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CANADA

Dear Dr. Redfield:

Manuscript number: 1219861

Thank you for sending us your manuscript "Absence of arsenate in DNA from arsenate-grown GFAJ-1 cells." We are potentially interested in publishing the paper as a Report, but we are not prepared to accept it in its present form. Please revise your manuscript in accord with the referees' comments (pasted below) and as indicated on the attached editorial checklist and marked manuscript (attached as separate files). I have also made some suggestions regarding shortening and clarification directly on the manuscript. Please also see our instructions for authors (which include a link to a template for Supplementary Materials http://www.sciencemag.org/site/feature/contribinfo/prep/index.xhtml). Because of the nature of the reviewers' comments and revisions required, we may send the revised manuscript back for further review. A revision that does not meet the reviewers' and our expectations will be rejected.

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I look forward to receiving your revised manuscript. Please let me know if I can be of assistance.

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Sincerely,

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Reviews for 1219861 Reaves

Reviewer 1

We live in interesting times. In the case of the Science paper by Wolfe-Simon et al , a minor storm was generated because virtually everyone with some understanding of microbial physiology was convinced these conclusions were unwarranted (judging from comments published in the journal and in the public press). The objections were many and varied. Since the claims of arsenate replacing phosphate in DNA were extraordinary, some scientific response in print seems reasonable. The many objections to the original publication suggest an editorial lapse on the part of Science, that should be also be addressed. Perhaps this manuscript gains weight as a way for Science to cover its backside. It seems a pity that one can sell newspapers by both committing and reporting a crime.

I was convinced at the first reading of the original paper that something was wrong here, but it seems right to address outrageous claims in broad daylight with some actual data. The manuscript by Redfield et al hits the high points by addressing the issue of DNA composition and showing no significant evidence for arsenate incorporation. I'm convinced that they got it right and that Science ought to print this in the interests of Truth and Beauty. The only weakness of the paper, is that the growth behavior of the strain couldn't be reproduced, raising questions about the relevance of the DNA analysis since doubters can always say that different conditions or a different version of the strain explain the failure. The finding of a growth requirement however is a significant contribution.

Despite the problems that might keep this debate alive, I vote for publication – maybe in a short form. However, since Science remains a general science journal, this article ought to help the uninitiated reader over the high points in the controversy and come clean about reservations in interpreting the data. I list a few points below that I think would help.

The strain itself The original claims were made for a particular isolate (GFAJ-1), which accordingly is the center of attention. A note that they got this strain from authors of the original article (graciously acknowledged in the text) would be appropriate. This lets the reader know that there's some reason to expect that the chain-of-evidence is intact. I had to compare the acknowlegment with the full authorship of the original paper.

Old medium AML60 How about a line (or even a reference) for this medium and what's in it --- including its elemental content of As and P. Is it a minimal salts medium that normally contains phosphate, but now has it dropped out??? Wolfe-Simon et al said that GFAJ-1 grew slowly on AML60 "when phosphate was not added", but did better with added arsenate (containing unspecified PO4). This could be stated more clearly, I think. Along with the fact that no report is made here of growth on AML60 plus arsenate alone (and without the gluamate added phosphate that later came into the act).

My personal take on the original paper was that inorganic salts are notoriously difficult to purify to high standards and all commercial phosphate and arsenate samples are likely to be contaminated significant levels of the other salt. This could be mentioned, but more important is to make clear who did what.

When you say that -P medium (center of page 2) actually contains 4µM phosphate, is that your measurement or theirs?? When you say cells grew on medium with high phosphate but NO arsenate, you sin in the opposite direction. How much arsenate contaminates that high phosphate? Maybe high phosphate provides the needed level of arsenate.

The growth behavior The commentary on the growth with various media is, of course complicated by the fact that the previous study and this one find different growth results. This is unfortunate because

now one can worry about whether the strain is really the same or if the media used were somewhat different. At this point the matter will be hard to settle.

