a data set of cell dimensions for both strains collected from a single research group builds confidence in the comparisons. SA:V ratios decreased with increasing growth rate because the cell length and width increased (Fig. 2C) (1, 2). NCM3722 cells are smaller than MG1655 at low growth rates, leading to higher SA:V ratios (Fig. 2B, 2C). The different SA:V ratios are proposed to play a role in strain phenotypes. NCM3722 has a specific growth rate on glucose salts media that is ∼40% faster than MG1655 ($0.97 \pm 0.06\ h^{-1}$ vs. $0.69 \pm 0.02\ h^{-1}$, respectively; Fig 2A, supplementary data S1, S2). MG1655 displays an acetate overflow metabolism at growth rates $\geq 0.4 \pm 0.1\ h^{-1}$ while NCM3722 displays acetate overflow at growth rates $\geq 0.75 \pm 0.05\ h^{-1}$, which are faster than μ_{max} for MG1655 (Fig. 2A, supplementary data S1). Additionally, MG1655 has a cellular volume that is approximately two times larger than NCM3722 at a growth rate of 0.65 h^{-1}, whereas MG1655 has a SA:V ratio ∼30% smaller than NCM3722 at this growth rate (Fig. 2B, supplementary data S2). We present evidence that the cell SA:V, as well as the membrane capacity to host proteins, correlate with the phenotypic properties of the strains, suggesting these biophysical properties are important for analyses of cell biology.

A.

Strain	μ_{max} (h^{-1})	Overflow Initiation (h^{-1})	Yield g (g^{-1})
MG1655	0.69 ± 0.02	≥ 0.4 ± 0.1	0.40 ± 0.02
NCM3722	0.97 ± 0.06	≥ 0.75 ± 0.05	0.45 ± 0.04

Experimental data references in Supplementary Material S1.

B.



C.

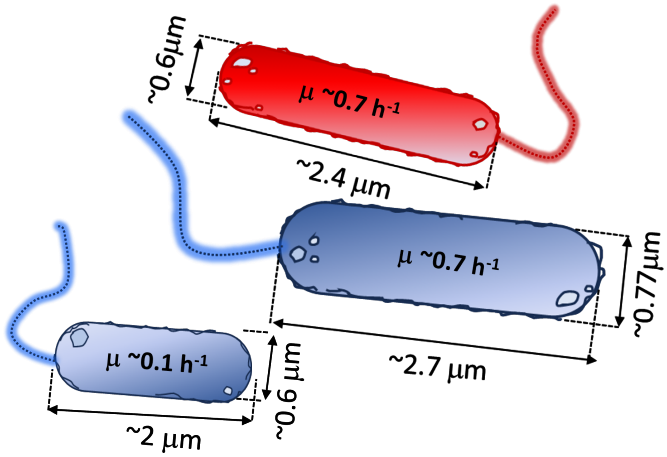


Figure 2: *E. coli* K-12 strains MG1655 and NCM3722 have distinct phenotypes and geometries. A) Comparison of strains MG1655 and NCM3722 based on maximum specific growth rate on glucose minimal medium, growth rate at which acetate overflow initiates on glucose minimal medium, and biomass yield on glucose ($g\ biomass\ (g\ glucose)^{-1}$). **B)** Experimental surface area to volume (SA:V) ratios for *E. coli* K-12 strains MG1655 and NCM3722 as a function of specific growth rate. *E. coli* geometry data from Si et al. 2017 (2). **C)** Graphical representation of differences in cell geometry (cell diameter and length) as a function of *E. coli* strain (blue = MG1655, red = NCM3722) and specific growth rate.

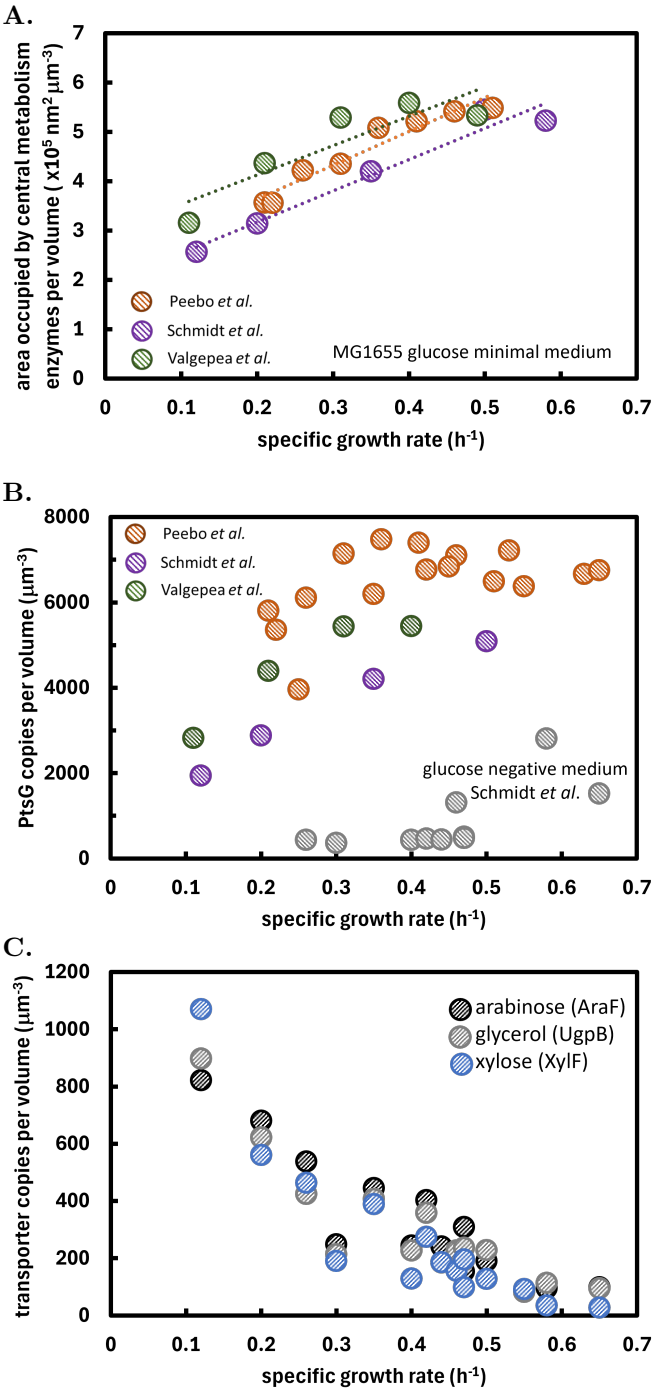


Figure 3: Dynamic membrane proteome of *E. coli* MG1655 as a function of growth rate and environment. A) The capacity of the *E. coli* MG1655 inner membrane to host proteins, expressed here as nm^2 occupied by central metabolism enzymes per volume (μm^{-3}). Data from *E. coli* cultures grown on glucose salts medium. Proteomics data from (36-39). Enzyme properties and calculations can be found in supplementary data S1, S3, and S4. Analysis was limited to cultures grown on glucose minimal medium. Calculations were not possible for complex medium because the consumption rates of the numerous substrates were unknown. **B)** Glucose transporter (PtsG) copy number per cell volume as a function of growth rate and growth medium. Gray data points are from glucose negative medium. Proteomics data from (36-39). Supplementary data S3. **C)** Alternative, high affinity sugar transporter copy number per cell volume as a function of growth rate during chemostat growth on glucose containing medium. The specific substrate for each transporter was not present in the medium. Proteomics data from (36-39). Supplementary data S3.

Membrane proteome is dynamic with respect to growth rate and environment

The *E. coli* membrane proteome is dynamic as a function of growth rate and environment (35-39) (Fig. 3A, 3B, 3C). The membrane hosting of central metabolism proteins (protein crowding) including glucose transport, electron transport capacity, and ATP synthase per cell volume increased with growth rate (Fig. 3A) (36-39) (supplementary data S3). In fact, the protein hosting increased faster than the decrease in SA:V (Fig. 2B), resulting in a net gain of membrane catalytic potential per cell volume (protein crowding multiplied by SA:V) with growth rate. Note both sets of data are plotted on a volume basis to facilitate comparisons. The areal densities of central metabolism proteins for *E. coli* K-12 were determined using three proteomics data sets (36-39) and cell geometry (2). Copy number of membrane-associated proteins was converted into an occupied surface area per cell volume using enzyme properties and cell geometry (2, 36-39) (supplementary data S3). The presented trends are for glucose minimal salts media. Faster growth rates were reported in media containing multiple substrates, but the studies did not include detailed substrate uptake data, precluding its inclusion in the current analysis. The presented analyses pooled data for *E. coli* K-12 strains MG1655 and BW25113 which are closely related and have similar growth rates, cell geometries, and phenotypes (33). For simplicity they are collectively referred to here as MG1655.

