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

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Concerns have been raised about our recent study suggesting that arsenic (As) substitutes for phosphorus in major biomolecules of a bacterium that tolerates extreme As concentrations. We welcome the opportunity to better explain our methods and results and to consider alternative interpretations. We maintain that our interpretation of As substitution, based on multiple congruent lines of evidence, is viable.

Our study (*1*) described the ability of a known microbe, a member of the Gammaproteobacteria (strain GFAJ-1), to survive and grow in the presence of 40 mM arsenate (As) when cultured without deliberately added phosphate (P). We hypothesized that GFAJ-1 incorporates As into biomolecules in place of P, based on evidence ranging from mass spectrometry and x-ray spectroscopy to classical techniques in microbiology. Here, we address specific concerns raised about our procedures and interpretation (*2*–*9*).

The foundation of our study was a suite of microbial growth experiments demonstrating extreme As tolerance and enhanced growth in +As/–P media as compared with –As/–P controls (*1*). These experiments have been criticized because of the presence of trace P in the experimental media. Benner (*3*) suggests that this contamination came as an impurity with the As added to +As/–P experiments and that the level of contamination is unknown. If this were so, the experiments would be fundamentally flawed; however, to clarify the methods described in the Supporting Online Material (SOM) accompanying our original study (*1*), medium mineral salts were initially made up as –As/–P, without added vitamins or glucose. The P content of the batch of this mixture used in all experiments was 3.7 ± 0.8 μM (±2 SD; table S1, 5 April 2010 batch) (*1*). For every experiment, vitamins, glucose, and either P or As were added to this mixture, as described in "Materials and Methods" in the SOM (*1*). In the two batches of medium to which we added As along with glucose and vitamins, we measured P contents of 2.9 ± 0.6 and 2.7 ± 0.6 μM (table S1) (*1*). These values are statistically indistinguishable from the P content of the –As/–P mineral salt medium. Therefore, the P background presumably came from the mineral salts, not from the added As, and was effectively identical in all treatments (~3 μM).

Several comments suggest or imply that this background P was sufficient to sustain growth in the +As/–P experiments (*2*, *3*, *5*, *6*, *8*, *9*). We do not find these arguments compelling given the poor growth in the control experiment compared with the +As/–P experiment, both of which contained the same P background. Any alternative hypothesis must account for this basic observation.

Foster (*9*) hypothesizes that the enhanced growth we observed upon addition of As compared with the –As/–P control can be explained if we inadvertently selected for a microbe that retains a high-affinity P uptake mechanism (Pst) that is stimulated by arsenate. If operational, we would expect evidence of arsenate detoxification processes, such as arsenate reduction or methylation, because all phosphate-requiring systems would be flooded with the incoming arsenate (*10*). However, our x-ray absorption near-edge structure (XANES) and extended x-ray absorption fine structure (EXAFS) data do not show a change in the redox state of As from As(V) to As(III), which suggests that no such biologically mediated reactions occur under the aerobic conditions of growth (*10*–*13*). Methylated As species also were not indicated by our data (*1*, *14*, *15*); x-ray spectra do not indicate direct As-C bonds that would include methylated As species such as arsenobetaine

Table 1. Various bond-length distances of typical phosphate-containing biomolecules between phosphorus, oxygen, and carbon atoms as compared with measured values for a known arsenic-containing compound and whole GFAJ-1 cells. These structures were taken directly from the Protein Data Bank (www.pdb.org) (*29*) and are "ligand structures" standards in the PDB, whereas 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 of (*1*). References noted as indicated above for data retrieved from the literature.

	Type			
Compound (PDB ID)	**P-O**	**P-C**	**P-P**	**P-C_2**
Adenosine triphosphate (ANP)	1.69 1.75 1.76	2.91	3.24	–
Nicotinamide adenine dinucleotide (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 (*30*)	–	1.91		–
GFAJ-1 whole cells (*1*)	1.73	2.35		2.92

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(Table 1), nor a pattern of As-S bonds that would characterize thioarsenate [for a review, see (*16*)]. Furthermore, this hypothesis does not account for the evidence we observe of As in cell fractions and biomolecules that are normally only associated with P. Nevertheless, direct investigations of As detoxification and P uptake pathways in GFAJ-1 are important avenues for future investigation.

