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GFAJ-1 Is an Arsenate-Resistant, Phosphate-Dependent Organism

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The bacterial isolate GFAJ-1 has recently been proposed to substitute arsenic for phosphorus to sustain growth. We have shown that GFAJ-1 is able to grow at low phosphate concentrations (1.7 µM), even in the presence of high concentrations of arsenate (40 mM), but lacked the ability to grow in phosphorus-depleted (<0.3 µM), arsenate-containing medium. High resolution mass spectrometry analyses revealed that phosphorylated central metabolites and phosphorylated nucleic acids predominated. A few arsenylated compounds, including C6 sugar arsenates, were detected in extracts of GFAJ-1, when GFAJ-1 was incubated with AsO_4^{3-}, but further experiments showed they formed abiotically. Inductively coupled plasma mass spectrometry confirmed the presence of phosphorus and the absence of arsenic in nucleic acid extracts. Taken together, we conclude that GFAJ-1 is an arsenate-resistant, but still a phosphate-dependent bacterium.

The discovery of GFAJ-1, a Gammaproteobacterium that was claimed to use arsenic instead of phosphorus has challenged the universal role of phosphorus in biology (*1*), although the arguments used in the study were doubted (*2–9*). Here, we combine classical physiological experiments with high-resolution mass spectrometry, as well as inductively coupled plasma optical emission and -mass spectrometry (ICP-OES / ICP-MS) to provide evidence that GFAJ-1 is highly resistant to arsenate, yet still requires phosphate for growth.

To understand the physiological properties of GFAJ-1, we studied the organism's phosphate-dependency in greater detail. Using reagents of the highest purity available for medium preparation we reduced the phosphorus-background in the minimal medium below the detection limit (<0.3 µM phosphorus), which is an order of magnitude less than detected by Wolfe-Simon *et al*., i.e., 2.7-3.2 µM phosphorus impurity (*1*). In the phosphorus-depleted medium (<0.3 µM) we made, no growth of GFAJ-1 was observed; however, the amount of growth of GFAJ-1 correlated with the amount of external phosphorus added in form of phosphate, up to a concentration of 20 µM beyond which other nutrients might become limiting (*10*) (Fig. 1, A and B). Notably, 1.7 µM phosphate was sufficient to sustain growth of GFAJ-1 similar to that remaining as impurity in the arsenate-grown culture media published by Wolfe-Simon *et al*. (*1*). From this observation we conclude that cultures in the previous study might have grown on trace amounts of phosphate rather than on the arsenate provided. Indeed, when GFAJ-1 was tested for growth, by optical density measurements and direct cell counts, on arsenate (40 mM) using phosphorus-depleted medium (<0.3 µM), no growth was observed unless trace amounts of phosphate were added (Fig. 1C). Inductively coupled plasma mass spectrometry (ICP-MS) was used to follow the fate of trace phosphate during growth of GFAJ-1 in arsenate-containing medium. The concentration of phosphorus in the medium supernatant decreased with time and became enriched in the cellular fraction, in parallel with the growth of GFAJ-1, as shown by optical density and cell-number counting (Fig. 1D). The accumulation of phosphorus in the cellular fraction during growth of GFAJ-1 in these experiments provided additional evidence that GFAJ-1 is a phosphate-dependent organism, even when cultivated in the presence of high arsenate concentrations.