E. coli MG1655 synthesized more glucose transporter complexes (PtsG) per volume with increasing growth rate (Fig. 3B). However, *E. coli* cultures grown in the absence of glucose expressed much lower numbers of PtsG complexes. The proteomics data are from chemostat cultures, suggesting the cells have a finite surface area and prioritize environmentally relevant membrane proteomes at high growth rates (Fig. 3B). Meanwhile, *E. coli* grown on glucose at low dilution rates synthesized increasing levels of alternative, high affinity substrate transporters for arabinose, glycerol, and xylose as the dilution rate decreased (Fig. 3C). These substrates were not present in the media and the phenotypes likely represented a hedge strategy preparing the energy limited cells for alternative substrates, should they become available (35, 36) (supplementary data S3). These unused proteins were only synthesized at low dilution rates where we propose there was excess capacity for the membrane to host proteins.

We propose that finite membrane surface area per *gcdw* ($sMSA$) and membrane capacity to host proteins preclude the simultaneous and constitutive expression of all membrane proteins at all growth rates. The dynamic nature of the membrane proteome is hypothesized to reflect the substantial constraint it places on *E. coli* phenotypes. The remainder of the study presents theory and data to support this proposal.

Box 1: The membrane surface area required for a phenotype can be tabulated by considering enzyme fluxes and enzyme properties. Literature reviews of enzyme parameters used in this study can be found in supplementary data S4. The surface area required for enzyme i , per $gcdw$, is calculated as:

$$SA_{Ei} = \left(\frac{\nu_i \gamma_i}{\tau_i \varepsilon_i}\right)\left(\frac{\omega_i}{\sigma_i}\right)\left(\frac{mol}{10^3\ mmol}\right)\left(\frac{h}{3600\ s}\right)(N_{Avo}) \quad \text{(Eqn. 1)}$$

Variable	Definition	Units
SA_{Ei}	Surface area occupied by enzyme i	$nm^2\ (gcdw)^{-1}$
ν_i	Flux of reaction i	$mmol\ (gcdw \cdot h)^{-1}$
γ_i	Membrane area occupied by single enzyme i	$nm^2\ (enzyme)^{-1}$
τ_i	Enzyme turnover number (a.k.a. k_{cat})	$molecules\ (enzyme \cdot s)^{-1}$
ε_i	$S\ (S+K_M)^{-1}$: Enzyme i active site saturation, where S is substrate concentration	Unitless (0 - 1)
σ_i	Membrane allosteric regulation	Unitless (0 - 1)
ω_i	Enzyme overcapacity factor	Unitless (≥ 1)
N_{Avo}	Avogadro's number	$molecules\ (mole)^{-1}$

Membrane protein crowding is quantified as the total membrane surface area used by central metabolism enzymes. It is determined by summing the contributions from all active enzymes and applying biophysical parameters (reviewed in supplementary data S4). Eqn. 2 quantifies membrane protein crowding as the fraction of membrane surface area (f_{MSA}) occupied by active central metabolism enzymes. Conversions include, from left to right, water content of live cells, density of live cells, growth rate dependent SA:V ratio of cells ($c_1 \cdot \mu + c_2$), and unit conversions. δ_i accounts for nonidealities in surface area usage including misfolded proteins or misassembled protein complexes:

$$f_{MSA} = \sum_i \left\{ SA_{Ei}\left[\frac{gcdw}{3.85\ g\ wet\ cell}\right]\left[\frac{1.1\ g\ wet\ cell}{cm^3\ wet\ cell}\right]\left[\frac{\mu m^3\ wet\ cell}{(c_1 \cdot \mu + c_2)\ \mu m^2}\right]\left[\frac{cm}{10^4 \mu m}\right]\left[\frac{cm^2}{10^{14}\ nm^2}\right] + \delta_i \right\} \quad \text{(Eqn. 2)}$$

Eqn. 2 can be arranged to define the surface area resource MRE_i (membrane real estate for enzyme i) which is incorporated into the presented metabolic model. θ is a constant comprised of biophysical parameters and conversion factors (note: c_1 has units of h, c_2 is unitless):

$$f_{MSA} = \sum_i \left[\left(\frac{\nu_i \gamma_i}{\tau_i \varepsilon_i}\right)\left(\frac{\omega_i}{\sigma_i}\right)\left(\frac{\theta}{c_1 \cdot \mu + c_2}\right) + \delta_i\right] = \sum_i \left[\nu_i MRE_i \left(\frac{\theta}{c_1 \cdot \mu + c_2}\right) + \delta_i\right] \quad \text{(Eqn. 3)}$$

where $\theta = 0.048\ (molecules \cdot h \cdot gcdw)\ (mmol \cdot s \cdot nm^2)^{-1}$.
Summing δ_i over all the enzymes quantifies the f_{MSA} nonideality offset Δ:

$$(f_{MSA} - \Delta)\left(\frac{c_1 \cdot \mu + c_2}{\theta}\right) = \sum_i \nu_i MRE_i \quad \text{(Eqn. 4)}$$

Eqn. 4 may be rearranged as:

$$(f_{MSA} - \Delta)\left(\frac{c_2}{\theta}\right) = \sum_i \nu_i MRE_i - (f_{MSA} - \Delta)\left(\frac{c_1 \cdot \mu}{\theta}\right) \quad \text{(Eqn. 5)}$$

Collecting constants results in the linear constraint accounting for finite surface area:

$$sMSA' = \sum_i \nu_i MRE_i' - \beta' \cdot \mu \quad \text{(Eqn. 6)}$$

The units of Eqn. 6 may be converted for interpretability, multiplying by 1 $molecule\ (s \cdot mmol)^{-1}$:

$$sMSA = \sum_i \nu_i MRE_i - \beta \cdot \mu \quad \text{(Eqn. 7)}$$

Variable	Definition	Units
$sMSA$	Specific membrane surface area available for enzymes	$nm^2\ (gcdw)^{-1}$
MRE_i	Membrane area used by active enzyme i	$(h \cdot nm^2)\ (mmol)^{-1}$
β	Growth rate dependent change in surface area due to change in SA:V	$(h \cdot nm^2)\ (gcdw)^{-1}$

The constraint was applied by including an appropriate MRE_i cost to membrane-associated reactions and incorporating the β parameter into the biomass synthesis reaction. Note, the SA:V slope (c_1) is negative, making the contribution of $\beta \cdot \mu$ positive in Eqn. 7. See supplementary data S4 and S5 for parameter values and literature reviews.

Systems biology of cellular SA:V ratios and membrane protein crowding

The foci of this study are the cellular SA:V, inner membrane protein hosting capacity, the areal requirements of inner membrane associated proteins, and the collective role of these biophysical parameters on phenotype. The specific Membrane Surface Area ($sMSA$, units: $nm^2\ (gcdw)^{-1}$) required for membrane-associated enzyme fluxes was quantified using enzyme properties including flux per g cell dry weight (cdw), surface area requirements ($nm^2\ enzyme^{-1}$), and k_{cat} ($molecules\ (s \cdot enzyme)^{-1}$). Total $sMSA$ required for a phenotype was tabulated by summing the contribution of all active membrane-associated enzymes and by account-

ing for the growth rate dependent SA:V ratio. Mathematical development is presented in Box 1 while parameters are reviewed in supplementary data S1 and S4. Concisely, the catalytic potential of the cell membrane, on a per *gcdw* basis, strongly influences what phenotype is possible. The catalytic potential of the membrane is positively correlated with the cellular SA:V and the membrane protein crowding.

The $sMSA$ constraint was evaluated using a metabolic model of *E. coli* (40, 41) and flux balance analysis (FBA) (42). The $sMSA$ constraint was implemented by incorporating a membrane surface area requirement for each membrane-associated flux, termed MRE_i (membrane real estate) (Box 1) (15). Flux through membrane-associated enzymes was constrained by a finite $sMSA$ pool which changed in magnitude based on growth rate dependent SA:V ratios and protein crowding (quantified as the fraction of the Membrane Surface Area occupied by central metabolism proteins, f_{MSA}) (supplementary data S5, Box 1). The $sMSA$ balance was enforced concurrently but orthogonally to standard FBA mass balances ($mmol\ (gcdw \cdot h)^{-1}$). The *in silico* theory was termed specific Membrane Surface Area-constrained Flux Balance Analysis (*sMSAcFBA*) (42). This novel formulation contrasts with previous studies where the weights on the membrane-associated fluxes were the molecular masses of the protein, which correlates with enzyme volume (10, 19, 24, 25, 43), as opposed to the surface area occupied as in the presented work (Fig. 1C). The presented analyses did not consider cytosolic protein investment nor cytosolic macromolecular crowding to isolate the influence of the membrane catalytic constraint on phenotypic predictions.

