nature portfolio

Peer Review File

Engineering a phi15-based expression system for stringent gene expression in Pseudomonas putida

Corresponding Author: Professor Rob Lavigne

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 study constructed a phi15 expression system in P. putida, offering a homogeneous and tunable response, which will provide researchers with the flexibility to fine-tune the system output according to their specific requirements. The optimized strains of P. putida, namely P15, P15-L, P15-GD, and P15-LGD, present promising prospects for diverse applications in biotechnology and synthetic biology. Overall, it's an interesting job, but some issues need to be rationalized.
1. The text lacks line numbers, making it difficult to describe the specific location.
2. There are some grammar or formatting errors, for example, P2, “is undoubtedly the foundation of the Standard European Vector Architecture”, the sentence is missing a subject. Also, there’s extra space or missing space in some sentences.
3. Please indicate the full name when the abbreviation first appears, for example, inducer 3-methylbenzoate (3mBz).
4. Why did the author choose P. putida SEM11 as the expression host? Is strain SEM11 a genome-reduced KT2440? I wish to know its background. Also, please describe the characteristics of this strain when mentioning it for the first time.
5. Fig. 3 is missing. In this version, Fig. 3 and Fig. 2 are the same. Since I can’t see Fig. 3, thus I have some questions, such as, how to understand “A weaker RBS and start codon to drive phi15rnap expression improves the inducibility of the phi15 expression system”?
6. What does “degenerate variants (RBS-C and RBS-D)” mean? The author didn’t tell the details. Also, why could the degenerated RBS improve the inducibility of the system more than tenfold?
7. Fig. 4, Genomic integration of P14c-BCD22-phi15lys(G3RQ) in P. putida SEM11 improves the inducibility of the phi15 expression system. However, Fig. 4a shown the map of KT2440.

Reviewer #2

(Remarks to the Author)
Summary

The authors present the careful development and testing of an optimized expression system for Pseudomonas putida that does not rely on the T7 RNAP that is toxic to Pseudomonas. The study is well-designed and the experiments appropriately conducted. The materials and methods are described in sufficient details. Statistical analyses are performed and described appropriately. The paper is well-written and the author’s reasoning for experiments and the description and discussion of the results is easy to follow. The developed system should be of high relevance for the field.

The optimization strategy involved selecting an inducible system to drive phi15 RNAP expression, genomic integration, adding a phi15 lysozyme, inhibiting phi15 RNAP at basal levels, and including a growth-decoupling module – all in great detail, with control experiments, and comparison to existing systems.

The authors eventually developed four platform strains with modifications of the system that result in a more or less tight system, higher expression levels, and growth decoupling, which can be selected for specific purposes and needs of a given application or question. Further possible improvement strategies are discussed.

Finally, the system was tested with a relevant gene-of-interest to produce fluorinase in comparison to other established expression systems as benchmarks, which showed that an increase in product formation was achieved with the optimized

system.

The results are discussed appropriately and put in the wider context.

Figure 2 and 3 are identical, Figure 3 is missing!

Specific

Abstract:
- add “T7 phage polymerase“, „phi15 phage polymerase“ as used later in the text
- “application in Pseudomonas putida” as no other species have been tested here

Introduction:
Page 2
- Align the use of Pseudomonas and Pseudomonas putida and do not use interchangeable, as this work is specific for P. putida and cannot be generalized without further experimentation
- Mid of second paragraph: remove have sentence after reference 20.
- Third paragraph: I think it should be rephrased to say that a reliable expression system is remarkably underrepresented instead of “the T7 phage RNA polymerase…”

Page 3
- Introduce (abbreviations) SEVAtile/Golden Standard (give references)
- Introduce abbr msfGFP

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Figure S2: There are no panels, remove “a)” and explain what is shown left and right.
Check all Figures and Suppl Figures for decimal delimitator “,” vs “.”

Reviewer #3

(Remarks to the Author)
The manuscript ‘Engineering a phi15-based expression system for stringent gene expression in Pseudomonas putida’ focuses on establishment of a phi15-based expression system in Pseudomonas putida as a replacement of T7 platform having issues of cytotoxicity. The authors demonstrated that the phi-15 based expression system increases the protein yield by 2.5-5fold compared to traditional expression systems.
Overall, the study contains development of a new expression system that will be of interest in to the field of protein expression in Pseudomonas putida.
However, several aspects of the study need to be addressed.
Comments:
1. Overall presentation needs to be improvised: Figure 3 is missing in the manuscript and figure 2 is tagged as figure 3 which makes line number 342 to 423 inconclusive. Erroneous sentence construction in line number 48 and 341.
2. In figure 2, fluorescence/OD values remain identical with increasing

