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Response to Comments on "A Bacterium That Can Grow Using Arsenic Instead of Phosphorus"

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ABSTRACT

Concerns have been raised about our recent study describing a bacterium that can grow using arsenic (As) instead of phosphorus (P). Our data suggested that As could act as a substitute for P in major biomolecules in this organism. Although the issues raised are of investigative interest, we contend that they do not invalidate our conclusions. We argue that while no single line of evidence we presented was sufficient to support our interpretation of the data, taken as an entire dataset we find no plausible alternative to our conclusions. Here we reply to the critiques and provide additional arguments supporting the assessment of the data we reported.

Our study (*1*) described the ability of a known microbe, a member of the Gammaproteobacteria identified as strain GFAJ-1, to survive and grow in the presence of 40 mM arsenate (As) when cultured without any deliberately added phosphate (P). Our data suggested that GFAJ-1 appeared to incorporate As into biomolecules where we would only expect to find P. We presented six lines of evidence ranging from mass spectrometry and X-ray spectroscopy to classical techniques in microbiology. Members of the scientific community have raised concerns about our conclusions (*2–9*). Here we address comments that have been raised in response to our study (*1*), noting that some of these concerns will only be resolved through further research.

One concern is the amount of phosphate "background" present in our media and potential P impurities found in our reagents (*2*, *3*, *5*, *8*). As noted in the "Methods" section of the Supporting Online Material (SOM) (*1*), all treatments (+As/-P, -As/+P, and -As/-P) were prepared complete with vitamins and glucose. The value reported for background P was 3.1 μM. We emphasize that for every experiment, medium mineral salts for a single experiment were initially made up as -As/-P and therefore the background was identical in all treatments for a given experiment. To prepare this stock medium for experimental use, we took a single batch prepared as -As/-P and added glucose and vitamins. We then added either arsenate or phosphate [see "Methods" in SOM, (*1*)]. Thus, all treatments contained the same level of P impurity coming from major salts [table S1, (*1*)]. Our analyses of the arsenate stock solution showed that it would contribute P below the level of detection (< 0.002 μM). Thus when this arsenate was added to the -As/-P batches to make the +As/-P medium (final concentration 40 mM arsenate), the arsenate contributed very little P to any P impurity coming from other salts. Data in our paper clearly showed that strain GFAJ-1 was unable to utilize this P impurity [≤ 3.1 μM, see figure 1, A and B,

(*1*)] to support growth in the absence of added arsenate regardless of how much that impurity might have been (*1*). Table S1 (*1*) showed that even after the addition of glucose, vitamins, and arsenate, the background P impurity remained essentially the same when compared to the -As/-P condition.

We calculated whether the total observed intracellular elemental P content measured in +As/-P grown cells was enough to supply the P needed to construct a bacterial cell's biomass (Table 1). The +As/-P grown cells contained 0.019 % P by dry weight on average. Based on known distributions of P in other microbes, a cell allocates 10% of its total dry weight in RNA, 2.5% in DNA and 0.6% in ATP (*10, 11*). Moreover, the percent of DNA is also likely to be growth-rate independent (*11*). Known P content of these various fractions are 8.7%, 8.7% and 18% respectively (*12*). Thus a "normal scenario", ignoring P contributions to protein phosphorylation, would require at least 1.3% P by dry weight, a value 70 times greater than our measurements (Table 1). We also repeat this estimate taking an ultra-low P scenario and find that the cells would require at least 0.13% P by dry weight to construct cellular biomass (Table 1). This estimate still assumes an order of magnitude higher P than the content we measured in the +As/-P treatment. In contrast, the -As/+P grown cells had 0.54% by dry weight, which is similar in magnitude to the P pool size for the "normal" condition in Table 1. We cited the average required value of P for microbes as 1- 3% by weight yet recognize that this may not be the only possibility as microbes living under P limitation *in situ* would be more appropriate (*6*). Recent evidence (*13*) suggests that in P-limited environments, microbes may be able to grow at a total P of 0.5% by weight, which compliments our findings.

We recognize that these calculations do not fully support our conclusions, but they were not intended to do so. They were meant to serve as a departure point for conducting further analyses of the cells themselves. When combined with the EXAFS data (*1*), we see these results as an indication that intracellular As was in a structural environment consistent with a replacement of P. We recognize that this could be interpreted to mean that As was not incorporated in nucleic acids (Table 1), but given the additional evidence provided by our NanoSIMS data (which showed elemental As associated with purified DNA fractions) along with our radiolabeled study (indicating that As was associated with all cellular fractions), we concluded that As is likely located within DNA as well as other biomolecules such as proteins and lipids.