E. coli MG1655 growth rate and biomass yield are constrained by membrane surface area, protein crowding, and maintenance energy

Cell growth requires fluxes of nutrients across the membrane for biomass synthesis and for maintenance energy generation. Intracellular reactions for macromolecular synthesis are well characterized while maintenance energy requirements are still largely a black box (44). Maintenance energy is cellular energy consumed for functions other than the direct production and polymerization of biomass components (45). The theoretical impact of finite $sMSA$ on these fluxes was quantified for *E. coli* strain MG1655 growing on glucose salts medium (Fig. 4A). Briefly, a matrix of biomass synthesis rates (q_x) and maintenance energy fluxes (q_{ATP}, aggregate of growth- and nongrowth-associated maintenance energy (GAM, NGAM, respectively)) was defined and applied as FBA flux constraints. Each parameter combination comprised a unique q_x and q_{ATP}, while an $sMSA$ optimized phenotype was identified by minimizing glucose uptake ($f_{MSA} = 0.07$, Fig. 4A, Box 1). This analysis used an 'unbiased' approach to maintenance energy fluxes to illustrate the effect of $sMSA$ on *in silico* phenotypes. Maintenance energy magnitude is largely a parameter fitting exercise requiring substantial assumptions like respiration efficiency; additionally, the magnitude of maintenance energy changes with environment (29, 46-48). For example, *E. coli* cultures grown aerobically on glucose under phosphate or iron limitation or grown anaerobically on glucose appear to have substantially different maintenance energy fluxes than reference, aerobic glucose-limited *E. coli* cultures (49-52). With the data presented, a reader can apply a maintenance energy vs. growth rate relationship of their choosing to recreate a metabolic solution space of interest (Supplementary Data S6). The plot uses an f_{MSA} of 0.07 because, as shown below, this corresponds with the *E. coli* K-12 maximum growth rate on glucose minimal medium and thus represents a convenient reference point. The supplementary material presents MATLAB code to recreate the analysis with any f_{MSA} value from 0.01 to 0.2 (Supplementary Data S9).

Metabolic processes competing for a finite pool of $sMSA$ resulted in rate-yield tradeoffs (16, 53). Phenotypes with high biomass yields ($g\ biomass\ (g\ glucose)^{-1}$) required larger investments of $sMSA$ for high efficiency electron transfer chain (ETC) components, quantified by the P/O number (Fig. 4B). The P/O number is the ratio of ATP produced via respiration per two electrons transferred to O_2. *E. coli* has the genetic potential to operate ETC configurations with P/O numbers from ∼0 to 2 (54-56). ETC efficiency is not influenced by substrate-level phosphorylation such as ATP synthesis associated with acetate overflow. Phenotypes with low growth rates and small maintenance energy fluxes can support high P/O numbers because of the modest $sMSA$ requirements for transporters and ATP synthase complexes (upper left Fig. 4B, supplementary data S6). Phenotypes with high growth rates require larger investments of $sMSA$ for glucose transporters (PtsG) (Fig. 4C, supplementary data S6). These tradeoffs generate gradients in other phenotypic properties including the potential for maintenance energy fluxes (q_{ATP}) (Fig. 4A), use of parallel ETC NADH dehydrogenase enzymes Nuo (Fig. 4D) or Ndh II (Fig. 4E), and acetate overflow metabolism ($q_{acetate}$) (Fig. 4F). Secretion of acetate generates ATP via substrate-level phosphorylation and reduces the need for $sMSA$-intensive ETC components and ATP synthase complexes (15, 16). Presented q_{ATP} accounts for the capacity to generate the sum of growth- and nongrowth-associated maintenance energy (GAM and NGAM, respectively) but does not include ATP required to polymerize monomers into macromolecules for biomass synthesis as this energetic cost is included in the biomass synthesis reaction (supplementary data S5).

As a control, the same matrix of biomass synthesis fluxes and maintenance energy fluxes was analyzed without the $sMSA$ constraint (Fig. 4G). Without the constraint, there was no rate-yield tradeoff, no maximum growth rate was identified, and the simulations permitted maintenance energy fluxes more than 10-fold higher than with the $sMSA$ constraint at fast growth rates.

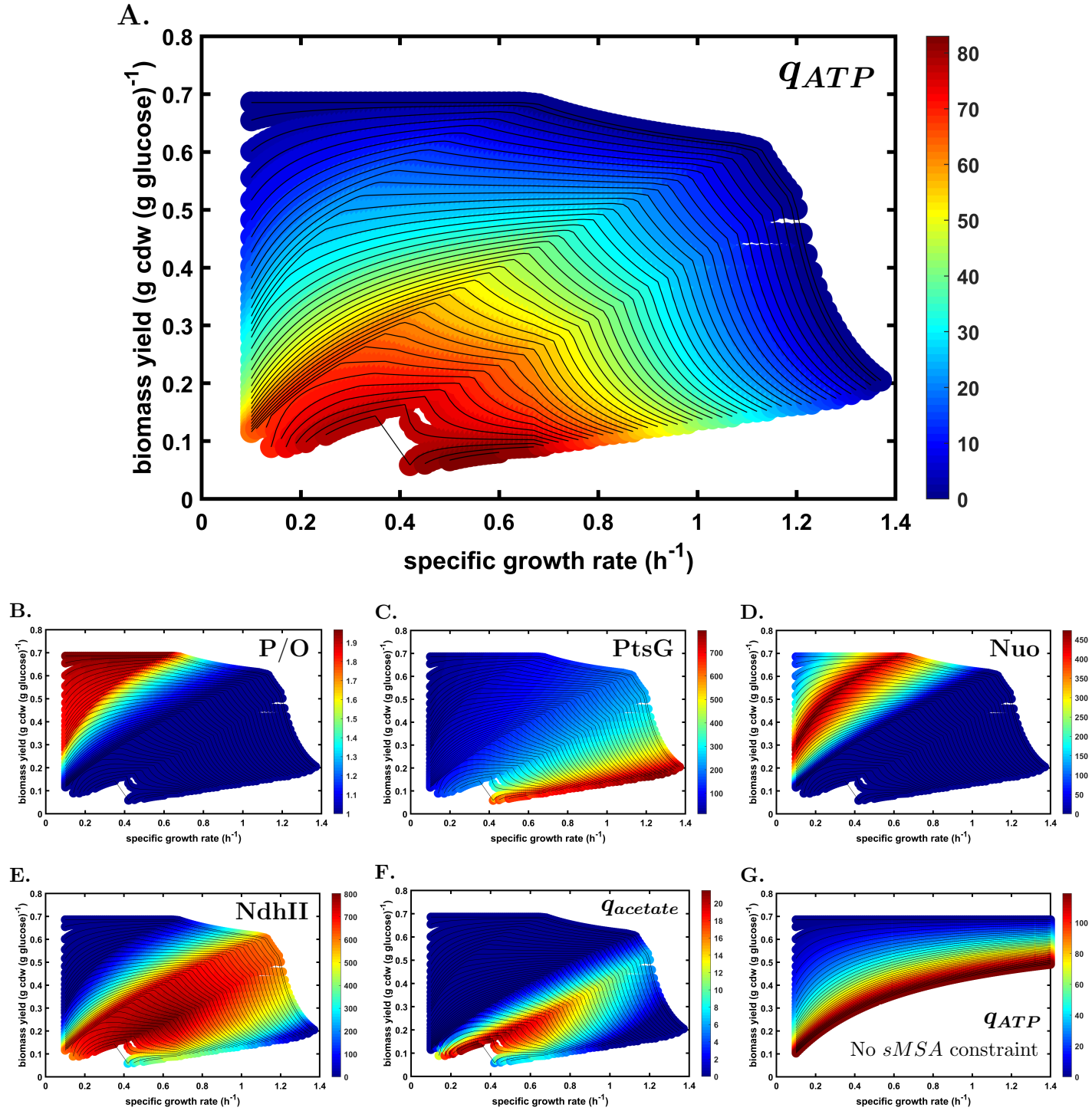


Figure 4: Predictive growth rate – biomass yield phenotype space for *E. coli* strain MG1655 constrained by membrane surface area and membrane protein crowding. A) Potential for maintenance energy fluxes ($mmol\ ATP\ (gcdw \cdot h)^{-1}$) shown by the color gradient (protein crowding, $f_{MSA} = 0.07$). Black contour lines are maintenance energy isoclines. Maintenance energy flux accounts for ATP in excess of ATP used for macromolecule polymerization. **B)** Color gradient represents the P/O number which quantifies the efficiency of oxidative phosphorylation. **C)** Color gradient quantifies the areal density of glucose transporter PtsG (complexes μm^{-2}). **D)** Color gradient quantifies areal density of the electron transport chain component Nuo (complexes μm^{-2}).**E)**$Colorgradientquantifiesarealdensityoftheelectrontransportchaincomponent NdhII(complexes\mu m^{-2})$. **F)** Color gradient quantifies overflow metabolism $q_{acetate}$ ($mmol\ acetate\ (gcdw \cdot h)^{-1}$). **G)** Control simulation unfettered by the $sMSA$ constraint. Color gradient quantifies potential for maintenance energy generation (q_{ATP}).

μ_{max} is a Pareto tradeoff between biomass production and maintenance energy generation

Finite $sMSA$ may necessitate phenotypic tradeoffs, providing a new lens with which to interpret maximum growth rate. Consensus extracellular fluxes were assembled for *E. coli* strain MG1655 growing on glucose salts media (Fig. 5A, supplementary data S1). Data span growth rates from 0.1 h^{-1} to μ_{max} (6, 38, 43, 57, 58). $sMSA$ theory was applied integrating the experimental fluxomics data (Fig. 5A), with growth rate dependent changes in: SA:V, protein crowding (f_{MSA}, 0.02-0.09), and PtsG enzyme saturation (Fig. 5B). These data sets were all applied as constraints to *sMSAc-FBA* analyses which optimized the maximum possible maintenance energy generation (q_{ATP}) as a function of growth rate (Fig 5B, 5C, 5D). q_{ATP} was calculated 100 times for each growth rate by randomly and independently perturbing each experimental consensus flux within an assumed error interval (±7% of flux) and using these perturbed fluxes as the *sMSAc-FBA* reaction constraints. Data points in Fig. 5C – 5E show the mean from 100 simulations; error bars represent the standard deviation.