If the strain requires some carbon (glutamate), I presume that glutamate was added to all of the media as stated in the text. That point might be added to Figure 1, to make it clear that the tests were made in a way that is different from that in the original paper.

Bottom of page 3. "We believe that our AML60 + 3µM phosphate replicates Wolfe-Simon et al's -P conditions." Does this mean that all the DNA prepared and analyzed in the rest of the paper was prepared using medium supplemented with 3µm phosphate so that phosphate is "sufficient to support moderate growth" (from page 2)?

This is disturbing when taken with the finding that adding arsenate made no difference in the growth (once AML60 was supplemented with glutamate plus 3µm phosphate).

I understand that one has to grow cells to analyze, but it does look strange. One might think it better to stack the cards in favor of arsenate rather than against it. If in their hands, high arsenate substituted for what is now concluded to be an organic, was that verified here? That is there was no growth on AMS60 plus phosphate (without organic), but was there growth on AML60 + high arsenate? (without glutamate)

Top of page 4 "Our + As and –As cultures grew to similar densities……." Does this mean using AMS60 supplemented with glutamate and 3µm phosphate?? A skeptic (not I) might say, "Hey, you gave them phosphate, why should arsenate help now?" Also was glutamate tested for PO4 contamination -- it could have been precipitated from a phosphate buffered or neutralized solution.

DNA analysis This all seems fine, given that the cells were prepared in a fair way.
The differences in growth behavior raise questions, but I suppose you have to get material somewhere.

You cut DNA to mono- or di-nucleotide phosphates (or arsenates) with P2 and snake venom nucleases, which, I suppose, might not work with arsenate and might leave longer oligos- would they have been seen in the mass spec analysis??

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Reviewer 2

The experiments have been carefully done, and the conclusions unambiguous. However, the reporting of the testing for arsenate of arsenate-DNA is rather laborious; it could be made much sharper by proper consideration of limit of detection (see below). The work is novel in that it produces definitive proof that GFAJ-1 cells do not need arsenate to live, and that arsenate is not incorporated into DNA. This was not an unexpected result, but nevertheless it was important to do the experiments, and to do them well.

Because the manuscript is refuting previously published results by Wolf-Simon et al that were both highly novel and controversial, the value of the work rests largely on the rigor of the experiments and data interpretation. The experiments were described in the following headings:

Growth properties of GFAJ-1
Initial attempts to grow the cells following the procedure of Wolf-Simon et al were unsuccessful, after which the authors checked that their organism was in fact the correct strain (it was). This reviewer would like to see this confirmation up front, before discussing the discrepancy in results. Subsequent experiments were performed with the addition of 1 mM glutamate, which resulted in excellent growth. Nevertheless their results did not agree with those of Wolf-Simon et al. Importantly, Reaves et al showed that GFAJ-1 does not require arsenate for growth. However, the authors present no empirical data on the

actual concentrations of arsenate and phosphate in their low exposure ("nil") media, although they suggest that their unsupplemented medium contained about 1 µM contaminating phosphate based on their observed growth at 3 µM and some results from Wolf-Simon et al. This is a small weakness in the work.

Analysis of GFAJ-1 DNA
This section had two parts. First, the stability of DNA was investigated: The authors perform careful purification experiments to isolate the DNA from the cells exposed to various As/P combinations, and then show that there was no evidence of arsenate-dependent DNA breakage. The DNA was stable to hydrolysis even when unprotected by other macromolecules. Second, the authors look directly for evidence of arsenate-DNA species by LC/MS examination of arsenate and arsenate nucleotides in their enzymatically digested purified DNA. The work is again carefully done – essentially the authors could find no evidence for the presence of arsenate-DNA in the purified DNA fractions. Although the experiments are thorough, the description is a bit labored and doesn't follow usual practice in reporting analytical data which detracts from the scientific quality of the work (see below).

