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Peer Review File

The double-edged role of FASII regulator FabT in *Streptococcus pyogenes* infection

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript titled, "The double-edged role of FASII regulator FabT in Streptococcus pyogenes infection" by Lambert et al investigates the dichotomy of GAS FabT mutants emerging in vivo in the context of these mutants being defective in pathogenesis. The authors test this dichotomy in a FabT H105Y point mutant that results in altered membrane lipid composition relative to the WT strain, validating that this mutation has a functional impact. The mutant resulted in lower overall burden during ex vivo growth on human decidua, human cells as well as cell conditioned supernatants. The growth kinetics revealed that the H105Y mutant showed rapid mortality compared to the WT strain. The authors suggest that these defects in overall growth of the mutant could explain its virulence defects in vivo observed in prior studies. They argue that the cost for these mutants is higher metabolic consumption that does not translate into increase in bacterial yield. In contrast, the lipid rich environments confer a growth advantage to these mutants providing a clue as to their emergence. The authors show that supplementation of exogenous fatty acids results in the emergence of FabT variants. This is an interesting study focused on an important topic which has implications for other bacterial species as well. The experiments are well-designed and the manuscript is well written. However, concerns regarding data overinterpretation in some instance exist. Further, certain controls are missing, addition of these will add more rigor to the study.

Major Comments –
1. Lines 131-140, Fig S4: The data convincingly shows that the fab genes are co-transcribed. However, there is not enough data to support authors' conclusions that these genes are also differentially regulated. Can the authors quantify this via qRT-PCR?
2. Fig 2 and Supp Table 4: The conclusions from RNA-seq dataset are unclear –
a. Why is there no difference in fab gene expression in mFabT vs the WT when grown in THY? It is clear that this has a functional impact since there is a difference in membrane lipid composition as shown by the authors?
b. Some of the virulence genes are differentially regulated in mFabT relative to the WT in THY media. If these are due to changes in fab gene expression, it is unclear why there is no difference in the expression of these genes in the THY-tween condition? Should the effects not be exacerbated in this case?
c. Lines 158-160: The H105Y point mutant still seems to regulate fab locus as evidenced by membrane lipid composition and fab gene expression. As such it does not "lose control of FASII", and it still acts a repressor. Please rephrase.
d. The complete RNA-seq dataset is not included in the manuscript – it will be worthwhile to include this as well.
3. Lines 217 – 218: There is no difference in the CFU count of the mutant vs WT at 4hr but a slight reduction at 8h. The authors conclude that the mutant grows slower than the WT. However, it is possible that the mutant growth peaks more quickly (ie before 8 hrs) and it starts to die by this time point. In agreement, the authors see more dead mFabT cells at the 8h time point relative to the WT cells. It will be worth to test another time point between 4 and 8hrs to be able to conclude that the mutant strain grows slower than the WT.
4. Lines 266-269: What is the frequency of mutation in FabT in the tested conditions? What was the total number of colonies screened? How many of those harbored a mutation (and which one)?
5. Lines 268-269: Do these mutations matter? Do they impact the function of FabT? Do they influence either fab gene expression or membrane lipid composition?

Minor Comments –

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2. Lines 108-110: Is there any difference in viable CFU count between mFabT and WT cells at the OD tested? Is it possible that there is lower overall lipid yield in mFabT cells grown in THY just because there are fewer viable cells in this condition?
3. Lines 116-118: How would this result in a reduced overall membrane lipid content?
4. Lines 173-174: Is the thickness referring to the average thickness over the biomass or

maximum thickness of these structures?
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7. Lines 235, 248-250: The lower proportion of eFA incorporation into the membrane seems to be a result of continued FASII activity in the mutant and not really the mutant being "less responsive" or "defective" in eFA incorporation. Current phrasing seems misleading.

Reviewer #2 (Remarks to the Author):

The manuscript by Lambert et al. tried to reconcile the spontaneous selection of fabT (a FASII fatty acid synthesis pathway repressor) mutants in Streptococcus pyogenes in vivo, even though such mutants are less virulent. They thoroughly characterized a representative fabT mutant and found that fabT mutant (mFabT) has a growth advantage in lipid (saturated fatty acid)-rich muscle over WT that is dependent on active FASII, but they grow poorly on tissues, cells, or other supplements that are presumably lacking lipids. Mechanistic insights were provided with RNA sequencing, lipidomics, metabolomics, and other phenotype characterization using in vitro and ex vivo models. The significance of this work lies in that it can potentially explain the occurrence of non-virulent mFabT mutant in vivo, but I have some major concerns that need to be addressed.

