Economics of Membrane Occupancy and Respiro-fermentation

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 January 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest, and they were generally supportive. However, they raise a series of important concerns, which, we would ask to address in a revision of this work.

In general, the reviewers recognized that the membrane economics model presented in this work could represent a valuable advance. Nonetheless, they had a series of concerns that will require additional clarification, discussion, and in some cases additional analyses, to address. I highlight here some points of particular importance:

1. Point #3 by reviewer #2 seems to require additional statistical analysis of the data from Vemuri et al. If the reduction in yield prior to acetate production is non-significant, then this should be acknowledged in the manuscript and related claims should be tempered.

2. Reviewer #2 felt that additional detail was needed regarding the metabolic model used, the cytochrome relative cost calculations, and the figures. If the metabolic model employed here was modified from Feist et al., then we ask that you supply the full model as supplementary material, and, ideally, also submit this modified model to a public database like BioModels. For the figures, the editor agrees that additional description is needed. In addition, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). We feel that this sort of figure-associated data would be particularly appropriate for this work, and we encourage you to submit supplemental data files for appropriate figure panels. Guidelines have been pasted below.

3. Last reviewer indicated that care should be taken to use cautionary language regarding the conclusiveness of these results (given the lack of direct experimental verification).

We ask that you also address the following format and content issues when submitting your revised work:

1. Please provide three to four 'bullet points' highlighting the main findings of your study.
2. Please provide a 'standfirst text' summarizing the study in one or two sentences (approx. 250 characters).
3. Please provide an extended synopsis as separate file in LaTeX, RTF or MS Word format.
4. Please provide a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight your paper on our homepage.

PLEASE NOTE As part of the EMBO Publications transparent editorial process initiative (see http://www.nature.com/msb/journal/v6/n1/full/msb201072.html), Molecular Systems Biology now publishes online a Review Process File with each accepted manuscript. Please be aware that in the event of acceptance, your cover letter/point-by-point document will be included as part of this file, which will be available to the scientific community. More information about this initiative is available in our Instructions to Authors. If you have any questions about this initiative, please contact the editorial office (msb@embo.org).

If you feel you can satisfactorily deal with these points and those listed by the referees, you may wish to submit a revised version of your manuscript. Please attach a covering letter giving details of the way in which you have handled each of the points raised by the referees. A revised manuscript will be once again subject to review and you probably understand that we can give you no guarantee at this stage that the eventual outcome will be favorable.

Yours sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #1 (Remarks to the Author):

Referee report for ms "Economics of the membrane occupancy and the respiro-fermentation" by Zhuang et al.

The authors present a study on respiro-fermentative metabolism in E. coli. They develop a mathematical model to explain observed patterns in E. coli ATP production, which is based on a cost-benefit analysis for expressing enzymes. In particular, costs are assumed to be driven by the limited capacity of membranes to hold trans-membrane enzymes. Sugar import and respiratory enzymes therefore compete for space on the membrane, while fermentation enzymes do not. I have reviewed an earlier version of this paper and some of my comments have already been addressed.

The theoretical framework is technically solid and well-motivated, and can reproduce a wide range of experimental observations. Importantly, the paper presents a promising way to overcome the limitations associated with FBA. While key assumptions of FBA have been criticized, it remains one of the most successful frameworks in the field. The manuscript by Zhuang et al. is not simply criticizing FBA, but demonstrates how to go beyond its limitations. A similar approaches has been put forward before ("FBA with molecular crowding", see Beg et al, PNAS 2007), but the assumptions of Zhuang et al. are clearly closer to reality, at least for the system under investigation. I therefore believe that this paper is of substantial importance for the field and strongly recommend it for publication in MSB.

I have only one comment: It would be great to see how different membrane compartments for respiration and sugar import due to the presence of mitochondria will affect the model predictions for eukaryotes. While such an analysis does not need to be presented at the same level of detail as given for E. coli, I would definitely recommend an extensive verbal discussion in the Discussion Section.

Reviewer #2 (Remarks to the Author):

The authors propose a hypothesis that could explain a number of physiological observations made in E. coli. The core of the hypothesis is that the growth rate of E. coli is limited by the available membrane surface, and hence, maximal growth is only attained by proper allocation of the limited space to the membrane-located enzymes. The authors claim this membrane constraint explains, among other observations, yield decrease at high glucose concentrations, as well partial fermentation and concomitant acetate formation.

The idea follows up on a number of papers from other labs that try to explain physiological observations from an optimization principle using more fundamental constraints than those applied in FBA. The idea is appealing, and the ensuing model seems to fit experimental data. However, essential details are lacking in the current manuscript. In particular, the calculation of crucial model parameters, as well as the mathematical description of the model are not traceable. A number of other details need explanation.

1. The description of the model is incomplete. Too often, a similar situation has led to the conclusion that results from a published model could not be reproduced in another laboratory. Therefore, it is strongly recommended to deposit complete software code, SBML files, or publish a complete mathematical description. For example, the authors state that the genome scale model by Feist was used "as a platform", leaving, however, unclear to what extent it was modified for their simulations. The authors should guarantee that, without having to make guesses about the model structure, their results can be checked and reproduced by others, now and in the future.

2. Although it is stated in the main text (page 15, "Calculating the relative membrane costs in E. coli") that the calculation of the relative costs for Cyo, Cyd-I, Cyd-II is given in the Supplementary material, it can not be found there. This is a crucial step and must be included! Especially, since the calculation of the relative cost for the glucose transporter, illustrated in the same paragraph, leaves the conclusion that, strictly, not Equation 8 which is an inequality, but an equality was used. The authors should make this explicit, because the assumption is, basically, that the cell always hits the limit of this inequality. Knowing this, suspicion arises about figure 4 (see comment below), which seems to show that the limit of equation 8 is not hit under all experimental conditions. So, what is the basis for assuming that it does under the conditions used for the calculations of the membrane costs?

3. The statements on pages 4 and 8 that reduction of yield starts prior to the onset of acetate production is not warranted by the experimental data from Vemuri et al. If the authors insist on making this claim, and the fact that their model explains it, they should provide statistical evidence that the perhaps extremely tiny decrease from the maximum yield BEFORE the GUR=5 mmol/gdw.hr point (just one measurement, it seems) is statistically significant, given the experimental error. Without this information the statement is just not credible.

4. Figure 1: An explanation of figures 1D and 1E can not be found in the main text. The description in the legend leaves essentially no clues. It seems that parts D and E can be omitted without serious consequences.

5. Figure 3: the origin of the experimental data is obscure. Searching through the text was in vain. The most obvious place to state the origin of data would be the legend to this figure. Also, why not explicitly state which subunits are represented by the different symbols?

6. Figure 4: this figure needs explicit explanation of symbolism. A) What does the central circle represent? B) State the meaning of abbreviations "Glc", "Ac" and "EtOH"; C) The arrows on Glc and O2 seem to indicate consumption/production by the enzymes, so why does the arrow point towards O2 (it's a substrate!) D) What do the bold line segments in the outer circle indicate? Does it

show that under these conditions the membrane is not fully occupied? This is that an important and noteworthy observation, also in the light of comment 2 above.