Specific comments.
Figure 1: y axis (400-580 nm)
Figure 1: 250 or 1500 = +P (unclear).

Figures 3 & 4: present mostly negative results. Would be more informative to calculate quantification limits and give quantities for arsenate and arsenate species in the text. For example, page 6 last para, "some free arsenate"……"incorporation of this amount"……."this arsenate was largely removed" ……"persisting arsenate was detectable"……"Thus we conclude that at least most of the arsenate we detected…."The case would be much stronger if quantities were assigned.

Figure 4 legend:
(1) At least one of the two m/z values (330 or 347) is wrong-
(2) What is meant by "….matches the known retention time of dAMP" Was this not checked?

Table 1 (and Supplementary Table S1)
It appears that there is a trace of arsenate contamination in the analytical scheme. I notice that they use glass-distilled water which might be the source. It does not impact on the results but the authors make the scheme unnecessarily complicated by attaching arsenate values to mostly blank levels. From Supplementary table S1, it is clear that anything under about 2 x 10-8 M (peak area about 500 units) cannot be detected (let alone quantified). Replicate water blanks range from 192 to 980 units, from which a Limit of Quantification could be calculated. Any sample under the LOQ should simply be reported as such and not assigned a (probably false) value. This would simplify the table and provide more rigor to the data analysis.

Supplementary materials
Culture methods, line 5: Na3PO4 and Na3AsO4

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Reviewer 3

Comments to the authors:

The present manuscript by Reaves et al. questions the recently published study by Wolfe-Simon et al. that claimed to provide evidence for the presence of arsenate in biomolecules that normally contain phosphate, including nucleic acids and proteins. This is therefore a very relevant study with regard to the Wolfe-Simon paper that initiated and stimulated many controversial discussions amongst scientists from various disciplines. The major results from the present study are i) that strain GFAJ-1 does not grow in the

absence of phosphate when arsenate is added, ii) that growth of strain GFAJ-1 at low phosphate concentrations is not stimulated by arsenate, iii) that only a very low amount of As was detected in DNA from cultures grown in the presence of arsenate, and iv) that these concentrations of As in DNA were much lower than the ones suggested before by Wolfe-Simon et al.

Although on first view the manuscript therefore provides convincing data that not only questions but actually contradicts the claims from the Wolfe-Simon study, there are still several questions that have to be answered before this work can be published. These questions focus on the following points that are explained in detail below: i) the presence of glutamate in the growth medium, ii) the missing phosphate quantification, iii) cultures that were used as inoculum were pregrown with 40 mM arsenate, and iv) the detection of low amounts of arsenate in the DNA.

1) Do the authors have an explanation for why they were unable to grow GFAJ-1 in the basal AML60 medium even in the presence 1.5 mM phosphate? This is surprising since this medium was shown to work perfectly in the Wolfe-Simon et al. study. The authors speculate that a “growth-permitting contaminant” present in the phosphate salt used in the Wolfe-Simon et al. study might be responsible for that difference (page 4, first paragraph). Do they have any suggestions what this contaminant could be? Since the addition of glutamate significantly improved growth in the Reaves et al. medium, this contaminant must also have been present in their glutamate (or it is glutamate itself? although I don’t believe that to be the case) or the glutamate must have compensated for the lack of this growth-essential compound. The role of glutamate needs to be clarified - see also the following comments.

2) Reaves et al. explain that the addition of glutamate improved growth. However, for the reader in order to understand why this is the case it would be important to know what the basic energy-yielding metabolism under these cultivation conditions is. What is the electron donor and acceptor? Is it glucose and O2 as in the Wolfe-Simon et al. study? This should be mentioned in the beginning of the manuscript.

