Author's response to reviews

Title: Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when Burkholderia cenocepacia grows in cystic fibrosis sputum

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Author's response to reviews: see over
Dear Dr. Alam,

Re: Revision of MS:1729810633206106
Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when *Burkholderia cenocepacia* grows in cystic fibrosis sputum

Pavel Drevinek, Matthew T.G. Holden, Zhaoping Ge, Andrew Jones, Ian Ketchell, Ryan T Gill and Eshwar Mahenthiralingam

We thank the journal and reviewers for a prompt and helpful review of our study. We are very pleased that all the reviewers have recognised that our first description of the use of a microarray to examine gene expression in sputum of *Burkholderia cenocepacia* is of significant importance to the field of cystic fibrosis infection.

We have responded to all the comments from review and the manuscript has been revised in accordance to their wishes (reviewer comments are shown in italics below with our response following in normal font). We have also included a merged file with the submission (Revision_changes.doc) so that all the revisions can be easily tracked during review. Changes made to the manuscript are as follows:

*Reviewer 1: Tom Coenye*

*Reviewer's report:*

*Minor Essential Revisions*

1. *It is unclear how the authors determined that the patients investigated were chronically infected with ET12 strains identical to strain J2315 (page 7, line 132). This information needs to be included in the manuscript or an appropriate reference (e.g. to an earlier epidemiological study) needs to be provided.*

The strain identity of the infecting ET12 isolates was determined by the UK CF Microbiology Reference Centres in Colindale and Edinburgh UK as part of their routine surveillance of CF population. The actual patients sampled and their strains have not been specifically described in previous papers, however, the procedures and traits used to define strains of the *B. cenocepacia* ET12 lineage are well known to the field being: (i) conservation of genetic fingerprint with other ET12 strains, (ii) presence of the *Burkholderia cepacia* epidemic strain marker and (iii) presence of the cable pilus gene.

These features of the ET12 strain have been summarised in detail in two of the reviews we have cited (references 1 and 2). Therefore to inform the reader of how the strains were...
shown to be genetically related to ET12 we have changed the sentence as follows to insert these references:

“In this first complete study of B. cenocepacia sputum-transcriptomics, we were able to work with CF sputum samples in a non-sterilized, close to native form since the CF individuals examined were chronically infected with ET12 strains that were determined by microbial reference centres using standard procedures [1, 2] to be genetically identical to our experimental strain J2315.

2. Confusion may arise regarding the methodology used for normalisation of the qPCR data. I would strongly recommend to include fold-expression data based on only one type of normalisation. Both types of normalisation obviously show the same trend and mentioning both makes the manuscript (especially Table 1) unnecessarily complicated. I personally would prefer to include data based on the delta delta Ct method, if the E values are sufficiently close to 2.

We included results from both RQ-PCR normalization methods to show their slight discordance especially for high values of fold change. Nevertheless, the difference in normalization methods was always kept within one order of magnitude and was virtually identical for low values. Therefore, we are happy to reduce the amount of data provided in Table 1 and in agreement with the reviewer’s suggestion we have removed the results obtained by Pfaffl method.

3. On page 14, lines 306-307 the authors mention “the twofold filter”. However, this twofold filter is not mentioned in the section on microarray analysis. Is there a direct correlation between genes identified by the twofold filter and genes identified with the statistical significance filter of p < 0.05? This should be clarified.

Both filters (the statistical significance and the fold-change) were applied together to obtain the final genelists. We have realised that by mistake while we did include this information in Results, it was not described in the Methods. We have therefore modified a relevant section of the Methods to incorporate the filter description:

“Initially, the three patient-specific biological replicates were each tested using Student’s one-sample t-test and their changes in gene expression were defined by fold change relative to the gene expression level in self-hybridization assay; this analysis was performed with a two-fold change filter applied and no multiple testing correction or statistical significance filter of p < 0.05 adopted.”