1. The author generated a delta-fabT deletion mutant to compare with the endogenously selected fabT mutant, called mFabT. However, while mFabT grew similarly to WT in THY and THY-Tween, delta-fabT grew slower than the WT. If mFabT indeed have a loss of its FASII repressor function as the delta-fabT, why these two strains grow differently compare with WT? The same growth or live/dead analyses should be done for mFabT and delta-fabT for experiments in Supplementary Fig. 2.
2. In the analysis of membrane lipids by mass spectrometry, the fatty acid compositions (only the sum of all fatty acid carbon and unsaturation were shown in Supplementary Table 2) of major lipids were not determined to show that the differences in FA composition between WT and mFabT were reflected in the lipids. MS/MS fragmentation of major lipids is needed to show the specific FA composition.
3. If mFabT grew similarly as the WT, why it has about 60% of overall lipids compared to WT?
4. Differences in the images in 3B between WT and mFabT are not clear. In fact, the 1 hr and 4 hr images for mFabt are the same.
5. The authors showed that mFabT has a growth advantage in lipid-rich muscle over WT, but they grow poorly on human decidua, human endometrial cells, and undifferentiated and differentiated keratinocytes. However, the lipid content in these tissues and cells was not determined in comparison with muscle (the authors claimed it has 15% fats).
6. The authors claimed that the growth defect of mFabT is due to the over-consumption of some amino acids and hexoses relative to WT, but what is the explanation for mFabT leading to the over-consumption of amino acids and hexoses? It is not clear from RNAseq results that changes in gene expression of amino acid or hexose metabolism were observed. Furthermore, based on the data in Supplemental Table 5, the levels of those significantly changed amino acids (Asn, Ile, Lys, Ser) actually did not decrease that much (mostly <10%; Ser actually did not decrease) relative to Conditioned Supernatant (SN). This seems to contradict the authors' claim that mFabT over-consumes these nutrients.
7. It is puzzling to see that C14:0 and C16:0 inhibit WT growth, but not mFabT growth, because C16:0 is a major fatty acid of WT GAS. On the other hand, it would be revealing to also examine the effect of C18:0 and unsaturated FA, such as C18:1, on the growth of WT and mFabT strains because both are part of host FA composition. Does exposure to C18:1 also select for fabT mutation?
8. In the figure legend of Fig. 5d, it seems that White and black arrows are labeled wrong in the legend: white should be inhibition, and black should be growth, according to the main text.
9. Is it possible that there is another factor in the muscle that inhibits the bacterial growth and that such factor has synergy with decreased activity or the inhibition of FASII by platensimycin?

Reviewer #3 (Remarks to the Author):

The fatty acid synthesis pathway FASII in Streptococcus pyogenes is feedback-controlled by the FabT repressor. FabT defects have been linked to reduced virulence in animal models, but spontaneous fabT mutants can arise in vivo. In the manuscript titled "The double-edged role of FASII regulator FabT in Streptococcus pyogenes infection," Lambert et al elucidate the apparent contradiction between the emergence of attenuated fabT variants in vivo and the need for an active FabT repressor during infection.

The authors conducted detailed analyses of the features of both wild-type (WT) bacteria and a representative fabT mutant under different conditions relevant to host infection. They found that the fabT mutant is metabolically inefficient, which prevents it from causing infection. Additionally, environments rich in saturated fatty acids exert selection pressure against WT bacteria expressing active FabT. The authors demonstrated that the growth of fabT mutants is stimulated in lipid-rich muscle sources in a FASII-dependent manner, while the growth of WT bacteria is inhibited.

Therefore, the authors confirmed that FabT represents a promising target for new therapeutics against specific Gram-positive pathogens, including Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus pneumoniae, and Enterococcus faecalis. This study contributes significantly to our understanding of the biological function of FabT. However, I have some concerns regarding the work that I would like the authors to address.

Main questions
1. Are fabT(H105Y) mutants and delFabT mutants similar in their regulation of fatty acid synthesis? If they are, please provide the explanation for the growth discrepancy in THY-Tween medium.
2. Please test the phenotype of fabT(T65M) and determine if its phenotype is consistent with that of fabT(H105Y).
3. Please provide the growth analysis of fabT(H105Y) in THY and THY-Tween medium.

Minor questions
1. Line 23, the fatty acid (FA) synthesis pathway FASII...., correct to the type II fatty acid (FA) synthesis pathway (FASII)...
2. In the “Bacterial strains and culture conditions” section, change mg.ml-1, µg.ml-1, ng.ml-1 to mg/ml, µg/ml, ng/ml.
3. Line 462, “bacterial bacterial...” delete one.
4. In Supplementary Fig. 1a, the unsaturated fatty acids synthesized by FabM are cis, not trans.

We thank the reviewers for their provocative comments, suggestions and questions, which we believe have led to an improved manuscript. All the comments are addressed (in blue), and experiments were added to answer questions when useful.

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Major Comments –
1. Lines 131-140, Fig S4: The data convincingly shows that the fab genes are co-transcribed. However, there is not enough data to support authors' conclusions that these genes are also differentially regulated. Can the authors quantify this via qRT-PCR?