Alternative hypotheses must also be reconciled with simple calculations that suggest that the total observed intracellular elemental P content measured in +As/–P–grown cells was insufficient to supply the P needed to construct a bacterial cell's biomass (Table 2). The P contents (% dry weight) of the two batches of +As/–P–grown cells were 0.027 ± 0.012% (±2 SD) and 0.012 ± 0.003% (±2 SD) [June and July batches, respectively; see SOM for (*1*)]. Based on known distributions of P in other microbes, a cell on average allocates ~10% of its total dry weight in RNA, ~2.5% in DNA, ~0.6% in adenosine triphosphate (ATP), and ~3% in lipids (*17*, *18*). The percentage of DNA is likely to be growth-rate independent (*18*). The P contents (% dry weight) of these fractions are 8.7%, 8.7%, 18%, and 5%, respectively (*19*). Thus, a "normal" scenario, ignoring P contributions to protein phosphorylation, would require at least 1.3% P, a value at least 50 times greater than we observed (Table 2). An exaggerated "ultra-low" P scenario, presuming no ATP, no P lipids, 1/10 and 1/5 of "normal" RNA and DNA, respectively, predicts that cells require at least 0.13% P by dry weight to construct cellular biomass (Table 2). This estimate is a factor of ~5 to 10 higher than the P content measured in our +As/–P treatments. Hence, the P content of these P-starved organisms seems exceptionally low.

Cotner and Hall (*6*) propose that P contents as low as 0.03% P by dry weight are possible for bacteria adapted to P depletion (*20*). This proposal is based on measurements of individual cells observed in populations that average 0.5% P by dry weight. This population average, rather than the P content of extreme individuals in a community, seems the more relevant comparison to our bulk biomass data. This population average is similar to the 0.2 to 0.4% P values in chemostat-grown bacterial isolates reported by Cotner *et al.* (*20*). We find it notable that these values are also similar to the P contents in our –As/+P–grown populations, which ranged from 0.23 to 0.66% by dry weight, and that they lie between our "normal" and "ultra-low" scenarios (Table 2). Most important, however, these P contents are all significantly higher than the highest values for bulk P content in our +As/–P experiments (0.036 ± 0.008%). Therefore, the data reported by Cotner and Hall (*6*, *20*) seem to support the argument that the cells in our As-rich, P-depleted experiments contained unusually small quantities of P.

Csabai and Szathmáry (*2*) critique our presentation of the data from these experiments (*2*). They correctly point out that the average As contents and As/P ratios of the two batches of +As/–P experiments were very different and that problems can arise when averaging together such disparate data. Thus, in Table 3 we have reorganized the data from table S1 in (*1*) to avoid averaging. This revised presentation shows that the results were reasonably reproducible within each experiment. The uncertainties are smaller than the average at the ±2 SD level for the July experiment and at the ±1 SD level for the June experiment. The variability in the total As content between the two +As/–P experiments was most likely the result of collection during stationary growth phase when, as we noted in (*1*), the +As/–P cells were physiologically impaired. It is possible that the integrity of their membranes was compromised, leading to the release of cell constituents, including As-containing compounds, after repeated centrifugation/washings (*1*). In this context, variability of As contents between experiments is unsurprising. Similarly, sample preparation issues probably account for the variability of As contents among the four replicate measurements of cells from the June 2010 +As/–P experiment [table S1 in (*1*)]; each replicate was a separate biomass sample, and the analytical reproducibility of each of these replicates was within 10%, as indicated. Consequently, arguments built on the assumption that As contents are quantitatively correct [as in (*2*)] are tenuous. The same might be true of P contents, but the relatively low variability in the total P data suggests that intracellular P was more strongly biochemically retained compared with intracellular As. Hence, despite such complications, we find the very low P contents discussed above intriguing, as is the observation that the As cell concentrations are greater, and the P concentrations lower, in every measurement of the +As/–P condition compared with any of the –As/+P cell measurements.