Next we addressed the question of whether arsenate enters intermediates of the central carbon metabolism of GFAJ-1 and how this might affect the organism's metabolome. For that purpose, cells of GFAJ-1 were grown on the minimal medium described by Wolfe-Simon *et al*. with 10 mM glucose (*1*) in the absence or presence of 40 mM arsenate and trace amounts of phosphate. Cells were collected in mid-log phase through fast filtration and washed rapidly at 24°C on the filter to remove inorganic salts of the medium, before metabolites were extracted and analyzed by a high resolution mass spectrometry platform that allows detection of compounds in the femto/attomole range (*11*). Metabolite peaks were searched against a modified EcoCyc-metabolite database (*12*) that has been systematically extended by all the arsenylated and arsinylated metabolites theoretically possible (e.g., glucose-arsenate, glucose-arsenite), as well as permutations of mixed phosphorylated, arsinylated or arsenylated species to detect modified bis- or tris-phosphate metabolites (e.g., different ADP-, or ATP-species). Features that matched a database entry with an accuracy $<1 \times 10^{-3}$ atomic mass units were assigned as potential metabolites, and their retention time was compared to the known retention times of corresponding phospho-metabolite standards for further characterization. With the caveat that the metabolome of GFAJ-1 might have been perturbed to some extent by the short washing step, we found that when GFAJ-1 was grown in the presence of arsenate, most core metabolites (nucleotides, sugar phosphates, etc.) were only detected in their phosphorylated, but not arsenylated form (Table 1 and tables S1 to S4). Moreover, the absolute concentrations of most phospho-metabolites did not differ between GFAJ-1 cells grown in the presence or absence of arsenate (Table 1). Notably, the levels of nucleotide-trisphosphates (ATP, CTP, GTP, UTP), as a measure for cellular energy status, were similar between both growth conditions, although some nucleotide-bisphosphates (ADP, CDP, GDP), but not their corresponding monophosphates, appeared elevated in GFAJ-1 cells grown in the presence of arsenate. The elevated nucleotide-bisphosphate levels might result from a higher energy demand of GFAJ-1 cells when grown in the presence of arsenate (e.g., due to ATP-dependent detoxification mechanisms, such as active export of arsenate), or might point to the formation of transient, instable nucleotide-bisphosphate-arsenate species, as proposed before (*13*). Nevertheless, our results indicated that the core-metabolism of GFAJ-1 is based on phospho-metabolites, independent of its growth condition without major perturbation of most core metabolite levels (except above discussed nucleotide-bisphosphates) as a consequence of arsenate addition to the medium, which strongly argues against the use of arsenate to replace phosphate in GFAJ-1.

Yet, we observed hexose-arsenate in metabolome extracts of arsenate-grown cells of GFAJ-1 that co-eluted with the glucose-phosphate standard, as well as a potentially bisarsenylated hexose species (Table 1). However, the retention time of the latter deviated from that of fruc-