We thank Reviewer for this suggestion. A main insight in Fig. S4 is that a predicted double FabT binding site precedes only the transcript encoding *fabK* (Fig. 2a schema); this correlates to qRT-PCR results in revised Supplementary Fig. 4a (also relevant to point 2). In THY, *fabK* is the only gene differentially expressed in the mFabT *versus* WT strain, as we reported in manuscript Ref 2. These results demonstrate a differential regulation between *fabK* and the other FASII genes The text was updated to include this information, which was added in Supplementary Figure 4a.

2. Fig 2 and Supp Table 4: The conclusions from RNA-seq dataset are unclear –
a. Why is there no difference in fab gene expression in mFabT vs the WT when grown in THY? It is clear that this has a functional impact since there is a difference in membrane lipid composition as shown by the authors?

The data is clarified in answer to Reviewer's correct comment: RNAseq values for FASII gene differences were not statistically significant in THY. We now present the values and corresponding p-values in Supplementary Table 4, legend. As mentioned for point 1, qRT-PCR in Revised Supplementary Fig 4 shows that the differences in the *fabK* to *fabM* ratio are significant, and resolves the discrepancy raised by Reviewer.
Of note, the qRT-PCR tests show that *fabK* expression is 4-fold greater in mFabT than in the WT in THY. Increased *fabK* expression can account for the difference in length and saturation observed between mFabT and WT strains: FabT expectedly has little effect on FASII expression in the absence of exogenous FAs; nevertheless, the double FabT binding site upstream of *fabK*, and the presence of low amounts of free FAs, are suggested to explain greater susceptibility to the FabT status.

b. Some of the virulence genes are differentially regulated in mFabT relative to the WT in THY media. If these are due to changes in fab gene expression, it is unclear why there is no difference in the expression of these genes in the THY-tween condition? Should the effects not be exacerbated in this case?

We thank Reviewer for this question. In THY medium, the mFabT mutation led to longer FA chain lengths (determined as the C18 to C16 ratio; Supplementary Table 1) than in WT. In THY-Tween, these ratios are the same in mFabT and WT (it increases for WT). In keeping with the higher C18:C16 ratio in WT, expression of several virulence genes in THY-Tween is decreased (Supplementary Table 4). In the revised manuscript, we point out these considerations, indicate that virulence genes lack FabT binding motifs, and suggest that expression differences may be an indirect consequence of these membrane differences.

c. Lines 158-160: The H105Y point mutant still seems to regulate fab locus as evidenced by membrane lipid composition and fab gene expression. As such it does not "lose control of FASII", and it still acts a repressor. Please rephrase.

To be sure of the correct wording, we performed direct comparisons of mFabT and Δ*fabT* membrane FA profiles in the absence or presence of eFAs (in revised Supplementary Table 1 and new Supplementary Fig. 6). In the presence of exogenous FAs C18:1Δ9 source (Tween 80) or C14:0, Δ*fabT* produces a greater proportion of longer chain FAs, and incorporates less C18:1Δ9 than the mFabT strain. We modified the phrasing as recommended by Reviewer to now write that FabT^{H105Y} is defective for FASII repression. Note that this relates to Reviewer 3 Main comment #1.

d. The complete RNA-seq dataset is not included in the manuscript – it will be worthwhile to include this as well.
Accordingly, transcriptomic data was deposited in the Mendeley database https://data.mendeley.com/datasets/68bhhsy2p4/1 .

3. Lines 217 – 218: There is no difference in the CFU count of the mutant vs WT at 4hr but a slight reduction at 8h. The authors conclude that the mutant grows slower than the WT. However, it is possible that the mutant growth peaks more quickly (ie before 8 hrs) and it starts to die by this time point. In agreement, the authors see more dead mFabT cells at the 8h time point relative to the WT cells. It will be worth to test another time point between 4 and 8hrs to be able to conclude that the mutant strain grows slower than the WT.

The possibility that mFabT growth peaks and then decreases was evaluated using the Live-Cell Analysis System (IncuCyte®), which detects both live and dead bacterial bodies. Growth of WT and mFabT was followed on conditioned supernatants in triplicate experiments (shown in revised Supplementary Fig. 2f). The number of mFabT cells continued increasing, indicating that new bacteria continue being generated throughout the growth period, even if bacteria are dying. This added experiment shows that there is no peak in the number of mFabT bacteria.

4. Lines 266-269: What is the frequency of mutation in FabT in the tested conditions? What was the total number of colonies screened? How many of those harbored a mutation (and which one)?