Reviewer #3 (Remarks to the Author):

The authors propose that there are inherent limitations on the protein content of bacterial membranes which, in some cases, limit the rate at which substrates or oxygen can be utilized. The hypothesis provides a mechanistic explanation to several puzzling phenomena including:
1) Why does E. coli take up sugar faster under anaerobic conditions compared to aerobic conditions?
2) When the cytochromes are removed and the resultant strain is evolved, why does it take up glucose as fast aerobically as anaerobically?
3) Why do cells ferment when respiration is an option?
4) Why is acetate produced during E. coli fermentations when sufficient oxygen and glucose are available for complete respiration?
5) Why are inefficient cytochrome oxidases even present in E. coli?
6) Why does the specific glucose uptake rate decrease in an arcA mutant (Nikel et al, J Bact, Sept 2009, p5538-5548)? - This is not an example in the current paper, but cytochrome oxidases (particularly cytochrome bo oxidase) are repressed by the regulatory gene, arcA. In trying to find a counter example to the authors' theory, I uncovered additional support. If these up-regulated cytochrome oxidases take up precious cellular membrane space, less room would be available for glucose transporters.

One can rationalize each of these phenomena individually. Until reading this manuscript, I had yet to encounter a central theory that provides a mechanistic explanation for all of them. Thus it is possible that the authors have uncovered an extremely important fundamental constraint that governs bacterial metabolism and physiology. It is also possible, though less likely, that this theory is nothing more than a convenient modeling trick that enables better consistency of FBA predictions and experimental data. While I am still not completely convinced without an experimental study specifically geared towards disproving (or proving) this theory, I do recommend publication due to 1) the thought-provoking nature of this hypothesis and 2) the abundance of results that are consistent with it.

I have a few points that I would like to see addressed prior to publication.

1) The secretion of many fermentation products, particularly organic acids, requires membrane-bound transporters. This would place a constraint on fermentation rates just as cytochrome oxidases place a limit on specific oxygen uptake rate. Nevertheless, perhaps organic acid transporters take up far less volume than sugar transporters (particularly the multi-subunit PTS-system for glucose uptake in E. coli) and thus have a small cost. In any event, I would like to see at least some discussion on the role of metabolite exporters in constraining fermentative metabolism.
2) We are given no information on what % of the membrane surface is occupied by glucose transporters and cytochrome oxidases. Additionally, we are given no information on what % of the membrane proteins are glucose transporters and cytochrome oxidases. If only 0.1% of the total surface area (or surface proteins) comprises glucose transporters and cytochrome oxidases, then this theory would seem far less physiologically relevant than if >20% was occupied by such proteins. On p. 4, the authors argue that FBAwMC cannot predict acetate production if the ETS enzymes are removed from the formulation - "membrane-bound enzymes consume little intracellular volume". If ETS enzymes comprise only a minor portion of the total protein content or surface area of the membrane, they should be removed from the current formulation too.
3) The authors must be very careful, and they are for the most part, not to sell the current hypothesis as absolute fact. This is an interesting theory but is nowhere near completely proven. For example, on p. 8, 1st paragraph, last sentence, I recommend changing "which confirms our hypothesis" to "which supports our hypothesis". Additionally, I recommend changing the first line of the discussion from "Our simulations showed that...." to "Our simulations are consistent with the hypothesis that..." Additionally, the claim in the Abstract that "we were able to accurately predict all the observed changes in physiology" should be toned down. Surely there are some physiological changes that are not predicted by this modeling framework.
4) In the abstract, there seems to be a grammatical error (subject-verb agreement). I believe the statement should read, "we proposed that a bacterial cell optimally manages the occupancy of its

cytoplasmic membrane."
5) On p. 8, the authors state that "the new modeling framework - FBA with membrane economics (FBAME) - predicted that ...". They should clarify that such quantitative agreement with uptake rates of glucose and oxygen required tuning the membrane cost parameters with experimental data.
6) I would like the authors to comment on the applicability of this theory to eukaryotic organisms. As the authors note in the first line of the abstract, the puzzling phenomenon of simultaneous fermentation and respiration is observed in non-bacterial systems including yeasts and cancer cells. Can this theory explain why ethanol is formed during yeast fermentation in the presence of oxygen when glucose is in excess? In this case, cytochrome oxidases are on the mitochondrial membrane, not the cytoplasmic membrane. Does this invalidate the current theory?

Other comments:

1) The "Economics of the Membrane Occupancy and the Respiro-fermentation" hypothesis is essentially a form of crowding. However, whereas the authors of the FBAwMC work argue that this molecular crowding occurs in the cytoplasm, Zhuang et al., argue that this crowding occurs at the membrane. In my opinion, this is an extremely important distinction and FBAwMC does not detract from the novelty of this work. Zhuang et al. give proper credit to the FBAwMC authors for proposing that crowding is an important phenomenon while highlighting why they feel that membrane crowding is more physiologically relevant than crowding in the cytoplasm.
2) The main utility of constraint-based metabolic models, in my opinion, is to generate testable hypotheses. The proposed theory serves this purpose very well. For example, the first question listed above is highly important as volumetric productivity of industrial fermentations is directly related to how fast production organisms metabolize substrate. This paper suggests that perhaps productivities of bacterial fermentations under aerobic or microaerobic conditions can be increased by down-regulating costly cytochrome oxidases. Perhaps this would work, perhaps not, but scientific understanding of bacterial physiology will grow either way.

1st Revision - authors' response 25 February 2011

Editor's Comment:

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the three referees whom we asked to evaluate your manuscript. As you will see from the reports below, the referees find the topic of your study of potential interest, and they were generally supportive. However, they raise a series of important concerns, which, we would ask to address in a revision of this work. In general, the reviewers recognized that the membrane economics model presented in this work could represent a valuable advance. Nonetheless, they had a series of concerns that will require additional clarification, discussion, and in some cases additional analyses, to address. I highlight here some points of particular importance:

- We thank the editor for his comments during this review process.
- We have made revisions to our manuscript and addressed all of the reviewer 's comments point by point as detailed below.

1. Point #3 by reviewer #2 seems to require additional statistical analysis of the data from Vemuri et al. If the reduction in yield prior to acetate production is non-significant, then this should be acknowledged in the manuscript and related claims should be tempered.

- The reviewer is correct to suggest statistical analysis for this yield reduction.
- We found that while we cannot claim that the yield decrease prior to acetate secretion is statistically significant, we are certain that acetate secretion is not the sole contributor to the overall yield decrease.
o A subtle decrease in yield was observed prior to the onset of acetate secretion in two out of three chemostat experiments (between D=0.2 hr^{-1} and D=0.3 hr^{-1}, Figure SI-6); however, this

decrease is not statistically significant when taking all three experiments into account.
o On the other hand, a statistically significant (T-test, P=0.08) yield decrease is observed between D=0.3 hr^{-1} and D=0.4 hr^{-1} (Figure SI-6). The acetate secretion began at D=0.4 hr^{-1}at a minute level of 0.0152 mmol/gdw/hr. The overall yield decrease between D=0.3 hr-1 and D=0.4 hr^{-1} (0.014 g/mmol based on average yields) is much larger than the yield decrease due to acetate secretion (0.0039 g/mmol, based on stoichiometric calculation using FBA), indicating that the respiratory efficiency decreased independently of acetate secretion.
o FBAME explains this decrease in respiratory efficiency: as the membrane becomes saturated, the costly Cyo is replaced by the cheaper but less efficient Cyd-II, leading to an increased ATP production rate at the expense of respiratory efficiency. The timing of this replacement is dependent on the membrane cost parameters - it is possible that this replacement occurs more gradually in vivo.
o The replacement of Cyo by Cyd-II is also evident from gene expression data (Figure 3A, B from main manuscript).
- We have included this discussion in the Supplementary Information under the section heading "Evidence for Decrease in Respiratory Efficiency".
- We have tempered our claim in the main manuscript: instead of claiming that the yield decreased prior to acetate production, we claim that the respiratory efficiency decreased independent of acetate production.
o On page 4, line 25, we modified the sentence to "However, there is some experimental evidence (Supplementary Information) suggesting that the efficiency of the respiratory pathway itself may be compromised due to the utilization of less-efficient dehydrogenases and cytochromes."
o One page 9, line 2, we modified the sentence to "and the utilization of Cyd-II explains the subtle decrease in respiratory efficiency observed in experiments"

