nature portfolio

Peer Review File

Plasmid-encoded phosphatase RapP enhances cell growth in non-domesticated Bacillus subtilis strains

Corresponding Author: Professor Xiongfeng Dai

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This interesting manuscript by Zhu et al. describes the identification of the plasmid-encoded RapP protein as a factor that enhances growth due to the effective suppression of the phosphorelay and thus a more pronounced separation of growth-related gene expression from stationary phase gene expression. The plasmid carrying the rapP gene is present in non-domesticated wild type strains of Bacillus subtilis but not in the laboratory strain 168. As a result, the laboratory strain exhibits a reduced growth rate as compared to the wild strain and has a competitive disadvantage on minimal media.

General comments:
#1 The lab strain was probably evolved on complex medium with all components available. Under such conditions, there might be no fitness difference. Please include such an experiment using LB for example!

Specific comments
#1 l. 18: What is meant by "in such case"? I is not clear whether there is a link between RapP and regulation here.
#2 l. 19 ff: "Our study reveals a delicate strategy": please be explicit about this "delicate strategy"!
#3 l. 26: "nutrient statues", better "nutrient availability"
#4 l. 29, l. 64 and throughout: "in such case", better "therefore"
#5 l. 36/37: Please do not generalize the role of cAMP, this molecule indicates lack of glucose in E. coli, but this role is not conserved outside the enteric bacteria. If you want to use cAMP as an example, please specifiy that this is the case for E. coli!
#6 l. 63/64: Is comI located on the chromosome or on pBS32? The sentence is not clear. Please define the differences between the three strains clearly.
#7 l. 79, Fig. 1D: Please include strain 168 expressing RapP to make sure that RapP alone is sufficient for the effect. The same for fig. 1F.
#8 l. 90, Fig. 2: Is the same true in the background of 168? Please put these results in the main document not in the supplement!
#9 l. 121: Surfactin or other antibiotics are not cofactors and should not be classified as such!
#10 l. 152 and throughout: please avoid the terms "growth defect" or even "strong growth defect", better "slower growth" or "reduced growth" or "growth advantage" for strains carrying RapP
#11 Fig. 5B: Please provide a clearer legend for the x axis: xth what? Please provide information on the medium used in the competition experiment!
#12 l. 177: "need-based regulation of adaptive responses", better "to prevent the harmful expression of"
#13 l. 229: better "that is mainly triggered"
#14 l. 243: better "is widely recognized"
#15 l. 261/262: The authors should mention that genome-reduced strains of B. subtilis derived from 168 were already shown to have superior capacities for the production of foreign secreted proteins (PMID 30540431).

Reviewer #2

(Remarks to the Author)
General comments:

Zhu and collaborators started from the observation that the wild type Bacillus subtilis strain 3610, the ancestor of strain 168 and most laboratory strains used to study genetics of B. subtilis, presented a higher growth rate than strain 168 when grown in various minimal medium.
The authors recently showed that the absence of Spo0A (0A), the master regulator of stationary phase events in this bacterium, leads also to higher growth rate of B. subtilis, an observation they could link to a reallocation of resources from cell adaptation toward metabolic pathways and ribosome production (Zhu, 2023 ; 10.1126/sciadv.adg9733).
The rapP gene, carried by a natural plasmid of strain 3610 but absent in 168, was an obvious candidate since it encodes a phosphatase known to inactivate 0A (Parashar, 2013), and its deletion have been shown by others to slow down growth rate of 3610 (Nordgaard, 2021).
Zhu and collaborators confirmed that the rapP gene was sufficient to explain the growth difference, and that its effect depends on 0A. Logically, in agreement with their previous findings with 0A, they showed reallocation of resources +/- rapP via a proteomic approach, and changes in 0A-dependent phenomenon (expression of sporulation genes). They also introduced the rapP gene in domesticated strains (devoid of its plasmid carrier) which, as expected, reduced leaky expression of 0A-dependent genes and increased their growth rate.

Overall, the article is quite well written and easy to read and the authors provided a profusion of figures and panels, to illustrated in details their observations.
I found however the introduction too short, minimalistic, lacking a lot of necessary information about growth and stationary phase events in B. subtilis, Spo0A, its activation via the phosphorelay and the Rap phosphatase all things that will be useful along the paper for readers not on the topic.

This is especially problematic for RapP itself, who is the main focus of this article, for which most of the literature seems to have been ignored with a single citation (Parashar et al., 2013), while this gene was the subject of several publications during the past decade. I should add that the publications are of importance for the present study since RapP impact on 0A-dependent stationary phase events (biofilm, competence and sporulation) was shown as early as 2013 by Parashar et al., completed by works from Bendori et al., 2015 and Pollack et al., 2015. Parashar also shown that RapP dephosphorylates Spo0F (thus inactivating Spo0A), and Nordgaard et al.,2021 reported the slower growth of 3610 when rapP was deleted, an important finding for this work that Zhu and coworkers reported here too. Of these four publications, only Parashar is cited here but only for a strain construction (!). This is a main issue, not only because it does not give the credits to other teams that worked on it, and because it reduces the clarity of the article, but also because it hides the facts that the rapP gene was an obvious choice when looking for the origin of the growth difference between the strains.

On the same line, their own recent works (Zhu et al., 2023) is very parsimoniously cited while knowing this article enlighten these results very differently. And this is my main concern: the works seems to have been done with great care and as said generated a profusion of data and figures. But in the light of their Science Advances paper and the works of others before, most present results were highly predictable (when not known) and does not bring a lot of new findings and in my opinion is of limited interest and impact.
I will come back to this point in more details below along other minor comments.

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Comments:

Fig1 (legend): please indicate what are the error bars (SD to the mean?) and number of replicates. Panels C & D could probably be pooled together (redundant info between them) and possibly even with panel A

Fig1B: a small suggestion : of the five strains, all but 168 are direct parents varying only at the level of the plasmid (cured or mutated), while 168 is genetically distant (having additional mutations and indels in its chromosome). I would make this apparent on the schema somehow, to distinguish it from the others.

Line76-80 : this section definitely can't neglect the previous finding that rapP deletion reduces growth rate of 3610 (Nordgaard et al., 2021)

Fig1.E: "To gain a mechanistic insight into the positive effect of rapP on cell growth, we measured the ribosome content of B. subtilis as ribosome synthesis lied at the core of bacterial growth control". I am not convinced this experiment brings any information, much less mechanistic details. The correlation between the abundance of ribosomes (here reported ass the RNA/protein ratio) and the growth rate is a known thing (Klumpp et al., 2008, 2009), and this correlation is maintained here across conditions (media) and strains. Since the mutant grow slower, the ratio is lower but it gives no additional information about RapP mode of action. If the correlation has been lost, this would have brought a fresh lead, but as it is, it is rather a control that does not add more insight into the phenomenon.

