



**Open Access** This file is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. In the cases where the authors are anonymous, such as is the case for the reports of anonymous peer reviewers, author attribution should be to 'Anonymous Referee' followed by a clear attribution to the source work. The images or other third party material in this file are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

## REVIEWER COMMENTS

### Reviewer #1 (Remarks to the Author):

The investigators used complementary methods and found that arginine biosynthesis is linked to antibiotic tolerance in *S. aureus* grown as a biofilm. In particular, the group focused on argGH, an operon encoding the last two steps of arginine biosynthesis. While performing a series of robust experiments, they found that free arginine concentration is depleted during biofilm formation and a mutation in argGH increases tolerance. Further, restriction of arginine induces tolerance as does depletion of other amino acids that are typically not synthesized such as valine and proline. In addition, using fairly novel techniques, they found that inhibition of protein synthesis induced antibiotic tolerance. Overall, the manuscript has some impactful aspects regarding possible treatment modalities and, in addition, lends some light onto possible reason why *S. aureus* has been selected to be an arginine auxotroph and does not grow when arginine is depleted.

### Major comments:

1. During growth in a biofilm, arginine is depleted and this may be linked to its catabolism in an ADI-dependent manner. This should potentially be confirmed or tested. If this is the case, one might predict that an ADI knockout is less tolerant than WT grown in a biofilm.
2. It seems that the relationship between antibiotic tolerance observed during these experiments and the stringent response should be confirmed and investigated. At this point, it is inferred but it would bolster the story if the group could document that the stringent response is activated and that tolerance is dependent upon the stringent response.

### Reviewer #2 (Remarks to the Author):

The manuscript by Freiberg et al. employs unbiased proteomic and Tn-Seq approaches to explore *S. aureus* biofilm tolerance to antibiotics. Their findings reveal that biofilm cells adjust their metabolism to deplete intracellular arginine when exposed to antibiotics. This depletion of arginine leads to the cessation of protein biosynthesis, ultimately causing antibiotic tolerance.

The study offers valuable mechanistic insights and represents a significant contribution to our comprehension of antibiotic tolerance. The article is well-written, concise, and largely aligns with the current understanding of arginine metabolism in *S. aureus*. Below, I note a few areas that could further strengthen the conclusions of the manuscript.

### Major concerns:

1. The authors have effectively demonstrated that protein synthesis is arrested in media lacking arginine, leading to antibiotic tolerance. However, it remains unclear whether biofilms challenged with antibiotics also experience such protein arrest. The authors appear to assume that protein arrest may have been initiated as they did not detect intracellular arginine in antibiotic-challenged biofilms. However, to strengthen this conclusion, the authors need to confirm that biofilms treated with antibiotics indeed undergo protein arrest in an arginine-dependent manner when compared to untreated biofilms of the

same age. This would be central to their conclusions.

2. The authors have speculated that *S. aureus* may have evolved to use arginine depletion to induce tolerance as there are multiple pathways that can deplete arginine quickly from the extracellular environment. However, it is not clear to me that arginine is taken up at a higher rate compared to other amino acids when subject to antibiotic challenge.

a. The authors could potentially use toxic arginine analogues to determine arginine uptake. Alternately, quantifying arginine uptake directly would also work.

b. Would increasing arginine concentrations in the culture media decrease antibiotic tolerance?

3. The discovery that ArgGH is downregulated during antibiotic treatment is intriguing, especially since flux through this pathway is typically not observed unless CcpA is mutated. The significance of this finding should be discussed further in light of what is understood about its regulation.

4. In line 138, the paragraph concludes that arginine is the sole amino acid depleted in cells. However, based on Figure 2C, it appears that Glutamate, Histidine, and Tryptophan are also depleted. Please modify the conclusion to accurately reflect the data.

5. I have concerns about the glutamate levels shown in Figure 2C. Glutamate is typically the most abundant amino acid in cells, so it would be unusual if it is depleted, especially when glutamine levels are still maintained high. Please reconfirm whether glutamate levels have indeed decreased.

6. It is essential for the authors to genetically complement the phenotypes of various mutants they have observed to strengthen their findings.

Minor concerns:

1. Lines 84-87: While I appreciate the inclusion of antibiotic concentrations in the materials and methods section, it would be helpful to also mention the antibiotic concentrations in the main text and in parentheses show how many times the MIC this concentration of drug represents.