To examine the incorporation of As in biomolecules, we extracted nucleic acids from cells. Redfield (*8*) questions the quality of our DNA/RNA extraction procedure. This procedure begins with cells collected by centrifugation and then triple-washed with a solution of mineral salts identical to the AML60 medium that the cells are grown in but with no added glucose, vitamins, arsenate, phosphate, or trace metals (*1*). The pellet was 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) extractions to remove impurities such as unincorporated arsenate. The

Table 2. Calculated estimates of intracellular P content. Cellular P content discussed in (*19*), and DNA and RNA estimates based on (*18*).

	Ultra-low	Normal
% RNA	1	10
% DNA	0.5	2.5
% ATP	0	0.6
% P lipids	0	3
% RNA-P	0.086	0.86
% DNA-P	0.043	0.22
% ATP-P	0	0.11
% P-lipid-P	0	0.15
% P estimate	0.13	1.3

Observed % P in +As/–P cells	
June	July
0.027 ± 0.006	0.012 ± 0.002

Ultra-low estimate/observed	
June	July
4.8	11

Table 3. Intracellular elemental profile of strain GFAJ-1. Concurrent experiments are shown together, with the number of replicates (*n*) indicated. Concentrations are reported as averages of *n* replicates. When *n* = 4, the range of the replicates is given in parentheses. When *n* = 2, the two replicate measurements are reported in parentheses. Cells grown and prepared with trace metal clean techniques. Data are from inductively coupled plasma mass spectrometry analyses from (*1*).

Condition (*n*)	As (% dry weight)	P (% dry weight)	As:P
+As/–P (4)	0.37 (0.11–0.62)	0.027 (0.023–0.036)	4.9–26.6
+As/–P (4)	0.010 (0.009–0.011)	0.012 (0.011–0.014)	0.76–0.97
–As/+P (2)	0.0006 (0.0005, 0.0006)	0.45 (0.23, 0.63)	0.001–0.002
–As/+P (2)	0.0015 (0.0015, 0.0015)	0.64 (0.63, 0.65)	0.0022–0.0023

interface between the aqueous and organic phases, where we would expect to find particulate impurities, appeared clear. We therefore concluded that three phenol:chloroform steps were sufficient 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, repelleted, dried, and resuspended in ultra-clean water (Fisherbrand, BP2484-100). After agarose gel loading buffer was mixed with the resuspended pellet, the DNA/RNA was electrophoresed on a 1% gel. Arsenate in the spent culture medium should have been removed by washing of the cells before extraction. Moreover, because both arsenate and DNA are negatively charged molecules, there should be little As sorbed to the DNA pellet after the purification process.

Our $^{73}AsO_4^{3-}$ experiment confirms that significant As is extracted into the organic fractions and that 11% of the total radiolabel associated with the cell pellet was associated specifically with the DNA/RNA fraction (*1*). This proportion of As seems too large to represent residual inorganic As contamination after multiple washing and extraction steps. Furthermore, our interpretation of our EXAFS data is that they are consistent with intracellular arsenic in the form of As(V) bound to about four O atoms and further bound to C atoms in secondary coordination shells, rather than being free in solution as an ion. Although it is possible that additional arsenic as residual inorganic arsenate was present in our whole-cell samples after washing, the spectra we obtained for GFAJ-1 indicate bond distances similar to phosphate bond distances in many phosphate biomolecules (Table 1) and are thus suggestive of As present in arsenoester-like compounds.

Our interpretations of EXAFS and radiolabel results are supported by high-resolution secondary ion mass spectrometry (NanoSIMS) analysis of whole cells and electrophoresed DNA/RNA gel bands (*1*). Redfield is concerned that because DNA was not purified from the agarose gel, most of the measured C comes from the agarose (*8*). In response, we first note that this imaging mass spectrometry technique uses a high-energy ion beam to break molecular bonds, detecting the resultant monatomic ions (e.g., $^{75}As^-$) sputtered from a discrete spot of sampled material. Because of variability in sputtering efficiency from spot to spot, NanoSIMS elemental ion counts are normalized to the counts of an ion representative of the sample matrix. In organic matrices, such as DNA embedded in agarose gel, the normalizing ion is typically $^{12}C^-$. Therefore, the reported $^{12}C^-$ ion counts were not assumed to represent cell stoichiometry, so this concern is not relevant to the way in which we used the $^{12}C^-$ data (*1*). Further, because the ion yield differs for each element, it is not valid to compare NanoSIMS As:C ratios to P:C ratios, as attempted by Borhani (*5*). Cross-element comparisons, typically as concentrations, require a measure of relative sensitivity for each ion derived from bulk gel measurements [e.g., relative sensitivity factor $(RSF)_{X/C}$; SOM in (*1*)], as well as the assumed gel C content and wet/dry ratio. However, it is true that quantitative comparison of As:C in the +As/–P and –As/+P DNA samples is inconsistent with wholesale substitution of As for P (*5*). We did not mean to imply otherwise (*1*).