Positive slopes between growth rate and maintenance energy flux (Fig. 5C) indicate that both rates can theoretically increase simultaneously without penalty, as the membrane can accommodate a balanced increase of substrate transporters, ETC complexes, and ATP synthase complexes. A slope of zero indicates that the growth rate can increase with no reduction in q_{ATP}; the membrane hosts more substrate transporters while holding the other processes constant. A negative slope indicates that the growth rate can increase only with a concurrent decrease in q_{ATP}; the increased substrate transporter demand requires a decrease in ETC components and ATP synthase complexes. Negative slopes define a Pareto front between simultaneous increases in growth rate and q_{ATP} (Fig. 5C, 5D) (59).

Membrane protein crowding (f_{MSA}) has the potential to constrain the energetic efficiency of respiration by limiting the ability of the cell to extract useful energy from substrate. For example, there was ∼4-fold increase in predicted MG1655 maintenance energy potential (10 ± 3 vs. 41 ± 4 $mmol\ ATP\ (gcdw \cdot h)^{-1}$) for the same consensus fluxes ($\mu = 0.5\ h^{-1}$, $q_{glucose} = 7.2 \pm 0.5\ mmol\ (gcdw \cdot h)^{-1}$, $q_{acetate} = 1.70.1\ mmol\ (gcdw \cdot h)^{-1}$) when the enforced protein crowding increased from 4% to 9% ($f_{MSA} = 0.04, 0.09$) (Fig. 5D, supplementary data S6). The difference in q_{ATP} potential is based on the availability of $sMSA$ required to use higher efficiency ETC components and assemble more ATP synthase complexes. We hypothesize that *E. coli* operates at a maximum membrane protein capacity as this would significantly increase the possible maintenance energy generation, thereby improving fitness.

Experimental *E. coli* MG1655 cultures have a μ_{max} of 0.69 ± 0.02 h^{-1} on glucose salts media (Fig. 2A, blue shaded area, Fig. 5D) which coincides with the start of a negative slope between growth rate and q_{ATP} ($f_{MSA} = 0.07$). The predicted $f_{MSA} = 0.07$ is lower than values estimated by Szenk et al. (15). The current study adjusted enzyme k_{cat} values to a temperature of 37°C, resulting in some faster k_{cat} values and therefore smaller f_{MSA} requirements (60). All reactions in the *in silico* model were balanced for atoms and electrons (supplementary material S5). Therefore, all simulations necessarily balanced consensus fluxes with appropriate CO_2 (necessary for carbon balance) and O_2 (necessary for electron balance) fluxes as no other electron acceptors were available in the simulations (61).

Submaximal P/O numbers optimize energy conserving potential of finite membrane surface area

E. coli has a branched ETC that can convey electrons using multiple enzymatic routes to acceptors including O_2 (54-56). These enzymes can conserve varying amounts of substrate chemical energy by converting it into chemiosmotic energy stored as proton motive force. As mentioned previously, *E. coli* ETC components can operate at P/O numbers between ∼0 and 2 (54-56). P/O number can significantly influence maintenance energy calculations (47). *E. coli* cultures typically operate at submaximal P/O numbers in the range of 1 to 1.5 even at O_2 sufficiency (reviewed in supplementary data S4). Few theories have been proposed to explain this phenotype (62, 63). We hypothesize $sMSA$ theory is relevant to the experimentally observed submaximal P/O numbers.

Data from four proteomics studies (36-39) and enzyme k_{cat} numbers were used to calculate experimental P/O numbers as a function of growth rate (Fig. 5E, supplementary data S6, S7). Theoretical growth rate dependent P/O numbers were calculated using *sMSAc-FBA* from experimental consensus fluxes, SA:V ratios, and growth rate dependent membrane protein crowding (Fig. 5E, supplementary data S6, S7). *In silico* P/O numbers at μ_{max} were comparable to the experimental values (1.04 ± 0.01 and 1.01-1.07, respectively). We propose the experimentally observed submaximal P/O numbers represent an optimized use of the finite $sMSA$ that enables optimal substrate fluxes for growth and cellular energy generation and now present additional evidence for this conclusion. Predicted P/O numbers increase rapidly as growth rate decreases. We propose slow-growing cells are not substantially constrained by $sMSA$ as the slow growth rate does not require as many glucose transporters, ETC components, or ATP synthase complexes (Fig. 5B). Slow growing cells have sufficient $sMSA$ to permit the expression of alternative substrate transporters such as those shown in Fig. 3C.

Membrane surface area and membrane protein crowding predict onset of overflow metabolism.

The intersection of metabolism, membrane protein crowding, and cellular geometry predicts the onset of acetate overflow based on optimization of finite $sMSA$ (Fig. 5B). The presented analysis is designed to further test the $sMSA$ theory by highlighting its potential significance as a mediator