2. Line 99: Please provide a reason for why the essentiality of 227 genes is uncertain.

3. Lines 117-120: Please specify whether the arc genes that are noted (based on proteomics and Tn-seq analysis) are located on the chromosomal DNA or on the ACME island.

4. Line 171: Change 'there as' to 'there was.'

5. Line 352: It appears that the reference to Figure S1 here may not be correct. Please verify and correct if necessary.

Reviewer #3 (Remarks to the Author):

In the work by Freiberg et al. the authors seek to unravel how antibiotic tolerance is induced in *Staphylococcus aureus* biofilms. Using a powerful combination of quantitative proteomics and transposon sequencing, the authors show that arginine metabolism, specifically the depletion of arginine, can induce antibiotic tolerance via inhibition of protein synthesis. Overall, the topic is important and the experiments, for the most part, are well designed with appropriate controls.

The main limitation of the study is that the implications of the discoveries remain to be shown. While the in vitro data are strong, the in vivo data are not.

Specific Comments:

1. What type is the solid-air interface biofilm modeling regarding *S. aureus* pathobiology? What type of biofilm is formed (e.g., eDNA, PNAG, protein based)?
2. In vivo experiments: It was unclear why the authors chose an SSTI model. What are they modeling? Does *S. aureus* form biofilms in this model? Why not use the more clinically relevant biofilm model established in the Skaar lab (PMCID: PMC3721972)?
3. Line 241: Why were the cultures diluted 2:1 (JE2 to argH::Tn) instead of 1:1?
4. Line 247: *S. aureus* skin infection between male and female mice. This is known and it should include the appropriate citation (PMCID: PMC5760295).
5. The in vivo data show a modest phenotype using a genetic "lock" strain that shows strong phenotype in vivo. So, either the in vitro pathway is not at play in vivo or the in vivo model is not appropriate to study biofilms.
6. Line 250: As written the statement is misleading—there was no statistical significance in females, while only a modest difference in males.
7. Line 254: Stats are only shown for the vehicle control.
8. Lines 256-257: Another interpretation of the in vivo data is that arginine and citrulline are present in vivo and might have nothing to do with *S. aureus* pathobiology or the in vitro biofilm data.
9. Line 547: "infections" should be injections.

## RESPONSE TO REVIEWERS

We greatly appreciate the careful consideration given to our manuscript by all three reviewers. Their comments have greatly improved both the quality of our manuscript and the strength of our conclusions. Highlighted below are the specific changes we have made in response to the follow reviewer comments.

## REVIEWER COMMENTS

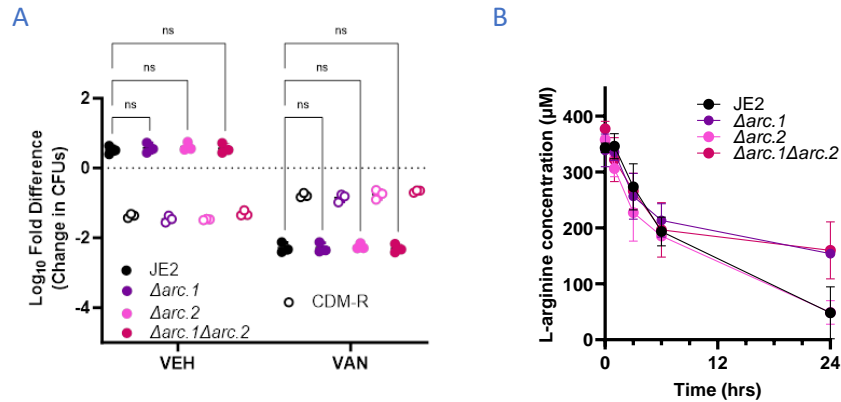
Reviewer #1 (Remarks to the Author):

The investigators used complementary methods and found that arginine biosynthesis is linked to antibiotic tolerance in *S. aureus* grown as a biofilm. In particular, the group focused on argGH, an operon encoding the last two steps of arginine biosynthesis. While performing a series of robust experiments, they found that free arginine concentration is depleted during biofilm formation and a mutation in argGH increases tolerance. Further, restriction of arginine induces tolerance as does depletion of other amino acids that are typically not synthesized such as valine and proline. In addition, using fairly novel techniques, they found that inhibition of protein synthesis induced antibiotic tolerance. Overall, the manuscript has some impactful aspects regarding possible treatment modalities and, in addition, lends some light onto possible reason why *S. aureus* has been selected to be an arginine auxotroph and does not grow when arginine is depleted.