4. I was a bit surprised to see both semi-quantitative PCR and qPCR being used in the same study. Why was the expression of BCAL1165 not investigated with qPCR as well? My understanding is that semi-quantitative PCR is more a “quick & dirty” approach. This seems to be confirmed by the data in Fig 1: there appears to be a (very) faint signal in lane 2 after 35 cycles, while there appears to be no signal at all after 40 cycles. If there is a specific reason why this gene
was only investigated with this method this should be mentioned. If not I would strongly recommend to include qPCR data for this gene as well.

We agree that RQ-PCR is more appropriate approach for fold change evaluation than semi-quantitative PCR. For this reason, we also analyzed as many genes as possible with RQ-PCR as a more accurate measure of differential regulation. However, while up-regulated genes were analyzable in real time PCR format without major difficulties, this analysis failed for down-regulated gene BCAL1165 because of PCR sensitivity issues. Therefore, to prove its low expression, we performed the semi-quantitative PCR. This alternative method verified our microarray data very well and show that the BCAL1165 transcript was very low in abundance and downregulated in comparison to our control gene. We believe it will be valuable for future users of the B. cenocepacia microarray to be aware that in certain circumstances a semi-quantitative PCR may be very useful to corroborate microarray data.

To acknowledge the reviewer’s comment about a specific reason for using semi-quantitative PCR for BCAL1165 and not RQ-PCR we have revised the following sentence:

“Semi-quantitative PCR was initially applied to BCAL0270, encoding a putative ferric reductase transmembrane protein (36 x upregulated), and BCAL1165, a conserved hypothetical gene (6 x downregulated; RQ-PCR analysis of this gene was not possible due to limited PCR sensitivity).

5. Other comments
-Can the authors clarify whether patients 5 and 6 (both infected with P. aeruginosa) were coinfected with B. cenocepacia? If so, this should be clearly mentioned in the text (page 9, lines 16-170).

Patients 5 and 6 were infected with P. aeruginosa only and we have clarified this as follows:

“Subsequently, along with sputum 2 and 3, three additional sputum samples from three further CF patients were examined using quantitative PCR to validate the microarray observed gene expression: patient 4 (sputum 4) was infected with B. cenocepacia and clinically stable; patient 5 (sputum 5) was infected only with P. aeruginosa and exacerbating, and patient 6 (sputum 6) was also only infected with P. aeruginosa but stable at the time the sample was provided (Table 1).”

-Define the abbreviation BSM when it is first used (page 9, line 186).

This term is used in full (but without introduction of abbreviation) in the paragraph preceding the one which the reviewer refers to. So we have added the abbreviation to the sentence in this section to correct our mistake:
“Each sputum sample was stored frozen at -20°C and upon arrival to the research laboratory, they were thawed, diluted in a minimal basal salts medium (BSM).....”

-Include the power output (in watt) during sonication (page 10, lines 203-204).

We added practical information on sonicator amplitude and power to the text as follows:

“To increase RNA yield, we performed an additional sonication step at the beginning of RNA isolation (10 x 10 second pulses, each at 5 µm amplitude, with a Sanyo Soniprep 150 Watt sonicator).

Discretionary Revisions

6. The authors could include patient history data (including antibiotic use) in a separate Table.

Part of patients’ characteristics has already been mentioned in the original Table 1 (infection and clinical status). However, we agree that patient information is quite important in the whole context of the study and could be provided in a more convenient way. Therefore, we have introduced the data as a new Table 1, and corrected the numbering, position and titles of all subsequent tables accordingly. Overall this change produces a better flow to the tables in the manuscript. A sentence has been introduced in the methods to describe the new Table 1:

“For each patient the clinical condition, bacterial infection and antibiotics administered have been summarised in Table 1.”

Reviewer 2: Mark Thomas
Reviewer’s report:
Generating a picture of the global changes in Burkholderia cepacia complex (Bcc) gene expression that occur during growth in CF sputum would be a key step in the process leading to the identification of genes that are crucial for Bcc survival and virulence in the CF lung. To this end, Drevinek and colleagues describe the assessment and validation of a newly designed B. cenocepacia microarray. The authors have chosen at the outset to assess the performance of their new microarray on cells growing in CF sputum. I would have been tempted to start by altering a more controllable parameter such as temperature or iron availability. Nevertheless, the authors have done a good job, and a number of genes have been flagged as potential players in CF infection that will warrant further study.

