A Bacterium That Can Grow by Using Arsenic Instead of Phosphorus

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Life is mostly composed of the elements carbon, hydrogen, nitrogen, oxygen, sulfur and phosphorus. Although these six elements make up nucleic acids, proteins and lipids and thus the bulk of living matter, it is theoretically possible that some other elements in the periodic table could serve the same functions. Here we describe a bacterium, strain GFAJ-1 of the Halomonadaceae, isolated from Mono Lake, CA, which substitutes arsenic for phosphorus to sustain its growth. Our data show evidence for arsenate in macromolecules that normally contain phosphate, most notably nucleic acids and proteins. Exchange of one of the major bio-elements may have profound evolutionary and geochemical significance.

Biological dependence on the six major nutrient elements carbon, hydrogen, nitrogen, oxygen, sulfur, and phosphorus is complemented by a selected array of other elements, usually metal(loid)s present in trace quantities that serve critical cellular functions, such as enzyme co-factors (*1*). There are many cases of these trace elements substituting for one another. A few examples include the substitution of tungsten for molybdenum and cadmium for zinc in some enzyme families (*2, 3*) and copper for iron as an oxygen-carrier in some arthropods and mollusks (*4*). In these examples and others, the trace elements that interchange share chemical similarities that facilitate the swap. However, there are no prior reports of substitutions for any of the six major elements essential for life. Here we present evidence that arsenic can substitute for phosphorus in the biomolecules of a naturally-occurring bacterium.

Arsenic (As) is a chemical analog of phosphorus (P), which lies directly below P on the periodic table. Arsenic possesses a similar atomic radius, as well as near identical electronegativity to P (*5*). The most common form of P in biology is phosphate (PO_4^{3-}), which behaves similarly to arsenate (AsO_4^{3-}) over the range of biologically relevant pH and redox gradients (*6*). The physico-chemical similarity between AsO_4^{3-} and PO_4^{3-} contributes to the biological toxicity of AsO_4^{3-} because metabolic pathways intended for PO_4^{3-} cannot distinguish between the two molecules (*7*) and arsenate may be incorporated into some early steps in the pathways [(*6*) and refs therein]. However, it is thought that downstream metabolic processes are generally not compatible with As-incorporating molecules because of differences in the reactivities of P- and As-compounds (*8*). These downstream biochemical pathways may require the more chemically stable P-based metabolites; the lifetimes of more easily hydrolyzed As-bearing analogs are thought to be too short. However, given the similarities of As and P, and by analogy with trace element substitutions, we hypothesized that AsO_4^{3-} could specifically substitute for PO_4^{3-} in an organism possessing mechanisms to cope with the inherent instability of AsO_4^{3-} compounds (*6*). Here, we experimentally tested this hypothesis by using AsO_4^{3-}, combined with no added PO_4^{3-}, to select for and isolate a microbe capable of accomplishing this substitution.

Geomicrobiology of GFAJ-1. Mono Lake, located in eastern California is a hypersaline and alkaline water body with high dissolved arsenic concentrations (200 µM on average, *9*). We used lake sediments as inocula into an aerobic defined artificial medium at pH 9.8 (*10, 11*) containing 10 mM glucose, vitamins, trace metals but no added PO_4^{3-} nor any additional complex organic supplements (e.g. yeast extract, peptone) with a regimen of increasing AsO_4^{3-} additions initially spanning the range 100 µM to 5 mM. These enrichments were taken through many decimal-dilution transfers greatly reducing any potential carryover of

autochthonous phosphorus (*11*). The background PO_4^{3-} in the medium was 3.1 (± 0.3) μM on average, with or without added AsO_4^{3-}, coming from trace impurities in the major salts (*11*) (table S1). The sixth transfer of the 5 mM AsO_4^{3-} (no added PO_4^{3-}) condition was closely monitored and demonstrated an approximate growth rate (μ) of 0.1 day^{-1}. After 10^{-7} dilutions, we used the 5 mM AsO_4^{3-} enrichment to inoculate an agar plate that contained the same chemical composition as the artificial medium. An isolated colony was picked from the agar plates, reintroduced into an artificial liquid medium with no added PO_4^{3-} where we then progressively increased the AsO_4^{3-} concentration to determine the optimal level for growth. Currently this isolate, strain GFAJ-1 identified by 16S rRNA sequence phylogeny as a member of the Halomonadaceae family of Gammaproteobacteria (see fig. S1) (*11*), is maintained aerobically with 40 mM AsO_4^{3-}, 10 mM glucose and no added PO_4^{3-} (+As/-P condition). Members of this family have been previously shown to accumulate intracellular As (*12*).