Questions have been raised as to the stability of arsenate esters (*3*, *5*, *7*). We appreciate the importance of this issue (*14*), but the rapid hydrolysis times (*15-17*) are of doubtful relevance to the case of arsenate bound in long chain polyesters or nucleotide di- or tri-esters, and it is the latter that are most relevant. For example, Kay (*18*) demonstrated the incorporation of radiolabeled arsenate into nucleotides of tumor cells as resolved by paper chromatography. Additionally, Geraldes *et al.* (*19*) showed by NMR that these compounds not only form but may have slower hydrolysis rates than estimated by others (*15-17*). These studies directly look at arsenate incorporation into relevant compounds and support our conclusions. Beyond them we are not aware of any measurements of the stability of such molecules in an aqueous environment. Baer and others (*15*) showed that the hydrolysis rates for these simple alkyl triesters of arsenate decreased with increasing complexity of the alkyl substituent (methyl > ethyl > n-pentyl > isopropyl). Aside from these two additional references there has been no work on the hydrolysis rates of arsenate-linked nucleotides or other biologically relevant moieties.

If the hydrolytic rate trend (*16*) continues to higher-complexity organics, such as those found in biomolecules (*15*), it is conceivable that arsenate-linked biopolymers might be more resistant to hydrolysis than previously thought. The work of Kay (*18*) and Geraldes *et al.* (*19*) strongly suggests this is the case and hence one cannot directly extrapolate (*3*) from very simple arsenate esters to biomolecules. The small model compounds so far investigated (*15-17*) are relatively flexible and can easily adopt the ideal geometry for water to attack the arseno-ester bond. Arsenate esters of large bio-molecules, however, are likely to be more sterically hindered leading to slower rates of hydrolysis.

It has been suggested (*3*) that if arsenate were to substitute for phosphate, entire biosynthetic pathways would need to be either completely different or operate in ways currently unknown. Again, we make no attempt at this stage to propose a mechanism, but we do note that there is evidence that arsenate is indeed "mistaken" for phosphate by many known pathways (*14*) and these examples motivated the empirical approach in (*1*). In Table 2, we summarize many of the known biochemical pathways that appear to incorporate some arsenate. In some cases, these pathways may lead to the instability of the system.

Concerns have been raised about the quality of our DNA/RNA extraction procedure (*3*, *8*). Our protocol begins with cells collected by centrifugation and then triple-washed with mineral salts (no added glucose, vitamins, arsenate, phosphate or trace metals). These are then subjected to a standard DNA/RNA extraction protocol, which included first a phenol (pH 6.6) extraction followed by multiple phenol:chloroform (pH 6.7) steps to remove impurities, such as

unincorporated arsenate. The interface between the various solutions, where we would expect to find contaminating biological and other impurities, was clean (i.e., it did not have notable particles or hazy appearance). After three phenol:chloroform steps, we concluded that this number of steps was enough to remove any impurities. We then continued with a single chloroform step followed by cold precipitation using sodium acetate and ultra chilled (-70°C) 100 % ethanol. The DNA/RNA pellet was collected by centrifugation and washed with 70% ethanol, re-pelleted, dried and resuspended in ultra-clean water (Fisherbrand, BP2484-100). After mixing in agarose gel loading buffer, DNA was electrophoresed on a 1% gel, further separating the DNA from impurities. Any residual arsenate contamination from the medium itself would have been removed by washing of the cells prior to extraction. Moreover, only if As was incorporated into a lipid or protein would it have partitioned into the phenol, phenol:chloroform, or chloroform fractions as evident in our radiolabel $^{73}AsO_4^{3-}$ results (see below).

To further dispel the notion that the arsenic measured by NanoSIMS (see below) in a DNA gel band [Fig 2A, (*1*)] was a "contaminant", our radiolabeled $^{73}AsO_4^{3-}$ experiment showed that of the total radiolabel associated with the cell pellet, 11.0% was associated with the DNA/RNA fraction. This indicated that we should expect some arsenate in the total pool to be associated with nucleic acids. From our data set, the exact concentration of As in DNA of GFAJ-1 cannot be determined; it was not our original intention to be able to make this estimate. The strongest evidence for the incorporation of As into nucleic acids is the EXAFS data. We obtained EXAFS evidence which showed that intracellular arsenic was As(V) bound to about four O atoms and further bound to C atoms in secondary coordination shells, and was not free in solution as an ion.

This result is consistent with the As(V) in an organic molecule with bond distances in an analogous chemical environment to phosphate in many phosphate biomolecules (Table 3, *1*).