FabT^{T65M} was the sole mutation arising during the THY-C14:0 liquid culture procedure (8 clones sequenced, all the same mutation) and a single mutant, mutation FabT^{G99S}, was found when

sequencing the six clones appearing during selection on C14:0 solid medium. This is now stated in the Methods section.
We note that mutation frequency is difficult to estimate. Growths were carried out for 60 hours before obtaining mutants. When 60 h growth was done in THY-C14:0 liquid medium, a single mutation, found in eight sequenced clones, was obtained from a culture initially seeded with $4x10^6$ cfus, suggesting that sister mutant strains emerged prior to isolation. Our best approximation would then be $\leq 2.5x10^{-7}$. When the selection was performed directly on THYA-C14:0, a single mutant clone appeared, suggesting a frequency of $6.6x10^{-8}$. We can thus grossly estimate the mutation frequency to be around 10^{-8}. We decided not to discuss mutation frequencies in the manuscript due to the uncertainty of these calculations.

5. Lines 268-269: Do these mutations matter? Do they impact the function of FabT? Do they influence either fab gene expression or membrane lipid composition?

We addressed this important question by a new experiment (Supplementary Fig. 6 in revised manuscript, as summarized below). Triplicate cultures of the 2 isolated *fabT* mutants ($FabT^{T65M}$ and $FabT^{G99S}$) and controls (WT, mFabT, Δ*fabT*) were prepared in BHI medium supplemented or not with 100 µM C14:0. Consistently with results in Fig. 5b, C14:0 addition blocked WT but not mFabT growth; we now add that growth of all *fabT* mutants was unaffected by C14:0 addition. Despite poor growth of the Δ*fabT* strain, C14:0 did not affect growth. FA profiles of the WT strain showed significant incorporation of C14:0 (~53% of total FAs), in contrast to the 4 tested mutant strains. These results answer Reviewer's question, and strengthen the relevance of the *fabT* mutants arising in the presence of saturated FAs as preventing toxicity.

Minor Comments
1. Lines 89-91: Please show data comparing the growth of mFabT and WT strains in both THY and THY-Tween as shown for ΔfabT. This data seems to be missing from the manuscript.

This information is presented in revised Supplementary Fig. 2a-b.

2. Lines 108-110: Is there any difference in viable CFU count between mFabT and WT cells at the OD tested? Is it possible that there is lower overall lipid yield in mFabT cells grown in THY just because there are fewer viable cells in this condition?

The lipid yield would expectedly be similar regardless of the live/dead bacterial status. Nevertheless, we observed no difference in viable CFU counts in THY and THY-Tween between mFabT and WT cells at the tested ODs (shown for information below).

Table. OD_{600} and the corresponding CFU/ml of samples analyzed for lipids.

	OD_{600}				CFU/ml			
	Cultivated in THY		Cultivated in THY-Tween		Cultivated in THY		Cultivated in THY-Tween	
	WT	mFabT	WT	mFabT	WT	mFabT	WT	mFabT
Sample n°1	0.505	0.550	0.586	0.512	3.70E+07	5.35E+07	3.90E+07	9.35E+07
Sample n°2	0.547	0.527	0.492	0.546	5.20E+07	9.10E+07	5.43E+07	7.53E+07
Sample n°3	0.463	0.576	0.55	0.608	2.54E+07	5.10E+07	6.03E+07	2.18E+07
Sample n°4	0.535	0.506	0.509	0.579	7.43E+07	1.07E+08	6.17E+07	9.87E+07

3. Lines 116-118: How would this result in a reduced overall membrane lipid content?

We cannot definitively answer this question, but suggest that the different FA composition might affect extraction efficiency. We feel that this information deserves mention in the text, and we note that further study will be needed to understand this observation.

4. Lines 173-174: Is the thickness referring to the average thickness over the biomass or maximum thickness of these structures?

We refer to the average thickness of the bacterial structure over the endometrial tissue. This is now specified in Fig. 3c right legend. Information is now added in Materials and Methods to indicate how measurements were performed.

5. Lines 194: missing reference to Fig 3e.

Corrected.

6. Lines 227-231: Is it possible that the higher metabolic consumption is due to the faster growth of the mutant relative to WT but a lower bacterial yield just results from higher mortality of this strain? It is worth discussing this possibility.

We thank Reviewer for suggesting discussion of this point, which is also addressed in revised manuscript and Supplementary Fig. 2f (also see point 3 above). Our studies did show greater mFabT mortality than WT in nutrient-limited medium (e.g., conditioned supernatants). However we have no evidence for its faster growth, but rather a continued growth with accrued mortality that is markedly greater than in the WT (revised Supplementary Fig. 2f). We now add "Futile energy loss in mFabT might be further due to expression changes associated with its altered membrane FA composition, and continued FASII synthesis, leading to increased mortality in nutrient-limited biotopes. These factors might account for the diminished capacity of *fabT* mutants to cause infection (Supplementary Fig. 2f).".