2. Reviewer #2 felt that additional detail was needed regarding the metabolic model used, the cytochrome relative cost calculations, and the figures. If the metabolic model employed here was modified from Feist et al., then we ask that you supply the full model as supplementary material, and, ideally, also submit this modified model to a public database like BioModels. For the figures, the editor agrees that additional description is needed. In addition, we provide a new functionality that allows readers to directly download the 'source data' associated with selected figure panels (e.g. <http://tinyurl.com/365zpej>). We feel that this sort of figure-associated data would be particularly appropriate for this work, and we encourage you to submit supplemental data files for appropriate figure panels. Guidelines have been pasted below.

- We have included additional details in describing the model itself as well as the calculation of the relative cost.
- We will submit the metabolic model we used along with our revision as a downloadable MATLAB file to be used along with the COBRA toolbox.
- We will submit the experimental data used in our analysis as source data for the figures as shown in the example.

3. *Last reviewer indicated that care should be taken to use cautionary language regarding the conclusiveness of these results (given the lack of direct experimental verification).*

- We agree that this study lacks direct experimental verification and have made the changes suggested by the reviewer in order to remain cautionary in our language.

Reviewer #1 ‘s Comment:

The authors present a study on respiro-fermentative metabolism in E. coli. They develop a mathematical model to explain observed patterns in E. coli ATP production, which is based on a cost-benefit analysis for expressing enzymes. In particular, costs are assumed to be driven by the limited capacity of membranes to hold trans-membrane enzymes. Sugar import and respiratory enzymes therefore compete for space on the membrane, while fermentation enzymes do not. I have reviewed an earlier version of this paper and some of my comments have already been addressed.

The theoretical framework is technically solid and well-motivated, and can reproduce a wide range

of experimental observations. Importantly, the paper presents a promising way to overcome the limitations associated with FBA. While key assumptions of FBA have been criticized, it remains one of the most successful frameworks in the field. The manuscript by Zhuang et al. is not simply criticizing FBA, but demonstrates how to go beyond its limitations. A similar approaches has been put forward before ("FBA with molecular crowding", see Beg et al, PNAS 2007), but the assumptions of Zhuang et al. are clearly closer to reality, at least for the system under investigation. I therefore believe that this paper is of substantial importance for the field and strongly recommend it for publication in MSB.

- We thank the reviewer for both this and the previous review. The reviewer 's previous review has provided valuable feedback for us to strengthen our manuscript.

I have only one comment: It would be great to see how different membrane compartments for respiration and sugar import due to the presence of mitochondria will affect the model predictions for eukaryotes. While such an analysis does not need to be presented at the same level of detail as given for E. coli, I would definitely recommend an extensive verbal discussion in the Discussion Section.

- Given that the membrane constraint is mechanistic in nature, it should affect eukaryotes cells as well as prokaryotes. Eukaryotes are generally larger than prokaryotes. The increase in volume offers eukaryotes the needed 3-dimensional real estate necessary for the greater level of complexity and organization; the downside is that increase in volume leads to a significant reduction in S/V ratio. The necessity for additional membrane real estate in larger cells is a possible explanation for the ubiquitous existence of mitochondria in eukaryotes.
- In eukaryotes, the cytochromes are located in the mitochondrial inner membrane whereas the glucose transporters are located in the cytoplasmic membrane. Interestingly, a positive correlation between the respiratory rate and the mitochondrial membrane area has been observed in both S. cerevisiae (Visser et al. 1995) and hummingbirds (Bicudo and Zerbinatti, 1995), and numerical analysis suggests that the glucose uptake rate of S. cerevisiae may be limited by the cytoplasmic membrane area (Phillips and Milo, 2009).
- While these reports suggest that the mitochondrial and cytoplasmic membrane area may be important constraints of eukaryotic physiology, additional studies are required to determine whether the membrane economics theory alone is sufficient to explain the respiro-fermentation phenomenon in eukaryotes. Nonetheless, one can certain imagine a case where the mitochondrial membrane is filled with cytochromes and the cell is forced to ferment for further ATP production.
- We are currently working on modeling eukaryotes using the membrane constraint and plan to discuss this in more detail in a future publication.
- We have included some of these discussions in the revised manuscript (page 12, under the section name "membrane constraints in eukaryotes").

Reviewer #2 (Remarks to the Author):

The authors propose a hypothesis that could explain a number of physiological observations made in E. coli. The core of the hypothesis is that the growth rate of E. coli is limited by the available membrane surface, and hence, maximal growth is only attained by proper allocation of the limited space to the membrane-located enzymes. The authors claim this membrane constraint explains, among other observations, yield decrease at high glucose concentrations, as well partial fermentation and concomitant acetate formation.

The idea follows up on a number of papers from other labs that try to explain physiological observations from an optimization principle using more fundamental constraints than those applied in FBA. The idea is appealing, and the ensuing model seems to fit experimental data. However, essential details are lacking in the current manuscript. In particular, the calculation of crucial model parameters, as well as the mathematical description of the model are not traceable. A number of other details need explanation.

- We thank the reviewer for the comments. We have made some modifications to our manuscript based on the comments, including more detailed description of the modeling framework

and parameter calculations. We will address the comments point by point in the following sections.

- We also thank the reviewer for bringing the BioNumbers paper to our attention. It has really helped us addressing some comments from reviewer #3.

1. The description of the model is incomplete. Too often, a similar situation has led to the conclusion that results from a published model could not be reproduced in another laboratory. Therefore, it is strongly recommended to deposit complete software code, SBML files, or publish a complete mathematical description. For example, the authors state that the genome scale model by Feist was used "as a platform", leaving, however, unclear to what extent it was modified for their simulations. The authors should guarantee that, without having to make guesses about the model structure, their results can be checked and reproduced by others, now and in the future.

- We have indeed modified the Feist model (iAF1260) in order to include the cytoplasmic membrane budget constraint, as well as the Cyd-II reaction (which was missing in the iAF1260 model).
- The Cyd-II reaction was also introduced to the FBA model and the FBAwMC model we used in order to compare these three models fairly. The introduction of this reaction made no difference to FBA predictions (which does not use less-efficient pathways) and made negligible difference to the FBAwMC predictions.
- We have added the detailed description of how we modified the iAF1260 model in the SI.
- We have uploaded the MATLAB/COBRA Toolbox script we used to create the FBA^{ME} model, as well as the resultant COBRA Toolbox model file.
- We have changed the sentence referred to by the reviewer as follows: "To illustrate that the "membrane economics" theory could satisfactorily explain the physiological changes associated with the respiro-fermentation phenomenon in E. coli, we modified the genome-scale metabolic model of E. coli (Feist et al, 2007) to include the cytoplasmic membrane constraint." (page 7, line 11).