3) If I understand it correctly the only/main difference between the cultivation medium used by Wolfe-Simon et al. and by Reaves et al. is the glutamate in the medium. This again raises a few questions: does the glutamate undergo any interactions, e.g. complex formation, with the arsenate and could this prevent arsenate from being incorporated into biomolecules? If the glutamate is used as a carbon (and/or nitrogen) source, how does the arsenate interfere with the utilization of the glutamate? A quick literature search in Web of Science showed that at least for plants some inhibitory effects seem to be published. Maybe this is worth checking to clarify the role of glutamate and the effect of arsenate on glutamate utilization.

4) There is a discrepancy regarding the glutamate concentration used in the medium: in the manuscript (page 3) the authors state that 1 mM glutamate was used. In the method section (page 19) they state that 5 mM glutamate was used. Please clarify.

5) Additionally, the chemical formulas of the phosphate and arsenate salts used (page 19) are incorrect. Please correct them.

6) Reaves et al explain that the low P/high As medium used by Wolfe-Simon et al. contained about 4 µM P and that cultures with low P/high As showed significant growth only due to the presence of the contaminating P stemming from the As salt. Actually, in the Wolfe-Simon et al. study it is mentioned that at least 3 µM P was present both in the presence but even in the absence of arsenate. Reaves et al. in their study now followed growth of GFAJ-1 by quantification of optical densities in cultures amended with different concentrations of phosphate (all without arsenate, see Figure 1). From the cell densities they obviously determined/calculated cell numbers, compared these cell numbers to the Wolfe-Simon et al., study, and concluded that in their own medium approximately 1 µM phosphate was present as a background concentration (in medium without phosphate amendment). They then conclude that “GFAJ-1

does not require arsenate for growth in -P medium". I have some comments regarding these experiments and the conclusion:

o The estimation of the background concentration of 1 µM phosphate in their medium is based on comparisons of cell numbers from their study to the data provided in the Wolfe-Simon et al. study. First I think it is absolutely required to analyze/measure the phosphate concentration present in their medium (without P amendment) since this is the crucial parameter. The phosphate concentration should not only be estimated indirectly from cell numbers. Second, the authors need to provide a figure correlating OD with cell numbers to demonstrate how they determined the cell numbers. Third, they need to give the initial cell numbers (i.e. the cell number inoculated). Fourth, the conclusion regarding the arsenate requirement is not fully justified as long as they don't provide the data for growth experiments done at low P concentrations that are amended with arsenate. Providing cell numbers for such experiments will allow to evaluate whether under P-limiting conditions arsenate increases the growth yield or not. The authors state in the text (that there is no increase) but I would like to see the actual data.

o In addition to cell numbers, protein content, i.e. a measure of absolute amount of biomass needs to be provided. Depending on the growth conditions (more or less P) the cells could be either small or large, i.e. the same cell number could still mean a very different absolute amount of biomass present.

o I recommend that the authors do a back of the envelope calculation of whether the determined cell numbers (and the amount of biomass determined in mg/mL) could be supported by the 3 µM phosphate supplied.

o To determine which fraction of the phosphate present was built into the biomolecules, experiments using isotope- or radiolabeled phosphate would help and allow to distinguish phosphate being recycled from the inoculated cells and phosphate built into newly formed / grown cells.

7) Regarding Figure 1: i) the figure legend needs to be improved. It doesn't contain all information necessary to fully understand what is presented here (for example that the "-P" and "+P" refers to the conditions in the Wolfe-Simon et al. study), ii) why is the OD measured over such a large wavelength range and not at a specific wavelength?, iii) how do you explain the OD curves for the 250 and 1500 µM phosphate setups that are below the 70 µM phosphate setups? Is there a toxic effect of the phosphate?

8) Regarding the DNA stability experiments (Figure 2): in addition to the prominent >30kb band that is shown in Fig. 2A, there are two additional bands above this band. These two bands can be seen nicely in Fig. 2B in all 4 samples. In the figure it looks like these bands are more prominent in the +As/+P and the +As/-P setups than in the two other setups. Is this correct? Additionally, it looks like the main thick band is slightly weaker in these two lanes than in the two other lanes, correct? If yes, do the authors have an explanation for that? Could this have something to do with the high As concentrations present in these setups?