We thank the reviewer for recognizing that we have done a good job in examining gene expression in sputum. However, we can also add that during development of the microarray, a simple heat-shock experiment was performed as a controllable parameter with which to assess the performance of the microarray. The results of this simple experiment demonstrated that the microarray was performing adequately. Hence in this
study we specifically set out to examine gene expression in sputum as being one of the major unknown areas of *B. cenocepacia* pathogenesis.

**Minor revisions:**

1. My 'take' on the **semi-quantitative RT-PCR result** (Figure 1) **is that it shows expression of the constitutively expressed house-keeping gene, BCAL1861, is downregulated in sputum medium relative to BSM. How do the authors explain this?**

The housekeeping gene BCAL1861 was selected as a suitable candidate for a control gene after very careful analysis of all the microarray data. No significant fold change was observed in BCAL1861 for any of conditions examined (all sputum samples and BSM replicates).

The semi-quantitative PCR analysis presented indicated relative, but not absolute changes in expression (up-regulated vs. down-regulated) of the test genes (BCAL1165 and BCAL0270) in relation to the BCAL1861 house-keeping gene for a single respective condition. This was done for both growth conditions, sputum and BSM, for the semi-quantitative PCR. However, it is less accurate to directly compare gene expression across growth conditions (as the reviewer is trying to do), than within conditions, hence interpreting that the control gene is actually down-regulated in sputum.

The observed change of BCAL1861 in sputum vs. BSM was may have been the result of slightly less cDNA being present in the samples recovered from sputum compared to those from the BSM-grown cultures. As with RQ-PCR where delta Ct method is applied, pre-normalization would be required before performing a direct comparison of cycle numbers for semi-quantitative PCR. Therefore to clarify that the BCAL161 control gene was not down-regulated in sputum we have added the following to the results section on semi-quantitative PCR:

“Using pooled cDNA from the microarray experiments, in the sputum growth condition BCAL0270 was consistently amplified after 5 fewer cycles in comparison to the *phaC* (BCAL1861) house-keeping control gene while in contrast, BCAL1165 was not amplified until 35 cycles in comparison to the control gene BCAL1861 (Figure 1); this corroborated the microarray data showing that BCAL0270 was upregulated and BCAL1165 downregulated in sputum. During growth in BSM signals for BCAL270, BCAL1165 and the control *phaC* gene all appeared after 30 cycles (Figure 1) indicating similar levels of relative expression for all these genes in this growth condition. Although a signal for the control gene was absent in sputum yet present in BSM at 30 cycles of amplification (Figure 1), this probably resulted from less cDNA template being present in the sputum-recovered samples, as the microarray-observed gene expression had shown that the *phaC* control gene was stable in expression relative to all other genes for both sputum and BSM growth.”
2. Does the fact that detection of the BCAL0270 transcript occurs after five fewer cycles than the BCAL1861 transcript actually mean that it is upregulated in sputum medium? For example, if the control house-keeping transcript was of low abundance, then transcripts originating from the test gene still may be detectable before that of the house-keeping gene, even if the test gene has not derepressed. Surely it is the difference between the number of cycles of amplification required to detect the test transcript under either condition that is important? This could be compared with the ratio of cycles for the house-keeping gene transcript amplified under both sets of conditions.

From the microarray observed gene expression across all growth conditions we know that the control gene was within the top 25% of all constantly expressed genes in terms of abundance. The level of raw fluorescence signal on the microarrays was taken into account as another factor for selection of a suitable control gene. Irrespective of growth condition, BCAL1861 possessed a raw signal lying above the 75th percentile of all genes with unaltered expression (fold change 0.667-1.334; ~2,500 genes). This indicated that the BCAL 1861 control gene was not a transcript of low abundance.