2. Although it is stated in the main text (page 15, "Calculating the relative membrane costs in E. coli") that the calculation of the relative costs for Cyo, Cyd-I, Cyd-II is given in the Supplementary material, it can not be found there. This is a crucial step and must be included! Especially, since the calculation of the relative cost for the glucose transporter, illustrated in the same paragraph, leaves the conclusion that, strictly, not Equation 8 which is an inequality, but an equality was used. The authors should make this explicit, because the assumption is, basically, that the cell always hits the limit of this inequality. Knowing this, suspicion arises about figure 4 (see comment below), which seems to show that the limit of equation 8 is not hit under all experimental conditions. So, what is the basis for assuming that it does under the conditions used for the calculations of the membrane costs?

- We agree with the reviewer that the original description of the calculation of the relative membrane cost parameters is unclear, potentially leading to misunderstanding of our parameter estimation process.
- We did not use Inequality 11 (this was erroneously cited as Eq 8 in the original manuscript) directly as an equation with the assumption that the limit of the inequality is hit (i.e., assuming equality). Rather, Inequality 11 is used as a constraint to depict the scaled available membrane area as an upper bound (this constraint is what differentiates FBA^{ME} from FBA). Having the growth rates and uptake rates of different cytochrome knockouts allowed us to vary the membrane cost parameters of glucose transporter and cytochrome oxidases one by one until our model is capable of predicting the measured uptake rates for each mutants.
- Unsaturated membrane only occurs at very low glucose uptake rate. In wild-type E. coli, the saturation occurs at GUR above 3.2 mmol/gdw/hr. However, in NDH-I knockout strains (strains used to determine the cytochrome cost parameters), saturation occurs at GUR above 2.2 mmol/gdw/hr; this is because NDH-II is non-energetic and more ETC flow is required to satisfy maintenance requirement. Since the lowest experimental GUR used to determine the parameter is 2.3 mmol/gdw/hr, it is safe to assume the membrane is saturated.
- We have added a detailed section in SI describing the parameter estimation process.

3. The statements on pages 4 and 8 that reduction of yield starts prior to the onset of acetate

production is not warranted by the experimental data from Vemuri et al. If the authors insist on making this claim, and the fact that their model explains it, they should provide statistical evidence that the perhaps extremely tiny decrease from the maximum yield BEFORE the GUR=5 mmol/gdw.hr point (just one measurement, it seems) is statistically significant, given the experimental error. Without this information the statement is just not credible.

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4. Figure 1: An explanation of figures 1D and 1E can not be found in the main text. The description in the legend leaves essentially no clues. It seems that parts D and E can be omitted without serious consequences.

- Figures 1D and 1E are intended to show that conventional FBA never predicts the utilization of inefficient pathways such as Cyd-II, where as FBAME is capable of such predictions.
- We have included references for both of these figures.
- We have modified our manuscript at the page 8, line 26 to read: "In addition, FBAME was able to predict the reduction of the TCA cycle activities at higher uptake rates (Figure 3C,D) as well as the selective expression of Cyo and Cyd-II at lower uptake rates (Figure 1E, 3A,B), whereas conventional FBA cannot predict the expression of inefficient Cyd-II (Figure 1D)."
- We have included in the figure legend the sentence: "The shape of the solution space is different between FBA and FBA^{ME}; the utilization of Cyd-II is predicted by FBAME, but never predicted by FBA."

5. Figure 3: the origin of the experimental data is obscure. Searching through the text was in vain. The most obvious place to state the origin of data would be the legend to this figure. Also, why not explicitly state which subunits are represented by the different symbols?

- We have added the origin of the data and indicated which subunits are represented by which symbol in the legend of Figure 3 of the updated manuscript.

6. Figure 4: this figure needs explicit explanation of symbolism. A) What does the central circle represent? B) State the meaning of abbreviations "Glc", "Ac" and "EtOH"; C) The arrows on Glc and O2 seem to indicate consumption/production by the enzymes, so why does the arrow point towards O2 (it's a substrate!) D) What do the bold line segments in the outer circle indicate? Does it show that under these conditions the membrane is not fully occupied? This is that an important and noteworthy observation, also in the light of comment 2 above.

- We have redrawn this figure in order to make it more clear to the readers.
- See our response for comment #2 regarding the membrane saturation issue.

-

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6) Why does the specific glucose uptake rate decrease in an arcA mutant (Nikel et al, J Bact, Sept 2009, p5538-5548)? - This is not an example in the current paper, but cytochrome oxidases (particularly cytochrome bo oxidase) are repressed by the regulatory gene, arcA. In trying to find a counter example to the authors' theory, I uncovered additional support. If these up-regulated cytochrome oxidases take up precious cellular membrane space, less room would be available for glucose transporters.

One can rationalize each of these phenomena individually. Until reading this manuscript, I had yet to encounter a central theory that provides a mechanistic explanation for all of them. Thus it is possible that the authors have uncovered an extremely important fundamental constraint that governs bacterial metabolism and physiology. It is also possible, though less likely, that this theory is nothing more than a convenient modeling trick that enables better consistency of FBA predictions and experimental data. While I am still not completely convinced without an experimental study specifically geared towards disproving (or proving) this theory, I do recommend publication due to 1) the thought-provoking nature of this hypothesis and 2) the abundance of results that are consistent with it.

I have a few points that I would like to see addressed prior to publication.

- We thank the reviewer for the excellent comments. In particular, we thank the reviewer for the fantastic summary of our manuscript as well as uncovering additional support for our paper.
- We have included the arcA mutant case in a new sub-section in the Discussion section of the revised manuscript.
- We have addressed the reviewer ‘s comments point by point in the following sections.

1) The secretion of many fermentation products, particularly organic acids, requires membrane-bound transporters. This would place a constraint on fermentation rates just as cytochrome oxidases place a limit on specific oxygen uptake rate. Nevertheless, perhaps organic acid transporters take up far less volume than sugar transporters (particularly the multi-subunit PTS-system for glucose uptake in E. coli) and thus have a small cost. In any event, I would like to see at least some discussion on the role of metabolite exporters in constraining fermentative metabolism.

- We have indeed made the simplifying assumption that the transport of fermentation products out of the cell does not consume membrane area. The primary reason for this simplification is that we were unable to ascertain the membrane cost of acetate transport. The only known E. coli acetate transporter is a permease coded by ActP (EcoCyc, Gimenez 2003); however,

while it has been demonstrated that this protein is responsible for the uptake of acetate into the cell (Gimenez 2003), it is unclear whether this enzyme is used during acetate excretion. It is possible that fermentation products such as acetate are transported out of the cell through diffusion, a process that depends on the pH and the concentration difference between the two sides of the membrane (Gimenez 2003, Butler 2004).

- Nonetheless, we have simulated the glucose limited chemostat growth of E. coli using a FBA^{ME} model with assumed membrane costs for acetate transport. Our simulations suggest that as long as the acetate transport cost is below 7% of the glucose transport cost, the effect of its inclusion is negligible when the GUR is less than the experimentally measured maximum of 10.7 mmol/gdw/hr (Fig SI-5).

If we assume that acetate transporter for secretion is monomeric (that is the case for ActP), then its membrane cost should be much smaller than the large glucose transport systems (PtsG has 6 subunits per complex, PtsX has 7 subunits per complex) and the very large (22 subunits) ATP synthase required by respiration.