Our interpretation of EXAFS and radiolabel results are supported by NanoSIMS analysis of whole cells and electrophoresed DNA/RNA gel bands, an independent As and P analysis based on imaging mass spectrometry. NanoSIMS is a destructive technique, that breaks molecular bonds and allows detection of monatomic ions (e.g., $^{75}As^{-}$). Due to variability in sputtering efficiency, NanoSIMS elemental ion counts are typically normalized to C for organic matrices. NanoSIMS' strength lies in its ability to quantify relative differences in ion ratios with high sensitivity and ultra-small sample size. To estimate As and P concentrations in ppm [table S2, (*1*)], calculations involved the assumed gel carbon content and wet/dry ratio, plus a measure of relative sensitivity for each ion ($RSF_{X/C}$, SOM, *1*) derived from bulk gel measurements. As these estimates are inherently less precise, we chose to report simple ion ratios of As^{-}:C^{-} and P^{-}:C^{-} in the text. Blank gel samples came from outside an electrophoresis lane, and are considered a 'high estimate'; repeated analyses gave consistent blank P^{-}/C^{-} results (0.000538, 0.000699, 0.000695, 0.000833). For NanoSIMS images and whole cell data [Fig 2, S2, (*1*)], differences in scaling should not be misinterpreted (*2*), there is no assumption of 1:1 As for P substitution, and images contain equivalent pixel density. Images were scaled to show the data trends. Error bars (Fig S2) are calculated based on replicate measurements within individual cells. The numeric ranges of the minor axis of the two populations indicate that those error bars reflect the overall measurement precision. Therefore, the fundamental conclusion that the +As/-P cells have substantially less P and more As holds.

Our XANES and EXAFS data show no evidence for a change in the redox state of arsenic from As(V) to As(III), indicating the absence of any such biologically-mediated reactions under the aerobic conditions of growth (*20*-*23*). Arsenic-based lipids are known, but they contain methylated arsenic species, which were also not indicated by our data (*1, 24, 25*). Moreover, concerns regarding other known arsenic metabolites are not supported by our data. Our X-ray spectroscopy did not indicate direct As-C bonds (which would include other methylated arsenic species such as arsenobetaine, (Table 3) nor a pattern of As-S bonds that would characterize thioarsenate (for review see *23*).

Some argue that we have selected for a particularly efficient microbe that retains a high-affinity P uptake mechanism (Pst) that may be stimulated by arsenate but is also well suited to scavenge P (*3*, *4*, *9*). While this argument may explain the stimulatory affect of arsenate in the growth experiment, this neglects the entirety of our other data including the radiolabel, NanoSIMS and EXAFS results. Moreover, if we did select for a mutant that has lost the Pit system and maintained a particularly efficient Pst uptake pathway, we would also expect to see the upregulation of arsenate detoxification pathways and observe some level of arsenate reduction or methylation and/or other detoxification pathways expressed because all of phosphate-requiring systems would be flooded with the incoming arsenate (*23* and references therein). We have no evidence for these processes or other detoxification mechanisms as active in GFAJ-1 and rather show arsenate incorporated and associated various cellular fractions. Thus, while this interpretation is plausible, it is not evident from our data. We argue that when our data are taken collectively it leads to the conclusion that As can be found in cell fractions in biomolecules that are normally only associated with P.

The way we presented our data, with any attendant details of statistical analyses and processing, is in line with most other biochemistry and biogeochemistry papers of which we are aware (*2*). Indeed, we showed each datum in the SOM in an effort to be transparent about the trends and nature of our measurements. To addresses the comments (*2*), and better illustrate this point, we have reorganized our original "Table 1" (*1*) to show that there was good agreement within each experiment (Table 4 here). As we mention in our paper (*1*), the variability in the total As content of the +As/-P cells between the experiments is most likely due to collection during stationary growth-phase. The cells were collected via repeated washing and centrifugation, which could lead to a selective loss of various arsenic compounds. The relatively low variability in the total P content suggests that the small amount of intracellular P was more strongly biochemically retained when compared with the As-containing biomolecules (Table 4). In contrast, the integrity of the -As/+P cells appeared robust and thus intracellular P measured for these cells likely reflects their content and that P was functioning normally as part of either mobile or less mobile biomolecules in the cells.

Many groups are interested in the availability of strain GFAJ-1 and we will release samples to the community, initially plates from the Oremland lab, and then further disseminated via cultures collections (i.e., ATCC and DSMZ).