The difference in cycles between BCAL0270 and BCAL1861 does not actually say much about up-regulation of BCAL0270. The relevant information comes from a comparison of cycle number between up- and down-regulated genes per condition, or from a comparison of cycle number of the same gene between conditions. We can understand the reviewer’s concern, however, as they have also stated this correct way of interpreting the semi-quantitative PCR: “Surely it is the difference between the number of cycles of amplification required to detect the test transcript under either condition that is important?.” Analysis of the ratio of cycles for the house keeping gene will not help with interpretation of the semi-quantitative PCR as it is a relative measure of gene regulation and that is why we chose to also validate our data further with Real Time PCR as a more accurate measure of fold expression change (we have already clarified this in response to reviewer 1, point 4; see above).

To clarify the reviewer’s concern that the control gene *phaC* could have been a low abundance transcript we have added the following to the text:

“The gene *phaC* (BCAL1861), with constant expression in both control and test microarrays, was used as a control for all quantitative PCR work and amplified using previously described primers [16]; Table 2). Irrespective of growth condition, BCAL1861 possessed a raw fluorescent microarray signal lying above the 75th percentile of all genes with unaltered expression (fold change 0.667-1.334; among approximately 2,500 genes). This also demonstrated that the BCAL1861 transcript was highly abundant in all growth conditions.”

3. Why are the BCAL1861 data not included in Table 1, as it is stated in Table 2 that this was included in the real time analysis?
Since BCAL1861 served as a reference gene against which transcription levels of all other genes correlated to for real time PCR, its value for every sputum sample examined was equivalent to value of 1. Hence it was not included in Table 1 but was included in Table 2 to show the primers needed to amplify phaC during real time PCR.

4. As little as 5 microM iron is fully repressing for some iron-regulated genes in B. cenocepacia. That would suggest that the concentration of iron that the authors measure in BSM and diluted sputum (~35 microM) corresponds to repressing conditions, and may explain why they do not observe derepression of iron-regulated genes. Also note that there may be iron carry over from the LB, as the cells were not washed. It would have been useful to have determined the iron concentration in their neat (i.e. undiluted sputum), since the iron they measure in the diluted sputum may have largely originated from the diluent (BSM). At least we would know whether the results obtained for genes involved in iron homeostasis reflect the true situation in the CF lung.

It should be noted that although the iron concentration in sputum samples may correspond to a value that is known to support growth in laboratory media, its 'availability' may be restricted. For example, any free iron present in sputum is likely to be mopped up by lactoferrin, which binds iron very tightly (notwithstanding the fact that the freezing procedure that the sputum has been subjected to may inactivate this protein). Also, there is evidence that CF mucus has iron sequestering properties (Wang et al., 1996). NB FeIII is "readily utilised" if the bacteria produce siderophores.

The reviewer makes multiple comments in the above section and it is difficult to interpret which specific points need to be responded to. We have done the following in response to section 4 comments:

a. **Iron concentration in native sputum.** Unfortunately, we were unable to measure the iron concentration in undiluted sputum because of the requirements of the technique and device limitations. For accurate measurement, a solution has to be provided in a homogeneous form and this was not possible for native sputum which was a thick viscous non-homogenious mixture that varied greatly for each patient examined. After dilution to 10% and minimal homogenization we were able to obtain samples that could put through the iron analysis.

b. **Limited availability of iron in sputum.** The limited iron (Fe II) availability in sputum despite its presumed abundance was already discussed in the original manuscript (line 466): “only soluble ferrous ions can be readily utilized by bacteria. Hence, in our experimental model the ferrous form of iron was likely to have been lower in the 10% sputum medium than in the control BSM which contained solely soluble ferrous ions.” As the reviewer and we have already pointed out in the original paper there are many other studies which have examined the availability of iron in sputum. We feel it is beyond the scope of our
microarray-focussed study to further discuss this phenomenon, other than stating that our data is clearly showing that B. cenocepacia is responding to growth in sputum with gene expression that indicates iron is limited.

c. **Readily utilized iron.** By stating “only soluble ferrous ions can be readily utilized by bacteria” we had meant direct iron utilization without a need of specific chelate transporters. As the reviewer has noted this statement is not entirely accurate and therefore we have corrected it as follows: “However, the total iron measured in sputum is composed of both ionic forms, of which only the soluble ferrous ions can be readily utilized by bacteria without using siderophores”.