L88-95: as stated on my general comments, this section really lacks background information (about 0A, the phosphorelay and the Rap phosphatases) and references.
More importantly, their own recent results are just mentioned (ref 27 at L91) but not put in perspective with their current results. This is critical because since they already published that deleting spo0A reallocates resources to metabolisms allowing a faster exponential growth, thus anything decreasing 0A activation, including anything acting negatively on the phosphorelay (as Rap phosphatase) during exponential growth, will necessarily have a similar impact. Since RapP was previously shown by others to act on 0A via 0F and its absence to increase growth rate…
All previous findings should appear already in the introduction and the state of the art clearly depicted.

L97-109: Effect of rapP on the expression of sporulation genes:
The first result of this section (qPCR) showed that the absence of rapP "strongly stimulated" the 0A-dependent genes assayed, but the levels of induction are in truth extremely low, as it appears later with the b-galactosidase assay (lacZ). This is not obvious in the text and a bit misleading. One needs to be familiar with the assay and Miller units to realize that on panels Fig 3 E/F, the values are closed to the background, and it is indicated only in the Methods section that in the 3610 background, the levels were so low the author had to change detection method for fluorescence (panel C/D).
I think it is important to stress out in this result section that what they observed are (barely detectable) fluctuations of background levels of 0A activity and that the sporulation genes are followed only as proxy of 0A activity and not for the phenomenon of sporulation they are involved in. If not, one may be surprised that sporulation genes are "strongly" expressed during exponential growth while sporulation is supposed to occur in carefully designed media in stationary phase.

This observation should also be put in perspective with previously reported fluctuation of spo0A expression (and activation to low levels) during exponential growth (Mirouze et al., 2011).

Fig2: Panels A and B are ok but C and D are clearly sup materials.

Fig3 panels CDEF: There is no mention of replica, and error bars appear only for panel C without precision on what they represent (standard deviation to the mean?). Considering the low accuracy of the b-Galactosidase assay and that the values are in the very low ranges close to the threshold of detection, I would be very surprised if the error bars were small enough to be hidden by the symbols.

Also, there is something puzzling that is not addressed. There is a similar trend between the 2 background, 168 (Fig3 EF) and DS7906 (Fig3 CD), regarding the (almost perfectly linear!) anti-correlation between growth rate and spo genes leaky expression. Yet, for given growth rates (which, based on these results is related to 0A activity), the levels of expression of the reporters are quite different between 168 and DS7906 (reason they needed different methods to detect them in the 2 backgrounds).
But then, the 0A activity must be higher in 168 to affect the growth than in DS7906, or we could say that DS7906 growth is highly sensitive (more than 168) to extremely low fluctuation of 0A. This suggests that something else than 0A would play a role in this growth rate control (somehow counter-acting the 0A effect in 168). Since the effect of RapP seems to be entirely depending on 0F/0A, I would like to know how the authors explain this discrepancy.

L111: The title is misleading as it suggests RapP is specifically controlling resource reallocation thus independently of 0A, while its whole action is supposed to be going through 0A, and is necessarily affecting all 0A controlled phenomenon (as it was described before by others: competence, biofilm, sporulation…)

L114: "to gain a more comprehensive insight…"
Again, this is far fetch. The authors investigate the proteome changes (to check for resources reallocation) with and without rapP. Since they already showed by proteomic analysis that absence of 0A reallocate resources, and others before that RapP is a negative effector of 0A through dephosporylation of 0F, thus this investigation is rather a control, confirming an expected logical output. However it did not bring "a more comprehensive insight…" into the phenomenon.

L121: the description of results Fig4C is identical to those they reported for the mutant 0A published last year, which makes sense of course (since they are here looking at the effect of 0A being +/- activated). But again the fact that this was totally expected on the light of this previous results, are not mentioned here. The whole Fig4 is just confirmation of their previous results.

Fig 5I: When comparing the spore counts with the growth curves, the DS7906 culture has a strong head start with a high starting OD compared to reference DK1042. Please explain.

References :

Parashar, V., Konkol, M. A., Kearns, D. B. & Neiditch, M. B. A plasmid-encoded phosphatase regulates Bacillus subtilis biofilm architecture, sporulation, and genetic competence. 2013. J Bacteriol 195, 2437-2448, doi:10.1128/jb.02030-12.

Bendori S, Pollak S, Hizi D, Eldar A. The RapP-PhrP quorum sensing system of Bacillus subtilis strain NCIB3610 affects biofilm formation through multiple targets, due to an atypical signal-insensitive allele of RapP. 2015. J Bacteriol 197:592–602. doi:10.1128/JB.02382-14.

Pollak S., Bendori S., Eldar A. A complex path for domestication of B. subtilis sociality.2015. Curr Genet. Nov;61(4):493-6. doi: 10.1007/s00294-015-0479-9. Epub 2015 Feb 14.

Nordgaard M., Mortensen RMR, Kirk NK, Gallegos-Monterrosa R, Kovács AT. 2021. Deletion of Rap-Phr systems in Bacillus subtilis influences in vitro biofilm formation and plant root colonization. Microbiologyopen. 2021 Jun;10(3):e1212. doi: 10.1002/mbo3.1212.

Mirouze N., Prepiak P., and Dubnau A. 2011. Fluctuations in spo0A transcription control rare developmental transitions in

Bacillus subtilis. PLoS Genet. Apr;7(4):e1002048. Doi. 10.1371/journal.pgen.1002048

Reviewer #3

(Remarks to the Author)
In their interesting paper "A plasmid-encoded global growth accelerator in the ancestral Bacillus subtilis" the authors examined the growth behaviour (and "proteome allocation") of different B. subtilis wild type strains and observed that the ancestral NCBI3610 B. subtilis strain had a growth advantage compared to the auxotrophic domesticated B. subtilis 168 strain. Furthermore, they observed that the rapP gene encoded on a pBS32 plasmid is responsible for the growth advantage.
Subsequently they investigated sporulation-specific mutants and LacZ-reporter fusions, which suggested that rapP acts on this pathway, thereby influencing the growth enhancement, by possibly "accelerat(ing) cell growth by minimizing the leaky expressions of adaptive response pathways (such as sporulation) during exponential stage" (Fig 1-3)(Fig 8)
This hypothesis was supported by proteome MS experiments, whose comparison supported the upregulation of pathways important for growth, such as translation and the downregulation (already in early exponential phase??) of the mentioned "adaptive responsive pathways such as sporulation" in the presence of rapP (Fig 4). In addition, this RapP mediated "more tight" regulation during exponential growth did not to appear to negatively influence the cells ability to later transition into stationary phase developmental processes such as e.g. sporulation (Fig 5).
Additional support came from experiments, where inducible rapP gene was put in trans on the chromosome of Bs 168 and PY79. The expression of RapP rescued the fast growth phenotype, which dependent on the sporulation pathway (Fig 6) and resulted in similar patterns when measuring and comparing the proteome in early exponential phase by MS (Fig 7).