GFAJ-1 grew at an average μ_{max} of 0.53 day^{-1} under +As/-P, increasing by over 20-fold in cell numbers after six days. It also grew faster and more extensively with the addition of 1.5 mM PO_4^{3-} (-As/+P, μ_{max} of 0.86 day^{-1}, Fig. 1A, B). However, when neither AsO_4^{3-} nor PO_4^{3-} was added, no growth was observed (Fig. 1A, B). We include both optical density and direct cell counts to unambiguously demonstrate growth using two independent methods. Cells grown under +As/-P were oblong and approximately two by one microns when imaged by scanning electron microscopy (Fig 1C, *11*). When grown under +As/-P conditions, GFAJ-1 cells had more than 1.5-fold greater intracellular volume (vol. ≈ 2.5 ± 0.4 μm^3) as compared to -As/+P (vol. ≈ 1.5 ± 0.5 μm^3) (Fig. 1D) (*11*). Transmission electron microscopy revealed large vacuole-like regions in +As/-P grown cells that may account for this increase in size (Fig. 1E). These experiments demonstrated arsenate-dependent growth, morphological differences in GFAJ-1 driven by AsO_4^{3-} in the growth medium, and the fact that the level of PO_4^{3-} impurities in the medium was insufficient to elicit growth in the control (-As/-P).

Cellular stoichiometry and elemental distribution. To determine if GFAJ-1 was taking up AsO_4^{3-} from the medium, we measured the intracellular As content by ICP-MS (*11*). In +As/-P grown cells, the mean intracellular As was 0.19 (± 0.25) % by dry weight (Table 1), while the cells contained only 0.02 (± 0.01) % P by dry weight. This P was presumably scavenged from trace PO_4^{3-} impurities in the reagents; and not likely due to carryover given our enrichment and isolation strategy [see above, (*11*)]. Moreover, when grown +As/-P this intracellular P is 30-fold less than our measured P values for this microbe when grown -As/+P (see above) and far below the 1-3% P by dry weight required to support growth in a typical heterotrophic bacterium (*13*). By contrast, GFAJ-1

cells grown under -As/+P conditions had a mean P content of 0.54 (± 0.21) % by dry weight. There was variation in the total As content of the +As/-P cells, possibly a result of collection during stationary phase and losses during the repeated centrifugations and washing cycles due to the potential instability of the cellular structures given their swollen state (Fig. 2C, E). In contrast, the integrity of the -As/+P cells appeared robust (Fig. 2D) and thus intracellular P measured for these cells likely reflects their content. However, the low total intracellular P in +As/-P cells was consistently far below the quantity needed to support growth, suggesting that these low values are correct despite variation in data from the +As/-P cells. Low intracellular P in concert with high intracellular As was further confirmed by high-resolution secondary ion mass spectrometry and X-ray analyses as discussed below.

We used radiolabeled $^{73}AsO_4^{3-}$ to obtain more specific information about the intracellular distribution of arsenic (*11*). We observed intracellular arsenic in protein, metabolite, lipid and nucleic acid cellular fractions (Table 2). Stationary phase cells incorporated approximately a tenth of the total intracellular $^{73}AsO_4^{3-}$ label into nucleic acids but more than three quarters of the $^{73}AsO_4^{3-}$ into the phenol extracted "protein" fraction, with a small fraction going into lipids. We caution that the large "protein" fraction is probably an overestimate, as this extraction step likely contains numerous small, non-proteinaceous metabolites as well. To determine if this distribution pattern reflected a use of AsO_4^{3-} in place of PO_4^{3-} in DNA, we estimated the average sequenced bacterial genome to be 3.8 Mbps, which would contain approximately 7.5 x 10^6 atoms or 12.5 x 10^{-18} moles of P. Assuming one complete genome per cell, this would equal 0.39 fg of P in the genome. By ICP-MS, we measured about 9.0 fg P per cell in the -As/+P condition, which implies that only ~ 4% of total intracellular P is associated with the genome. Since these cells were harvested in stationary phase (*11*), the fraction of P associated with RNA is likely small (*14*). Hence, roughly 96% of P is presumably distributed between the "lipid" and "protein" fractions. If AsO_4^{3-} is substituting for PO_4^{3-} in DNA then we can assume that roughly the same fraction of the total intracellular AsO_4^{3-} would reflect a similar distribution to our estimated PO_4^{3-} distribution. The distribution of intracellular $^{73}AsO_4^{3-}$ in our experiments was consistent with these estimates. If AsO_4^{3-} is fulfilling the biological role of PO_4^{3-} then AsO_4^{3-} should act in many analogous biochemical roles including DNA, protein phosphorylation, small molecular weight metabolites (e.g. arsenylated analogs of NADH, ATP, and intermediates like glucose and acetyl-CoA) and phospholipids.