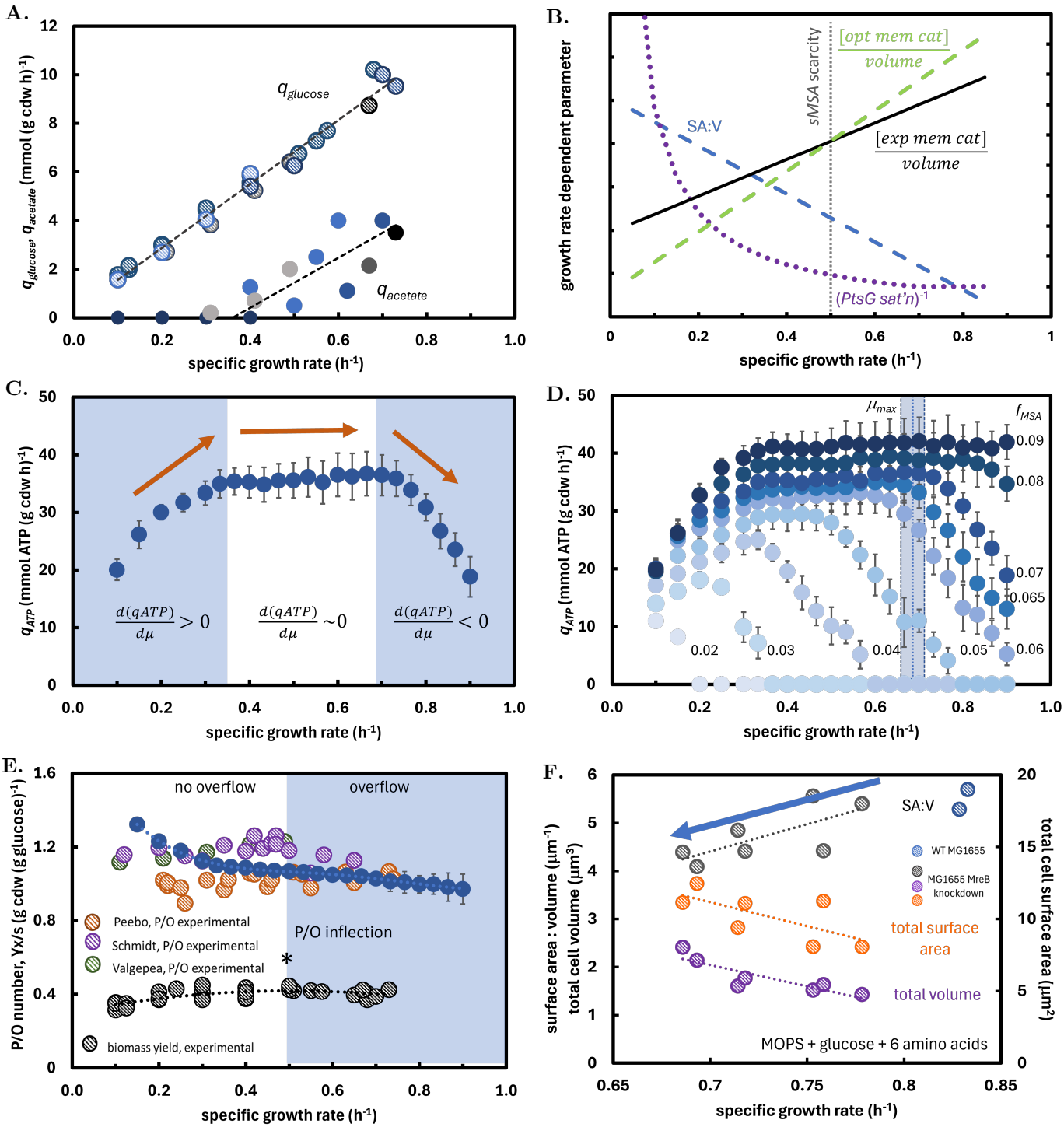


Figure 5: *E. coli* MG1655 phenotypic properties constrained by membrane surface area and protein crowding. A) Experimental data from *E. coli* K-12 strain MG1655 used to build consensus fluxes as a function of growth rate. Experimental data references can be found in supplementary material S1. **B)** Summary of biological processes integrated in $sMSA$ analysis including growth rate dependent changes in SA:V, *PtsG* glucose saturation, and membrane catalytic capacity per volume, optimal (opt mem cat) and experimental (exp mem cat). **C)** An example of the nexus of experimental consensus fluxes and $sMSA$ theory defines three regions based on the slope between the capacity to support biomass fluxes and maintenance energy fluxes. The negative slope defines a Pareto tradeoff. **D)** Maintenance energy potential of the *E. coli* MG1655 consensus fluxes as a function of membrane protein crowding quantified by f_{MSA}. A series of simulations was performed enforcing different membrane protein crowding (f_{MSA} = 0.02-0.09, or 2-9% of occupied membrane area). Error bars represent standard deviation of 100 simulations; see main text. A Pareto tradeoff between growth rate and maintenance energy flux occurs at the experimental μ_{max} for a f_{MSA} of 0.07. Shaded area quantifies experimental maximum growth rate and standard deviation. **E)** Predicted *E. coli* MG1655 P/O numbers (solid blue points) plotted with experimentally determined P/O numbers (striped points) as a function of growth rate. Predicted P/O numbers have an inflection point denoting a change in scarcity of the membrane surface area. Overflow metabolism occurs at severe surface area scarcity, right of inflection point. The P/O number inflection corresponds with higher experimental biomass yields on glucose. Biomass yield maximum highlighted with (*). Error bars represent standard deviation of 100 simulations using strain-specific consensus fluxes. Experimental proteomics data from (36-39). **F)** Experimental testing of $sMSA$ theory using an MG1655 strain engineered for titratable MreB expression to control cell SA:V ratios. Decreasing strain SA:V ratio resulted in slower growth rates on glucose medium supplemented with amino acids (gray data). The total volume (purple data) and total surface area (orange data) of the MG1655 strain expressing titratable levels of MreB decreased with growth rate. Data from (2).

of major phenotypic strategies. *sMSAc-FBA* was used to calculate growth rate dependent P/O numbers for strain MG1655 using the experimental consensus fluxes, SA:V, and growth rate dependent membrane protein crowding (Fig. 5A, Box 1, supplementary data S7).

The theoretical P/O number vs. growth rate curve was fit empirically (analyses of different empirical fittings are presented in supplementary data S7). The inflection point was determined from the second derivative with respect to growth rate. The inflection point identified the growth rate where $sMSA$ became sufficiently limiting to warrant the overflow metabolism. The inflection point at $\mu = 0.5\ h^{-1}$ correlates well with the experimental values for overflow onset, $0.4 \pm 0.1\ h^{-1}$ (Fig. 2A, supplementary data S1). At growth rates less than the inflection point, the analysis suggests cells have sufficient $sMSA$ to operate substrate transporters, ETC apparatus, and ATP synthase without requiring the overflow tradeoff (Fig. 5B, 5E). The P/O number inflection also coincides with the maximum experimental biomass yields (black circles), providing a basis for defining an optimal biomass phenotype (Fig. 5A, 5E, supplementary data S1).

Modulating *E. coli* MG1655 SA:V alters maximum growth rate in predictable manner

Si *et al.* (2) examined the effect of MG1655 SA:V on batch growth rates. A titratable MreB system was used to modulate *E. coli* geometry by changing the cell diameter and cell length and therefore changing SA:V ratios (1-3, 64). The batch growth rate on defined glucose medium supplemented with six amino acids was measured as a function of titrated SA:V. These experiments modulating the cell SA:V using the transcriptome while using the same growth medium (MOPS + glucose + 6 amino acids). In contrast, the data in Figure 2B was collected from wild type cultures subjected to different nutrient environments which provided a different mechanism of controlling growth rate and SA:V (31, 65). Cells with higher SA:V ratio grew faster than cells with lower SA:V ratios, supporting the $sMSA$ theory theory as the theory correlates higher membrane catalytic activity per $gcdw$ ((SA:V) $\cdot$ f_{MSA}) with faster substrate uptake, faster growth, and more efficient respiration (Fig. 5F). Interestingly, when the same data set was plotted as total cell surface area or total cell volume vs. growth rate, a negative relationship was observed (Fig. 5F) (1, 3, 66). The same study also analyzed a strain with a titratable FtsZ system. The strain did not achieve a substantial range of SA:V ratios, and the specific growth rates during batch growth were largely unchanged (supplementary data S2).

Testing $sMSA$ predictions using data from *E. coli* strain NCM3722

$sMSA$ theory, developed using *E. coli* strain MG1655 data, predicted several phenotypic properties based on SA:V ratios and membrane protein crowding (f_{MSA}). We propose if these phenotypic properties can be predicted for another *E. coli* strain, such as strain NCM3722, then the theory holds meritorious promise. Synthesizing the theoretical framework, we predict the following behaviors will hold for *E. coli* strain NCM3722: **prediction 1**, *sMSAc-FBA* using an f_{MSA} of 0.07 will predict μ_{max} at the Pareto front between biomass synthesis and maintenance energy generation; **prediction 2**, *sMSAc-FBA* will only predict an accurate μ_{max} when biologically relevant SA:V ratios are applied to the analyses; **prediction 3**, *sMSAc-FBA* will quantitatively predict P/O ratios highlighting the optimal use of finite membrane surface area and protein crowding; **prediction 4**, *in silico* P/O ratios, plotted as a function of growth rate, will have an inflection point that corresponds with the experimental onset of acetate overflow; **prediction 5**, the most efficient biomass yield on glucose will correspond with the inflection point of P/O number vs. growth rate, providing a basis for defining an optimal glucose phenotype; and **prediction 6**, culture growth rates will decrease in a fixed nutrient environment when the cellular SA:V ratios are decreased. These evaluations of $sMSA$ theory used the same metabolic model with the same enzyme parameters as applied for MG1655 simulations. However, strain-specific SA:V ratios (Fig. 2B) and consensus fluxes were applied (Fig. 6A).

E. coli NCM3722 data were assembled to develop consensus fluxes (6, 43, 67). The majority of the NCM3722 data were derived from a recombinant strain with a recombinant glucose uptake system (6). Briefly, the strain had the native PtsG glucose transport component deleted, and in place, a plasmid-based genetic construct with a titratable PtsG protein was used to control growth rate. The recombinant NCM3722 strain was grown in batch, and the glucose transporter was saturated (enzyme $K_M \sim 5\ \mu M$, initial medium glucose concentration $S \sim 11\ mM$) (Fig. 6B). The recombinant NCM3722 phenotype was applied to the *sMSAc-FBA* simulations using a PtsG enzyme saturation factor $\varepsilon_{PtsG} = 1$ (Fig. 6B). This was in contrast with data for MG1655 which was collected from wild type cultures with growth rates controlled by substrate concentration modulated by chemostat dilution rates (6, 38, 43, 57, 58). MG1655 simulations accounted for changing PtsG enzyme saturation (ε_{PtsG}) as a function of growth rate (Fig. 6B).

Testing prediction 1. NCM3722 data demonstrated trends analogous to MG1655, including the existence of a Pareto tradeoff between biomass synthesis and the capacity to generate maintenance energy. At a protein crowding of $f_{MSA} = 0.07$, the predicted μ_{max} of $0.95\ h^{-1}$ correlated closely with experimental μ_{max} of $0.97 \pm 0.06\ h^{-1}$ (red shaded area, Fig. 6C, supplementary data S6). Both strains had similar predicted protein crowding at their respective μ_{max} ($f_{MSA} = 0.07$) despite a 40% difference in maximum growth rate and substantial differences in SA:V ratios as a function of growth rate (Fig. 2B). Interestingly, strains MG1655 and NCM3722 are predicted to have a similar potential for q_{ATP} at their respective μ_{max} (37 ± 5 and $37 \pm 4\ mmol\ ATP\ (gcdw \cdot h)^{-1}$) despite the faster growth rate of NCM3722 (Fig. 2A).