7. Lines 235, 248-250: The lower proportion of eFA incorporation into the membrane seems to be a result of continued FASII activity in the mutant and not really the mutant being "less responsive" or "defective" in eFA incorporation. Current phrasing seems misleading.

The chapter heading now reads "Continued FASII activity in the mFabT mutant leads to lower eFA incorporation". We also simplify the concluding statement: "Poor eFA incorporation in the mFabT mutant thus correlates with continued expression of FASII genes."

Reviewer #2 (Remarks to the Author):

The manuscript by Lambert et al. tried to reconcile the spontaneous selection of fabT (a FASII fatty acid synthesis pathway repressor) mutants in Streptococcus pyogenes in vivo, even though such mutants are less virulent. They thoroughly characterized a representative fabT mutant and found that fabT mutant (mFabT) has a growth advantage in lipid (saturated fatty acid)-rich muscle over WT that is dependent on active FASII, but they grow poorly on tissues, cells, or other supplements that are presumably lacking lipids. Mechanistic insights were provided with RNA sequencing, lipidomics, metabolomics, and other phenotype characterization using in vitro and ex vivo models. The significance of this work lies in that it can potentially explain the occurrence of non-virulent mFabT mutant in vivo, but I have some major concerns that need to be addressed.

1. The author generated a delta-fabT deletion mutant to compare with the endogenously selected fabT mutant, called mFabT. However, while mFabT grew similarly to WT in THY and THY-Tween, delta-fabT grew slower than the WT. If mFabT indeed have a loss of its FASII repressor function as the delta-fabT, why these two strains grow differently compare with WT? The same growth or live/dead analyses should be done for mFabT and delta-fabT for experiments in Supplementary Fig. 2.

We thank Reviewer for this relevant comment. Please also see responses on this topic to Reviewer 1's #2c and #5, and Reviewer 3's #1 and #2. We added comparison of growth and FA composition of WT, mFabT, and Δ*fabT* strains, plus the *fabT* mutant strains isolated in this work. FA profiles of both mFabT and Δ*fabT* mutants differ strikingly from that of the WT. However, the Δ*fabT* shows a more pronounced skew toward longer saturated FAs compared to mFabT (C18:0 / C16:0 ratios are 3.7 and 2.3 respectively). As these species comprise about 50% of total FAs, these differences could impact bacterial physiology. Based on these differences, as described in the revised manuscript, we state that the mFabT point mutant is not totally inactive, which would explain Δ*fabT vs* mFabT growth differences. We feel that these experiments provide clear-cut results that resolve Reviewer's question. As the focus of this work is on arising *fabT* point mutants, we did not perform additional live/dead analyses. This data is in revised Supplementary Fig. 6, and the intermediate phenotypes of FabT point mutations (including others in revised Supplementary Fig. 6) compared to Δ*fabT* are now discussed.

2. In the analysis of membrane lipids by mass spectrometry, the fatty acid compositions (only the sum of all fatty acid carbon and unsaturation were shown in Supplementary Table 2) of major lipids were not determined to show that the differences in FA composition between WT and mFabT were reflected in the lipids. MS/MS fragmentation of major lipids is needed to show the specific FA composition.

MS/MS data showing fatty acid composition of MGDG, DGDG, and PG is presented in revised Supplementary Fig. 3. For cardiolipin phospholipids, fatty acid composition is not presented, as assignments were ambiguous, and further analysis would not add relevant information in the context of this work.

3. If mFabT grew similarly as the WT, why it has about 60% of overall lipids compared to WT?

We cannot definitively answer this question, but suggest that the different fatty acid composition might affect extraction efficiency. We feel that this information deserves mention in the text as a point of information, and we note that further study will be needed to understand this observation.

4. Differences in the images in 3B between WT and mFabT are not clear. In fact, the 1 hr and 4 hr images for mFabt are the same.

We thank Reviewer for highlighting the ambiguity in this figure. The images look alike because there was no growth and the images are from the same spot; arrowheads highlighting distinguishing differences have been added (Fig. 3b).

5. The authors showed that mFabT has a growth advantage in lipid-rich muscle over WT, but they grow poorly on human decidua, human endometrial cells, and undifferentiated and differentiated keratinocytes. However, the lipid content in these tissues and cells was not determined in comparison with muscle (the authors claimed it has 15% fats).

In the revised manuscript we provide additional information on lipid content in the two tested biotopes. The 15 % fat value is derived from the supplier's packaging as per initial text. We further added: "This is within the percent fat range estimated for human muscle (8-30 %; in contrast, uterine fluid lipid concentrations are >1000-fold lower [27,28])."