Our data suggested that arsenic was present in a number of biomolecules and in particular we sought to confirm the presence of arsenic in the DNA fraction. Initially, we

measured traces of As by ICP-MS analysis of extracted nucleic acid and protein/metabolite fractions from +As/-P grown cells (*11*) (table S1). We then used high-resolution secondary ion mass spectrometry (NanoSIMS) to positively identify As in extracted, gel purified genomic DNA (Fig. 2A). These data showed that DNA from +As/-P cells had elevated As and low P relative to DNA from the -As/+P cells. NanoSIMS analysis of the DNA showed that the As:P ratio on an atom per atom basis was significantly higher in the +As/-P versus -As/+P grown cells (Fig. 2A, *11*, table S2). Whether expressed as an ion ratio relative to C, ($^{75}As^-$:$^{12}C^-$, Fig. 2A) or $^{31}P^-$:$^{12}C^-$ (*11*) (table S2) or normalized by relative ion yield and expressed as a concentration in parts per billion (*11*) (table S2), we saw a similarly consistent trend, with significantly higher As in the +As/-P DNA, and higher P in the -As/+P DNA. In both cases, the non-amended element concentration was equal or less than background levels. These measurements therefore specifically demonstrated that the purified DNA extracted from +As/-P cells contained As. Our NanoSIMS analyses, combined with the evidence for intracellular arsenic by ICP-MS and our radiolabeled $^{73}AsO_4^{3-}$ experiments demonstrated that intracellular AsO_4^{3-} was incorporated into key biomolecules, specifically DNA.

Characterization of the intracellular arsenic chemical environment. We next used synchrotron X-ray studies to determine the speciation and chemical environment of the intracellular arsenic (*11*). Micro X-ray absorption near edge spectroscopy (μXANES) of +As/-P grown cells exhibited an absorption edge characteristic of As(V) coordination with no evidence of As(III) observed. Best fits of the micro extended X-ray absorption fine structure (μEXAFS) spectra are listed in Table 3 and shown in Figure 3. The first neighbor shell around the arsenic in +As/-P cells consisted of four oxygen ligands (Table 3), but has a second shell that is inconsistent with our As-Fe and As-S models, free arsenate ions or published spectra for organo-arsenicals (Fig. 3A) (*15, 16*). While other arsenical compounds, such as dimethylarsinate (DMA) also have As-O and As-C bonds, they have edge positions which are shifted to lower energy from the observed As(V) and have much shorter observed As-C bond distances (*16*). In contrast to the models, these As-O and As-C distances are consistent with that reported from the solved crystal structure of DNA for the analogous structural position of P relative to O and C atoms (Fig. 3A) (*16, 17*). Therefore, our X-ray data support the position of arsenate in a similar configuration to phosphate in a DNA backbone or potentially other biomolecules as well. These data also indicated evidence for the presence of arsenate in small molecular weight metabolites (e.g., arsenylated analogs of NADH, ATP, glucose, acetyl-CoA) as well as arsenylated proteins where arsenate would substitute for phosphate at serine, tyrosine and threonine residues (*1, 11*) (table S3). Micro X-ray

fluorescence data (μXRF) further confirmed our ICP-MS measurements and showed low background P which contrasted with regions of high arsenic correlated with high iron and zinc (Fig. 3B, fig. S2) (*11*). These latter two elements are routinely used as proxies for the presence of cellular material (such as C, N and O) in our experiments because these light elements could not be detected by X-ray fluorescence under our non-vacuum conditions. However, to further support the distribution of arsenic with cellular material, we used NanoSIMS to map cellular ion ratios of $^{75}As^-$:$^{12}C^-$ and $^{31}P^-$:$^{12}C^-$ (Fig. 2B-G, fig. S2) (*11*). These analyses confirmed, at a much finer resolution, the intracellular distribution of As with C in the +As/-P condition with a low background of P (Fig. 2B, D, F). This is in contrast to the intracellular distribution of P in -As/+P grown cells (Fig. 2C, E, G). Because the X-ray absorption data provided information about the average coordination of arsenic, our data identified a mixture of compounds in the cells. These results indicated that these compounds are dominated by arsenic(V)-oxygen-carbon coordinated structures and thus, the bonding environment we described is consistent with our NanoSIMS data (Fig. 2A) and can be attributed to DNA. In summary, these data show that arsenic is in the +5 redox state and bound to O and distal C atoms within acceptable covalent bond lengths identifying arsenate assimilated into biomolecules within the cells in specifically relevant coordination.

