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Absence of Detectable Arsenate in DNA from Arsenate-Grown GFAJ-1 Cells

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A strain of *Halomonas* bacteria, GFAJ-1, has been claimed to be able to use arsenate as a nutrient when phosphate is limiting and to specifically incorporate arsenate into its DNA in place of phosphorus. However, we have found that arsenate does not contribute to growth of GFAJ-1 when phosphate is limiting and that DNA purified from cells grown with limiting phosphate and abundant arsenate does not exhibit the spontaneous hydrolysis expected of arsenate ester bonds. Furthermore, mass spectrometry showed that this DNA contains only trace amounts of free arsenate and no detectable covalently bound arsenate.

Wolfe-Simon *et al.* isolated strain GFAJ-1 from the arsenic-rich sediments of California’s Mono Lake by its ability to grow through multiple subculturings in artificial Mono Lake medium AML60 that lacked added phosphate but had high concentrations of arsenate (+As/-P condition) (1). Because GFAJ-1 grew in -P medium only when arsenate was provided, and because substantial amounts of arsenate were detected in subcellular fractions, growth was attributed to the use of arsenate in place of phosphate. However, the basal level of phosphate contaminating the -P medium was reported to be 3 to 4 μM (1), which previous studies of low-phosphate microbial communities suggest is sufficient to support moderate growth (2). GFAJ-1 grew well on medium supplemented with ample phosphate but no arsenate (1500 μM PO₄, +P/-As condition), indicating that GFAJ-1 is not obligately arsenate-dependent.

Wolfe-Simon *et al.* (1) further inferred that arsenic was incorporated into the DNA backbone of GFAJ-1 in place of phosphorus, with an estimated 4% replacement of P by As based on the As/P ratio measured in agarose gel slices containing DNA samples. This finding was surprising because arsenate is predicted to reduce rapidly to arsenite in physiological conditions (3, 4) and because arsenite esters in aqueous solution are known to be rapidly hydrolyzed (5). We have now tested this report by culturing GFAJ-1 cells supplied by the authors (1) and by analyzing highly purified DNA from phosphate-limited cells grown with and without arsenate.

Wolfe-Simon *et al.* reported that GFAJ-1 cells grew very slowly in AML60 medium (doubling time ~12 hours) and that, when phosphate was not added to the medium, cells failed to grow unless arsenate (40 mM) was provided (1). However, although we obtained strain GFAJ-1 from these authors, in our hands GFAJ-1 was unable to grow at all in AML60 medium containing the specified trace elements and vitamins, even with 1500 μM sodium phosphate added as specified in (1). We confirmed the strain’s identity using reverse transcription–polymerase chain reaction and sequencing of 16S ribosomal RNA, with primers specified by Wolfe-Simon *et al.* (1); this gave a sequence identical to that reported for strain GFAJ-1. We then found that addition of small amounts of yeast extract, tryptone, or individual amino acids to basal AML60 medium allowed growth, with doubling times of 90 to 180 min. Medium with 1 mM glutamate added was therefore used for subsequent experiments (6).

With 1500 μM phosphate but no added arsenate (Wolfe-Simon *et al.*’s -As/+P condition), this medium produced ~2 × 10⁵ cells/ml, similar to the -As/+P yield obtained by Wolfe-Simon *et al.* (1). As expected, the growth yield depended on the level of phosphate supplementation (Fig. 1), with even unsupplemented medium allowing growth to ~2 × 10⁴ cells/ml. Because analysis by inductively coupled plasma–mass spectrometry (ICP-MS) showed that this medium contained only 0.5 μM contaminating phosphate, our supplementation with additional 3.0 μM phosphate replicates Wolfe-Simon *et al.*’s “-P” culture condition. The growth analyses shown in Fig. 1 were performed in the absence of arsenate and showed
that GFAJ-1 does not require arsenate for growth in media with any level of phosphate.

The cause of the discrepancies between our growth results and those of Wolfe-Simon et al. is not clear. The arsenate dependence they observed may reflect the presence in their arsenate (purity and supplier unknown) of a contaminant that filled the same metabolic role as our glutamate supplement. Our +As and −As cultures grew to similar densities, and we did not observe any cases in which +As cultures grew but −As cultures did not. The phosphate dependence we observed is also consistent with that expected from work on other species (2).