Major comments:

1. During growth in a biofilm, arginine is depleted and this may be linked to its catabolism in an ADI-dependent manner. This should potentially be confirmed or tested. If this is the case, one might predict that an ADI knockout is less tolerant than WT grown in a biofilm.

We agree with the reviewer that based on our data presented in this manuscript we would anticipate that the ADI pathway plays a role in arginine depletion and contributes to antibiotic tolerance in a biofilm. However, our preliminary data utilizing complete knockouts of both copies of the ADI pathways separately and in combination have not demonstrated a change in antibiotic tolerance (data shown below-A). Interestingly, there is still depletion of arginine from the media in these mutants, albeit less than in the wildtype when the native *arc* operon (*arc.1*) is deleted ( $\Delta arc.1$  and  $\Delta arc.1/\Delta arc.2$ , also shown below-B). Given that these ADI mutants also have the arginine importer, *arcD*, inactivated, these data suggest that there must be other efficient arginine import systems and ways for *S. aureus* to degrade arginine, even in the absence of the ADI pathway. This is particularly true for the double mutant,  $\Delta arc.1/\Delta arc.2$ , in which both copies of *arcD* were deleted. While this finding is intriguing and something we continue to pursue, these results are a preliminary step towards a full investigation of the ADI pathway along with alternative arginine degradation pathways, and therefore we think this falls outside of the scope of this manuscript. For this reason, we have not included these data in the revised version of the manuscript.



2. It seems that the relationship between antibiotic tolerance observed during these experiments and the stringent response should be confirmed and investigated. At this point, it is inferred but it would bolster the story if the group could document that the stringent response is activated and that tolerance is dependent upon the stringent response.

We appreciate the reviewer's interest in the relationship between antibiotic tolerance and the stringent response and agree that it was worthy of further investigation in this manuscript. To that end, we used a *relA::Tn* mutant (and relevant complementation strain) to show that the arginine-related effect on antibiotic tolerance is abrogated when the normal RelA (p)ppGpp synthetase function is disrupted. These results are now shown in a revised version of Figure 4F and text has been added to the results section (lines 253- lines 263 clean manuscript; lines 254-lines 264 marked-up manuscript):

"Inhibition of protein synthesis from amino acid starvation is known to induce the stringent response in *S. aureus*<sup>39</sup>. Since activation of the stringent response can induce tolerance to antibiotics, we hypothesized that this mechanism likely explained some, if not the majority, of the means by which arginine depletion ultimately leads to antibiotic tolerance<sup>40</sup>. To test this hypothesis, we utilized a *relA::Tn* containing a transposon mutation in the C-terminal domain of the RelA bifunctional (p)ppGpp synthetase/hydrolase<sup>33</sup>. Disruption of the C-terminal domain of RelA has been shown to preserve the hydrolase function of RelA, which is essential, but disrupts its normal synthetase function by preventing its interaction with the bacterial ribosome<sup>41</sup>. Consistent with a role for the stringent response in arginine-deprivation mediated antibiotic tolerance, there was no difference in vancomycin susceptibility related to the presence of arginine (Figure 4F). An arginine dependent effect on tolerance was subsequently restored by complementation of *relA* under its native promoter."

Reviewer #2 (Remarks to the Author):

The manuscript by Freiberg et al. employs unbiased proteomic and Tn-Seq approaches to explore *S. aureus* biofilm tolerance to antibiotics. Their findings reveal that biofilm cells adjust their metabolism to deplete intracellular arginine when exposed to antibiotics. This depletion of arginine leads to the cessation of protein biosynthesis, ultimately causing antibiotic tolerance.

The study offers valuable mechanistic insights and represents a significant contribution to our comprehension of antibiotic tolerance. The article is well-written, concise, and largely aligns with the current understanding of arginine metabolism in *S. aureus*. Below, I note a few areas that could further strengthen the conclusions of the manuscript.

#### Major concerns

1. The authors have effectively demonstrated that protein synthesis is arrested in media lacking arginine, leading to antibiotic tolerance. However, it remains unclear whether biofilms challenged with antibiotics also experience such protein arrest. The authors appear to assume that protein arrest may have been initiated as they did not detect intracellular arginine in antibiotic-challenged biofilms. However, to strengthen this conclusion, the authors need to confirm that biofilms treated with antibiotics indeed undergo protein arrest in an arginine-dependent manner when compared to untreated biofilms of the same age. This would be central to their conclusions.