Our data show arsenic-dependent growth by GFAJ-1 (Fig. 1). Growth was accompanied by arsenate uptake and assimilation into biomolecules including nucleic acids, proteins and metabolites (Table 1 and 2, Figs. 2 and 3). In some organisms, arsenic induces specific resistance genes to cope with its toxicity (*7*); while some dissimilatory arsenic-utilizing microbes can conserve energy for growth from the oxidation of reduced arsenic species, or "breathe" AsO_4^{3-}, as a terminal electron acceptor (*18*). Our study differs because we used arsenic as a selective agent and excluded phosphorus, a major requirement in all hitherto known organisms. However, GFAJ-1 is not an obligate arsenophile and it grew considerably better when provided with P (Fig. 1A, B). Although AsO_4^{3-} esters are predicted to be orders of magnitude less stable than PO_4^{3-} esters, at least for simple molecules (*8*), GFAJ-1 can cope with this instability. The vacuole-like regions observed in GFAJ-1 cells when growing under +As/-P conditions are potentially poly-β-hydroxybutyrate rich [as shown in other *Halomonas* species (*19*)] which may stabilize As(V)-O-C type structures because non-aqueous environments appear to promote slower hydrolysis rates for related compounds (*8*). We propose that intracellular regions or mechanisms that exclude water may also promote this stability.

We report the discovery of an unusual microbe, strain GFAJ-1, that exceptionally can vary the elemental composition of its basic biomolecules by substituting As for P. How arsenic insinuates itself into the structure of biomolecules is unclear, and the mechanisms by which such molecules operate are unknown.

References and Notes

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20. The authors wish to thank S. Benner, W. Hastings, I.L. ten Kate, A. Pohorille, B. Rosen, D. Schulze-Makuch and R. Shapiro for stimulating discussions. We thank G. King, A. Oren and L. Young for constructive criticisms of earlier drafts of this manuscript, and S. Baesman, M. Dudash, and L. Miller for technical assistance. Strain GFAJ-1 is available upon request for other researchers to investigate. Sequence data are deposited with GenBank (accession HQ449183). Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource (SSRL), a division of SLAC National Accelerator Laboratory and an Office of Science User Facility operated for the U.S. Department of Energy Office of Science by Stanford University. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Basic Energy Sciences, Office of Biological and Environmental Research, and by the National Institutes of Health, National Center for Research Resources, Biomedical Technology Program. NanoSIMS analyses were performed under the auspices of the U.S. Department of Energy at Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. R.S.O. and J.F.S. were supported by NASA Exobiology. F.W.S. acknowledges support from the NASA Postdoctoral Program, NASA Astrobiology/Exobiology and the NASA Astrobiology Institute while in residence at the U.S. Geological Survey, Menlo Park, CA. The authors declare no conflicts of interest.

Fig 1. Growth, and electron microscopy of strain GFAJ-1. (**A** and **B**) Growth curves of GFAJ-1 grown on the defined synthetic medium amended with either 1.5 mM phosphate (solid circles), 40 mM arsenate (solid squares) or neither phosphate nor arsenate (open triangles). Cell growth was monitored both by an increase in (A) optical density and (B) cell numbers of the cultures. Symbols represent the mean ± the standard deviation of n=6 experimental and n=2 controls (A) and n=3 experimental and n=1 control (B). This was a single experiment with six replicates, however material was conserved to extend the duration of the experiment to allow material for cell counting samples. Scanning electron micrographs of strain GFAJ-1 under two conditions discussed in the text. (**C**) +As/-P and (**D**) -As/+P. Transmission electron micrography of +As/-P GFAJ-1 (**E**) showed internal vacuole-like structures. Scale bars are as indicated in the figure (*11*).