6. The authors claimed that the growth defect of mFabT is due to the over-consumption of some amino acids and hexoses relative to WT, but what is the explanation for mFabT leading to the over-consumption of amino acids and hexoses?
It is not clear from RNAseq results that changes in gene expression of amino acid or hexose metabolism were observed. Furthermore, based on the data in Supplemental Table 5, the levels of those significantly changed amino acids (Asn, Ile, Lys, Ser) actually did not decrease that much (mostly <10%; Ser actually did not decrease) relative to Conditioned Supernatant (SN). This seems to contradict the authors' claim that mFabT over-consumes these nutrients.

We thank Reviewer for raising these challenging questions. As noted, mFabT consumption of hexoses and the indicated amino acids by mFabT is 10% to 15% greater than in WT, yet mFabT grows poorly. Although the differences are modest, they are statistically significant; upregulation of purine synthesis in mFabT (Supplementary Table 4) further supports our statement that the mutant undergoes futile metabolic turnover that does not lead to growth.
In answer to Reviewer's question, two properties of mFabT are hypothesized to explain this futile cycle. 1- The high C18:C16 ratio in mFabT compared to WT (respectively 2.3 to 1) may reduce bacterial fitness by altering activities of membrane components, leading to greater mortality in nutrient-limited medium. 2- Lipid synthesis and FA availability reportedly play a role in regulating metabolic functions (new reference 24). In the revised manuscript, we propose that altered membrane lipid fatty acids and continued FASII synthesis might cause mFabT to be defective for coordinating membrane biogenesis to growth conditions, such that metabolite import remains active even if other growth factors are unavailable. We also note that increased metabolite consumption occurred after 8 h incubation, when growth presumably slows.

7. It is puzzling to see that C14:0 and C16:0 inhibit WT growth, but not mFabT growth, because C16:0 is a major fatty acid of WT GAS. On the other hand, it would be revealing to also examine the effect of C18:0 and unsaturated FA, such as C18:1, on the growth of WT and mFabT strains because both are part of host FA composition. Does exposure to C18:1 also select for fabT mutation?

We appreciate Reviewer's comments and questions on how different FAs affect bacterial physiology. Although C16:0 is a major GAS fatty acid, its presence in non-FA medium is equilibrated by ~70 % other FAs. When C16:0 is added to medium, its proportion in membrane FAs rises to over 60 % (Fig. 5c). We believe it is reasonable to propose that such high levels can perturb various bacterial functions. In experiments not included in this work, we tested C18:0, which had little effect on growth, possibly due to its hydrophobicity and thus poor access to bacteria.
Concerning unsaturated FAs, we observed that C18:1 (the major FA in Tween 80) and C17:1 were both efficiently incorporated into the WT strain without a negative effect on WT growth and FA profiles (Suppl Fig. 2, Suppl Tables 1 and 6, Fig 5A). As growth of WT and mFabT were equivalent, there would be no selective pressure to search for mutants.

8. In the figure legend of Fig. 5d, it seems that White and black arrows are labeled wrong in the legend: white should be inhibition, and black should be growth, according to the main text.

We thank Reviewer for highlighting this inversion, now corrected.

9. Is it possible that there is another factor in the muscle that inhibits the bacterial growth and that such factor has synergy with decreased activity or the inhibition of FASII by platensimycin?

We agree with Reviewer's suggestion that indirect effects, in addition to FASII inhibition by platensimycin or by FabT repression, may contribute to growth inhibition. In discussing Fig. 5, we now state: "We note that indirect effects, consequent to FASII inhibition by platensimycin and/or by FabT repression, may contribute to growth inhibition."

Reviewer #3 (Remarks to the Author):

The fatty acid synthesis pathway FASII in Streptococcus pyogenes is feedback-controlled by the FabT repressor. FabT defects have been linked to reduced virulence in animal models, but spontaneous fabT mutants can arise in vivo. In the manuscript titled "The double-edged role of FASII regulator FabT in Streptococcus pyogenes infection," Lambert et al elucidate the apparent contradiction between the emergence of attenuated fabT variants in vivo and the need for an active FabT repressor during infection.

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Therefore, the authors confirmed that FabT represents a promising target for new therapeutics against specific Gram-positive pathogens, including Streptococcus pyogenes, Streptococcus agalactiae, Streptococcus pneumoniae, and Enterococcus faecalis. This study contributes significantly to our understanding of the biological function of FabT. However, I have some concerns regarding the work that I would like the authors to address.

Main questions
1. Are fabT(H105Y) mutants and delFabT mutants similar in their regulation of fatty acid synthesis? If they are, please provide the explanation for the growth discrepancy in THY-Tween medium.

We thank Reviewer for this question, also addressed in responses to Reviewers 1 (Main comment #2c), and 2 (Main comment #1). Compared to WT, differences in FA profiles are more pronounced with Δ*fabT* than with mFabT (FabT^{H105Y}), as seen in THY, THY-Tween, and THY+C14:0 (revised Supplementary Table 1 and new Supplementary Fig. 6). Differences are consistent with a partial effect of FabT^{H105Y} compared to the absence of FabT (Δ*fabT*). For example, in THY-Tween, the C18:0 to C16:0 ratio was 2 in mFabT, and 5.8 in the Δ*fabT* strain, indicating more processive FASII activity in the latter. The difference between FabT point mutations and FabT deletion are highlighted in the revised manuscript.