We appreciate the reviewer's careful consideration of our central hypothesis and its implications. We agree that demonstration of the arrest of protein synthesis in an arginine-dependent manner, even in the presence of antibiotics, is central to our conclusion. To that end, we repeated the nascent protein labeling experiments previously included in the paper with the addition of vancomycin. These new results are now included in revised versions of Figure 4E and S8, which clearly show that even in the presence of vancomycin there is ongoing protein synthesis when arginine is present, but this protein synthesis is arrested in a similar manner when arginine is absent, including when vancomycin is present.

2. The authors have speculated that *S. aureus* may have evolved to use arginine depletion to induce tolerance as there are multiple pathways that can deplete arginine quickly from the extracellular environment. However, it is not clear to me that arginine is taken up at a higher rate compared to other amino acids when subject to antibiotic challenge.

a. The authors could potentially use toxic arginine analogues to determine arginine uptake. Alternately, quantifying arginine uptake directly would also work.

We appreciate the suggestions from the reviewer to evaluate arginine uptake. We attempted to evaluate arginine uptake using toxic arginine analogues, however, we have been unable to identify a truly toxic analog, as the analogues tried thus far (L-homoarginine, L-canavanine) did not cause any negative effects on *S. aureus* growth or survival in the presence or absence of low levels of arginine. It is unclear whether these analogues are not taken up by our *S. aureus* isolate, or if they are not incorporated into protein synthesis, but regardless this method of investigation has been unsuccessful.

We have, however, attempted to measure the uptake of arginine from the extracellular environment directly by quantifying its depletion from the extracellular media over time. These results are shown in a revised version of supplemental figure 6B, and clearly demonstrate that when homogenized biofilms are transferred to media containing arginine, the arginine is depleted over time. However, in the presence of vancomycin there is a lack of depletion of arginine from the media. The lack of arginine depletion is likely due, in part, to bacterial death in the presence of vancomycin, limiting the number of viable bacteria

available to uptake arginine. In conjunction with the findings in revised supplemental figure 8, which shows ongoing protein synthesis in the presence of vancomycin when arginine is available, we feel this provides strong support for our hypothesis that the inhibition of protein synthesis is the important aspect of arginine depletion. Although the data from our screens suggest that increased arginine degradation plays a role in increasing antibiotic tolerance, in this assay the depletion of arginine is likely not rapid enough to deplete arginine prior to the bactericidal effects of vancomycin. This supports the hypothesis that the presence of an existing arginine deplete environment within the intact biofilms (as shown in Figure 2C) predisposes the bacteria to high levels of antibiotic tolerance. The following text has been added to the manuscript (lines 198-201 clean manuscript; lines 199-202 marked-up manuscript):

“Homogenized biofilm bacteria deplete arginine from the extracellular media over 24 hours, but depletion of arginine is not seen in the presence of vancomycin, suggesting that at high levels of arginine bacteria are not able to deplete arginine fast enough to overcome the bactericidal effects of the antibiotic (Figure S6B).”

**b. Would increasing arginine concentrations in the culture media decrease antibiotic tolerance?**

Throughout our paper we use CDM media according to a published recipe which has an arginine concentration of 400  $\mu$ M. We have previously tested a 10x increase in the concentration of arginine and found that there was no increase in antibiotic susceptibility, suggesting there is a ceiling to this effect (data not shown). However, since a concentration of 400  $\mu$ M is already on the high end of physiologic concentrations and well above the typical plasma levels of arginine seen in humans, we instead tested lower concentrations of arginine. As expected, there was a concentration dependent effect on antibiotic tolerance with antibiotic killing decreasing as the concentration of arginine decreased. These data are now included in the paper in a revised version of Supplemental Figure 6, and text has been added to the results section describing these observations (lines 196-197 clean manuscript; lines 197-198 marked-up manuscript):

“Arginine restriction showed a dose-response effect as tolerance to vancomycin increased as the starting arginine concentration of the media decreased (Figure S6A)”

**3. The discovery that ArgGH is downregulated during antibiotic treatment is intriguing, especially since flux through this pathway is typically not observed unless CcpA is mutated. The significance of this finding should be discussed further in light of what is understood about its regulation.**