Testing prediction 2. Altering the applied NCM3722

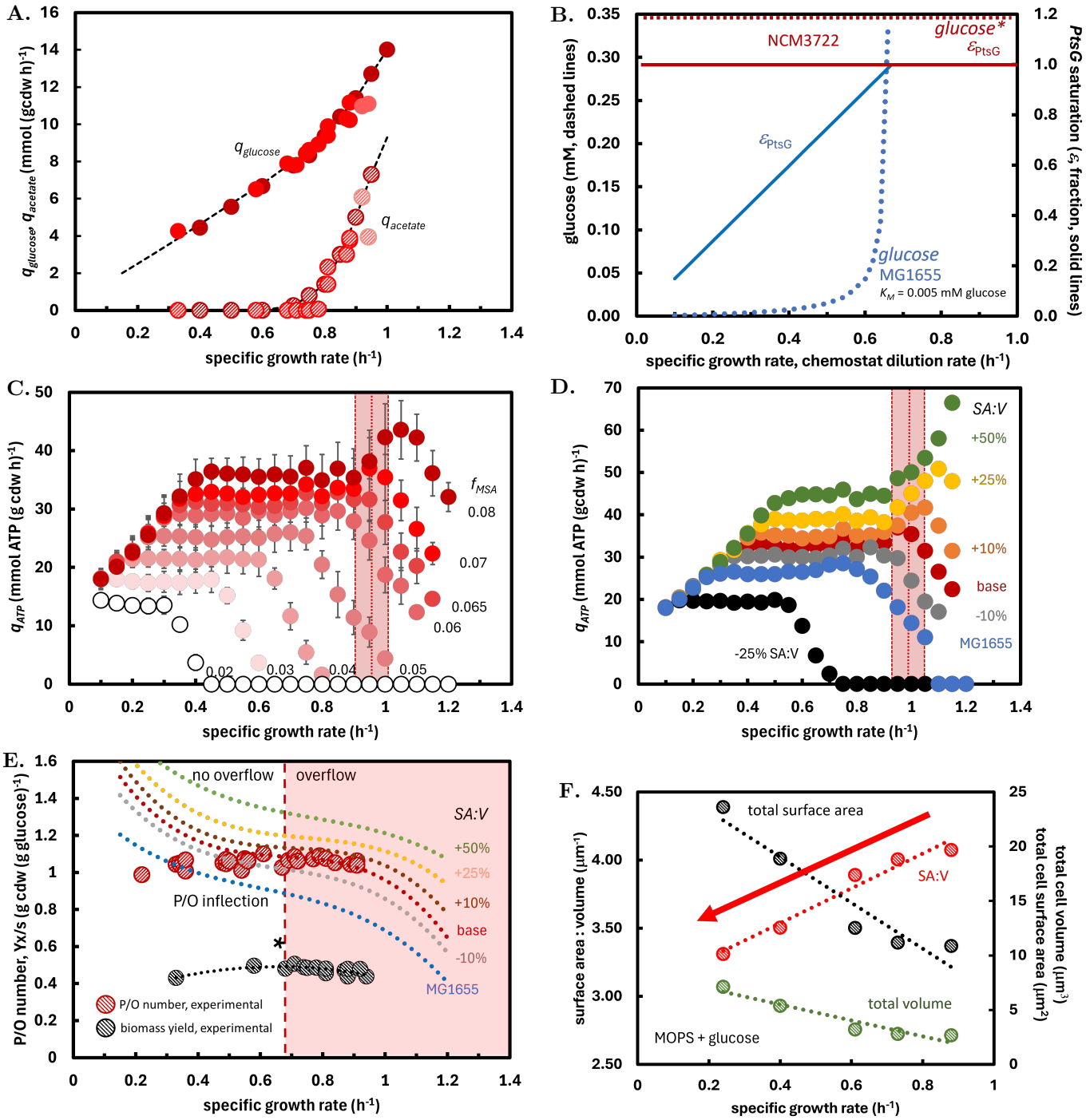


Figure 6: Testing $sMSA$ predictions using data from *E. coli* NCM3722. A) Experimental data from *E. coli* K-12 NCM3722 used to build consensus fluxes as a function of growth rate. Experimental data references can be found in supplementary material S1. **B)** Glucose transporter (PtsG) saturation (dotted lines) and as a function of growth rate for wild type MG1655 and recombinant NCM3722. Steady state glucose concentration vs. growth rate for wild type MG1655 and representative batch growth glucose concentration for recombinant NCM3722 cultures. NCM3722 cultures started at approximately 11 mM (off the chart). **C)** Maintenance energy potential of the *E. coli* NCM3722 consensus fluxes as a function of membrane protein crowding quantified by f_{MSA}. A series of simulations was performed enforcing different membrane protein crowding (f_{MSA} = 0.02-0.08, or 2-8% of occupied membrane area). Error bars represent standard deviation of 100 simulations; see main text. A Pareto tradeoff between growth rate and maintenance energy flux occurs at the experimental μ_{max} for a f_{MSA} of 0.07. Shaded area quantifies experimental maximum growth rate and standard deviation. **D)** Effect of applied SA:V ratios on the relationship between biomass synthesis and maintenance energy potential. Simulations fixed f_{MSA} at 0.07 and varied the SA:V from the experimentally determined relationship (±25% of the experimental values and the MG1655 SA:V relationship). Changing the applied SA:V changed the predicted μ_{max}. Data points represent the mean of 100 simulations. **E)** Predicted P/O numbers as a function of varying SA:V ratios and growth rate plotted with experimentally determined P/O number (supplementary data S7). Predicted P/O numbers have an inflection point denoting the change in scarcity of the membrane surface area. Overflow metabolism occurs at severe surface area scarcity, right of inflection point. The P/O number inflection corresponds with higher experimental biomass yields on glucose. Biomass yield maximum highlighted with (*). Experimental proteomics data from (58). **F)** Experimental testing of $sMSA$ theory using an NCM3722 derived strain engineered for titratable protein expression which controlled cell volume and SA:V ratios. Decreasing strain SA:V ratio slowed growth rates on glucose medium (red data). The total cell volume (green data) and total surface area (black data) decreased with increasing growth rate. Data from (68).

SA:V ratio while holding $f_{MSA} = 0.07$ constant caused the predicted μ_{max} to deviate from the experimental value. The experimental SA:V was varied from +25% to -25% (Fig. 2B), and the μ vs. q_{ATP} analysis was repeated (Fig. 6D, supplementary data S6). Additionally, the experimental MG1655 SA:V ratio was applied to the NCM3722 simulations (Fig 6D). The predicted μ_{max} changed in a predictable manner; increasing SA:V predicted faster growth before the Pareto tradeoff occurred. Applying an MG1655 SA:V ratio to the NCM3722 fluxes predicted a μ_{max} of 0.75 h^{-1}, a substantial deviation from the experimental μ_{max} of 0.97 h^{-1}.

Testing prediction 3. *sMSAc-FBA* predictions of NCM3722 P/O numbers were quantitatively consistent with experimental numbers when experimental NCM3722 SA:V values were applied (Fig. 6E, supplementary data S6). Analogous to the MG1655 analysis (Fig. 5E), the predictions suggest the submaximal P/O numbers are actually optimal uses of the constrained $sMSA$. Predicted P/O numbers varied substantially when the SA:V was increased or decreased (Fig. 6E). Applying the experimental MG1655 SA:V ratios with experimental NCM3722 fluxes did not accurately predict strain phenotype (Fig. 6E, supplementary data S6). The P/O number increased with decreasing growth rate, again suggesting $sMSA$ is a considerable constraint only at high growth rates.

Testing prediction 4. The inflection point in the NCM3722 P/O number vs. μ relationship predicted the onset of overflow metabolism. The analysis predicted acetate overflow at $\mu = 0.68\ h^{-1}$, which correlated reasonably well with the experimental onset at $0.75 \pm 0.05\ h^{-1}$. The P/O number vs. μ trends for seven different SA:V trends were considered (Fig. 6E, supplementary data S6). The trends demonstrate inflection points that were identified by taking the second derivative of the empirical polynomial fits. Altering the SA:V altered the predicted onset in a predictable manner. A summary of the predicted maximum growth rates and acetate overflow metabolism is presented in supplementary data S6.

Testing prediction 5. Experimental NCM3722 biomass yields on glucose were plotted along with the P/O numbers as a function of growth rate (Fig. 6E, supplemental data S1, S6). Analogous to MG1655 data, the maximal biomass yield corresponded closely with the inflection point for the P/O vs. μ relationship (0.66 vs. 0.68 h^{-1}, respectively). Finally, it should be noted that the *sMSAc-FBA* predictions were remarkably accurate despite the majority of the NCM3722 data being measured using a recombinant strain with an altered membrane proteome.

Testing prediction 6. Basan *et al.* examined a recombinant *E. coli* NCM3722 strain which had a titratable SA:V ratio based on the overexpression of "useless" *lacZ* protein (68). While this was not the original intent of the study, the data is useful for testing the *sMSA* theory. High expression levels of *lacZ* caused the cell size to increase. The published cell dimensions were converted into SA:V ratios assuming a rod-shaped cell (supplementary data S6). The recombinant strain was grown in a fixed nutrient environment (MOPS + glucose), while the SA:V changes were modulated through titratable protein overexpression. *E. coli* cultures with larger total cell volumes, and therefore larger total surface areas, had slower growth rates due to smaller SA:V ratios (Fig. 6F). The mechanism of cell SA:V manipulation used in Basan *et al.* (68) differed from the mechanism used in Si *et al.* (2). However, the changes in growth rate with SA:V were consistent across studies and were consistent with the presented $sMSA$ theory which proposes a positive correlation between membrane catalytic potential per *gcdw* (positive function of cellular SA:V and f_{MSA}) and growth rate. This sensitivity to SA:V was also tested theoretically in prediction 2.