concentration of Inducer in case of fluoride responsive riboswitch promoter in the absence of phi15 RNAP in case of both the medium. Discuss the probable reason.
3. In case of LacI/PlacUV5 promoter, at zero concentration of inducer the fluorescence/OD value is very low when the P inducible promoter is placed upstream of msfgfp directly. However, when the phi15rnap is under the control of the same promoter and msfgfp gene is under the control of Pphi15 promoter the leaky expression at zero inducer concentration is comparatively higher (for both the medium). Explain.
4. Figure 6 should include a control set with cells harboring T7 RNAP promoter at induced and uninduced condition and FI should have been provided to have a clear idea.
5. In figure 6, 5(6) FAM /OD 600 of P15 GD induced was approximately 30% higher than that of P15. Whereas, in fig 8b, 5' FDA ug/mg protein for the both are almost identical. Discuss the disparity.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have successfully addressed my comments and concerns.

Reviewer #2

(Remarks to the Author)
All comments were addressed appropriately, and after reviewing the correct Figure 3 in addition to the implemented changes, I do not have any further comments.

Reviewer #1 (Remarks to the Author):

This study constructed a phi15 expression system in P. putida, offering a homogeneous and tunable response, which will provide researchers with the flexibility to fine-tune the system output according to their specific requirements. The optimized strains of P. putida, namely P15, P15-L, P15-GD, and P15-LGD, present promising prospects for diverse applications in biotechnology and synthetic biology. Overall, it's an interesting job, but some issues need to be rationalized.

1. The text lacks line numbers, making it difficult to describe the specific location.

We believe this is a technical issue on the journals' end as line numbers are automatically added in the final PDF upon submission to the journals' portal and other reviewers (see comments reviewer 3) did have line numbering in their document.

2. There are some grammar or formatting errors, for example, P2, "is undoubtedly the foundation of the Standard European Vector Architecture", the sentence is missing a subject. Also, there's extra space or missing space in some sentences.

We have done a thorough pass to correct grammar and formatting errors.

3. Please indicate the full name when the abbreviation first appears, for example, inducer 3-methylbenzoate (3mBz).

Thank you for this remark. Missing full names have now been introduced throughout the text.

4. Why did the author choose P. putida SEM11 as the expression host? Is strain SEM11 a genome-reduced KT2440? I wish to know its background. Also, please describe the characteristics of this strain when mentioning it for the first time.

SEM11 is indeed a genome-reduced derivative of KT2440. We have added the genotype in the methods section to clarify this. We also clarified the choice for this strain in the results section by adding the statement "SEM11 was chosen for its potential as an enhanced production strain since it is a genome-reduced derivative of KT2440 with increased genome stability and generally improved growth and physiology parameters"

5. Fig. 3 is missing. In this version, Fig. 3 and Fig. 2 are the same. Since I can't see Fig. 3, thus I have some questions, such as, how to understand "A weaker RBS and start codon to drive phi15rnap expression improves the inducibility of the phi15 expression system"?

We apologize for mistakenly adding a duplicate figure to the final manuscript file. Figure 3 has now been replaced with the intended figure. We appreciate you taking the time to re-review the figure and the manuscript text that is associated to it.

6. What does "degenerate variants (RBS-C and RBS-D)" mean? The author didn't tell the details. Also, why could the degenerated RBS improve the inducibility of the system more than tenfold?

The RBS variants to which you refer are indicated in the newly added figure 3 together with associated data that demonstrates improved inducibility. We hope this sufficiently clarifies the missing context and apologise that it was not added in the initial version.

7. Fig. 4, Genomic integration of P14c-BCD22-phi15lys(G3RQ) in P. putida SEM11 improves the inducibility of the phi15 expression system. However, Fig. 4a shown the map of KT2440.

We have changed the map to the one of SEM11 for clarity. The sequence location remains unchanged as it involves a genome reduced strain of KT2440, making both maps valid to pinpoint the exact engineering location.

Reviewer #2 (Remarks to the Author):

Summary

The authors present the careful development and testing of an optimized expression system for Pseudomonas putida that does not rely on the T7 RNAP that is toxic to Pseudomonas. The study is well-designed and the experiments appropriately conducted. The materials and methods are described in sufficient details. Statistical analyses are performed and described appropriately. The paper is well-written and the author's reasoning for experiments and the description and discussion of the results is easy to follow. The developed system should be of high relevance for the field.

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The authors eventually developed four platform strains with modifications of the system that result in a more or less tight system, higher expression levels, and growth decoupling, which can be selected for specific purposes and needs of a given application or question. Further possible improvement strategies are discussed.

Finally, the system was tested with a relevant gene-of-interest to produce fluorinase in comparison to other established expression systems as benchmarks, which showed that an increase in product formation was achieved with the optimized system.