We appreciate the reviewer’s intrigue at our discovery regarding ArgGH expression in the context of antibiotic treatment. While the synthesis of arginine via this pathway is not observed in the absence of mutations, this has been typically attributed to deficiencies in enzymes upstream of citrulline in the arginine biosynthesis pathway. These deficiencies effectively restrict the ability of *S. aureus* to utilize glutamate or proline as a source for arginine synthesis in the absence of a mutation, but the ability of ArgGH to convert citrulline to arginine is consistent with this understanding of the repression of arginine biosynthesis in *S. aureus*. We do agree, however, that the decreased levels of ArgGH following antibiotic treatment in biofilms is surprising as the known mechanisms of transcriptional and post-transcriptional regulation of ArgGH levels would not explain this change. We have added the following section to expand upon the significance of our findings in light of the current understanding of ArgGH regulation (lines 401-421 clean manuscript; lines 404-424 marked-up manuscript):



The detection of differences in the levels of the ArgG and ArgH proteins in response to antibiotic treatment is particularly interesting in the context of the existing paradigm of regulation of this operon. The *argGH* operon is transcriptionally repressed under glucose replete conditions by CcpA and transcriptionally repressed under arginine replete conditions by AhrC<sup>37</sup>. Mutations in these repressors allow for the transcription of the *argGH* operon and the subsequent growth of *S. aureus* in the absence of arginine through the utilization of proline<sup>35</sup>. However, the absence of both glucose and arginine is not sufficient to overcome this repression in a wildtype background, suggesting there is also glucose independent repression due to CcpA<sup>36,37</sup>. Arginine and glucose both quickly become limited within the biofilm in the colony filter biofilm model, which explains why expression of ArgG and ArgH was observed. Although citrulline was able to rescue growth in the absence of arginine in an ArgH dependent manner (Figure S9), the presence of proline in the media was not sufficient to restore growth in the absence of arginine within a biofilm (Figure 2A & 2B), consistent with the current understanding that proline is not an adequate source for arginine biosynthesis due to glucose-independent repression of *putA* by CcpA<sup>36</sup>. Given this regulation pattern, it is unclear by what mechanism antibiotics would lead to lower levels of ArgG and ArgH within a biofilm as their transcription should remain de-repressed by the low glucose, low arginine environment. Another recently identified regulator of *argGH* is the small RNA *teg58*<sup>72</sup>. However, *teg58* is repressed by SarA<sup>72</sup>, and SarA expression is induced by vancomycin<sup>73</sup>, making *teg58* unlikely to be responsible for the decreased levels of the ArgG and ArgH proteins that are observed. It appears there is some yet unrecognized mechanism responsible for the decreased abundance of these enzymes, and it warrants further investigation as it may elucidate a further understanding of how *S. aureus* responds to antibiotics within a biofilm.

4. In line 138, the paragraph concludes that arginine is the sole amino acid depleted in cells. However, based on Figure 2C, it appears that Glutamate, Histidine, and Tryptophan are also depleted. Please modify the conclusion to accurately reflect the data.

We understand the confusion that arose from our statement in line 138, as we meant to suggest that all the amino acids *S. aureus* is unable to synthesize are detected in at least one of the conditions. We have edited the text to make that distinction more apparent:

“Furthermore, its availability is likely one of the growth-limiting factors within a biofilm, since all other essential amino acids for which *S. aureus* is auxotrophic were detected in at least one of the two media conditions (Figure 2C).”

5. I have concerns about the glutamate levels shown in Figure 2C. Glutamate is typically the most abundant amino acid in cells, so it would be unusual if it is depleted, especially when glutamine levels are still maintained high. Please reconfirm whether glutamate levels have indeed decreased.

We appreciate the reviewer drawing our attention to the unusual finding of glutamate depletion seen within this biofilm model. We confirmed with the VUMC analytical services core who performed the amino acid analysis that there was, in fact, no glutamate detected in these samples. At this time, we do not have a conclusive explanation for this finding, although we feel that it warrants further investigation in future work. We hypothesize that the depletion of glutamate is due to the conversion of glutamate into  $\alpha$ -ketoglutarate to replenish depleted intermediates in the TCA cycle via the anaplerotic reaction catalyzed by glutamate dehydrogenase (GudB). This enzyme has been shown to be important for growth in glucose deplete environments (Halsey *et. al.* 2017. *mBio*. PMID: 28196956) and our TnSeq experiments

(Supplemental Table S3) suggest it is important for fitness within our biofilm model, as well. Furthermore, GudB was detected within the proteome of our biofilm samples, while the glutamate synthase enzymes (GltB and GltD), which convert glutamine to glutamate, were not detected by proteomics. The glutamine synthase enzyme, GlnA, was also detected within the biofilm proteome, suggesting an explanation for the accumulation of glutamine in the absence of glutamate. We agree with the reviewer that this finding is interesting and worthy of study in future experiments, but we feel that it is outside of the scope of this paper.