2. Please test the phenotype of fabT(T65M) and determine if its phenotype is consistent with that of fabT(H105Y).

We incorporated Reviewer's suggestion by comparing growth and FA profiles of WT, mFabT, Δ*fabT*, and the two *fabT* mutants isolated on C14:0 (expressing FabT^{T65M} and FabT^{G99S}), in new

Supplementary Fig. 6. Of note, in answer to Reviewer, the strains expressing FabT^{H105Y} (in mFabT) and FabT^{T65M} showed equivalent growth and FA incorporation phenotypes.

3. Please provide the growth analysis of fabT(H105Y) in THY and THY-Tween medium.

The growth comparisons as specified are now provided in Supplementary Fig 2a,b.

Minor questions
1. Line 23, the fatty acid (FA) synthesis pathway FASII...., correct to the type II fatty acid (FA) synthesis pathway (FASII)...

Corrected

2. In the "Bacterial strains and culture conditions" section, change mg.ml-1, µg.ml-1, ng.ml-1 to mg/ml, µg/ml, ng/ml.

Done

3. Line 462, "bacterial bacterial..." delete one.

Corrected

4. In Supplementary Fig. 1a, the unsaturated fatty acids synthesized by FabM are cis, not trans.

While the FabM substrate is "trans" (as indicated), the product is "cis". We now specify 'cis' in describing the product in Suppl Fig. 1a legend.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have sufficiently addressed my concerns in the revised manuscript. The revisions have made this manuscript substantially stronger, and it now convincingly demonstrates the reasons for the dichotomy that authors aimed to understand.

Reviewer #2 (Remarks to the Author):

The authors have addressed many of my concerns, but some additional clarifications are still needed.

1. In the authors' response, they stated that "the delta fabT shows a more pronounced skew toward longer saturated FAs compared to mFabT (C18:0 / C16:0 ratios are 3.7 and 2.3 respectively)." However, neither Figure S6 nor Table S1 showed such large ratios.
2. The authors added the fatty acid composition in the labels of Figure S3, but still did not show representative MS/MS fragmentations. Furthermore, were all lipids analyzed in negative ionization mode? If so, one would expect cardiolipins to fragment into fatty acid anions in the negative mode. Furthermore, what molecular ions were used for each class of lipids?
3. The authors still did not demonstrate that lipid contents in human decidua, human endometrial cells, and keratinocytes are much less than that in human muscles to support their claim that it is the lipid content in the muscle that favors the bacterial growth in muscle. Although the authors added that "This is within the percent fat range estimated for human muscle (8-30 %; in contrast, uterine fluid lipid concentrations are >1000-fold lower)," uterine fluid is not the same as the human decidua tissue. And lipid contents in the cells used were not determined.
4. I am still not convinced by the authors' argument that the growth defect of mFabT is due to over-consumption of some amino acids and hexose. Again, the extent of changes of select amino acids (only Asn, Ile, Lys, Ser, but not other amino acids) is small even though statistically significant for Asn, Ile, and Lys. If there is indeed upregulation of uptake of these four amino acids, then measurement of their levels in the bacterial cells would be more informative than measuring their changes in the supernatant. The authors also argue upregulation in purine synthesis based on RNAseq, and in this case, measurement of purine metabolites would support their argument that "mutant undergoes futile metabolic turnover that does not lead to growth".

Reviewer #3 (Remarks to the Author):

The authors provided a reasonable explanation to the issue I was concerned about. I basically agree with the author's viewpoint. The authors have also made corrections to some errors I pointed out. I have no further comments.

Revised manuscript: '**The double-edged role of FASII regulator FabT in *Streptococcus pyogenes* infection' NCOMMS-24-09627-T**.

Below we address Reviewer 2's remaining 4 questions, and are grateful for the thoughtful comments and careful reading.

1. In the authors' response, they stated that "the delta fabT shows a more pronounced skew toward longer saturated FAs compared to mFabT (C18:0 / C16:0 ratios are 3.7 and 2.3 respectively)." However, neither Figure S6 nor Table S1 showed such large ratios.

We apologize for the error in the previous response to Reviewer 2, and note that the manuscript text is accurate. We should have referred to total C16 and C18 species, and not only the saturated FAs. The correct statement should read: "the delta fabT shows a more pronounced skew toward longer FAs compared to mFabT (C18 / C16 ratios are 3.7 and 2.3 respectively)."