- As such, we feel that our simplifying non-inclusion of the fermentation transport cost is justified.
- We have included this discussion in the "Model Simplification and Consequences" section of the SI.

2) We are given no information on what % of the membrane surface is occupied by glucose transporters and cytochrome oxidases. Additionally, we are given no information on what % of the membrane proteins are glucose transporters and cytochrome oxidases. If only 0.1% of the total surface area (or surface proteins) comprises glucose transporters and cytochrome oxidases, then this theory would seem far less physiologically relevant than if >20% was occupied by such proteins. On p. 4, the authors argue that FBAwMC cannot predict acetate production if the ETS enzymes are removed from the formulation - "membrane-bound enzymes consume little intracellular volume". If ETS enzymes comprise only a minor portion of the total protein content or surface area of the membrane, they should be removed from the current formulation too.

- Philips and Milo (2009) have estimated that glucose transporters cover 4% of the total surface area. This is a low estimate because it only considers the carbon requirement for glucose and ignores the energy requirement for glucose. Assuming 50% of the membrane is composed of lipids, then glucoses transporters can cover up to 8% of the available membrane area. (We would like to thank reviewer #2 for bringing this to our attention).
- We have made similar estimates for the fraction of membrane area occupied by glucose transporters and the ETC components:
  - At the highest oxygen uptake rate (18 mmol/gdw/hr), about 15% of E. coli ‘s cytoplasmic polypeptides are cytochromes.
  - Under microaerobic conditions, about 11% of E. coli ‘s cytoplasmic polypeptides are cytochromes.
  - At the optimal growth rate, based on simulated ATP requirement suggests that about 13% of cytoplasmic polypeptides are ATP synthase during optimal aerobic growth.
- Detailed description of our calculations is given in the section "Estimation of Membrane Protein Content" in Supplementary Information.
- We have included a section in the manuscript discussing these estimates.

3) The authors must be very careful, and they are for the most part, not to sell the current hypothesis as absolute fact. This is an interesting theory but is nowhere near completely proven. For example, on p. 8, 1st paragraph, last sentence, I recommend changing "which confirms our hypothesis" to "which supports our hypothesis". Additionally, I recommend changing the first line of the discussion from "Our simulations showed that...." to "Our simulations are consistent with the hypothesis that..." Additionally, the claim in the Abstract that "we were able to accurately predict all the observed changes in physiology" should be toned down. Surely there are some physiological changes that are not predicted by this modeling framework.

- We agree with the reviewer on using more cautious language in the manuscript.
- We have made the suggested changes in the revised manuscript.
- We have changed the wording of the abstract to "we were able to accurately predict a variety of observed changes".

4) In the abstract, there seems to be a grammatical error (subject-verb agreement). I believe the statement should read, "we proposed that a bacterial cell optimally manages the occupancy of its cytoplasmic membrane."

- We have made the suggested changes in the revised manuscript.

5) On p. 8, the authors state that "the new modeling framework - FBA with membrane economics (FBAME) - predicted that ...". They should clarify that such quantitative agreement with uptake rates of glucose and oxygen required tuning the membrane cost parameters with experimental data.

- We have changed the sentence to "Using relative membrane costs calculated from experimental data, we the new modeling framework - FBA with membrane economics (FBA^{ME}) - predicted that ..."

6) I would like the authors to comment on the applicability of this theory to eukaryotic organisms. As the authors note in the first line of the abstract, the puzzling phenomenon of simultaneous fermentation and respiration is observed in non-bacterial systems including yeasts and cancer cells. Can this theory explain why ethanol is formed during yeast fermentation in the presence of oxygen when glucose is in excess? In this case, cytochrome oxidases are on the mitochondrial membrane, not the cytoplasmic membrane. Does this invalidate the current theory?

- Given that the membrane constraint is mechanistic in nature, it should affect eukaryotes cells as well as prokaryotes. Eukaryotes are generally larger than prokaryotes. The increase in volume offers eukaryotes the needed 3-dimensional real estate necessary for the greater level of complexity and organization; the downside is that increase in volume leads to a significant reduction in S/V ratio. The necessity for additional membrane real estate in larger cells is a possible explanation for the ubiquitous existence of mitochondria in eukaryotes.
- In eukaryotes, the cytochromes are located in the mitochondrial inner membrane whereas the glucose transporters are located in the cytoplasmic membrane. Interestingly, a positive correlation between the respiratory rate and the mitochondrial membrane area has been observed in both S. cerevisiae (Visser et al. 1995) and hummingbirds (Bicudo and Zerbinatti, 1995), and numerical analysis suggests that the glucose uptake rate of S. cerevisiae is limited by the size of the its cytoplasmic membrane (Phillips and Milo, 2009).
- While these reports suggest that the mitochondrial and cytoplasmic membrane area may be important constraints of eukaryotic physiology, additional studies are required to determine whether the membrane economics theory is sufficient to explain the respiro-fermentation phenomenon in eukaryotes. Nonetheless, one can certainly imagine a case where the mitochondrial membrane is filled with cytochromes and the cell is forced to ferment for further ATP production.
- We are currently working on modeling eukaryotes using the membrane constraint and plan to discuss this in more detail in a future publication.
- We have included some of these discussions in the revised manuscript (page 12, under the section name "membrane constraints in eukaryotes".

Other comments:

1) The "Economics of the Membrane Occupancy and the Respiro-fermentation" hypothesis is essentially a form of crowding. However, whereas the authors of the FBAwMC work argue that this molecular crowding occurs in the cytoplasm, Zhuang et al., argue that this crowding occurs at the membrane. In my opinion, this is an extremely important distinction and FBAwMC does not detract from the novelty of this work. Zhuang et al. give proper credit to the FBAwMC authors for proposing that crowding is an important phenomenon while highlighting why they feel that membrane crowding is more physiologically relevant than crowding in the cytoplasm.

2) The main utility of constraint-based metabolic models, in my opinion, is to generate testable hypotheses. The proposed theory serves this purpose very well. For example, the first question listed above is highly important as volumetric productivity of industrial fermentations is directly related to how fast production organisms metabolize substrate. This paper suggests that perhaps productivities of bacterial fermentations under aerobic or microaerobic conditions can be increased by down-regulating costly cytochrome oxidases. Perhaps this would work, perhaps not, but scientific understanding of bacterial physiology will grow either way.

We thank the reviewer for these comments and we have included the practical implication in our revised manuscript.

2nd Editorial Decision 21 March 2011

Thank you again for submitting your work to Molecular Systems Biology. We have now heard back from the two referees who agreed to evaluate this revised study. In general the referees are supportive; however, an important concern regarding how the membrane cost parameters were estimated has arisen, which the reviewers feel must be addressed before this work would be appropriate for publication.

Reviewer #2 notes that it appears that the membrane costs cannot be uniquely estimated without assuming the percent saturation of the membranes -- a relatively strong assumption that s/he felt needs to be clearly acknowledged. More troubling perhaps, this reviewer's calculations suggest to him/her that different saturation levels may have been used when estimating the various costs.