To investigate the possible incorporation of arsenate into the GFAJ-1 DNA backbone, we purified and analyzed DNA from GFAJ-1 cells grown in four differentially supplemented versions of AML60 medium, matching those analyzed by Wolfe-Simon et al.—i.e., −As/−P: no arsenate, 3.5 μM phosphate; +As/−P: 40 mM arsenate, 3.5 μM phosphate; −As/+P: no arsenate, 1500 μM phosphate; +As/+P: 40 mM arsenate, 1500 μM phosphate. Initial purification of DNA consisted of two preliminary organic extractions, precipitation from 70% ethanol, digestion with ribonuclease and proteinase, two additional organic extractions, and a final ethanol precipitation (6). DNA was collected from 70% ethanol by spooling rather than centrifugation, because this reduces contamination with other substances insoluble in ethanol (7).

Wolfe-Simon et al. suggested that arsenate ester bonds in GFAJ-1 DNA might be protected from hydrolysis by intracellular proteins or compartmentalization of the DNA (8). We therefore tested whether purification exposed GFAJ-1 DNA to spontaneous hydrolysis. Gel analysis of DNA immediately after purification revealed fragments of >30 kb, whether cells were grown with limiting or abundant phosphate and with or without 40 mM arsenate (Fig. 2A). We also reexamined this DNA after 2 months of storage at 4°C. All preparations showed very similar-sized fragments of double-stranded DNA and of single-stranded DNA (Fig. 2, B and C), with no evidence of hydrolysis. Haemophilus influenzae DNA served as a control for gel migration, indicating that GFAJ-1 DNA is not associated with hydrolysis-protecting proteins or other macromolecules that might have persisted through the purification. Unless arsenate-ester bonds are intrinsically stable in DNA, our analysis estimates a minimum separation between arsenates in the DNA backbone of at least 25 kb, three orders of magnitude below that estimated by Wolfe-Simon et al.

Arsenate bonds that were stable to spontaneous hydrolysis should be detectable as free arsenate, arsenate-containing mononucleotides, or arsenate-containing dinucleotides after enzymatic digestion of purified DNA. We therefore used liquid chromatography–mass spectrometry (LC-MS) to analyze GFAJ-1 DNA for arsenate after digestion with P1 and snake venom nucleases (6). Relevant molecular species were identified by negative-mode, full-scan, high-mass resolution LC-MS analysis (6). This method was used to analyze two independent replicate DNA preparations from cells grown in either +As/−P or −As/+P medium and fractions from CsCl gradient analyses of these DNAs.

The initial DNA preparations of +As/−P DNAs contained some free arsenate anion (H₂AsO₄⁻) (Table 1), at levels similar to those reported by Wolfe-Simon et al. (1). This arsenate was largely removed by three serial washes with distilled water; digested washed DNA contained arsenate at a level slightly higher than in the water blank (Fig. 3 and Table 1). Thus, we concluded that most of the arsenate we detected after preliminary DNA purification arose by contamination from the arsenate-rich (40 mM) growth medium.

Further analyses compared the nucleases-digested and washed fractions obtained from

### Table 1. DNA, arsenate, and nucleotide content of samples measured by absorbance at 260 nm and LC-MS. AU, absorbance units.

<table>
<thead>
<tr>
<th>Sample</th>
<th>A₂₆₀(DNA)</th>
<th>Compound</th>
<th>Arsenate</th>
<th>dAMP</th>
<th>dAMA</th>
<th>dAMP-dAMP</th>
<th>dAMA-dAMP</th>
<th>Peak area, ion counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 (top)</td>
<td>0.03 (0)</td>
<td>0</td>
<td>0</td>
<td>154</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>#2</td>
<td>0.01 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>#3</td>
<td>0.02 (0)</td>
<td>226</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>#4</td>
<td>0.01 (0)</td>
<td>0</td>
<td>160</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>#5</td>
<td>0.01 (0)</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>#6</td>
<td>0.82 (4.5)</td>
<td>157</td>
<td>39,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>#7</td>
<td>1.12 (6.7)</td>
<td>373</td>
<td>52,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>#8 (bottom)</td>
<td>0.44 (1.9)</td>
<td>300</td>
<td>3,700</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>197</td>
<td></td>
</tr>
<tr>
<td>Water blank</td>
<td>0 (0)</td>
<td>329</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>−As/+P partial digest</td>
<td>(3.3)</td>
<td>515</td>
<td>0</td>
<td>0</td>
<td>21,000</td>
<td>210</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+As−P washed, digested DNA</td>
<td>(1.7)</td>
<td>2625</td>
<td>186,457</td>
<td>0</td>
<td>241</td>
<td>202</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+As−P whole DNA (1:10 dil.)</td>
<td>(1.7)</td>
<td>2794</td>
<td>562</td>
<td>0</td>
<td>781</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>+As−P wash of gDNA (300 μl)</td>
<td>0 (0)</td>
<td>9545</td>
<td>182</td>
<td>207</td>
<td>0</td>
<td>221</td>
<td></td>
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<tr>
<td>Arsenate standards (molar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1.66 × 10⁻³</td>
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<td></td>
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<td></td>
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<tr>
<td>1.66 × 10⁻²</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>1.66 × 10⁻¹</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1.66 × 10⁻⁰</td>
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<td></td>
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<tr>
<td>Expected if DNA As:P = 0.04</td>
<td>(6.7)</td>
<td>~122,000</td>
<td>&gt;0</td>
<td>&gt;0</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
CsCl isopycnic density gradient centrifugation of the DNAs (Fig. 3) (6). The arsenate detection limit for these measurements was \(5 \times 10^{-8} \text{ M}\) (table S1), a level that if present in the fractions with the most DNA would correspond to an As:P ratio of \(<0.1\%\), 50-fold lower than the 4\% ratio estimated by Wolfe-Simon et al. Although traces of arsenate (or a contaminant of mass similar to that of arsenate) were found in several fractions of the CsCl gradient, the arsenate peak did not exceed the limit of detection, and a similar-intensity signal at a mass-to-charge ratio \((m/z)\) of arsenate was observed in the water blank. There was no evidence that the arsenate trace comigrated with the DNA. In contrast, normal phosphate-containing deoxynucleotides were observed in rough proportion to the abundance of DNA throughout the gradient for both the +As/-P and –As/+P cells (Fig. 4A and table S2).