*Regarding the establishment of the reconstituted sputum medium I have a few questions/comments. I think it is important that the method for preparing the medium and setting up the cultures is clear.
5. Line 173, I am assuming that the frozen sputum sample was not lyophilised, since this is not stated?*

The reviewer is correct, we did not lyophilise sputum samples. This information has been clarified as follows:

“Each sputum sample was stored frozen in its native form at -20°C and upon arrival to the research laboratory,…..”

6. **Line 175, it is stated that the sputum concentration was 12.5% (w/v) in the initial homogenate. To what volume of this was 1 ml of overnight cells added? What is the fold dilution of the o/n bacteria in the sputum medium? The statement on line 188 does not make sense. Is the final concentration of sputum now 10%, as stated on line 468 (meaning that it was a 1 in 5 dilution, i.e. 1 ml cells plus 4 ml 12.5% sputum medium)?**

1 ml of the cell suspension was added to 4 ml of 12.5% sputum to get the final concentration of 10% w/vol. We realize that our original description of this in the methods may not have been entirely clear and therefore we have changed the section as follows:

“The pellet was resuspended in 3 ml BSM and 1 ml (~ 1 x 10⁸ colony forming units; cfu) inoculated into either: (i) 4 ml BSM (control) or, (ii) 4 ml BSM supplemented with homogenized CF sputum at 12.5% w/vol, giving a final sputum concentration of 10% w/vol in the 5 ml culture volume (test).”

7. **The initial OD of the diluted culture must have been quite high. How many doublings occurred in the new medium before gene expression was analysed? For example, if the starting OD was 0.2 and cells were harvested 0.6 OD units**
later (OD 0.8) that is two doublings. I assume that is enough time to allow adaptation to the new medium and turnover of any long-lived mRNAs.

The OD at the beginning of growth experiment in sputum (i.e., after adding cell suspension) was ~ 0.3-0.4. The final reading reached OD value of 0.9-1.0 and hence there was sufficient time for B. cenocepacia to grow and fully adapt its gene expression in sputum. To clarify this for the reviewer we have added the following to the methods section:

“For each experiment this meant that growth started at an OD_{600nm} of between 0.3 and 0.4 and was then harvested at an OD_{600nm} of between 0.9 and 1.0, providing sufficient time for B. cenocepacia to adapt is gene expression to the new growth condition. After growth, the cultures…”

Other minor comments:
Was P. aeruginosa present in any of the sputum samples infected with B.cenocepacia?

The patients were infected with B. cenocepacia only and this information has been clarified by the addition of the new Table 1 (made in response to reviewer 1, point 6).

Line 174 "...in a minimal salts medium (BSM) containing...”
We have added this abbreviation.

Line 339-340, delete "and were hypothetical in nature," (redundant)
Deleted as requested

Line 348 "semi-quantitative reverse transcriptase PCR" (?). Ditto line 353.
We have corrected our designations and abbreviations semi-quantitative reverse transcriptase PCR.

Lines 355-358, I presume the authors are referring to growth in sputum medium?
Please clarify
Only genes alter in expression in sputum were validated by quantitative PCR methods. To clarify this for the reader we have added the following:

“In order to validate microarray results, five genes with identified altered expression in sputum (4 overexpressed: BCAL0270, BCAM2753, BCAL1107 and BCAM1676; and 1 underexpressed: BCAL1165) were examined individually by using either semi-quantitative reverse transcriptase PCR (RT-PCR) or Real Time PCR (RQ-PCR) approaches.”