Molecular Systems Biology now encourages referees to comment on each other's reports, and during this process Reviewer #3 agreed with this concern, writing:

"I now understand and share reviewer #2's concern about the derivation of the cost parameters. In order for the cost coefficients to take on unique values, the Sum v_i * C_i <= 1 constraint must be binding (i.e., Sum v_i * C_i = 1). The authors should reveal the flux values for the GUR, cyd-I, cyd-II, and cyo for the simulations where C_(GUR), C_(cyd-I), C_(cyd-II), and C_(cyo) are estimated. If true, they should also mention the implicit assumption of 100% membrane saturation."

While both reviewers seem optimistic that this issue may be addressable with additional clarification, they both felt that this issue was of fundamental importance. As such, we may send any revised work back to one or both of these reviewers to confirm that this issue has been rigorously addressed.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #2 (Remarks to the Author):

Most of the questions that were raised about the initial version of the manuscript were properly addressed by the authors, except that the details of the "membrane cost" parameter estimation process revealed in the revised MS raises serious doubt about its validity. Correct membrane cost parameter values are at the hart of the model: they determine the predictions made by the model. This is why it is crucial to understand how these parameters are exactly determined, and why I describe my understanding of the process and my doubts in a somewhat lengthy discourse below: I want to make sure that we understand each other correctly. But briefly, my objections are:

1. The mathematical structure of the model does not allow unique determination of the cost parameters unless additional assumptions, not mentioned in the paper, are made.
2. The parameter values obtained and used in the paper seem to have an aspect of arbitrariness, perhaps due to the way the parameters are estimated, or the implementation of the model in Matlab.

The description of the parameter estimation procedure by the authors, in their letter as well as the revision, leaves the impression that it was a manual or perhaps programmatic scanning or trial and error procedure (the authors are not explicit here). This is fine, as long as it is guaranteed to lead to UNIQUE VALUES. Which is precisely the problem with the procedure revealed in the revision, because to my understanding it does not lead to unique values unless additional assumptions are made.

Let me first summarize the FBAME model of the authors in two equations:

1) dm/dt = Nv

2) Sum v_i * C_i <= 1 ("<=" means "less than or equal to")

Equation 1) is the classical FBA equation in matrix notation where m is a vector of metabolite concentrations, N the reaction stoichiometry matrix and v the vector of reaction rates. Assuming quasi steady-state for intracellular metabolites, and knowing a number of consumption and production rates of external metabolites allows a solution in which many of the rates v can be uniquely determined. Equation 2) is the membrane cost constraint for membrane-located reactions added by the authors in the current paper. It imposes additional constraints on the vector of rates v, once the cost parameters C_i are known. These cost parameters are estimated by the authors from a specific set of experiments. However, since Eq 2) (Eq. 11 in the paper) is an inequality, and since this is the only equation in which the cost parameters appear, they can not be determined uniquely. Eq 2) only imposes an upper bound to the cost parameters!

One could assume additionally that in the set of experiments used for estimating the cost parameters the membrane is completely filled with proteins leading to the equality:

3) Sum v_i * C_i == 1

which would, in principle, allow unique determination of the C_i. However, the authors mention nowhere that they make such an assumption. To the contrary, in their rebuttal they explicitly say that they use the inequality 2).

I would like to illustrate my objections by going through the estimation of three of the cost parameters:

- The authors deduce the C_{GUR} of the glucose uptake carrier from the experiment in which E. coli is grown anaerobically under glucose excess. In this experiment the rate (v_{GUR}) was equal to 18 mmol/gdw.h, and the autors deduce a C_{GUR} = 0.0556 gdw.h/mmol. This is actually equal to 1/18, and is hence equivalent with the equality 3). However, this value of C_{GUR} can not be unique, since it is not constrained by any other equation in the model and any positive value below 0.0556 would have satisfied the inequality 2). So the implicit assumption in the derivation of this parameter was that the membrane is saturated under the given experimental condition!

If it subsequently leads to quantitatively correct predictions, such an assumption is acceptable, as long as it is explicitly mentioned in the paper! Of course, the hypothesis needs to be proven experimentally at some point.

However, now turning to the other cost parameters I observe the following problems:

- C_{CYO} was derived from an experiment where the glucose uptake rate was equal to 2.3 mmol/gdw.h and the O_2 uptake rate was equal to 6.4 mmol/gdw.h. From their Matlab files it can be deduced that their model calculates with cytochrome oxidase rates in oxygen atom equivalents due to a stoichiometric parameter of 0.5 for O_2, so the cytochrome oxidase rate in the constraint would be 12.8 mmol/gdw.h. The C_{CYO} was determined by the authors to be 0.0658. Substituting in Sum v_i*C_i:

2.3x0.0556 + 12.8x0.0658 = 0.970

Although this satisfies the inequality, so would all positive values for C_{CYO} below 0.0681. Why

a total degree of membrane saturation of 0.97 was chosen is unclear.

- C_{CYD-1} was derived from an experiment where the glucose uptake rate was equal to 3.2 mmol/gdw.h and the O_2 uptake rate was equal to 8.8 mmol/gdw.h. The C_{CYD-1} was determined by the authors to be 0.0427. Substituting:

3.2*0.0556 + 17.6*0.0427 = 0.879

However, again any positive value below 0.0496 for C_{CYD-1} would have satisfied inequality 2), and the reason why particularly a value of the total degree of membrane saturation of 0.879 was chosen is unclear. My suspicion is that this apparently arbitrary choice for cytochrome oxidase parameters may have been an artifact of the parameter variation during fitting or of the way in which constraint 2) was implemented in the Matlab code of the model.

Finally, I would like to stress that I am in favor of publishing this idea. But I also think that the crucial numbers and details have to be correct before giving it a final destination.

Reviewer #3 (Remarks to the Author):

The authors have adequately addressed all of my concerns. I am particularly satisfied with the provided calculations and estimates describing how crowded the membrane is with transporters and respiratory chain enzymes. Clearly a significant portion of the membrane is comprised of these enzymes and it is reasonable to conclude that they compete for space. The commentary on how metabolism in eukaryotes may be affected by membrane crowding is thought-provoking and I look forward to follow-up studies aimed at addressing this in more detail. I also welcome the additional details that describe how the authors arrived at the membrane costs for the glucose transporter and cytochrome oxidases. This information, along with the supplementary materials, should make duplicating the simulations relatively straightforward.

2nd Revision - authors' response 08 April 2011

At the outset, we thank the reviewers for such an insightful and thorough review which has led to significant improvements in the paper. However, it seems that the clarity of our description of the parameter estimation process in the first revision was insufficient, leading to some confusions. We would like to address the points reviewer #2 and #3 brought up in this document.

Summary

- Reviewer #2 and #3 were absolutely correct in saying that unless the constraint $\sum C_i v_i$ is binding (equal to 1), we cannot acquire unique values for our Ci parameters.
- However, $\sum C_i v_i = 1$ cannot be assumed prior to simulation because this may lead to erroneous results when simulating certain experimental conditions (namely, conditions when the membrane is not saturated).
- To acquire unique values for Ci parameters, $\sum C_i v_i = 1$ can and must arise as a simulation result. This is the crux of the discussion; we will discuss this point at length.
- In all our simulations for parameter acquisition, FBAME predicts $\sum C_i v_i$ to be binding (equal to 1). This differs from Reviewer #2's calculation because he/she had used measured average oxygen uptake rates, which contained uncertainties. Instead, the predicted oxygen uptake rates should have been used in the calculation.

o Our selection criteria for the cytochrome C* parameter is that it must allow FBAME to simultaneously predict a growth rate of 0.15 hr-1 and an oxygen uptake rate that falls within one standard deviation of the measured average oxygen uptake rate. (0.15 hr-1 is the dilution of the

chemostat experiments we used to calibrate the cytochrome C* parameters).
o We realized that the wording of our first revision may be misleading, suggesting that we have only used oxygen uptake values instead of both growth rates and oxygen uptake values to calibrate the parameters, and that the oxygen uptake values were matched perfectly.
o We have reformulated our description, and we have revealed the predicted growth rates and fluxes during our calibration simulation (we thank reviewer #3 for this suggestion). We'll discuss these values later in this document.
- We have made significant modifications to Section 7 of the Supplementary Materials in hope to improve the clarity of our wordings.