The results are discussed appropriately and put in the wider context.

1) Figure 2 and 3 are identical, Figure 3 is missing!

Our sincere apologies for this. Figure 3 has now been replaced. See also response to reviewer 1, comment 5.

Specific

2) Abstract:

- add "T7 phage polymerase", „phi15 phage polymerase" as used later in the text

- "application in Pseudomonas putida" as no other species have been tested here

Thank you. We have adjusted the abstract with the proposed changes

Introduction:

3) - Align the use of Pseudomonas and Pseudomonas putida and do not use interchangeable, as this work is specific for P. putida and cannot be generalized without further experimentation

We concur with this comment. While the phi15 RNAP and lysozyme have been demonstrated in the past to also be functional in P. aeruginosa (Lammens et al. 2023, IJMS), we agree that the focus of the work is on P. putida in particular. We have therefore made changes to minimise the use of the more general Pseudomonas genus.

4) - Mid of second paragraph: remove have sentence after reference 20.

Thank you for pointing out this misplaced sentence, we have removed it accordingly

5) - Third paragraph: I think it should be rephrased to say that a reliable expression system is remarkably underrepresented instead of "the T7 phage RNA polymerase…"

We have rephrased the start of this paragraph accordingly, it now reads as "Despite these advancements, a powerful and reliable orthogonal expression system as it exists for Escherichia coli, remains remarkably underrepresented in P. putida research. In E.coli, the highly active single-subunit RNAP of phage T7 and its cognate 17-bp promoter(P_{T7}) have led to numerous SynBio applications…"

Page 3

6) - Introduce (abbreviations) SEVAtile/Golden Standard (give references)

- Introduce abbr msfGFP

References for SEVAtile and Golden Standard cloning have now been introduced when first mentioned, in addition to the references already used later down the text. Abbreviation on first use of msfGFP is also added.

7) Material and Methods:

- Numbering of Tables S is not appearing in correct order

We have corrected the numbering throughout the text.

8) - Page 7: M9 medium not listed

We have adapted the sentence on growth media to "Unless stated otherwise for specific assays, all strains were cultured overnight in LB…" and have clarified the composition of M9 medium on its first mention in the methods section (Section on Fluorescence intensity assays and growth curve monitoring)

9) - Page 8: abbreviations "3mBz" and "Rha" are explained later only; Refs are given as "[XY]", check for spacing between number and unit

Thank you for noticing these errors, they have now been fixed

10) - Page 10: de Bont medium not specified in "media" section

We have now added the medium composition upon first mention.

11) Results:

Page 12

- Last sentence from p11 to p12 is complex and hard to understand what “respectively” refers to. Consider splitting up.

This part is now clarified and reads as: “This effect was most noticeable for the XylS/Pm and LacI/PlacUV5 systems, where samples with phi15 RNAP respectively showed an 8- and 11-fold higher fluorescence level in LB medium for the highest inducer concentrations. This was even more pronounced in M9 medium with a respective 11 and 23-fold increase in fluorescence output.”

12) - …(results not shown), which can be most likely attributed to…” (is data shown actually? Fig 3B?), not able to compare due to missing Figure 3

The OD600 data for this particular experiment was not shown as there is already an extensive amount of data shown in figure 2. However, we have now added the datapoints in supplementary data and referred to it in the text, removing the ‘data not shown’ statement. In figure 3b we no longer observe growth defects as the expression construct is now present as single copy opposed to the vector-borne expression in figure 2. As mentioned in a previous comment, we have replaced figure 3 with the correct figure. We appreciate you taking the time to review the figure and the manuscript text that is associated to it.

13) - Page 22: end of paragraph “Figure 5a” instead of “c”

Thank you for pointing out this mistake, figure references are now corrected

14) - Page 24: second paragraph; “which were further characterized on the population…”

Corrected in the text

15) Figure 1: legend of symbols is missing; abbreviation RBS not used in Figure; The Figure legend states that the system was validated for P. aeruginosa but I do not think that this is presented in this study?!

We have now mentioned the use of the most important symbols in the figure caption and removed the reference to P. aeruginosa as this was referring to prior work.

16) Figure 6: “left” and “right” instead of “top” and “bottom” for panel “a”

Corrected

17) Figure numbers are mixed-up on last pages.

We thank the reviewer for pointing this out, we have updated the figure numbers.

18) Figure S1a: Vector-based expression seems to influence cell growth. Is this significantly different?

Indeed there is a significant difference in cell growth, statistics on the final cell density (OD_{600}) is displayed in the new figure 3.

19) Figure S2: There are no panels, remove “a)” and explain what is shown left and right.

Agreed and corrected

20) Check all Figures and Suppl Figures for decimal delimitator “,” vs “.”