Comments & question

This is a very interesting observation and a number of questions, which should at least be discussed in more details, are raised. It is for example not really addressed in the discussion how RasP could achieve this sophisticated influence on growth integrating it with the control of cell developmental pathways.
There are already some publications on this Rap/phr family of proteins and also some experiments with RapP has been performed. So the authors could discuss a little bit in detail what the "The RapP-mediated regulatory network" could be. I believe that it was already suggested in the literature that RapP can act on the sporulation pathway.

It would be great to know whether RapP is present and expressed and whether one can estimate its (relative) levels under the experimental conditions.
Since RapP family proteins are protein phosphatases of phosphorylated Histidine sidechains it would be very interesting to know whether a RasP active site point mutant, lacking its enzyme activity, behaves like a deletion mutant. Thereby one could estimate whether RasP works through its phosphatase activity or if also protein-protein interactions might be involved in this "network".

-The RapP activity on sporulation is more prominently investigated in this paper, it is my impression that other pathways such as competence are sometimes also implied, while the effect on biofilm formation is not very strong and motility, another adaptive pathway, is even upregulated.
-The proteome MS data is generated with cells early in exponential growth phase, however the first remnants of these pathways are detected by this method as "the leaky expressions of adaptive response pathways. Some comments whether this MS method is so sensitive that it can detect this? The mentioned number of 2500 identified proteins looks good.

It should also be mentioned that these different distinct "adaptive response pathways" result in heterogenous subpopulations which can be formed during transition to stationary phase cells. These subpopulations are not detected that well with promoter LacZ Fusions measuring the averaged whole cell population. For the detection of e.g. sporulation, competence, motility, biofilm reporter GFP fusion with subsequent Fluorescence microscopy or FACS cell sorting can be applied to estimate the formation of these subpopulation. I guess the sporulating subpopulation can also be (much) later identified by the formed spores.
With these reporter strains one might be able to estimate the extent of the finally formed subpopulations.

- In Fig 1B the authors should add the Names and genotype of the strains maybe below the graphs of the cells.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am happy with the authors' responses to my concerns.

Reviewer #2

(Remarks to the Author)
First, I would like to apology for the time taken for this review.

I acknowledge the authors for their detailed argumentation regarding the novelty of their contribution, and I think that the readers will now have the necessary references and background information to make their own opinion. I also thank the authors for the corrections introduced throughout the manuscript, and congratulate them for the clarity of their manuscript and the significant amount of work supporting this article.

Reviewer #3

(Remarks to the Author)
I am happy with the authors reply to my questions and comments. And I believe that this new revised version (based on all three Referee comments and suggestions) of this interesting manuscript is much improved.

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This interesting manuscript by Zhu et al. describes the identification of the plasmid-encoded RapP protein as a factor that enhances growth due to the effective suppression of the phosphorelay and thus a more pronounced separation of growth-related gene expression from stationary phase gene expression. The plasmid carrying the rapP gene is present in non-domesticated wild type strains of Bacillus subtilis but not in the laboratory strain 168. As a result, the laboratory strain exhibits a reduced growth rate as compared to the wild strain and has a competitive disadvantage on minimal media.

We thank the reviewer very much for his/her positive evaluation of our manuscript as well as various pieces of excellent suggestions below which further help us improve our manuscript.

General comments:
#1 The lab strain was probably evolved on complex medium with all components available. Under such conditions, there might be no fitness difference. Please include such an experiment using LB for example!

We thank the reviewer very much for this nice suggestion. In Figure S1 of the revised manuscript, we have now included such data. Indeed, 168 strain has the same growth rate as 3610 in LB rich medium.

Specific comments
#1 l. 18: What is meant by “in such case”? I is not clear whether there is a link between RapP and regulation here.

We thank the reviewer for pointing out this issue. We have changed the term “in such case” to “therefore” (line 18).

Our point is illustrated in Figure 8, during exponential growth when nutrient is still abundant, RapP accelerates cell growth by minimizing the leaky expressions of Spo0A-mediated adaptive response pathways (e.g., sporulation genes) during exponential stage and further maximizing the cellular budget of biosynthetic pathways, as shown in Figure 3 and Figure 4. Without RapP, the *rapP*-null DS7906 strain has an unnecessarily high investment on adaptive pathways, which limits the cellular resource for biomass growth and thus has a lower growth rate.

On the other hand, we further show that during late stage when the nutrients run out, the *rapP*$^+$ strain could substantially activate the adaptive response such as sporulation to adapt to the nutrient starvation condition (as shown in Figure 5). Therefore, it means that for RapP$^+$ strain, it could achieve fast growth during exponential growth (as RapP$^+$ minimize the unnecessarily adaptive response) but substantially activate adaptive response only when it is necessary (e.g., during nutrient starvation stage). Therefore, *rapP*$^+$ strain could prevent the premature expression of proteins related to adaptive response compared with *rapP*-null strain.

#2 l. 19 ff: “Our study reveals a delicate strategy”: please be explicit about this “delicate strategy”!

We thank the reviewer for pointing out this issue. We realize that the term “delicate strategy” is subjective, and therefore, we have re-phrased this sentence (line 21).

Our point is that RapP protein minimizes the leaky expressions of adaptive response pathways during exponential growth stage so that cellular resources for supporting biomass growth could be maximized (Figure 8A). In such case, the cellular resource competition between growth and adaptation during exponential growth stage (growth-adaptation trade-off) is avoided or constrained. Furthermore, the RapP$^+$ strain could substantially activate adaptive response for maintenance after entering into late starvation stage during which adaptive response such as sporulation become important (Figure 5 and depicted in Figure 8B). Therefore, its fitness during starvation stage is also guaranteed. Collectively, the presence of RapP protein allows *B. subtilis* to achieve fast growth without compromising its fitness during late starvation stage. Hence, the trade-off between growth-survival (adaptation) could be constrained and largely avoided.

#3 l. 26: “nutrient statues”, better “nutrient availability”

Yes, has fixed (now line 28)

#4 l. 29, l. 64 and throughout: “in such case”, better “therefore”

Yes, has fixed all related parts (now line 31, line 66 and etc.)

#5 l. 36/37: Please do not generalize the role of cAMP, this molecule indicates lack of glucose in E. coli, but this role is not conserved outside the enteric bacteria. If you want to use cAMP as an example, please specify that this is the case for E. coli!