6. It is essential for the authors to genetically complement the phenotypes of various mutants they have observed to strengthen their findings.

We agree with the reviewer that genetic complementation is an important part of validating our findings involving mutant strains. To address this, our *in vitro* experiments using an *argH* mutant in Figure 5B were also done with a complemented strain. These results are shown in Figure 5C and resemble the wildtype phenotype seen in Figure 5A. We have also added data showing the *argH* complemented strain uses citrulline for growth in the absence of arginine in a revised version of Figure S9. In the experiments employing a *relA* mutant (new Figure 4F), which we added in response to reviewer comments, we also genetically complemented the *relA* mutant and showed a restoration of the wildtype phenotype.

Minor concerns:

1. Lines 84-87: While I appreciate the inclusion of antibiotic concentrations in the materials and methods section, it would be helpful to also mention the antibiotic concentrations in the main text and in parentheses show how many times the MIC this concentration of drug represents.

In general, we choose antibiotic concentrations in light of peak serum concentrations and not with regards to how many fold in excess of the MIC they were. However, we have now added both the concentrations and their relationships to the MICs to the manuscript with the following sentences (lines 101-104):

“Antibiotic concentrations were 400 µg/ml for vancomycin, 20 µg/ml for ceftaroline, 20 µg/ml for linezolid, and 9 µg/ml for delafloxacin. These concentrations, with the except of vancomycin, were chosen based on published peak serum concentrations for standard clinical treatment doses<sup>25</sup>, and represent concentrations >200x, >20x, 10x, and >40x the MICs, respectively.”

2. Line 99: Please provide a reason for why the essentiality of 227 genes is uncertain.

For some smaller genes with small numbers of TA sites it can be difficult to confidently predict that they are essential and not missed due to limitations of the transposon library. This uncertainty is handled by the TRANSIT software by labeling these genes as uncertain to avoid misidentifying them due to limitations of the statistical power. We have attempted to better clarify this in the manuscript by adding the following (lines 116-117 clean manuscript; lines 118-lines 117-118 marked-up manuscript):

“Another 227 genes were labeled as “uncertain” as their small size limited the ability to confidently predict their essentiality.”

3. Lines 117-120: Please specify whether the *arc* genes that are noted (based on proteomics and Tn-seq analysis) are located on the chromosomal DNA or on the ACME island.

We have updated the text and the figure legend for Figure 1B to clearly reflect which set of *arc* genes are being referred to in each place. This section now reads (lines 134-137 clean manuscript; lines 135-138 marked-up manuscript):

“However, the enzymes responsible for degrading arginine via the arginine deiminase pathway, *ArcA*, *ArcB*, and *ArcC*, showed increased abundance during exposure to 3 out of the 4 antibiotics tested for both the operon located on the native chromosomal DNA and on the Arginine Catabolism Mobile Element (ACME) genomic island (Figure 1B).”

And the figure legend for Figure 1B states:

“The notation *arcA.1*, *arcB.1*, and *arcC.1* refer to the genes located on the chromosomal arginine deiminase operon while *arcA.2*, *arcB.2*, and *arcC.2* refer to the genes located in the arginine deiminase operon found on the ACME.”

4. Line 171: Change 'there as' to 'there was.'

We have made the corresponding change.

5. Line 352: It appears that the reference to Figure S1 here may not be correct. Please verify and correct if necessary.

We have corrected the reference to reflect that it is referring to Figure 1, instead.

Reviewer #3 (Remarks to the Author):

In the work by Freiberg et al. the authors seek to unravel how antibiotic tolerance is induced in *Staphylococcus aureus* biofilms. Using a powerful combination of quantitative proteomics and transposon sequencing, the authors show that arginine metabolism, specifically the depletion of arginine, can induce antibiotic tolerance via inhibition of protein synthesis. Overall, the topic is important and the experiments, for the most part, are well designed with appropriate controls.

The main limitation of the study is that the implications of the discoveries remain to be shown. While the in vitro data are strong, the in vivo data are not.

Specific Comments:

1. What type is the solid-air interface biofilm modeling regarding *S. aureus* pathobiology? What type of biofilm is formed (e.g., eDNA, PNAG, protein based)?