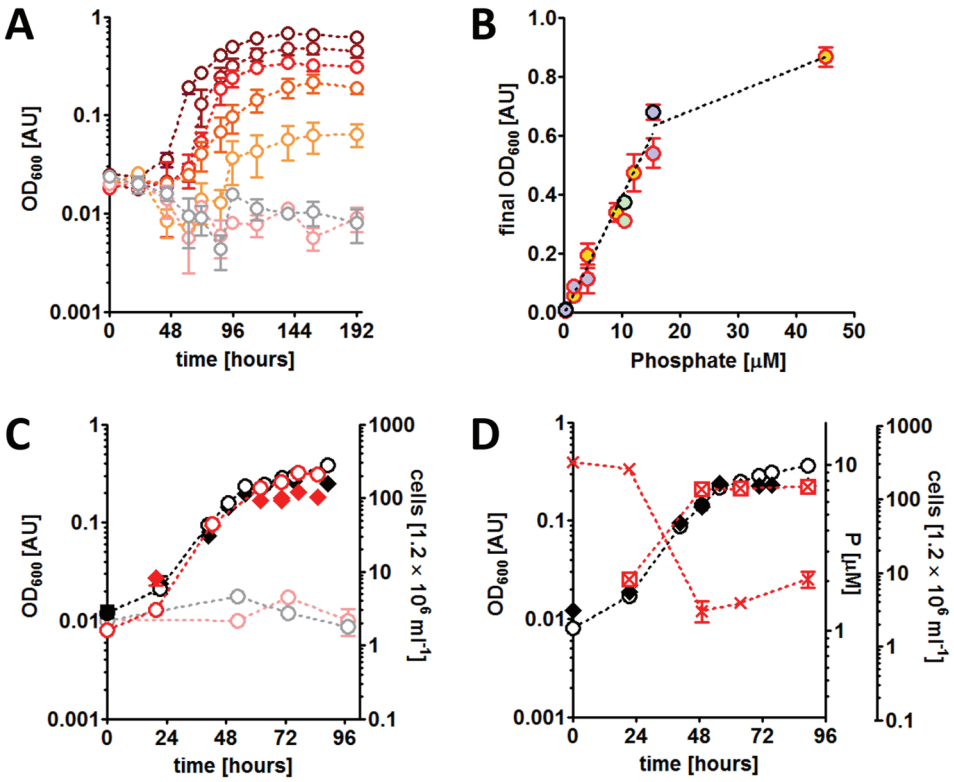

Fig. 1. Growth behavior of GFAJ-1 on minimal medium containing 10 mM glucose amended with different concentrations of phosphate and arsenate. Cell growth was followed by increase in optical density at 600 nm (OD_{600}, open circles), or direct cell counting (closed diamonds). (**A**) Phosphate-dependent growth of GFAJ-1, as determined by OD_{600}. Shown are triplicate growth curves of GFAJ-1 in the presence of 15.4 μM phosphate, as determined by elemental analysis (brown open circles); 12 μM phosphate, mixed from 15.4 μM phosphate medium and <0.3 μM phosphate medium (dark red open circles); 9 μM phosphate, mixed from 15.4 μM phosphate medium and <0.3 μM phosphate medium (red open circles); 4 μM phosphate, as determined by elemental analysis (light red open circles); 1.7 μM phosphate, as determined by elemental analysis (orange open circles); below 0.3 μM phosphate, as determined by elemental analysis (pale red-violet open circles); 40 mM arsenate, below 0.3 μM phosphate, as determined by elemental analysis (gray open circle). (**B**) Correlation of stationary phase OD_{600} and phosphate concentration in the medium. Shown are the results from three independently prepared batches of growth media and precultures. Red circles indicate the absence of arsenate in the growth medium. Black circles indicate the presence of 40 mM arsenate in the growth medium. Medium batch 1 (yellow filled circles); medium batch 2 (light blue filled circles); medium batch 3 (light green filled circles). (**C**) Phosphate-dependent growth of GFAJ-1 in the presence and absence of arsenate, as determined by OD_{600} and direct cell counting. Open circles indicate OD_{600} measurements, diamonds indicate direct cell counts. Shown are representative growth curves of GFAJ-1 cultures in the presence of 40 mM arsenate, 10 μM phosphate (black open circles, black closed diamonds); 10 μM phosphate, no arsenate (red open circles, red closed diamonds); 40 mM arsenate, phosphate below 0.3 μM (pale red-violet open circles); below 0.3 μM phosphate, no arsenate (gray open circles). (**D**) Dynamics of phosphorus during growth of GFAJ-1 in the presence of arsenate and limited amounts of phosphate. Shown are OD_{600} measurements (black open circles) and direct cell counts (black closed diamonds) of a GFAJ-1 culture in the presence of 40 mM arsenate and 10.4 μM phosphate, as well as the distribution of elemental phosphorus in the supernatant (red crosses), or the cellular fraction (boxed red crosses). Phosphorus concentrations were determined by ICP-MS.

Table 1. Selected core metabolites of GFAJ-1 cells actively growing on limiting amounts of phosphate in the absence or presence of 40 mM arsenate, and metabolic response upon incubation with 40 mM arsenate.

Metabolites (fmol/µl extract)	Cells grown with 9 µM P		Cells grown with 9 µM P 40 mM As		Medium treated filter		Cells grown with 10 µM P 6 s As incubat.		Cells grown with 10 µM P 360 s As incubat.	
Nucleotide-monophosphates										
AMP / dGMP	4.5	±1.2	5.2	±0.6	<0.3#		2.9	±<0.1	3.9	±0.8
CMP	1.3	±0.1	1.4	±0.3	<0.3#		1.0	±0.1	1.1	±0.2
UMP	0.7	±0.3	0.5	±0.1	<0.3#		0.2	±0.1	0.2	±0.2
Nucleotide-bisphosphates										
ADP	58	±16	141	±27	<0.8#		33	±5	58	±31
CDP	15	±1	27	±5	<0.5#		13	±2	16	±3
GDP	14	±2.4	31	±0.9	<0.4#		11	±3	14	±4
UDP	2.1	±0.2	4.5	±1.1	<0.2#		1.7	±0.2	2.2	±0.3
Nucleotide-trisphosphates										
ATP	145	±13	153	±27	<1.2#		166	±15	128	±29
CTP	21	±3	31	±4	<0.2#		25	±2	21	±2
GTP	22	±2	30	±4	<0.7#		25	±2	20	±4
UTP	18	±1	23	±7	<0.3#		18	±1	15	±2
Deoxynucleotide-phosphates										
dADP	1.2	±0.1	3.7	±1.2	<0.2#		0.7	±0.1	1.3	±0.8
dTDP	2.4	±0.2	6.1	±2.1	<1.1#		2.2	±0.5	4.0	±0.6
dTTP	32	±3	30	±10	<1.7#		28	±5	19	±3
Sugar-phosphates										
Hexose-phosphate	13	±3	13	±3	<0.9#		7.6	±0.1	10	±4
Hexose-bisphosphate	18	±3	18	±4	<0.9#		21	±3	29	±6
Phospho-gluconate	3.6	±0.1	2.9	±1.6	<0.2#		2.7	±0.2	4.0	±1.0
Nucleotide-sugar-phosphates										
UDP-hexose	42	±2	55	±11	<1.3#		36	±3	28	±2
UDP-*N*-acetyl-hexosamine	29	±2	37	±4	<1.1#		24	±2	22	±2
Other phospho-metabolites										
Methylerythritol-*cyclo*-P-P	5.2	±3.0	7.2	±0.7	<1.6#		5.7	±0.3	17	±3
Arsenylated analogs (rel. levels)										
Hexose-arsenate	<0.1		159	±18	215	±120	155	±81	74	±75
Hexose-bisarsenate	<0.1		4	±5	4	±2	18	±8	9	±9