Discussion

The rules of cell design remain elusive despite decades of research. For example, the design rules that link cell geometry and membrane protein capacity with central metabolism have not been defined previously on a quantitative basis for different strains, growing at different rates, with different SA:V ratios, and with different membrane crowding. Bacteria display exquisite control over cellular geometry and membrane properties which are proposed here to be biologically significant, as these properties are major mediators of possible phenotypes (1, 3, 17, 19). *E. coli* cell length and diameter increase with growth rate, reducing the SA:V ratio (Fig. 2B, 2C) (2, 12). However, as shown here, the reduction in enzymatic potential from decreases in SA:V is offset by an increase in the capacity of the membrane to host proteins (Fig 3A, supplementary data S3). This property is hypothesized to be critical to competitive phenotypes; if membrane protein content were not essential to fitness, this protein hosting would likely remain independent of, or decrease with, increasing growth rate (19). This membrane property was integrated into the new, molecular level theory which combines metabolism, cell geometry, and membrane protein crowding. $sMSA$ theory was used to predict maximum growth rates, acetate overflow metabolism, electron transport chain efficiency, and maintenance energy fluxes (6, 24, 43). The predictions were compared to experimental phenomics data, membrane proteomics data, and recombinant strains with titrated SA:V ratios (Fig. 5 and 6). In all cases, the theory was remarkably accurate without accounting for either cytosolic volume or cytosolic macromolecular crowding (6, 12, 24). The presented $sMSA$ theory is flexible and can be readily implemented with numerous systems biology approaches including biochemical pathways analysis (*e.g.* elementary flux mode analysis) (69), resource balance analyses (70, 71), growth law development (31), and other FBA-based approaches (43). Our research efforts are examining a number of these exciting avenues.

Much effort has documented the importance of volume-associated proteome investment and molecular crowding on phenotype (6, 12, 24). Volume-based processes are certainly central to phenotype (5, 6, 9), but we hypothesize that both surface area- and volume-associated processes are critical and concurrently influence phenotype (1). Surface area requirements for membrane enzymes do not correlate well

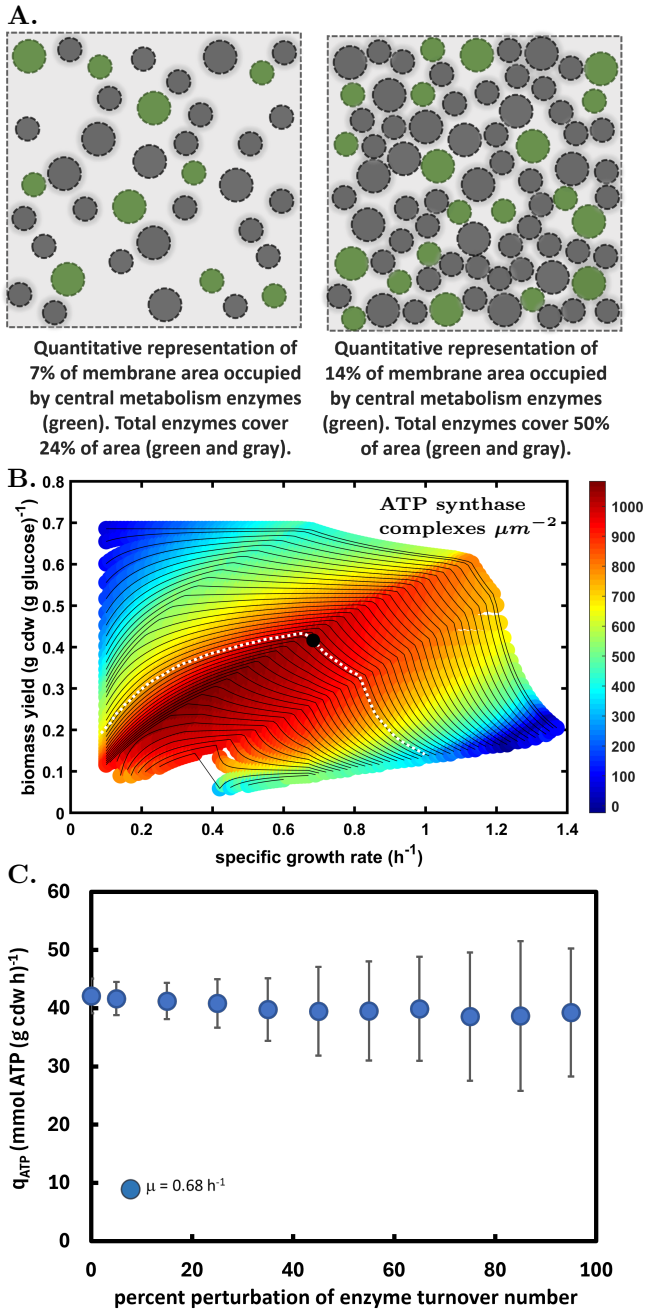


Figure 7: *sMSA* **theory topics and implications. A)** Quantitative representation of 24% and 50% of the membrane surface area being occupied by proteins. Areas are representative of bacteria *E. coli* and eukaryotic mitochondria inner membranes. **B)** Illustration of MG1655 experimental growth rate and biomass yield plotted in *sMSA* constrained phenotypic space. Dotted white line is a maintenance energy isocline. Third axis quantifies the density of ATP synthase complexes per membrane area. Experimental data suggests *E. coli* maximizes biomass yield for a specific maintenance energy requirement. **C)** Perturbation analysis of enzyme parameters and their effect on the potential for maintenance energy (q_{ATP}) production. Experimental fluxes from batch growth of *E. coli* MG1655 on glucose salts media with $f_{MSA} = 0.07$. Enzyme parameters were independently perturbed within ±95% (abscissa) while experimental fluxes were simultaneously and independently perturbed within ± one standard deviation. Each point is the mean of 100 simulations, and error bars represent one standard deviation.

with volume proxies like enzyme mass (Fig. 1C, supplementary data S1); membrane surface area requirements are instead proposed to be distinct biophysical properties which impose unique constraints (17, 24, 43). Tight regulation of cellular geometry and therefore SA:V ratio would arguably be futile if surface area-associated substrate transport and volume-associated protein synthesis were not also tightly regulated. If a single geometric aspect were limiting, evolution could select cells with changes in either cell length or cell diameter to overcome the surface area or volume limitation, respectively. A potential strategy to overcome the limits of geometry is to evolve membranes with higher protein hosting capacities. Mitochondria are eukaryotic organelles specialized in ATP generation (12). While the geometries of mitochondria are similar to bacteria, their membranes can host proteins at areal densities approximately two-fold higher than an *E. coli* membrane (24 ± 5.6 % vs. 40-50%) (4, 12, 72) (reviewed in supplementary data S4) (Fig. 7A). Mitochondria membranes have likely evolved the capacity to host high protein fractions while maintaining membrane integrity because they persist in the relatively constant, safe environment of the buffered eukaryotic cytosol. The role of cell geometries in different bacterial morphologies, *e.g.* bacilli vs. cocci, or the membrane protein capacities of Gram-negative vs. Gram-positive membrane architectures are open questions that can be addressed using *sMSAc-FBA* (12, 73).

sMSAc-FBA predictions assumed an idealized scenario including 1) all enzymes (except PtsG in MG1655 simulations) were saturated for substrate including ETC components and O_2 cytochromes, 2) cells produced optimal levels of each enzyme with no overproduction, 3) all protein complexes were perfectly assembled with exact subunit ratios, 4) all enzymes were flawlessly embedded in the membrane, 5) no allosteric regulation of enzyme activity occurred based on membrane curvature (3, 74), and 6) the enzyme k_{cat} numbers increased two-fold for every 10°C increase in temperature ($Q_{10} = 2$) (60). The curated proteomics data provided a basis for estimating deviations from this idealized scenario. Using a protein offset (Δ, Box 1) of ∼3% quantitatively aligns the *sMSAc-FBA* simulations with the experimental proteomics data without altering any presented interpretations or conclusions. The presented study implicitly included a perturbation analysis of Δ. For example, perturbing Δ for batch growth can be analyzed by examining results for f_{MSA} values larger or smaller than 0.07 (Fig. 5D, 6C).

Computational biology often assumes cells will adopt phenotypes that tend toward a maximum growth rate. This *in silico* growth rate is actually a relative maximum growth rate and not an absolute maximum growth rate. Fig. 7B plots the experimental MG1655 batch growth rate and biomass yield relative to the possible phenotypes based on the presented *sMSA* theory. The experimental data maps to a maximized biomass yield for a fixed maintenance energy demand, not a maximized growth rate. If the cell reduces maintenance energy demand, it could theoretically increase its growth rate while maintaining the same yield. For example, reducing the q_{ATP} requirement by

$8-10$ $mmol\ ATP\ (gcdw \cdot h)^{-1}$ could theoretically increase growth rate by 0.1 h^{-1} (shift experimental data point right $+0.1\ h^{-1}$ while keeping the same biomass yield). This proposed scenario, where a cell grows faster due to a decrease in the area associated with respiration activity (smaller q_{ATP}) and an increase in the area associated with substrate transporters, is consistent with some reported recombinant strain phenotypes (17, 75). Additionally, adaptive laboratory evolution can be used to create *E. coli* strains that grow faster than wild type cells, and some gene deletions can increase growth rate (76, 77). Minimizing maintenance energy demand through reductions in futile cycling may contribute to the phenotypes. These strains increase growth rates, but likely at the expense of robustness. The presented work provides a quantitative framework to assess the changes.