9) Regarding Figure 2: please explain (in the figure legend and in the text) what dsDNA and ssDNA stands for (single-stranded and double-stranded DNA). And explain why Figure C shows ssDNA (treatment at 95°C) and add this information to the gel (on the right side). Maybe not all readers are familiar with this. Please also explain the abbreviation "mos" (months?).

10) An additional question regarding these setups: the final cell numbers after incubation under the different growth conditions vary significantly as explained on page 3 of the manuscript. I assume that the DNA concentration in all lanes was normalized (the same amount of DNA was added to each lane). However, if there is no growth in -As/-P and +As/-P setups, as suggested by the authors, it means that in these setups mainly the DNA from the inoculated cells was present, correct? If this is true, than it would be good to know under what conditions the inoculum was cultivated. I initially assumed this was a -As/+P

culture. In the method section, however, the authors state that all cultures were inoculated from cultures that were grown with 40 mM arsenate and 3 µM phosphate. Even if the authors used only 1% inoculum this means that all (!!!) experiments contained at least 400 µM arsenate. As a consequence, basically all experiments that the authors did contained significant concentrations of arsenate and had the potential to build in arsenate into the biomolecules of growing cells (or interacting with the glutamate thus preventing uptake of arsenate into the biomolecules), correct? I strongly recommend repeating some of the crucial experiments using an inoculum culture that was not grown in the presence of As.

11) Along the same lines: what was the rationale for using frozen cells as inoculum?

12) The authors state in the abstract that "DNA purified from cells grown with limiting phosphate and abundant arsenate does not contain detectable arsenic". However, on page 6 of their manuscript they state that "persisting arsenate was detectable following digestion of the washed DNA" and refer to Figure 3 and Table 1. The data in Figure 3 indeed shows arsenate in DNA that stems from +As/-P cultures with DNA being washed and digested. From the comparison to the standards it looks like approximately 0.5 x 10-7 M (i.e. 0.05 µM) arsenate was present in this sample – a concentration close to the detection limit of the instrument and based on the calculation of the authors (that I couldn't really follow) 50-fold lower than reported by Wolfe-Simon et al. Although I agree that this amount of arsenate is really low – if it would be covalently bound to DNA, this result still means that at least small amounts of arsenate could be built into DNA, correct?

13) If the authors believe that the small amount of arsenate that they indeed found being associated with the DNA is simply arsenate sorbed to the DNA, why not isolate DNA (from As experiments) and incubate it with dissolved arsenate and then run the mixture through their complete DNA purification protocol? If there is any As left after the purification in the purified DNA, maybe even at concentrations that they observed in DNA from +As experiments, wouldn't this suggest that the As even in the +As experiments is probably sorbed As? In parallel, they could investigate the same mixture of As-free DNA with arsenate using the DNA purification protocol used in the original paper by Wolfe-Simon et al. The corresponding author of the present manuscript already criticized this simple purification protocol in a technical comment on the Wolfe-Simon paper (Redfield, R. J. (2011). "Comment on "A Bacterium That Can Grow by Using Arsenic Instead of Phosphorus"." Science 332(6034): 1149). Following this criticized protocol with an As-free DNA/arsenate mixture and quantifying the As remaining in the DNA after the purification protocol would show whether this procedure indeed leads to As associated with the DNA as suggested by Redfield in the technical comment.

14) The last paragraph discussing the "salt management challenge" needs to be revised. Better explanation is needed as to how this is related to the observations made by Wolfe-Simon et al. and/or Reaves et al.

15) Comment regarding the abstract: the question is not whether "arsenate is needed for growth of GFAJ-1 when phosphate is limiting" but whether this strain can use arsenate for growth, i.e. whether it can replace a fraction of the phosphate by arsenate and not all phosphate.

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END