The solid-air interface biofilm model used throughout this paper was chosen to best represent the biofilm seen in a chronic wound setting where the bacteria exist in a community at a solid-air interface. These biofilms consist of a mixture of eDNA, polysaccharides, and proteins. We have added confocal images of stained biofilms as a new supplemental figure 1 that highlight these features, and the following text is now included in the manuscript (Lines 87-88):

“*S. aureus* biofilms formed in this model have a matrix consisting of a mixture of bacteria, extracellular DNA (eDNA), polysaccharides, and protein (Figure S1).”

2. In vivo experiments: It was unclear why the authors chose an SSTI model. What are they modeling? Does *S. aureus* form biofilms in this model?

We apologize for failing to clearly explain our choice of an SSTI model in these studies. As mentioned above, our *in vitro* solid-air interface biofilm model most closely resembles a wound infection, hence our decision to utilize a skin infection model. Not only is *S. aureus* heavily associated with both acute and chronic wounds in humans, it is also known to form biofilms in mouse SSTI models. To clarify these points in the paper the following text has been added:

(lines 91-92) “This provides a useful *in vitro* model of the type of biofilm that might form in a wound infection, of which *S. aureus* is a frequent cause<sup>24-27</sup>”

and

(lines 286-288 clean manuscript; lines 287-289 marked-up manuscript) “Murine *S. aureus* SSTI models, including this tape-stripping model, promote *S. aureus* infections with local biofilm formation<sup>45-48</sup>.”

Additionally, we also utilized a *S. aureus* osteomyelitis model that further confirm our findings from the skin infection model, which is now shown as a revised version of supplemental figure 12.

Why not use the more clinically relevant biofilm model established in the Skaar lab (PMCID: PMC3721972)?

We appreciate the reviewer’s suggestion to test our hypotheses in another biofilm infection model and therefore we have included new experiments using our established osteomyelitis model in the revision. The results of this experiment are now shown in supplemental Figure 12 and are consistent with the relationship we observed in the SSTI model. In addition to the description of this model that was added to the methods section, the following text was added to the results section in the revision describing the outcome of this experiment (lines 310-318 clean manuscript; lines 311-319 marked-up manuscript):

“To further confirm the role of ArgH in antibiotic tolerance during biofilm-mediated infections, a murine osteomyelitis model with vancomycin treatment was also employed<sup>50</sup>. Biofilms play an important role in osteomyelitis and contribute to the high rates of antibiotic treatment failure seen in the treatment of this type of infection<sup>13</sup>. In this model, mice were inoculated directly in their femurs with JE2 and *argH*::Tn at a 2:1 WT:mutant ratio and treated with vancomycin for seven days beginning immediately at the time of infection. Consistent with the recalcitrance of osteomyelitis to vancomycin treatment, 7 out of the 10 mice treated with vancomycin remained

infected after 7 days of antibiotic treatment, despite starting therapy immediately (Figure S12A). Similar to what was observed in the SSTI model, the *argH*::Tn mutant had decreased fitness in the absence of antibiotics, but had a relative advantage during treatment with vancomycin (Figure S12B)."

3. Line 241: Why were the cultures diluted 2:1 (JE2 to *argH*::Tn) instead of 1:1?

We acknowledge that the decision to dilute our cultures to a 2:1 wildtype to mutant ratio for competition experiments was not explained in the paper. The reason for this ratio was because the *argH*::Tn mutant strain, which was the strain containing an antibiotic selection marker, was expected to increase in relative abundance with antibiotic treatment based on our *in vitro* experiments. The numbers of wildtype bacteria in the competition experiments were calculated by subtracting the number of bacteria with an antibiotic selection marker from the total number of bacteria recovered. This means the ability to accurately calculate the number of wildtype bacteria is limited when the majority of bacteria are carrying the resistance marker. We felt that a 2:1 ratio was biologically very similar to a 1:1 ratio as a starting point, however, it allowed us a much larger dynamic range to detect increases in the relative fitness of the mutant strain in the presence of antibiotics. To clarify this rationale in the manuscript, the following text has been added (lines 679-681 clean manuscript; lines 682-684 marked-up manuscript):

"This ratio was chosen, instead of a 1:1 ratio, as it allowed a greater dynamic range over which a relative survival benefit for the marked mutant strain could be assessed."

4. Line 247: *S. aureus* skin infection between male and female mice. This is known and it should include the appropriate citation (PMCID: PMC5760295).