Fig 2. NanoSIMS analyses of GFAJ-1: extracted DNA and whole cells elemental ratio maps. (**A**) Agarose gel loaded with DNA/RNA extracted from GFAJ-1 grown +As/-P (lane 2) and -As/+P (lane 3) as compared to a DNA standard (Lane 1). Genomic bands were excised as indicated and analysed by NanoSIMS. Ion ratios of $^{75}As^-$:$^{12}C^-$ of excised gel bands are indicated below with 2 sigma error shown (all values

multiplied by 10^{-6}). NanoSIMS images of whole GFAJ-1 cells grown either +As/-P (**B**, **D**, and **F**) or -As/+P (**C**, **E**, and **G**). The ion ratios of $^{75}As^-$:$^{12}C^-$ [(B) and (C)], $^{31}P^-$:$^{12}C^-$ [(D) and (E)], and secondary electron, SE [(F) and (G)]. Ratios in B, C multiplied by 10^{-4} and D, E multiplied by 10^{-3}. The color bars indicate measured elemental ratios on a log scale as indicated. Length scale is as indicated on images (*11*).

Fig 3. X-ray analysis of GFAJ-1 +As/-P described similarity of As coordinated like P in DNA. (**A**) EXAFS comparisons of the Fourier transformed data for two model compounds, As-S and As-Fe, whole GFAJ-1 cells (washed and fixed) and a fit of DNA with arsenic replacing phosphorus, *in silico*. Identification of each spectrum is indicated on the figure and from top to bottom are As-S, As-Fe, GFAJ-1 data (collected on whole cells) and fit to the GFAJ-1 data (in red). (**B**) XRF maps indicated the correlation between arsenic (As), iron (Fe) and zinc (Zn) and not with phosphorus (P) with some variability but consistent with the trend that these elements are often found together (See figure S3 in the SOM for element correlation plots). The length scale bar in the "Zn" quadrant, of the maps is as designated and applies to all parts of the figure. Given the spatial resolution of these images, the structures identified as containing high As, Fe, and Zn are aggregates of cells. Ranges as indicated in the color bar run from cold to hot, in units of $\mu g\ cm^{-2}$, as follows: As, 0 to 1.6; P, 0 to 40; Fe, 0 to 32.1, and Zn, 0 to 2.8. Standards were used to calibrate signal and background (*11*).

Table 1. Bulk intracellular elemental profile of strain GFAJ1.*

	(% dry weight)		
Condition (n)	As	P	As:P
+As/-P (8)	0.19 ± 0.25	0.019 ± 0.0009	7.3
-As/+P (4)	0.001 ± 0.0005	0.54 ± 0.21	0.002

*Cells grown and prepared with trace metal clean techniques (*11*). Number in parentheses indicates replicate samples analyzed.

Table 2. Intracellular radiolabeled $^{73}AsO_4^-$ arsenate distribution*

Solvent (subcellular fraction)	Cellular radiolabel recovered (% of total)
Phenol (protein + s.m.w. metabolites)	80.3 ± 1.7
Phenol:Chloroform (proteins + lipids)	5.1 ± 4.1
Chloroform (lipids)	1.5 ± 0.8
Final aqueous fraction (DNA/RNA)	11.0 ± 0.1

*All major cellular sub‑fractions contained radiolabel after cell washing procedures. Small molecular weight metabolites (s.m.w. metabolites) potentially include arsenylated analogs of ATP, NADH, acetyl‑CoA and others (*11*). Standard error shown.

Table 3. Results of fitting arsenic K-edge EXAFS of GFAJ1.*

Type	Number	R	σ^2
As-O	4.2 (0.6)	1.73 (2)	0.003 (2)
As-C	2.5 (0.5)	2.35 (4)	0.003 (2)
As-C	2.2 (0.5)	2.92 (6)	0.003 (2)

*Details for table: S02=1, global amplitude factor and E0= 13.97, offset for calibration. Type, the coordination type; Number, the coordination number; R, interatomic distance; σ^2, the measure of the static disorder of the shell. See Table S2 for comparison to P in P-containing biomolecules (*11*).

A

1   2   3
+As/-P   -As/+P

$^{75}As^-:^{12}C^-$ 13.4 (±2.5)   6.9 (±1.6)

+As/-P   -As/+P   Ratios

$^{75}As^-:^{12}C^-$

B   C

$^{31}P^-:^{12}C^-$

D   E

SE

F   G

A

FT magnitude

As-S

As-Fe

GFAJ (data)
Fit

$\sim$Å (r + θr)

B

As

P

Fe

Zn

10µm