We thank the reviewer for pointing out this important issue. We have specified that it is applicable to *E. coli* (now line 38).

#6 l. 63/64: Is comI located on the chromosome or on pBS32? The sentence is not clear. Please define the differences between the three strains clearly.

We are sorry for the unclearness here. The *comI* is located on pBS32, and we have re-adjusted the sentence (line 64 to 66, also shown in Figure 1B). The difference between 168 strain and 3610 strain has been described in line 52 to 55.

#7 l. 79, Fig. 1D: Please include strain 168 expressing RapP to make sure that RapP alone is sufficient for the effect. The same for fig. 1F.

We thank the reviewer very much for this suggestion. In Figure 6 and Figure 7, we have integrated an artificial RapP-expressing cassette in the chromosome of 168 strain and has found that RapP complementation indeed substantially accelerates the growth of strain 168 (Figure 6B). Since the RapP data for 168 strain is systematically described in Figure 6 and 7, it is technically difficult to move it directly to Fig. 1. But we have shown it in Figure 6D of the revised paper and we can find that RapP indeed enables 168 strain to achieve comparable growth rates with DK1042 strain.

For the RNA-protein data, we have actually shown it in Figure 6E, 6F. We found that RapP complementation increased the ribosome contents (reflected by RNA/protein ratio) of both 168 and PY79 strains in ribose medium (Figure 6E). In Figure 6F, the increase of ribosome content could quantitatively explain the increase of growth rate as their relations completely overlapped with the linear relation between RNA/protein ratio and growth rate (R-line of the new Figure 1D, note that the original 1C and 1D have been combined in new 1C). Therefore, the increase of RNA-protein ratio could quantitatively explain the growth acceleration.

#8 l. 90, Fig. 2: Is the same true in the background of 168? Please put these results in the main document not in the supplement!

Yes, we have moved it to Figure 2B. In addition, original panel C and panel D have been moved to Figure S5.

#9 l. 121: Surfactin or other antibiotics are not cofactors and should not be classified as such!

Yes, we are sorry for this and have removed the term of cofactors (line 138) and describe them as secondary metabolites. The co-factor classification is generated automatically by the proteomap websites based on the KEGG categorization. We have also noted this issue in the legend of Figure 4C.

#10 l. 152 and throughout: please avoid the terms “growth defect” or even “strong growth defect”, better “slower growth” or “reduced growth” or “growth advantage” for strains carrying RapP

Yes, we have fixed all the related terms (e.g., line 172 in revised paper).

#11 Fig. 5B: Please provide a clearer legend for the x axis: xth what? Please provide information on the medium

used in the competition experiment!

Yes, we have fixed the legend and added the medium information to the legend. The xth denotes the eight red time points throughout the growth curve shown in Fig. 5A. We measured the cell fraction sequentially at these eight time points. The co-cultures were cultured in mannose minimal medium.

#12 l. 177: “need-based regulation of adaptive responses”, better “to prevent the harmful expression of”

Yes, we have changed to “prevent the premature expression of proteins related to adaptive response” (now line 200).

#13 l. 229: better “that is mainly triggered”

Yes, fixed (now line 273).

#14 l. 243: better “is widely recognized”

Yes, fixed (now line 287).

#15 l. 261/262: The authors should mention that genome-reduced strains of B. subtilis derived from 168 were already shown to have superior capacities for the production of foreign secreted proteins (PMID 30540431).

We thank the reviewer very much for providing this valuable references. We have cited it (line 304).

We thank the reviewer very much again for his/her positive evaluation of our paper as well as valuable and excellent comments and suggestions, which help us a lot to improve our manuscript.

Reviewer #2 (Remarks to the Author):

General comments:

Zhu and collaborators started from the observation that the wild type Bacillus subtilis strain 3610, the ancestor of strain 168 and most laboratory strains used to study genetics of B. subtilis, presented a higher growth rate than strain 168 when grown in various minimal medium.
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This is especially problematic for RapP itself, who is the main focus of this article, for which most of the literature seems to have been ignored with a single citation (Parashar et al., 2013), while this gene was the subject of several publications during the past decade. I should add that the publications are of importance for the present study since RapP impact on 0A-dependent stationary phase events (biofilm, competence and sporulation) was shown as early as 2013 by Parashar et al., completed by works from Bendori et al., 2015 and Pollack et al., 2015. Parashar also shown that RapP dephosphorylates Spo0F (thus inactivating Spo0A), and Nordgaard et al.,2021 reported the slower growth of 3610 when rapP was deleted, an important finding for this work that Zhu and coworkers reported here too. Of these four publications, only Parashar is cited here but only for a strain construction (!). This is a main issue, not only because it does not give the credits to other teams that worked on it, and because it reduces the clarity of the article, but also because it hides the facts that the rapP gene was an obvious choice when looking for the origin of the growth difference between the strains.

On the same line, their own recent works (Zhu et al., 2023) is very parsimoniously cited while knowing this article enlighten these results very differently. And this is my main concern: the works seems to have been done with great care and as said generated a profusion of data and figures. But in the light of their Science Advances paper and the works of others before, most present results were highly predictable (when not known) and does not bring a lot of new findings and in my opinion is of limited interest and impact.
I will come back to this point in more details below along other minor comments.

We thank the reviewer very much for his/her comprehensive summary and perspective of our paper as well as his/her critical comments of our manuscript. The reviewer has made great efforts to explain and elaborate related important issues, for which we highly appreciate. First, we are sorry for not citing enough related references and providing enough background information in related places. In previous manuscript, we actually cited the authoritative review of Daniel Kearns, J Bacteriol 202, doi:10.1128/jb.00290-20 (2020), which has given a comprehensive summary of RapP regarding its interference on *spo0F-spo0A* signaling pathway. But we do agree with the reviewer that more related references of RapP should be cited. In the revised paper, we have cited all the references the reviewer mentioned and have provided enough background information in various places of the paper (see detailed response below).

Besides, we want to further clarify the important scientific contributions of this paper:

The conceptual advance of this paper over previous publication is that trade-off between growth and adaptation (survival) could be constrained. In Zhu et al 2023 focusing on 168 strain, *spo0A*-null mutant obtained improved growth performance but substantially compromised adaptability or competitiveness compared with the parental 168 strain due to resource re-allocation from adaptive pathways to biosynthetic pathways, suggesting a fundamental trade-off between growth and adaptation. In contrast, our current manuscript shows that RapP allows the ancestral *B. subtilis* strain to achieve need-based regulation of resource allocation ("do the right thing at the right time") (illustrated in Figure 8): minimizing the proteome burden of adaptive response to unleash the growth potential during exponential stage while substantially activate them during starvation stage. Therefore, bacteria could achieve rapid growth during exponential stages without compromising the long-term fitness during late starvation stage (Figure 5). Therefore, the trade-off issue for *spo0A*-null strain is addressed in the ancestral strain with the assistance of RapP, as demonstrated in our study (we have elaborated related points in the manuscript and discussion part such as line 176 to 178; line 240 to 253).