Binding-ness of the Membrane Constraint Civi

Mathematically, the formulation of FBAME is:

maximize growth rate flux
such that

$$\sum \frac{dm}{dt} = Nv = 0 \quad \text{(R1)}$$

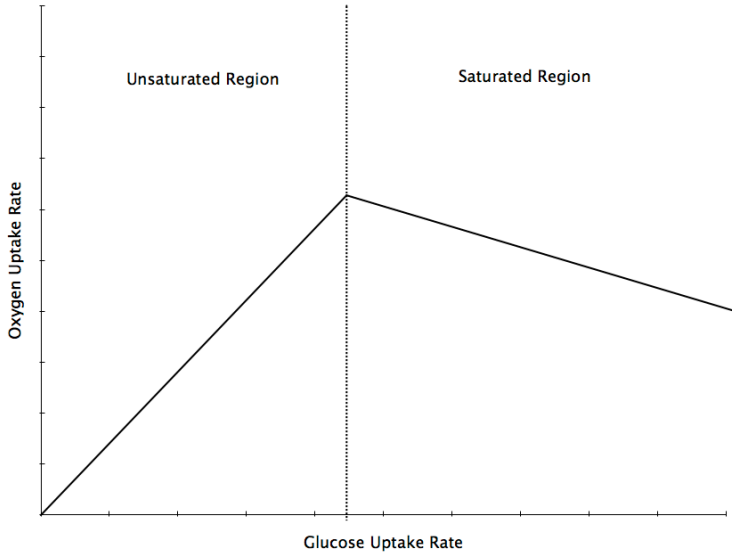
$$\sum v_i \, C_i^* \leq 1 \quad \text{(R2)}$$

$$v_{glc} \leq v_{glc}^{max} \quad \text{(R3)}$$

- R1 represents the reaction network. "=0" is the pseudo-steady-state assumption. R1 provides no information regarding the reaction fluxes.
- R2 is the membrane constraint.
- R3 is the glucose uptake constraint.
o This constraint is used when simulating the glucose-limited growth in a chemostat. (eg. when we 're calibrating for cytochrome costs).
o This constraint is not used when simulating batch growth where the glucose uptake rate is not limiting. (eg. when we're calibrating for glucose transporter cost).

In cases where R3 is not used, the membrane constraint $\sum C_i v_i$ (R2) is the only constraint that limits the fluxes through the metabolic network. In this case, it makes no difference if an "=" sign or an "<=" sign was used, because we know implicitly that the linear program solver will return an optimal solution where the constraint Civi is binding. In this case, the question of whether $\sum C_i v_i = 1$ is assumed or not is an issue of semantics; however, in cases where R3 is used, the assumption of $\sum C_i v_i = 1$ can create real problems.

In cases where R3 is used, there 's no way for us to determine whether R2 or R3 is the limiting constraint. We'll illustrate this with the following figure:



This figure is an illustration of the glucose uptake rate vs oxygen uptake rate graph for a knockout strain containing only 1 cytochrome grown in glucose-limited chemostat. In the region before the dotted line, the membrane is unsaturated and Civi<1; in the region after the dotted line, the membrane is saturated and Civi=1. During the calibration process for the cytochrome costs, since we do not know the C* parameters before hand (hence no values on the axis), we cannot know which region does a given set of experimental data fall into.

If the experiment falls into the saturated region:
- R2 is the limiting constraint
- Similar to the case where R3 is not used, we know implicitly that FBA^{ME} will return an optimal solution where $\sum C_i v_i = 1$.
- In this case, we are able to acquire unique values for C* parameters.

However, if the experiment falls into the unsaturated region:
- R3 is the limiting constraint
- FBAME will also work if $\sum C_i v_i <= 1$ is used, but we will not acquire unique values for the C* parameters through this simulation.
- However, in this case, if $\sum \mathbf{C_i v_i = 1}$ is assumed, FBAME will never correctly predict the growth rate and oxygen uptake rate because this assumption does not agree with the reality that the membrane is unconstrained.

o As a side note, conventional FBA will predict the result just fine in this case since the membrane constraint is not limiting.

Since we cannot determine which is the case, we do not feel comfortable making the assumption that $\sum C_i v_i$ is binding and setting $\sum C_i v_i = 1$. However, we can tell very easily which is the case after we run the simulations; we were fortunate that all the experiments we used for calibration purpose falls into to the saturated region as the conventional FBA cannot predict the growth rate and the oxygen uptake rate correctly.

Despite our arguments above, we realize that this is an issue of semantics: we have modified the manuscript to be as forthcoming as we can about this issue; however, if the reviewers feel strongly that we should call this an implicit assumption, we will.

Estimation of C^*_{GUR}
- Under anaerobic glucose-excess condition, E. coli has an oxygen uptake rate of 18 mmol/gdw/h and a growth rate of 0.45 hr-1 (Portnoy 2008).
- The glucose uptake flux is left unconstrained to simulate the glucose excess condition (R3 is not used). We manually varied C* until FBA^{ME} predicted the correct oxygen uptake rate.
- At C^*_{GUR}=0.0556 g h/mmol, FBA^{ME} predicted an oxygen uptake rate (OUR) of 18

mmol/gdw/hr and a growth rate of 0.44 hr^{-1}.

- In this case, the membrane is predicted to be saturated: $\sum v_i C^*_i = 0.0556 \times 18 = 1$. As mentioned above, this is a simulation result, not an assumption. viC*i is equal to 1 because the growth of the organism is limited by membrane availability, and the organism will use every bit of the available membrane to express proteins beneficial to its growth.

o It is important to note that the value "18" here is the predicted oxygen uptake rate, not the measured uptake rate.

Estimation of C^*_{CYO}

- In the Bekker (2009) study used to determine the cytochrome costs, the cytochrome knockouts are grown with a fixed growth rate of 0.15±0.1 hr-1 in glucose-limited chemostats. For the Cyo strain (cyd1 cyd2), the measured oxygen uptake rate was 6.4±0.4 mmol/gdw/h, and the measured glucose uptake rate was 2.3 mmol/gdw/h.
- To determine C^*_{CYO}, we constrained the glucose uptake rate to 2.3 mmol/gdw/hr (R3), and manually varied C* until FBA^{ME} predicted the both the oxygen uptake rate and the growth rate correctly.

o This description of this process was poorly worded in the last revision: we believe it had erroneously implied that only the measured oxygen uptake rate was used to determine C*CYO. We apologize for the confusion.

- At $C^*_{CYO} = 0.0658$ g h/mmol, FBA^{ME} predicted that the Cyo strain would have a growth rate of 0.15 hr-1 and an oxygen uptake rate of 6.7 mmol/gdw/hr. 6.7 mmol/gdw/hr is within one standard deviation of the measured oxygen uptake rate, which is 6.4±0.4 mmol/gdw/h.

o The value $C^*_{CYO} = 0.0658$ g h/mmol is unique in that it is the only value that allowed FBAME to predict a growth rate of 0.15 and an oxygen uptake rate that falls within one standard deviation of the measured oxygen uptake rate.