Line 556, is growth in CF sputum going to be as fast as it is in pure sputum?
The has been no research on the growth rate of B. cenocepacia in CF or pure sputum and one would imagine this would be a very difficult parameter to measure. By using the term “rapid growth” in this section we were only trying to refer to the fact that B. cenocepacia was growing exponentially and in a planktonic state; the cable pilus may not be needed in
this state, but may be expressed in other states of growth such as stationary phase or as a biofilm. We have altered this section slightly by removing the term “rapid” and adding “CF” so that its meaning is clear:

“Nearly the entire cable pilus gene cluster was significantly downregulated (cblS, BCAM2758; cblD, BCAM2759; cblA, BCAM2761 and cblB, BCAM2762; Table 4) suggesting that during growth in CF sputum these surface structures are not essential. In contrast, the cable pilus adherence-mediating 22 kDa adhesion protein (adhA; BCAM2143) [36], was found to be upregulated (Table 4).

Incidently, how did the growth rates in the two media compare?

B. cenocepacia generally grew better in the sputum supplemented BSM as this was effectively richer in nutrients than the minima salts BSM medium with glucose. However, the exact growth rates varied between sputum samples hence we cannot systematically report on growth rate within the context of this study.

Line 565, BCAL3521
Corrected.

Line 599, what do the authors mean by ‘interesting’? This is a bit vague.
We have removed “particularly interesting” and replaced them with “notable:”

“Many bacterial exoproducts are known to play important roles in infection and two proteases with altered gene expression were notable in the context of B. cenocepacia virulence in CF.”

Line 609, BCAL0269-0270
Corrected

Please shorten the Discussion

Without any specific guidance from the reviewer as to which aspects of the discussion to shorten it is very difficult to respond to this suggestion. We feel that our discussion is balanced and compares the significance of the most important findings in our study. The journal editor or the two remaining reviewers have not suggested any abbreviation of our manuscript and therefore we have chosen to leave the discussion content in its original form.

Discretionary revisions:
Line 328, I presume there are multiple copies of rRNA operons in B. cenocepacia (as there are in many bacteria). This would make the observation that only two rRNA genes are upregulated in sputum medium odd (the different rRNA operons are co-regulated in E.coli). The authors could comment on this observation.

The B. cenocepacia genome contains 6 rRNA clusters in total. While 5S rRNA sequence is identical for all six of these copies and is represented on the chip by a single probe, the 16S and 23S both have several slight sequence variants. Therefore, 16S is represented with 3 probes and 23S with 5 probes on the microarray. Our data showed statistically
significant signals for the 5S rRNA and one of 16S probes. This indicated that expression of all the operons had changed in the same manner despite the fact that not all probes had reached a threshold for statistical significance.

Table 1: I would like to have seen some down-regulated genes included in the data set in Table 1 as well. Also, to validate the iron-limited and oxidative stress conditions, it would have been useful to have included in the analysis genes that are known to be regulated under these conditions (orb, kat etc). What does the house-keeping gene do under these conditions?

We have discussed the limitations of the analysis of down-regulated genes by RQ-PCR in relation to Reviewer 1, point 4 above. Because of these limitations in the ability to amplify down-regulated genes and low abundance transcripts, we carried out the semi-quantitative RT-PCR to prove gene repression. As for using other control genes for testing of iron-limited and oxidative stress conditions, we were dealing with a situation where there were no obvious candidates, such as orb or kat available. As mentioned in the original text, these previously characterized genes remained unaffected in sputum (line 439 for orb and line 500 for kat). Other genes which could be considered “controls” (since they had altered their expression under iron-limited conditions) showed either quite low fold change (eg. pchD, ~ 2-fold) or belonged to the same category as the tested BCAL0270 gene (such as genes with poorly described behaviour which included for example BCAL0273, cyaY). Using these genes as reference genes for the iron-restricted or oxidative stress condition was therefore not possible.

Reviewer 3: Leo Eberl
This reviewer was highly complementary of our study and did not suggest any amendments.

In addition to the changes made above, we have now removed the supplementary tables from the manuscript since these will be included as online data in an Excel Spreadsheet format in the final manuscript. Overall, we have revised the manuscript fully in response to all the comments of each reviewer and feel that we have greatly enhanced the clarity of our study. We leave the revised manuscript for further consideration and look forward to hearing from the journal as to whether it is now suitable for publication. Please feel free to contact me if you have any questions about the revisions made.

Yours truly,

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