There are three additional contributions:

(1) We have systematically reported the substantial differences in growth physiology between the domestic 168 strain and its ancestral 3610 strain in various nutrient conditions, showing that 3610 strain has a more robust growth performance than 168 strain on various media. The laboratory domesticated strain 168 is not only the earliest sequenced model gram-positive bacterium but also the most intensively studied object in the *B. subtilis* community. 168 strain plays a fundamental role in both theoretical and application fields (including synthetic biology) of *Bacillus* community. Therefore. our study will naturally remind people to look back into the ancestral strain and pay more attention to it, which we think is very meaningful for the Bacillus community. Our data of

using RapP to accelerate the growth of 168 and PY79 would also be promising for the field of synthetic biology and metabolic engineering (discussed in line 299 to 313). Actually, when we studied this project, we first wondered whether the growth differences between 168 and 3610 were due to some mutations in the chromosome and has spent a long time trying to find out the genes. It is until after we have measured the growth rate of DS2569, we began to realize that it is related to pBS32 and further identified *rapP* gene as the major contributor (it is counterintuitive for us at the beginning because plasmid is generally proposed to impose metabolic burdens, please see point 3th below).

(2) We find that RapP represents a global strategy to delay the commitment to sporulation in the ancestral strain, which has been lost in the domesticated strain. Studies have shown that domesticated *B. subtilis* strain employs cannibalism to delay the commitment to the highly energy-cost sporulation process (the pioneering work of Richard Losick in Science (2003) 301, 510-513; Cell (2006) 124, 549-559; reviewed in *FEMS Microbiol Rev* (2011)35, 415-424), which is beneficial for the bacterial fitness. In Figure 5, our work shows that the ancestral *B. subtilis* strain uses RapP as a highly effective strategy to delay the premature entering into sporulation (even though *rapP*-null strain has higher cannibalistic proteins than *rapP*$^{+}$ strain in the mannose medium we used (Figure 4J), which would be of important scientific values for the *B. subtilis* community. This point has been elaborated in line 273 to 282 in discussion part.

(3) By systematically exploring the growth promotion effect of RapP, we revealed an important physiological function of pBS32 and have provided a novel example of plasmid-conferred growth advantage. As mentioned in the final conclusion part of the authoritative review of Daniel Kearns, J Bacteriol 202, doi:10.1128/jb.00290-20 (2020), the authors wondered the physiological importance of pBS32 *"Does its presence confer physiological benefits to the cell, and if so, which of the gene products are selectively advantageous?"* We show here that RapP in pBS32 confers a substantial growth advantage for the ancestral strain on various growth media and has quantitatively established the relation between proteome resource allocation with cell growth. On the other hand, the physiological function of plasmid has been widely concerned due to its tight relation with the spread of antibiotic resistance (***Nat Rev Microbiol*** (2024) 22, 18-32, (2024)). It is generally proposed that the plasmids generally impose fitness cost and physiology burdens on the host (***Nat Rev Microbiol*** (2021) 19, 347-359). In contrast to numerous cases of plasmid costs, here we provide a counterintuitive example of plasmid carriage that could confer fitness (growth) advantage to the host cells, which provides a new insight into the physiology of plasmids. This point is elaborated in line 284 to 296 in discussion part.

Overall, we hope the reviewer could now appreciate the important scientific contributions of our manuscript and thanks the reviewer very much again for his/her highly valuable suggestions and discussions on our study.

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Comments:

Fig1 (legend): please indicate what are the error bars (SD to the mean?) and number of replicates. Panels C & D could probably be pooled together (redundant info between them) and possibly even with panel A

Yes, the error bars are SD to the mean and we have included the information in the legend. We have also combined panel C and D while for panel A, we want to first emphasize the growth difference between 3610 and 168, which is the key finding that inspires us to work on this project.

We also realized that some small panels are redundant and have also combined them (e.g., we have combined the original two qPCR panels for mannose and ribose media into one panel, Fig. 3A).

Fig1B: a small suggestion: of the five strains, all but 168 are direct parents varying only at the level of the plasmid (cured or mutated), while 168 is genetically distant (having additional mutations and indels in its chromosome). I would make this apparent on the schema somehow, to distinguish it from the others.

We thank the reviewer for this nice suggestion, and yes, we have modified it in the current version in Figure 1B and have also described it in the legend.

Line76-80: this section definitely can't neglect the previous finding that rapP deletion reduces growth rate of 3610 (Nordgaard et al., 2021)

We really thank the reviewer for providing this valuable reference and we have cited it (Ref 39 in line 80). We also pointed out here that our work is a systematic study (especially at the background of growth difference of 168 vs 3610) and have shown that RapP could account for all the growth promotion effect of pBS32 (Figure 1C).

Fig1.E: "To gain a mechanistic insight into the positive effect of rapP on cell growth, we measured the ribosome content of B. subtilis as ribosome synthesis lied at the core of bacterial growth control". I am not convinced this experiment brings any information, much less mechanistic details. The correlation between the abundance of ribosomes (here reported ass the RNA/protein ratio) and the growth rate is a known thing (Klumpp et al., 2008, 2009), and this correlation is maintained here across conditions (media) and strains. Since the mutant grow slower, the ratio is lower but it gives no additional information about RapP mode of action. If the correlation has been lost, this would have brought a fresh lead, but as it is, it is rather a control that does not add more insight into the phenomenon.

We thank the reviewer for sharing this interesting perspective. We have first removed the term of "mechanistic insight" (line 87). So basically, the linear correlation between ribosome content and growth rate ("R-line") can be used as an indicator of cellular resource allocation (Scott 2010 Science; also refers to two recent reviews; Scott and Hwa 2022 Nat Rev Microbiol; Zhu & Dai 2020 Trends Biochem Sci). Ribosome synthesis lies at the core of bacterial cellular resource allocation. Here we observe that *rapP*-null strain follows the same R-line as *rapP*$^+$ strain. However, we can see that the ribosome content of *rapP*$^+$ strain at each medium is shifting to higher values than *rapP*-null strain, suggesting that *rapP*$^+$ strain has a resource re-allocation from somewhere else (we know later in Fig. 4 that it is *spo0A*-mediated adaptive response) to ribosome synthesis (biosynthetic pathway) to support higher growth rates, further laying the foundation for the follow-up proteome allocation study in Fig. 4. In addition, the slope of R-line is an indicator of translation elongation rate (Scott et al 2010), excluding the possibility that RapP affect growth rate via affecting the translation elongation rate. We thank the reviewer for his/her understanding on this issue.