We have added the appropriate citation to the manuscript.

5. The *in vivo* data show a modest phenotype using a genetic "lock" strain that shows strong phenotype *in vivo*. So, either the *in vitro* pathway is not at play *in vivo* or the *in vivo* model is not appropriate to study biofilms.

While we agree that there is a strong and striking phenotype *in vitro* and only a modest phenotype *in vivo*, we still feel that this is an appropriate model and our paper support the finding that this pathway is important during antibiotic treatment. As the reviewer recommended, we also utilized an osteomyelitis model (now included in supplemental Figure 12) and found a similar phenotype to the SSTI model where there is decreased fitness of the *argH*::Tn mutant in the absence of antibiotics, relative to the wildtype, but a relative improvement in the fitness of *argH*::Tn when the mice are treated with vancomycin. Given the complexities of the *in vivo* environment, with the presence of a host immune response and host-mediated nutrient limitations, it is not surprising that there was only a small difference between the two strains when there were multiple other factors influencing the bacteria's survival besides just arginine availability.

6. Line 250: As written the statement is misleading—there was no statistical significance in females, while only a modest difference in males.

We have revised this statement to specify that this is referring to a combined analysis of male and female mice pooled together. While we initially powered our experiment to detect a difference only in the combined analysis, to address this comment we repeated the experiment with a third group of thirty mice for a total of 45 mice per sex. As shown in the revised supplemental figure 10, in independent analyses both male and female mice now show statistically significant differences in the fitness of the *argH*::Tn mutant relative to the wildtype based on the presence or absence of vancomycin.

We have updated the manuscript text at previous line 250 (now lines 299-303 clean manuscript; lines 300-304 marked-up manuscript) to better reflect which comparisons are being described. The new text now reads:

“Among all mice (male and female combined), in the absence of antibiotic treatment, there was a significant decrease in fitness of the *argH*:Tn mutant relative to the parental control at both 2 DPI and 4 DPI (one-sample Wilcoxon test,  $p=0.0066$  and  $p<0.0001$ , respectively) (Figure 5D), in line with previous studies showing a virulence defect in an *argH* mutant during infection<sup>35</sup>. A similar phenotype was observed when the male and female mice were analyzed individually (Figure S10).”

7. Line 254: Stats are only shown for the vehicle control.

We apologize for our inconsistencies in which stats were referenced and which were shown. We have updated our figures to clearly reflect the Wilcoxon signed rank test p values throughout the *in vivo* experiments.

8. Lines 256-257: Another interpretation of the *in vivo* data is that arginine and citrulline are present *in vivo* and might have nothing to do with *S. aureus* pathobiology or the *in vitro* biofilm data.

While we agree that arginine and citrulline are present *in vivo* (we have subsequently quantified their concentrations in the mouse skin tissue and those data are now shown in a new version of supplemental figure 11), the phenotype we observe with the mutant strain *in vivo* provides a convincing argument for at least some role of arginine metabolism in *in vivo* antibiotic tolerance. Since ArgH has been demonstrated to play a role in the conversion of citrulline to arginine *in vitro* in this and other manuscripts, and it has no other known roles besides the production of arginine, we feel it is hard to conceive of the *in vivo* phenotype observed being due to anything besides the conversion of citrulline to arginine. However, we acknowledge that there are likely other factors contributing to antibiotic tolerance during *S. aureus* infections, and have therefore revised these lines to represent a more modest conclusion:

“These experiments support a role for the conversion of citrulline into arginine by ArgGH in influencing antibiotic tolerance during an infection.”

9. Line 547: “infections” should be injections.

Thank you, we have made the corresponding correction.

## REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Thank you for addressing my concerns with the manuscript, I have no further comments. Excellent work.

Reviewer #2 (Remarks to the Author):

The authors have done an excellent job addressing my comments. The study is robust and highlights a significant adaptation in *S. aureus* biofilms, wherein depletion of intracellular arginine reserves enhances antibiotic tolerance by limiting protein synthesis. The mechanism is well-developed with strong links to stringent response.

Reviewer #3 (Remarks to the Author):

The authors are to be commended for addressing all the reviewer's critiques. The revised manuscript is indeed much improved. While I still have concerns about the in vivo relevance of the current findings, only time will tell if indeed restriction of arginine is at play during antibiotic tolerance in *Staphylococcus aureus* during human SSTI. Regardless, I think this is a nice study that is appropriate for publication in *Nature communications*.