2. The authors added the fatty acid composition in the labels of Figure S3, but still did not show representative MS/MS fragmentations. Furthermore, were all lipids analyzed in negative ionization mode? If so, one would expect cardiolipins to fragment into fatty acid anions in the negative mode. Furthermore, what molecular ions were used for each class of lipids?

All raw data from MS/MS fragmentations are now accessible in the Mendeley database in MZML open-source format (https://data.mendeley.com/datasets/cf578v8d8b/1). Lipids were analyzed in the negative ionization mode. CL fragmentation did not lead to unambiguous FA assignments, and were therefore not shown, as already mentioned. The adducts observed for molecular ions were: CH3COO- for MGDG, CH3COO- and H- for DGDG, H- for PG and CL.

3. The authors still did not demonstrate that lipid contents in human decidua, human endometrial cells, and keratinocytes are much less than that in human muscles to support their claim that it is the lipid content in the muscle that favors the bacterial growth in muscle. Although the authors added that "This is within the percent fat range estimated for human muscle (8-30 %; in contrast, uterine fluid lipid concentrations are >1000-fold lower)," uterine fluid is not the same as the human decidua tissue. And lipid contents in the cells used were not determined.

Two points are of note: First, GAS infections are mainly extracellular [1] (Barnett et al, Streptococcus pyogenes: Basic Biology to Clinical Manifestations), and thus would not access intracellular lipids. To our knowledge, lipids in decidual tissue are mainly intracellular; in contrast, most fat accumulation in muscle is intermuscular, which would offer direct lipid access to extracellular GAS. Accordingly, our experimental approach used conditioned supernatant and surrounding tissue, where GAS were shown to multiply (revised manuscript reference 23)
For information to Reviewer, normal decidual tissue comprises ~1.6 % lipids (Staff et al, Am J Obstet Gynecol (1999) 180: 587[2]; similar to the average cell lipid content), compared to ~15 % in the tested muscle. We now state: 1- line 63 "GAS… causing mainly extracellular infections" [1]; we refer to the 2- line 351 "lipid-rich inter-muscular environment"; and note 3- line 353 "uterine fluids environments where GAS multiply".

4. I am still not convinced by the authors' argument that the growth defect of mFabT is due to over-consumption of some amino acids and hexose. Again, the extent of changes of select amino acids (only Asn, Ile, Lys, Ser, but not other amino acids) is small even though statistically significant for Asn, Ile, and Lys. If there is indeed upregulation of uptake of these four amino acids, then measurement of their levels in the bacterial cells would be more informative than measuring their changes in the supernatant. The authors also argue upregulation in purine synthesis based on RNAseq, and in this case, measurement of purine metabolites would support their argument that "mutant undergoes futile metabolic turnover that does not lead to growth".

Please note that we did not propose that the “growth defect of mFabT is due to overconsumption of some amino acids and hexose”, as Reviewer writes. Rather, we propose that membrane alterations and continued FASII synthesis are the primary causes for increased mortality in nutrient-limited biotopes, by failing to stop metabolic consumption after 8 h growth (see manuscript p12). We feel that our results, as supported by revised reference 25, convincingly favor this proposal. Reviewer suggested that we measure intracellular metabolite accumulation; however, imported energy sources would be rapidly metabolized, and even if measurable, would require onerous and complex methods that may not work, and would not contribute to the already statistically significant data already presented.

1 Barnett, T., Indraratna, A. & Sanderson-Smith, M. in *Streptococcus pyogenes: Basic Biology to Clinical Manifestations* (eds J. J. Ferretti, D. L. Stevens, & V. A. Fischetti) Ch. 13, (2022).

2 Staff, A. C., Ranheim, T., Khoury, J. & Henriksen, T. Increased contents of phospholipids, cholesterol, and lipid peroxides in decidua basalis in women with preeclampsia. *Am J Obstet Gynecol* 180, 587-592, doi:10.1016/s0002-9378(99)70259-0 (1999).

REVIEWERS' COMMENTS

Reviewer #2 (Remarks to the Author):

The authors have addressed my concerns. I just have one more small suggestion, in the authors' response, they stated that "... we did not propose that the “growth defect of mFabT is due to overconsumption of some amino acids and hexose”; however, their section title is "Faster metabolic turnover in mFabT generates a growth defect during infection", which indicates the primary role of the amino acid and hexose consumption. The authors might consider revising the section title to reflect their true intention, i.e., "Rather, we propose that membrane alterations and continued FASII synthesis are the primary causes for increased mortality in nutrient-limited biotopes, by failing to stop metabolic consumption after 8 h growth". The authors might also want to revise the corresponding sentence in the text accordingly.

September 10, 2024

As stated in our letter to Editor of Aug 24, we took into account Reviewer's optional suggestion to revise a section title, changed from "Faster metabolic turnover in mFabT generates a growth defect during infection" to "The mFabT mutant exhibits greater metabolic turnover in infection conditions".