Table 1. Calculated estimates of intracellular P content.[*]

"Best case" scenario for P		"Normal" scenario for P	
1	% RNA	10	% RNA
0.5	% DNA	2.5	% DNA
0	% ATP	0.6	% ATP
0	% P-lipids	3	% P-lipids
0.086	% RNA-P	0.86	% RNA-P
0.043	% DNA-P	0.22	% DNA-P
0	% ATP-P	0.11	% ATP-P
0	% P-lipid-P	0.15	% P-lipid-P
ESTIMATED			
0.13	total %P	1.33	total %P
OBSERVED		**OBSERVED**	
0.019	%P +As/-P cells	0.019	%P +As/-P cells
0.54	%P -As/+P cells	0.54	%P -As/+P cells
EST / OBS		**EST / OBS**	
6.8		70.2	

[*] Cellular P content discussed in (*12*) and DNA and RNA estimates based on (*11*).

Table 2: Evidence of arsenate substitution for phosphate by modern, extant biochemical processes[†]

Reaction or Enzyme	Phosphate compound	Arseno-analog	Reference
Adenylate deaminase	5'AMP	5'AMAs	(*27*)
Adenylate kinase	AMP	5'AM(CH_2)As	(*30*)
Aspartate aminotransferase	pyridoxal phosphate	pyridoxal arsenate	(*31*)
Chloroplastic electron transport	ATP	ADP-As	(*32*)
Hexokinase	ATP	ADP-As	(*33, 34*)
Human red blood cell sodium pump	P_i	As_i	(*35*)
Mitochondrial O_2 consumption	P_i	As_i	(*36*)
Myokinase	AMP	AMAs	(*27*)
Ribonucleic acid synthesis	AMP/GMP/CMP/UMP	AMAs/GMAs/CMAs/UMAs	(*18*)
RNA Polymerase	pyrophosphate	pyroarsenate	(*37*)
R. rubrum light induced phosphorylation	ADP+P_i	ADP+As_i	(*38*)
Phosphoenolpyruvate mutase	phosphonopyruvate	arsenopyruvate	(*39*)
Phosphotransacetylase	P_i	As_i	(*40*)
Protein phosphorylation	P_i	As_i	(*18*)
Protein synthesis	ATP hydrolysis	ADP-As hydrolysis	(*34*)
Purine nucleoside phosphorylase	P_i	As_i	(*41*)

[†]Table based on literature complied in (*14*).

Table 3. Various bond length distances of typical phosphate containing biomolecules between phosphorus, oxygen and carbon atoms as compared to measured values for a known arsenic-containing compound and whole GFAJ-1 cells. ‡

Compound (PDB ID)	***Type*** **P-O**	**P-C**	**P-P**	**P-C$_2$**
ATP (ANP)	1.69 1.75 1.76	2.91	3.24	-
NAD (NAD)	1.65 1.78	2.70	2.86	4.24
Glucose-6-phosphate (B6G)	1.49 1.62	2.45	-	3.86
acetyl-CoA (ACO)	1.50 1.64	2.49	2.63	3.65 3.91
Glycogen synthase kinase-3β inhibitor complex(3F88)	1.46 1.55 1.56 1.58	2.51	-	3.35 3.38
DNA (7BNA)	1.47 1.58	2.52 2.66	-	3.36 3.76 3.97 4.14
RNA (3MQK)	1.47 1.48 1.60	2.59 2.66	-	3.55 3.91 3.92
	As-O	***As-C***		***As-C***
Arsenobetaine (*28*)	-	1.91		-
GFAJ-1 whole cells (*1*)	1.73	2.35		2.92

‡These structures were taken directly from the protein databank (www.pdb.org, *29*) and are "ligand structures" standards in the PDB while 3F88 is an example of a phosphorylated protein. They have been identified in a range of biomolecules. This is a modified version of Table S3 (SOM of *1*). References noted as indicated above for data retrieved from the literature.

Table 4: Intracellular elemental profile of strain GFAJ-1.[§]

	(% dry weight)		
Condition (n)	**As**	**P**	**As:P**
+As/-P (4)[1]	0.372 ± 0.25	0.027 ± 0.006	14.0
+As/-P (4)[2]	0.010 ± 0.0007	0.012 ± 0.0001	0.87
-As/+P (2)[1]	0.0006 ± 0.0001	0.45 ± 0.31	0.001
-As/+P (2)[2]	0.0015 ± 0.00001	0.64 ± 0.015	0.002

[§]Cells grown and prepared with trace metal clean techniques. Concurrent experiments shown together: [1]Experiment 1 and [2]replicate experiment run at separate time. Number in parentheses indicates replicate samples analyzed. Data from ICP-MS analyses from (*1*).

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