L88-95: as stated on my general comments, this section really lacks background information (about 0A, the phosphorelay and the Rap phosphatases) and references.
More importantly, their own recent results are just mentioned (ref 27 at L91) but not put in perspective with their current results. This is critical because since they already published that deleting spo0A reallocates resources to metabolisms allowing a faster exponential growth, thus anything decreasing 0A activation, including anything acting negatively on the phosphorelay (as Rap phosphatase) during exponential growth, will necessarily have a similar impact. Since RapP was previously shown by others to act on 0A via 0F and its absence to increase growth rate…
All previous findings should appear already in the introduction and the state of the art clearly depicted.

We really thank the reviewer for pointing out this important issue. We have now added related background information and have also cited all related references here (0A, phosphorelay and RapP phosphatases in line 96 to 101). Zhu et al 2023 is mentioned in line 103-104 and 108.

L97-109: Effect of rapP on the expression of sporulation genes:
The first result of this section (qPCR) showed that the absence of rapP "strongly stimulated" the 0A-dependent genes assayed, but the levels of induction are in truth extremely low, as it appears later with the b-galactosidase assay (lacZ). This is not obvious in the text and a bit misleading. One needs to be familiar with the assay and Miller units to realize that on panels Fig 3 E/F, the values are closed to the background, and it is indicated only in the Methods section that in the 3610 background, the levels were so low the author had to change detection method for fluorescence (panel C/D).
I think it is important to stress out in this result section that what they observed are (barely detectable) fluctuations of background levels of 0A activity and that the sporulation genes are followed only as proxy of 0A activity and not for the phenomenon of sporulation they are involved in. If not, one may be surprised that sporulation genes are "strongly" expressed during exponential growth while sporulation is supposed to occur in carefully designed media in stationary phase.
This observation should also be put in perspective with previously reported fluctuation of spo0A expression (and

activation to low levels) during exponential growth (Mirouze et al., 2011).

We thank the reviewer for pointing out this important issue. We have now mentioned related content in the manuscript (line 116 to 119) that what we observed was largely basal expression levels of sporulation (very low in activity) and we measured it to monitor the fluctuation of Spo0A activity during exponential stage under various nutrient conditions. We have also cited and discussed our finding in perspective with Mirouze et al 2011 (line 126 to 130).

Fig2: Panels A and B are ok but C and D are clearly sup materials.

Yes, we have put C and D into sup materials (Figure S5). We also realized that some small panels are redundant and have also combined them (e.g., we have also combined the original two qPCR panels for mannose and ribose media into one panel, Fig. 3A).

Fig3 panels CDEF: There is no mention of replica, and error bars appear only for panel C without precision on what they represent (standard deviation to the mean?). Considering the low accuracy of the b-Galactosidase assay and that the values are in the very low ranges close to the threshold of detection, I would be very surprised if the error bars were small enough to be hidden by the symbols.

We are sorry for not providing this information. In previous manuscript, *spoIIE-lacZ* data in DK1042 and DS7906 have several biological replicates and have error bars (original panel C, now panel B). We have now added more biological replicates into other panels. Specially, we have now re-measured the 168 data using the same MUG assay (see responses below).

Also, there is something puzzling that is not addressed. There is a similar trend between the 2 background, 168 (Fig3 EF) and DS7906 (Fig3 CD), regarding the (almost perfectly linear!) anti-correlation between growth rate and spo genes leaky expression. Yet, for given growth rates (which, based on these results is related to 0A activity), the levels of expression of the reporters are quite different between 168 and DS7906 (reason they needed different methods to detect them in the 2 backgrounds).
But then, the 0A activity must be higher in 168 to affect the growth than in DS7906, or we could say that DS7906 growth is highly sensitive (more than 168) to extremely low fluctuation of 0A. This suggests that something else than 0A would play a role in this growth rate control (somehow counter-acting the 0A effect in 168). Since the effect of RapP seems to be entirely depending on 0F/0A, I would like to know how the authors explain this discrepancy.

We thank the reviewer for pointing out this issue and we are sorry for the confusion here. There are actually no big differences between the *spoIIE* & *spoIIG* promoter activities of 168 and DS7906. The reason is that: the result of 168 was done much earlier before the work of DS7906 and DK1042. We did see the very low activity of 168, which was actually close to the detection limit of ONPG. Then when we measured the promoter activities for DS7906 and DK1042, we did not use ONPG and directly changed to MUG. MUG is a highly sensitive fluorescent substrate, being much more sensitive than ONPG and could give more reliably quantitative data.

We have now re-done the experiments of 168 strain using MUG, the same as DS7906. Actually, compared 168 with DS7906 (panel B vs panel D; and panel C vs panel E), we can find that the two strains generally give comparable results.

L111: The title is misleading as it suggests RapP is specifically controlling resource reallocation thus independently of 0A, while its whole action is supposed to be going through 0A, and is necessarily affecting all 0A controlled phenomenon (as it was described before by others: competence, biofilm, sporulation…)

Yes, we have modified this title (line 131).

L114: “to gain a more comprehensive insight…”
Again, this is far fetch. The authors investigate the proteome changes (to check for resources reallocation) with and

without rapP. Since they already showed by proteomic analysis that absence of 0A reallocate resources, and others before that RapP is a negative effector of 0A through dephosporylation of 0F, thus this investigation is rather a control, confirming an expected logical output. However it did not bring "a more comprehensive insight…" into the phenomenon.

Yes, we have removed this sentence (line 132).

L121: the description of results Fig4C is identical to those they reported for the mutant 0A published last year, which makes sense of course (since they are here looking at the effect of 0A being +/- activated). But again the fact that this was totally expected on the light of this previous results, are not mentioned here. The whole Fig4 is just confirmation of their previous results.

We thank the reviewer for pointing out this issue, and we have now pointed out its consistence with Zhu et al 2023 Sci Adv (line 165 to 168). Figure 4 is still important as it quantitatively evaluates the effect of RapP to see whether its interference on Spo0F-Spo0A signaling pathway is strong enough to cause a global resource re-allocation as found in *spo0A* knockout. Without the resource allocation study of Figure 4, we would be unable to obtain the conclusion that RapP could globally downregulate the *spo0A*-mediated adaptive response.

Fig 5I: When comparing the spore counts with the growth curves, the DS7906 culture has a strong head start with a high starting OD compared to reference DK1042. Please explain.

We thank the reviewer for pointing out this issue. The reason is that: during the experiments of Fig. 5I, since DS7906 strain grows much slower than DK1042 strain, overnight culture of DS7906 in minimal medium was still at the range of exponential stage and could start at a high initial OD while the culture of DK1042 had reached saturation. Therefore, DK1042 culture needed to be inoculated at a low initial OD.