Likewise, no arsenate-conjugated mono- or dinucleotides were detected by exact mass (Fig. 4, B and D). Although retention time and ionization efficiency could not be validated with standards for these molecules, their behavior, if the molecules were stable, would be expected to resemble that of their phosphorylated analogs sufficiently to allow detection. Finally, an enrichment of deoxynucleosides per nanogram of DNA obtained from GFAJ-1 grown in the +As–P condition, relative to either –As/+P or –As–P conditions, could indicate nicked DNA resulting from arsenate-ester hydrolysis. However, we did not detect any enrichment despite detecting deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine (fig. S1 and table S2). Thus, although we detected arsenate associated with GFAJ-1 DNA, we found no evidence for arsenate bound sufficiently tightly to resist washing with water or able to comigrate with the DNA in a CsCl gradient. Differences in DNA purity can readily explain the conflict of these results with Wolfe-Simon et al.’s claim that GFAJ-1 uses arsenate to replace scarce phosphate in its DNA.

Our LC-MS analyses rule out incorporation of arsenic in DNA at the \(<0.1\%\) level, and a much lower limit is suggested by our gel analysis of DNA integrity. Given the chemical similarity of arsenate to phosphate, it is likely that GFAJ-1 may sometimes assimilate arsenate into some small molecules in place of phosphate, such as sugar phosphates or nucleotides. Although the ability to tolerate or correct very-low-level incorporation of arsenic into DNA could contribute to the arsenate resistance of GFAJ-1, such low-level incorporation would not be a biologically functional substitute for phosphate, and thus would have no appreciable effect on the organism’s requirements for phosphate.

From a broader perspective, GFAJ-1 cells growing in Mono Lake face the challenge of discriminating an essential salt (PO\(_4^\text{3-}\), 400 \text{μM}\) from a highly abundant but toxic chemical mimic (AsO\(_3^\text{3-}\), 200 \text{μM}). Similar salt management challenges are encountered by many other microorganisms, such as those growing in environments...
with scarce potassium and plentiful ammonia (9). Organisms typically adapt to such conditions not by incorporating the mimic in place of the essential salt but by enriching for the salt at multiple stages, from preferential membrane transport to the selectivity of metabolic enzymes. The end result is that the fundamental biopolymers conserved across all forms of life remain, in terms of chemical backbone, invariant (10–12).

References and Notes

6. Materials and methods are available as supplementary materials on Science Online.

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Methods and Materials
Fig. S1 References
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The Structure and Catalytic Cycle of a Sodium-Pumping Pyrophosphatase

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Membrane-integral pyrophosphatases (M-PPases) are crucial for the survival of plants, bacteria, and protozoan parasites. They couple pyrophosphate hydrolysis or synthesis to Na+ or H+ pumping. The 2.6-angstrom structure of Thermotoga maritima M-PPase in the resting state reveal a previously unknown solution for ion pumping. The hydrolytic center, 20 angstroms above the membrane, is coupled to the gate formed by the conserved Asp243, Glu244, and Lys70 by an unusual “coupling funnel” of six α-helices. Comparison with our 4.0-angstrom resolution structure of the product complex suggests that helix 12 slides down upon substrate binding to open the gate by a simple binding-change mechanism. Below the gate, four helices form the exit channel. Superimposing helices 3 to 6, 9 to 12, and 13 to 16 suggests that M-PPases arose through gene triplication.