Reported enzyme k_{cat} numbers vary substantially between published studies (supplementary data S1) (78). The presented study performed a literature survey of k_{cat} numbers and applied the same median k_{cat} values to simulations for MG1655 and NCM3722 (supplementary data S4). The robustness of the presented results was analyzed by perturbing the MRE_i parameter which accounted for enzyme properties including k_{cat}, degree of saturation, and surface area requirements (Box 1). The MRE_i parameter was randomly and independently perturbed within ±95% of the base value. Experimental fluxes were also perturbed randomly and independently within a standard deviation of the measured flux. Each scenario was analyzed with 100 simulations (Fig. 7C) (supplementary data S8). Capacity for q_{ATP} was robust to perturbations; calculated q_{ATP} varied $\leq$ 10% for random perturbations of all MRE_i parameters up to ±95% of the base value (Fig. 7C) (supplementary data S8). $sMSA$ theory is robust to the presented assumptions, likely based on the compensating nature of the redundant and parallel structures in metabolic networks (79). Given the robustness of the results, it is probable the parameters can be used in other animalcules with reasonable accuracy.

Additional robustness analyses examined perturbing one MRE_i parameter at a time to assess the effect on q_{ATP}. These analyses considered five membrane-associated enzymes which accounted for $>$ 80% of the predicted surface area during batch growth (ATP synthase, glucose transporter (PtsG), ammonium transporter (AmtB), NADH dehydrogenase (Nuo), and cytochrome oxidase (Cyo); supplementary data S8). Each enzyme MRE_i was perturbed ±35% to increase or decrease the $sMSA$ requirements, respectively. ATP synthase accounted for 45% of the surface area utilized by central metabolism enzymes and was most sensitive to MRE_i perturbations. Increasing the ATP synthase MRE_i parameter 35% while holding all other MRE_i parameters constant decreased the potential for q_{ATP} fluxes by ∼26%, whereas decreasing the MRE_i parameter 35% increased the potential for q_{ATP} fluxes by ∼8%. Perturbing the MRE_i parameter for the other four enzymes individually ±35% did not change the capacity for q_{ATP} fluxes more than ±5% (supplementary data S8).

Biological optimality principles are of keen interest as they illuminate the (pseudo) rules of life and are readily implemented using systems biology methods. The presented study demonstrates that the commonly observed submaximal P/O numbers are likely not suboptimal strategies. Instead, they represent an optimal tradeoff between surface area requirements for substrate transporters and ETC components. Other commonly observed submaximal, yet likely optimized, phenotypes include overflow metabolisms occurring at both high (6, 80) and low growth rates (57), use of the Entner-Doudoroff (ED) glycolysis rather than Embden-Meyer-Parnas (EMP) glycolysis (79), and use of a partial citric acid cycle under nutrient limitation (81). Numerous *in silico* studies have replicated the overflow and ED vs. EMP glycolysis phenotypes using a variety of criteria (6, 14-16, 43, 79, 82). It is probable that many of these criteria are concurrently relevant to cell geometry (24, 43). All the criteria explicitly or implicitly define tradeoffs between resource sufficiency and resource scarcity and can be viewed through a lens of cellular economics. The relative value of a limiting resource is higher than for resources present in excess. Therefore, fitness is increased when use of the limiting resource is optimized (40, 79). The presented $sMSA$ theoretical framework quantifies a metabolic resource defined by cell geometry and membrane protein crowding and elucidates cell design rules which govern selection of competitive phenotypes.

Materials and Methods

Enzyme parameters

Computational parameters including enzyme k_{cat} (i.e. turnover) numbers, enzyme surface area, cellular water content, and cell density for *E. coli* were estimated using literature reviews. Parameter values and references are provided in supplementary data S4. k_{cat} numbers were temperature corrected to 37°C using a Q_{10} number of 2 (60). Analyses assumed all membrane-associated enzymes were saturated ($\varepsilon_i = 1$) during batch growth, as typical concentrations of medium components during batch growth are ∼2 orders of magnitude greater than the average K_M values of *E. coli* enzymes, ∼0.1 mM. During simulation of MG1655 chemostat growth, the saturation parameter (ε_i) for glucose transport (PtsG) was adjusted based on dilution rate.

Metabolic model

The *E. coli* metabolic model was based on published models (40, 41). Every model reaction was balanced for atoms and electrons. The biomass reaction was constructed using theory developed by Neidhardt *et al.*, as described previously (40, 83). The biomass macromolecular composition on a dry mass basis was 78% protein, 10% RNA, 6% DNA, and 6% lipid and polysaccharide (83), the elemental composition was $C_1H_{1.96}O_{0.52}N_{0.28}P_{0.03}$, and the degree of reduction was 4.24 oxidizable electrons per carbon mole of biomass (NH_3 basis). The biomass ATP demand for monomer synthesis and macromolecule polymerization was 36.9 mol ATP per kg dry biomass (40). The biomass reaction did not account for any maintenance energy requirements as those

fluxes were calculated separately. The maximum potential for maintenance energy fluxes (q_{ATP}) was calculated from experimental data by constraining exchange fluxes to consensus data and maximizing flux through the ATP hydrolysis reaction (reaction identifier: $ATPm$). Thus, the calculated maintenance energy fluxes account for both growth- and nongrowth-associated ATP (GAM, NGAM). The same *E. coli* model was used for both *E. coli* strain MG1655 and NCM3722 simulations while applying strain-appropriate SA:V ratios and consensus data (supplementary data S1, S2, S4, S5). Unless otherwise noted, enzyme saturation (ε_i), enzyme over capacity factor (ω_i), and allosteric regulation based on membrane properties (σ_i) were set to 1.

The metabolic model is provided in supplementary data S5. All flux balance analysis simulations used COBRA Toolbox (42) with Gurobi as the solver. FBA is a linear algebra based method of interrogating stoichiometric metabolic models (84). By assuming a (quasi) steady state, FBA identifies a network flux distribution that optimizes an objective function while applying user defined constraints such as bounds on the flux permitted through particular reactions (84). The FBA linear optimization problem is often formalized as:

$$\begin{aligned} &\text{maximize } Z = c^T \nu \\ &\text{subject to } Sv = 0 \text{ and } lb_i \leq v_i \leq ub_i \end{aligned} \qquad \text{(Eqn. 8)}$$

where S is the stoichiometric matrix and defines the stoichiometry of all metabolites in all reactions, ν is the vector of reaction fluxes, and c is the vector specifying the objective function Z such as the flux toward maintenance energy. Fluxes through each reaction ν_i were constrained by assigned lower (lb_i) and upper flux (ub_i) bounds (84).

A summary of the code used in the current study can be found in supplementary data S9. Additional *sMSAcFBA* analyses, code, and reviewer comments from previous submissions can be found at (85).

Algorithm:
1. Perturb consensus fluxes (Fig. 5A) randomly and independently within +/- error interval (μ^*, $q^*_{glucose}$, $q^*_{acetate}$)
2. Solve separate flux balance analysis problem for f_{MSA} = 0.01 - 0.2 by:
   maximize $v_{\text{ATP maintenance}}$
   subject to $Sv = 0$
   $v_{j,biomass} = \mu^*$
   $v_{j,glucose} = q^*_{glucose}$
   $v_{j,acetate} = q^*_{acetate}$
   $lb_k \leq v_k \leq ub_k$ for $k \neq j$ (lb, ub, lower and upper bounds)
   $\varphi(f_{MSA}) \geq \sum_i v_i MRE_i - \beta(fMSA) * \mu*$ (defined in Box1)
3. Repeat 100 times
4. Tabulate mean and standard deviation

Data Availability: All MATLAB code, models, and supplementary data can be found at on github. `github.com/rosspcarlson/carlson2026_sMSAcFBA.git`

Author Contributions: R.P.C. conceived project, performed theory synthesis, developed code, analyzed data, wrote document, T.G., M.G.B., C.B.P., W.R.H., and R.M. analyzed data, developed interpretation, read and edited document, A.E.B. conceived project, developed code, analyzed data, read and edited document.

Competing Interest Statement: The authors have no competing interests.

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Summary of Supplementary Materials

Supplementary Data S1: Literature survey of extracellular flux data for *E. coli* K-12 strains MG1655 and NCM3722 including consensus data sets. Comparison of enzyme volume and membrane surface area requirements for 22 central metabolism enzymes.

Supplementary Data S2: Literature values for cell geometry as a function of growth rate for *E. coli* K-12 strains MG1655 and NCM3722. MreB and FtsZ titration data for MG1655. Protein inflating data for NCM3722.

Supplementary Data S3: *E. coli* K-12 membrane proteomics data from three independent studies collated by Belliveau *et al.* 2021.

Supplementary Data S4: Literature review of enzyme and biophysical parameters used in study.

Supplementary Data S5: *In silico*, stoichiometric model and associated data files.

Supplementary Data S6: Summary of simulation output for *E. coli* K-12 strains MG1655 and NCM3722.

Supplementary Data S7: Summary of experimental and *in silico* P/O number calculations.

Supplementary Data S8: Analysis of maintenance energy fluxes as a function of growth rate, membrane surface area, protein hosting capacity, and P/O number. Perturbation analysis of enzyme *MRE* parameters.

Supplementary Data S9: Summary of MATLAB code used for each figure in study.

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