Analysis of DNA separated from agarose would be a useful future experiment, because it could yield quantitative As:C and P:C ratios using NanoSIMS. This procedure would also minimize the challenges arising from the lack of a representative agarose gel blank (*2*, *5*). The "blank" samples in table S1 (*1*) came from outside an electrophoresis lane, so may not be appropriate controls for the within-lane samples. Repeated analyses of the blank did give quite consistent $^{31}P^-/^{12}C^-$ results (5.38×10^{-4}, 6.99×10^{-4}, 6.95×10^{-4}, and 8.33×10^{-4}). Hence, we reported an average blank in the interest of full disclosure, considering it a high estimate (*1*).

In addition to these procedural issues, the plausibility of our interpretations has been examined from the perspective of basic chemical principles. Some of these examinations are supportive. For example, recent quantum mechanical calculations indicate that As replacement of P in DNA would not alter the geometry and backbone structure of the DNA double helix (*21*, *22*). Others are critical. For example, Schoepp-Cothenet *et al.* (*4*) argue that the cellular environment is too reducing for arsenate compounds to persist. However, as noted above, we did not observe As(III) compounds in our EXAFS data as predicted by this suggestion. Hence, reduction is either very slow or the thermodynamic redox argument is not correct for the intracellular redox potential and/or the chemical form(s) of As in GFAJ-1.

The arguments by Benner and others about the stability of arsenate esters (*3*, *5*, *7*), based on the rapidity of hydrolysis of small model compounds (*23*–*25*), challenge the plausibility of As-substituted biomolecules and biosynthetic pathways (*26*). However, we note that arsenate esters of large biomolecules are likely to be more sterically hindered, leading to slower rates of hydrolysis than occurs in small compounds, which are relatively flexible and can adopt a geometry that allows water to attack the arseno-ester bond. There is little literature on the stability of arsenate bound in long chain polyesters or nucleotide di- or triesters, which are more relevant to our studies, but there is evidence that the hydrolysis rates for simple alkyl triesters of arsenate decrease with increasing complexity of the alkyl substituent (methyl > ethyl > n-pentyl > isopropyl) (*23*). It is therefore conceivable that arsenate-linked biopolymers are more resistant to hydrolysis than generally assumed, perhaps sufficiently so for an As-adapted organism to cope with some degree of As substitution. Consistent with this possibility, Geraldes *et al.* (*27*) showed by nuclear magnetic resonance that arsenate esters with glucose have surprisingly slow hydrolysis rates. Intriguingly, Kay (*28*) demonstrated the incorporation of radiolabeled arsenate into nucleotides of tumor cells, as resolved by paper chromatography, at rates consistent with the biosynthesis of DNA and RNA (and even protein) and not simple adsorption. Finally, as we noted previously (*1*), GFAJ-1 may have evolved specific strategies to cope with this issue, such as stabilizing structures.

We look forward to working with our peers to replicate our observations and to test our hypotheses along the lines suggested by Oehler (*7*) and others. To these ends, samples of GFAJ-1 are available to the community on request as plates from the Oremland laboratory, pending wider dissemination via culture collections [American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)] (*31*).

References and Notes

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31. Cultures of GFAJ-1 were submitted to the ATCC and DSMZ culture collections on 21 March 2011 and will be available from these sources within several months. Until then, samples of GFAJ-1 are available to the community from the Oremland laboratory upon completion of a materials transfer agreement, which is required by the U.S. Geological Survey for the transfer of bacterial cultures.

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