Found in plants, protozoans, bacteria, and archaea, membrane-integral pyrophosphatases (M-PPases) contain 14 to 17 transmembrane (TM) helices (1) and link pyrophosphate (PPi) hydrolysis or synthesis to sodium or proton pumping (2). PPases are essential to drive anaerobic reactions such as DNA synthesis to completion. In contrast to the soluble PPases, M-PPases recycle part of the free energy of PPi hydrolysis to generate electrochemical potential across biological membranes. In plants, they are vital for maturation and enhance survival under abiotic stress conditions (drought, anoxia, cold) (3). They also are important for proliferation of disease causing protozoa (4).

Unlike the rotary F-type adenosine triphosphatases (F-ATPases), M-PPases are dimeric (5), and, unlike the P-type ATPases, there is no phosphorylated enzyme intermediate (6). M-PPases can be divided into three functional classes: K2−-independent proton pumps and K2−-dependent sodium and proton pumps, both of which require potassium for maximal activity (2). The resting enzyme state is EMg2+, and two more metal ions bind with substrate, Mg2+PPi (7).

We have solved the structure of the Na+-pumping M-PPase of Thermotoga maritima (TmPPase) in the resting state (TmPPase:Ca2+:Mg2+ at 2.6 Å resolution (Rwork/Rfree = 20.5/24.5%) and with product bound (TmPPase:P2−:Mg2+) at 4.0 Å (Rwork/Rfree = 29.5/36.5%) (tables S1 and S2, Fig. 1, and fig. S1). In addition, the tungstate derivative used to solve the structure corresponds to the TmPPase:Mg2+:P2−:state (8), thus mapping out the catalytic cycle. TmPPase is a dimer of two very similar (rmsd/Cα 0.58 Å) monomers, each with 16 TM helices (Fig. 1). The helices extend up to 25 Å from the membrane bilayer on the cytoplasmic side but end near the bilayer on the periplasmic side (Fig. 1 and fig. S2). The dimer interface comprises helices 10, 13, and 15 (fig. S2) and a short antiparallel β sheet in the sixth periplasmic loop between TM12 and 13. Although the protein is an oblong dimer, residue conservation (fig. S3) shows that the pump is located entirely within a single monomer. It had been suggested that TmPPase was structurally similar to the F-ATPases (9), but a DALI search (10) shows that the only homologous structure is the recently published mung bean H+-pumping K+-dependent PPase (VrPPase) (11).

The unusual active site has four distinct regions: the hydrolytic center, some 20 Å above the membrane surface; a “coupling funnel”; the gate (closed in our structure) just below the membrane surface; and an exit channel for Na+ ions (Fig. 2A). The distance from the hydrolytic center to the gate is thus about 20 Å. Six helices, 5-6, 11-12, and 15-16 form the hydrolytic center and coupling funnel, whereas only helices 5, 6, 12, and 16 form the gate and channel, creating an internal “symmetry mismatch” (12) between the two regions. Conserved charged residues, many of which have been extensively mutated in M-PPases (fig. S4 and table S3) line the hydrolytic center, coupling funnel, and gate. Their positioning confirms that M-PPases arose by gene triplication (13). Helices 3 to 6, 9 to 12, and 13 to 16 of TmPPase, which carry all of the functional residues (Fig. 2), share a similar structural motif (Fig. 1) not found in other proteins except the VrPPase. The structural alignments align key conserved residues (fig. S2C) that cluster in the active site.

Our native 2.6 Å structure has one Ca2+ and one Mg2+ ion bound. The Ca2+ ion is coordinated by the conserved Asp688, Asp992, and Asp660 which are positioned by Lys662, Lys664, and Lys895 (Fig. 2C and fig. S1), whereas the Mg2+ ion is coordinated by Asp852 and Asp865 (fig. S1). The VrPPase structure, crystallized in the presence of Mg2+ and the competitive inhibitor imidodiphosphate (PNP) (11), contains two ions with about the same protein coordination (VrPPase M3 and M2) that are coordinated to the PNP inhibitor. We thus assign our Ca2+ as M1 (their M3) and our Mg2+ as M2 (their M2) because these are the two metal ion bounds in the resting enzyme and because the Ca2+ is multivalently coordinated in both structures (11), unlike M2, which only binds Asp852(SO4)(7) in VrPPase (VrPPase numbers in parentheses). In addition, G453, which inhibits TmPPase, binds at these two sites (fig. S5). The