References:

Parashar, V., Konkol, M. A., Kearns, D. B. & Neiditch, M. B. A plasmid-encoded phosphatase regulates Bacillus subtilis biofilm architecture, sporulation, and genetic competence. 2013. J Bacteriol 195, 2437-2448, doi:10.1128/jb.02030-12.

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Pollak S., Bendori S., Eldar A. A complex path for domestication of B. subtilis sociality.2015. Curr Genet. Nov;61(4):493-6. doi: 10.1007/s00294-015-0479-9. Epub 2015 Feb 14.

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Mirouze N., Prepiak P., and Dubnau A. 2011. Fluctuations in spo0A transcription control rare developmental transitions in Bacillus subtilis. PLoS Genet. Apr;7(4):e1002048. Doi. 10.1371/journal.pgen.1002048

We thank the reviewer again for providing these valuable references and have cited all of them.

We thank the reviewer very much again for his/her valuable and excellent comments and suggestions, and especially for sharing so many interesting and meaningful scientific perspectives with us.

Reviewer #3 (Remarks to the Author):

In their interesting paper "A plasmid-encoded global growth accelerator in the ancestral Bacillus subtilis" the authors examined the growth behaviour (and "proteome allocation") of different B. subtilis wild type strains and observed that the ancestral NCBI3610 B. subtilis strain had a growth advantage compared to the auxotrophic domesticated B. subtilis 168 strain. Furthermore, they observed that the rapP gene encoded on a pBS32 plasmid is responsible for the growth advantage. Subsequently they investigated sporulation-specific mutants and LacZ-reporter fusions, which suggested that rapP acts on this pathway, thereby influencing the growth enhancement, by possibly "accelerat(ing) cell growth by minimizing the leaky expressions of adaptive response pathways (such as sporulation) during exponential stage" (Fig 1-3)(Fig 8)
This hypothesis was supported by proteome MS experiments, whose comparison supported the upregulation of pathways important for growth, such as translation and the downregulation (already in early exponential phase??) of the mentioned "adaptive responsive pathways such as sporulation" in the presence of rapP (Fig 4). In addition, this RapP mediated "more tight" regulation during exponential growth did not to appear to negatively influence the cells ability to later transition into stationary phase developmental processes such as e.g. sporulation (Fig 5).
Additional support came from experiments, where inducible rapP gene was put in trans on the chromosome of Bs 168 and PY79. The expression of RapP rescued the fast growth phenotype, which dependent on the sporulation pathway (Fig 6) and resulted in similar patterns when measuring and comparing the proteome in early exponential phase by MS (Fig 7).

We thank the reviewer very much for his/her positive evaluation of our manuscript as well as various pieces of excellent suggestions below which further help us improve our paper.

Comments & question

This is a very interesting observation and a number of questions, which should at least be discussed in more details, are raised. It is for example not really addressed in the discussion how RasP could achieve this sophisticated influence on growth integrating it with the control of cell developmental pathways.
There are already some publications on this Rap/phr family of proteins and also some experiments with RapP has been performed. So the authors could discuss a little bit in detail what the "The RapP-mediated regulatory network" could be. I believe that it was already suggested in the literature that RapP can act on the sporulation pathway.

We thank the reviewer for pointing out this important issue. Our work has shown that the growth promotion effect of RapP is mediated by Spo0F-Spo0A signaling pathway (Figure 2 and Figure 6H, 6I). Previous studies have shown that RapP functions by targeting the Spo0F protein (Parashar et al 2013, Ref 40). Therefore, it will negatively affect the phosphorelay from Spo0F to Spo0B and finally to Spo0A. Spo0A (Spo0A-P is the active form) is a master regulator of sporulation and various adaptive pathways in *B. subtilis* (introduced in line 96 to 99 of the revised paper). In Figure 3, Figure 4 and Figure 7, we indeed show that RapP could effectively down-regulate various Spo0A-mediated adaptive responses (e.g., sporulation, surfactin and antibiotic biosynthesis, competence, etc).

Therefore, the global picture is depicted in Figure 8: RapP protein, by interfering with the Spo0F-Spo0A signaling pathway, substantially reduces the cellular investment on Spo0A-mediated adaptive response pathways during exponential growth so that the cellular budget of biosynthetic pathways (e.g., ribosome) could be maximized to support rapid growth of the ancestral RapP^{+} *B. subtilis* strain.

In the revised paper, we have now further elaborated related information regarding the effect of RapP on gene expression and resource allocation via targeting Spo0F-Spo0A signaling pathway (please see line 244 to 272)

It would be great to know whether RapP is present and expressed and whether one can estimate its (relative) levels under the experimental conditions.

We thank the reviewer for pointing out this important issue. Our proteomic study could detect the absolute cellular abundances of RapP. As shown in Figure 7B, the mass fraction of RapP in the proteome of DK1042 is 0.04% and 0.06% in mannose and ribose media, respectively. In addition, in our experiment of the artificial RapP expression in 168

strain, the RapP abundance is also ~0.06% (also ribose medium), being comparable with the native levels of ancestral *B. subtilis* strain.

Since RapP family proteins are protein phosphatases of phosphorylated Histidine sidechains it would be very interesting to know whether a RasP active site point mutant, lacking its enzyme activity, behaves like a deletion mutant. Thereby one could estimate whether RasP works through its phosphatase activity or if also protein-protein interactions might be involved in this "network".

We thank the reviewer for this nice suggestion. Based on the study of Parashar et al 2013 (Ref 40), we studied the effect of RapP E49A (phosphatase inactive form) on cell growth. From Figure S3, we do see that de novo expression of native RapP in a plasmid could completely restore the growth defect of DS7906. Meanwhile, the RapP E49A could also, albeit not fully, restored the growth defect. This suggests that phosphatase activity of RapP on Spo0F is important for its growth acceleration effect. Meanwhile, other effect, e.g., the direct steric binding of RapP (inactive E49A form) to Spo0F could also interfere with the Spo0F-Spo0A signaling pathway (described in line 82 to 87).

-The RapP activity on sporulation is more prominently investigated in this paper, it is my impression that other pathways such as competence are sometimes also implied, while the effect on biofilm formation is not very strong and motility, another adaptive pathway, is even upregulated.