#Below detection limit.

tose-1,6-bisphosphate, which makes it questionable that it represented an arsenate analog of the glycolytic intermediate. The origin of arsenylated hexoses in metabolome extracts of arsenate grown GFAJ-1 cells was subsequently addressed in more detail. Notably, hexose-arsenate as well as bisarsenylated hexose were also detected in control extracts of filters that had been mock-treated with arsenate amended, glucose containing, growth medium, indicating abiotic formation of these arsenylated hexoses (Table 1). This finding is in line with thermodynamic considerations and previous reports indicating spontaneous formation of glucose-arsenate from arsenate and glucose in solutions of alkaline pH (*14*). Consequently, the bisarsenylated hexose species we detected is likely to be abiotically formed glucose-bisarsenate, or an unspecific glucose-arsenate adduct rather than an arsenate analog of fructose-1,6-bisphosphate.

Although our experiments indicated that hexose-arsenate is preformed abiotically in the growth medium, we could not exclude the possibility that some might be produced biotically, e.g., through transient ADP-arsenate as suggested before (*13*). Hence, to reduce the background levels of abiotically formed hexose-arsenates in metabolome preparations, a more stringent washing step with water was included during cell collection. The modified protocol changed the metabolite pool sizes, particularly of hexose-phosphate, which decreased about tenfold (table S5). However, when GFAJ-1 cells, grown in the presence of arsenate, were extracted with this stringent protocol, we were not able to detect any arsenylated hexoses (table S5). Similarly, when 10 mM glucose was added to the washing solution to minimize depletion effects, we did not observe the formation of hexose-arsenates beyond small abiotic background levels in extracts of GFAJ-1 cells grown in the presence of arsenate (table S5). Although we did not find evidence for the biotic formation of hexose-arsenates, GFAJ-1 cells remained metabolically active in these experiments, as demonstrated by addition of 10 mM ^{13}C-labeled glucose to the wash solution: about 80% of the hexose-phosphate pool and 60% of the fructose-1,6-bisphospate pool were exchanged with the ^{13}C-label, even during stringent washing, indicating active glycolysis (table S6). These results suggest that the biotic formation of glucose-arsenate is most likely negligible in GFAJ-1.

Table 2. Elemental analysis of nucleic acid preparations from GFAJ-1 grown on 10 µM phosphate in the absence or presence of 40 mM arsenate.

Sample	GFAJ-1 grown with 9 µM P	GFAJ-1 grown with 9 µM P 40 mM As
Nucleic acids (ng)	15,700 ±1,800	17,400 ±1,900
Phosphorus in nucleic acids (ng)	934 ±13	1,043 ±8
Arsenic in nucleic acids (ng)	<1[#]	<1[#]
As/P molar ratio	<0.001	<0.001

[#]Below detection limit.

To probe the metabolic plasticity of GFAJ-1, we analyzed its metabolome in response to perturbation with arsenate in the absence of phosphate, and without pre-adaption of the organism to high-arsenate concentrations. Cultures of phosphate pre-grown GFAJ-1 cells were exchanged with medium that contained 40 mM arsenate, but no (i.e., <0.3 µM) phosphate, and incubated for 6 and 360 s, respectively. To avoid depletion of glycolysis metabolite pool sizes that occurred during stringent washing (see above), the standard washing protocol was applied, resulting in the detection of abiotically formed arsenylated hexoses, as expected (Table 1). However, all other central metabolites were only detected in their phosphorylated form and their pool sizes remained virtually unchanged upon arsenate-incubation (Table 1). Most notably, neither the levels of trisphosphate nucleotides (e.g., ATP), nor the levels of glycolysis-derived phosphorylated metabolites (e.g., glucose-phosphate, fructose-1,6-bisphosphate) indicating energy status as well as metabolic activity of the cell changed (*15*). This suggested that, independent of its pre-adaption to arsenate, the pool sizes of core metabolites in GFAJ-1 are not affected by perturbation through arsenate or hexose-arsenate. At the same time, our experiments also did not indicate that GFAJ-1 possess a detectable downstream metabolism of these abiotically formed arseno-hexoses.