- In this case, as in the C^*_{GUR} case, the membrane is predicted to be saturated: $\sum v_i C^*_i = 0.0658 \times 6.7x2 + 0.0556 \times 2.3 = 1$.

o This value differs from Reviewer #2 ‘s calculation because he/she had used the measured average oxygen uptake rate (6.4) instead of the predicted oxygen uptake rate (6.7). This is partly our fault because we did not explicitly state the predicted oxygen uptake rate.

Estimation of $C^*_{CYD\text{-}I}$

- For the Cyd-I strain (cyo cyd2), the measured oxygen uptake rate was 8.8±1.3 mmol/gdw/h, the measured glucose uptake rate was 3.2 mmol/gdw/h, and the growth rate was again fixed at 0.15±0.1 hr-1.
- To determine C^*_{CYO}, we constrained the glucose uptake rate to 3.2 mmol/gdw/hr (R3), and manually varied C* until FBA^{ME} predicted the both the oxygen uptake rate and the growth rate correctly.
- At $C^*_{CYO} = 0.0427$ g h/mmol, FBA^{ME} predicted that the Cyd-I strain would have a growth rate of 0.15 and an oxygen uptake rate of 9.62 mmol/gdw/hr. 9.62 mmol/gdw/hr is within one standard deviation of the measured oxygen uptake rate, which is 8.8±1.3 mmol/gdw/h.
- In this case, as in the C^*_{GUR} case, the membrane is predicted to be saturated: viC*i = $0.0427 \times 9.62◊2 + 0.0556 \times 3.2 = 1$.

o Again, this value differs from Reviewer #2 ‘s calculation because he/she had used the measured average oxygen uptake rate (8.8) instead of the predicted value (9.62). We apologize for the lack of clarity in our previous revision.

Estimation of $C^*_{CYD\text{-}II}$

- For the Cyd-II strain (cyo cyd1), the measured oxygen uptake rate was 11.1±0.6 mmol/gdw/h, the measured glucose uptake rate was 6 mmol/gdw/h, and the growth rate was again fixed at 0.15±0.1 hr-1.
- During the calibration for Cyd-II, an additional rational was introduced:

o Unlike the mutant containing Cyo only, which grew at a very low glucose uptake rate, the knockout containing Cyd-II only grew at a high glucose uptake rate of 6 mmol/gdw/h and consumes oxygen at a rate of 11 mmol/gdw/h. At this glucose uptake rate, the "membrane economics theory" predicts that Cyo and Cyd-II share the cell membrane of wild-type E. coli. Assuming the genetic programming remain functional in the knockout strain (since the cells were not given time to evolve in the experiment), and expression level of Cyd-II is expected to be the same in the knockout strain as in the wild-type growing at a glucose-uptake rate of 6 mmol/gdw/h. Therefore, to calibrate for the Cyd-II cost, C*CYD-II value is varied until 11.1±0.6 mmol/gdw/h of oxygen is processed

through Cyd-II.
o At C^*_{CYD-II} = 0.0128 g h/mmol, FBAME predicted that the wildtype would have an oxygen uptake rate of 14.38 mmol/gdw/h. The flux through Cyo is 5.63 mmol/gdw/h, the flux through Cyd-I is 0, and the flux through Cyd-II is 23.12 mmol/gdw/h (11.55 mmol/gdw/h of oxygen, which falls within 11.1±0.6 mmol/gdw/h).
ï Here, $viC^*i = 5.63\times0.0658+23.12\times0.0128+6\times0.0556 = 1$
o As a validation, we used C^*_{CYD-II} = 0.0128 g h/mmol to predict the growth of Cyd-II mutant with oxygen uptake rate of 11.1 and glucose uptake rate of 6. FBAME predicted a growth rate of 0.152 hr-1.

Uncertainties in the Parameters
- There are uncertainties in the experimental data, particularly in the measured oxygen uptake rates. We have performed sensitivity analysis on the C* parameters to deal with this issue (see Supplementary Information).

Modifications in the Manuscript
- We have made significant modifications to Section 7 of the Supplementary Materials in hope to improve the clarity of our wordings.

3rd Editorial Decision 11 April 2011

Thank you again for submitting your revised work to Molecular Systems Biology. We have now heard back from the reviewer who agreed to evaluate your revised work. This reviewer is now supportive of publication, although s/he still feels that the assumptions underlying the membrane cost estimates could benefit from some additional clarification.

The editor found your explanations in your most recent response letter to be very helpful in clarifying these issues, and the editor asks that explanations of this nature be directly included into the main manuscripts results, discussion, and methods sections, as appropriate. Whether or not this is directly described as a "strong assumption," I think it will be important to explain to the readers that the membrane costs are estimated from conditions where membrane occupancy is believed to be fully saturated and limiting (as supported by your simulations), and that saturation is a requirement for unique cost estimation.

We ask that you address this in a final minor revision of this work, and also address the following format and content issues:

1. Please supply high-resolution images for each figure in the main manuscript in TIFF, EPS, or PDF formats. The resolution of Fig. 4, and to a lesser degree Fig. 3, is currently rather low.

2. Please supply a "thumbnail image" (width=211 x height=157 pixels, jpeg format), which can be used to highlight your paper on our homepage.

3. The supplemental figures do not need to be supplied separately if they are included in the supplementary information PDF.

4. Thank you for supplying the Matlab and COBRA source model files. If possible and appropriate, we strongly encourage you to also supply this model in SBML, and to submit it to a public repository like BioModels or JWS Online.

Please resubmit your revised manuscript online, with a covering letter listing amendments and responses to each point raised by the referees.

Thank you for submitting this paper to Molecular Systems Biology.

Yours sincerely,

Editor
Molecular Systems Biology

REFEREE REPORTS

Reviewer #2 (Remarks to the Author):

I do not agree with the conclusion by the authors that the implicit binding assumption for membrane saturation is a matter of semantics only, because I still do not understand how unique Ci* could have been arrived at from inequalities only, unless their binding was explicitly assumed (perhaps not by the authors but by the software?) during the calibration. But I think the problems have been discussed between reviewers and authors extensively enough, and should now be continued in the scientific community. The basic hypothesis of the paper, as well as the comparisons of its predictions with experimental data are certainly worth publishing.

3rd Revision - authors' response 26 April 2011

I am writing to submit the revised version of the manuscript titled "Economics of Membrane Occupancy and Respiro-fermentation" for publication in the Articles section of Molecular Systems Biology in the Subject Categories of "Metabolic and regulatory Networks" and "Cellular Metabolism".
Based on the reviewer ‘s comments, we have modified the materials and methods section (Page 16 Lines 6-19, Section on "Calculating the Relative Membrane Costs in E. coli") to include the discussion on membrane constraints. We have also included a sentence in the discussion to highlight the importance of measuring membrane saturation to directly validate this hypothesis. In addition, we have also modified the SI to include details on the parameter estimation process and have included a high resolution version of Figure 4. We have also uploaded an SBML file in the SI and will upload the file into the Biomodels database post publication.

We hope that with these changes, the revised manuscript is suitable for publication in Molecular Systems Biology.