We thank the reviewer for pointing out this issue and we have extensively discussed related content in line 255-272 of the revised paper. As we mentioned, RapP takes effect by targeting the Spo0F-Spo0A signaling pathway. Therefore, the adaptive response in this paper should be more specified as Spo0A-mediated adaptive response and we have specified in related parts of the revised paper in addition to Figure 8 (e.g., line 17 of abstract and also in discussion part). So basically, when we talk about adaptive response, we are not limited to one specific sector (as different adaptive pathways might in principle even affect the levels of each other due to competition for the limited numbers of Spo0A). Instead, we are focusing on the changes of various Spo0A-mediated adaptive pathways as a whole. We summed various adaptive pathways together and we could solidly get the conclusion that RapP causes a global downregulation of Spo0A-mediated adaptive pathways so that more cellular resources are available for supporting biomass growth (Figure 4K, 4L and 7G).

Among those various proteins regulated by Spo0A, the most well-known and typical adaptive response is sporulation as Spo0A is known as a master regulator of sporulation. In addition, this is particular important in Figure 5 where we studied the fitness of bacteria during long-term starvation as sporulation is the one of the most crucial adaptive responses of *B. subtilis* to survive during long-term starvation. It is also one of the major proteome sectors that affected by RapP (Figure 4H, 4I and 7E). That's the reason why we emphasize sporulation. We also acknowledge that Spo0A, as a master regulator, also regulates various other pathways (competence and cannibalism, etc) and it is quantitatively captured by our proteome data (Figure 4J and Figure 7F). However, the abundances of competence and cannibalism are much less than sporulation (only accounting for 0.1% to 0.2% of the whole proteome). Therefore, we take sporulation as a major example of adaptive response and have now also modified the caption of Figure 5 to specified "sporulation pathway".

We note that motility is even upregulated by RapP and the result is actually logical. First, although Spo0A-P, as a key regulator of stress response in *B. subtilis*, activate various adaptive pathways. It actually also represses the expression of a few other genes such as chemotaxis & motility (Ref 65). Therefore, it makes sense that RapP targets Spo0F-Spo0A signaling pathway and upregulates motility (line 270).

Biofilm, although also known to be positively regulated by Spo0A-P, was not quite affected by RapP in our case (Figure S8). We have also discussed the potential reasons: a major point is that although Spo0A-P positively regulate various adaptive response including sporulation, competence, cannibalism, biofilm, antibiotic biosynthesis, etc. It has been suggested that different adaptive pathways require different thresholds of Spo0A-P. In principle, different adaptive pathways could have direct competitions for the available cellular Spo0A-P, which could further affect the relative abundances of each other. These mechanisms could ultimately lead to the different sensitivities of each specific adaptive pathway to the effect of RapP.

We have extensively discussed related content in line 255-271 and thanks the reviewer again for pointing out this interesting and important issue.

-The proteome MS data is generated with cells early in exponential growth phase, however the first remnants of these pathways are detected by this method as "the leaky expressions of adaptive response pathways. Some comments whether this MS method is so sensitive that it can detect this? The mentioned number of 2500 identified proteins looks good.

We thank the reviewer for pointing out this issue. The "leaky expression" could also be viewed as basal expression. Our MS data, based on the recently-emerging 4D label-free MS, has high coverage and high reproducibility (Figure S6 and S12). When we talk about adaptive response pathways, we are not referring to one or two proteins. Instead, a specific adaptive response pathway often contains dozens of proteins together, and could be viewed as "sector" (please see the raw supplementary MS table). For example, the "sporulation sector" consists of about 100 proteins. We quantify the mass fraction of a whole sector and compare different conditions. The abundances of various sectors are highly accurate and reproducible (please see the very small deviation of the two biological replicates in Figure 4 (e.g., 4D and 4E).

It should also be mentioned that these different distinct "adaptive response pathways" result in heterogenous subpopulations which can be formed during transition to stationary phase cells. These subpopulations are not detected that well with promoter LacZ Fusions measuring the averaged whole cell population. For the detection of e.g. sporulation, competence, motility, biofilm reporter GFP fusion with subsequent Fluorescence microscopy or FACS cell sorting can be applied to estimate the formation of these subpopulation. I guess the sporulating subpopulation can also be (much) later identified by the formed spores.
With these reporter strains one might be able to estimate the extent of the finally formed subpopulations.

We really thank the reviewer for pointing out this interesting issue. We quite agree that population heterogeneity could occur during transition stage to late starvation stage (related to Figure 5). Actually, we have already measured the dynamic change of spore fractions (using classical heating shock analysis) throughout the whole process in Figure 5 I and 5J. We can clearly observe that DK1042 strain just exhibited a delay in entering into sporulation (red circles, Figure 5I) compared with DS7906 strain (pink squares, Figure 5I). However, although being delayed, spore fractions of DK1042 strain could further increase steadily to 70% during long-term starvation stages (eight days after nutrient depletion) while the spore fractions of DS7906 strain were maintained at 70% to 80% (Figure 5J). The delayed entering into sporulation of DK1042 compared with DS7906 is consistent with the trend of *spoIIE-lacZ* and *spoIIG-lacZ* reporter activities for which DS7906 exhibits a premature expression of sporulation genes compared with DK1042 (panel E versus G; panel F vs panel H in Figure 5). Therefore, we believe our current result is enough to address the reviewer's concern and thank the reviewer again for his/her understanding.

We also point out that the study on Figure 5 actually revealed that RapP represents a global strategy to delay the commitment to sporulation in the ancestral strain, which has been lost in the domesticated strain. Studies have shown that domesticated *B. subtilis* strain employs cannibalism to delay or avoid the commitment to the highly energy-cost sporulation process (the pioneering work of Richard Losick lab in Science (2003) 301, 510-513; Cell (2006) 124, 549-559), which is beneficial for bacterial fitness. In Figure 5, our work shows that actually the ancestral *B. subtilis* strain uses RapP as a more global strategy to delay the premature entering into sporulation (even though *rapP*-null strain has higher cannibalism proteins than *rapP*$^{+}$ strain in the mannose medium we studied, Figure 4J). which would be of important scientific values for the *B. subtilis* community. This point has been elaborated in line 273 to 282 in discussion part.

In summary, our study shows that DS7906 (*rapP*-null) has an unnecessarily, premature proteome investment on adaptive response (e.g., sporulation) even during exponential stage, which limits the cellular budget of biosynthesis and results in slow growth. In contrast, RapP allows *B. subtilis* to achieve need-based regulation of resource allocation ("do the right thing at the right time"): minimizing the proteome burden of Spo0A-mediated adaptive response to unleash the growth potential during exponential stage while substantially activate them during starvation stage (sporulation genes in Figure 5). Therefore, bacteria could achieve rapid growth during exponential stages without compromising the long-term fitness during late starvation stages (depicted in Figure 8).

- In Fig 1B the authors should add the Names and genotype of the strains maybe below the graphs of the cells.

We thank the reviewer for this nice suggestion and we have done so.

We thank the reviewer very much again for his/her positive evaluation of our paper as well as valuable and excellent comments and suggestions, which help us a lot to improve our manuscript.