All figures have been adapted with correct delimitators

Reviewer #3 (Remarks to the Author):

The manuscript 'Engineering a phi15-based expression system for stringent gene expression in Pseudomonas putida' focuses on establishment of a phi15-based expression system in Pseudomonas putida as a replacement of T7 platform having issues of cytotoxicity. The authors demonstrated that the phi-15 based expression system increases the protein yield by 2.5-5fold compared to traditional expression systems. Overall, the study contains development of a new expression system that will be of interest in to the field of protein expression in Pseudomonas putida. However, several aspects of the study need to be addressed.

Comments:

1. Overall presentation needs to be improvised: Figure 3 is missing in the manuscript and figure 2 is tagged as figure 3 which makes line number 342 to 423 inconclusive. Erroneous sentence construction in line number 48 and 341.

See also response to reviewer 1, comment 5. Figure 3 has now been replaced.

Line number 48 has been adapted to remove a misplaced sentence. Line 341 is a subtitle to clarify which part is being engineered in each step. We have made subtitles more visible by adding spacing.

2. In figure 2, fluorescence/OD values remain identical with increasing concentration of Inducer in case of fluoride responsive riboswitch promoter in the absence of phi15 RNAP in case of both the medium. Discuss the probable reason.

As reported before, the fluoride dependent riboswitch is strongly affected by context (Calero et al. 2020., Nat. Comms.). As it happens with other riboswitches, the start of the downstream ORF influences the folding of the RNA structure. As no induction was observed in either condition, we assume that neither the starting ORF of gfp nor phi15 are compatible with the riboswitch, resulting in a non-functional riboswitch-mediated expression of the genes of interest. We have following discussion in the text to clarify these results: "All systems, except the fluoride responsive riboswitch, demonstrate tunability with varying degrees. Likely the riboswitch is incompatible with msfgfp and phi15 as its functionality is strongly affected by sequence context, leading to fluoride independent expression."

3. In case of LacI/PlacUV5 promoter, at zero concentration of inducer the fluorescence/OD value is very low when the P inducible promoter is placed upstream of msfgfp directly. However, when the phi15rnap is under the control of the same promoter and msfgfp gene is under the control of Pphi15 promoter the leaky expression at zero inducer concentration is comparatively higher (for both the medium). Explain.

Any leaky expression of phi15rnap is amplified by the phi15 RNAP action as it, in turn, transcribes at high levels from the phi15 promoter. Since lacI/PlacUV5 is known to be quite leaky this effect is very pronounced for this specific setup. We have added a sentence in to the manuscript to underscore this.

4. Figure 6 should include a control set with cells harboring T7 RNAP promoter at induced and uninduced condition and FI should have been provided to have a clear idea.

The phi15 RNAP has already been compared to T7 in prior research (Lammens et al. 2023, IJMS). Since the different optimisation steps are tailored towards the phi15 RNAP there is no single T7 RNAP construct to campare to as results will be heavily dependent on what T7 RNAP is combined with (different RBS, terminators, induction system,…). For this reason we have made the comparison only to

the original phi15 system in this figure. To make comparison possible without impact of OD we have now included this data in a supplementary data file. We do agree that it is highly relevant to compare the final optimised system to what T7 expression can achieve. This is why in figure 8 the final P15 and P15-GD strains were compared to T7 based expression in a more industrially relevant example than msfGFP expression. This includes a T7 system with the inducible XylS/Pm system, mimicking closely the initial phi15-based setup. To emphasize that it is compared to T7 in figure 8, we adapted the caption and manuscript text.

5. In figure 6, 5(6) FAM /OD 600 of P15 GD induced was approximately 30% higher than that of P15. Whereas, in fig 8b, 5' FDA ug/mg protein for the both are almost identical. Discuss the disparity.

Since these assays differ strongly, the degree by which the two strains improve production between both assays cannot be directly compared. Due to the large difference in assay, any disparity is multifactorial and mostly assay related as it involves a different protein (msfGFP vs fluorinase), OD600 at time of induction (0.1 vs 0.5), detection method (enzymatic, vs fluorescence; in vivo vs in vitro), normalisation of data (OD600 vs mg protein) as well as other factors. Despite these differences both assays clearly show in their own way that the growth decoupler improves production of the desired protein while limiting biomass production. However, a direct comparison is not possible.

Response to referees

Reviewer #1 (Remarks to the Author):

The authors have successfully addressed my comments and concerns.

We thank the referee for the positive review.

Reviewer #2 (Remarks to the Author):

All comments were addressed appropriately, and after reviewing the correct Figure 3 in addition to the implemented changes, I do not have any further comments.

We thank the referee for their positive feedback and taking the time to review the new version with updated figure 3.