When our search for arseno-metabolites was extended beyond core metabolism, a total of six potentially arsenylated compounds were detected in extracts of GFAJ-1, although most of them only at low abundance, and not in every experiment or replicate (table S7). Note that two of the corresponding phosphate analogs are also not known to be of biotic origin, according to the EcoCyc-metabolite database (*12*). In order to understand whether formation of these compounds was a feature specific to GFAJ-1 or might be a more general phenomenon, we examined the metabolome of *Escherichia coli* in response to arsenate perturbation. In contrast to GFAJ-1, *E. coli* is able to cope only with low arsenate concentrations, when phosphate limited (*16*, *17*), and has not been reported to incorporate arsenate into its biomass. However, similarly to GFAJ-1, some putatively arsenylated compounds were detected at low abundance in metabolome extracts of arsenate-incubated *E. coli* (table S7), indicating that the presence of such low abundance, putatively arsenylated compounds is not specific to GFAJ-1 and thus might not be of physiological relevance. Since these compounds are apparently unlinked to central carbon metabolism, they might also have formed abiotically.

The apparent absence of arsenylated nucleotides and desoxynucleotides in GFAJ-1 under all conditions tested raises the question of how and whether arsenate would find its way into larger biomolecules (e.g., RNA, DNA). To answer this question, total nucleic acids were extracted from GFAJ-1 cells grown in the presence of 10 µM phosphate with or without 40 mM arsenate (fig. S1) and analyzed for their arsenic and phosphorus content, respectively, by ICP-MS. In line with the metabolome results in general and the lack of evidence for (alpha-)arsenylated nucleotides in particular, we did not find evidence for significant incorporation of arsenic into nucleic acids. The amount of arsenic in nucleic acid preparations was found to be below detection limit (Table 2), resulting in an arsenic to phosphorus ratio of less than 1 ‰, confirming that metabolism of GFAJ-1 is essentially based on phosphorylated compounds.

In conclusion, our experiments indicate that GFAJ-1 requires phosphorus for growth. We did not find evidence that arsenate can replace phosphate; however, in line with previous results, we confirm that GFAJ-1 is able to grow in the presence of very high arsenate and limiting phosphate concentrations (arsenate:phosphate ≈1:10,000), in contrast to other arsenate resistant strains that require much higher phosphate concentrations (typically arsenate:phosphate ≈1:10-1:100, *Aspergillus* sp. P37 ≈1:1,000) (*16*–*18*). Phosphorus incorporation studies and metabolome analysis indicated that the core metabolism of GFAJ-1 is based on phosphorylated metabolites, even when cells are grown at high concentrations of arsenate (40 mM) and low concentrations of phosphate (below 10 µM). Feeding of arsenate to GFAJ-1 leads to the abiotic formation of some arsenylated compounds (hexose-arsenates); yet, most of the phosphorylated metabolite pools do not change upon arsenate feeding. We note that the abiotic formation of hexose-arsenates observed by us might explain the results of Wolfe-Simon *et al*. in which the intracellular accumulation of arsenate organo-esters by GFAJ-1 was indicated by secondary ion mass spectrometry, and x-ray analysis (*1*). However, the fact that these abiotically formed arseno-compounds apparently were not metabolized by GFAJ-1 and that virtually no arsenic was detected in nucleic acid preparations of GFAJ-1 is in line with our conclusions that GFAJ-1 is an arsenate-resistant, yet phosphate-dependent organism. The molecular basis for arsenate resistance in GFAJ-1 might be the subject of further investigations, in particular given the finding that the concentrations of arsenic and phosphorous were found within the same order of magnitude in the cellular fraction of growing GFAJ-1 cultures, despite the ratio of phosphate and arsenate in the medium differing by four orders of magnitude (fig. S3).

References and Notes

1. F. Wolfe-Simon *et al*., A bacterium that can grow by using arsenic instead of phosphorus. *Science* **332**, 1163 (2011). doi:10.1126/science.1197258 Medline
2. S. A. Benner, Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-c (2011). doi:10.1126/science.1201304 Medline
3. D. W. Borhani, Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-e (2011). doi:10.1126/science.1201255 Medline
4. J. B. Cotner, E. K. Hall, Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-f (2011). doi:10.1126/science.1201943 Medline
5. I. Csabai, E. Szathmáry, Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-b (2011). doi:10.1126/science.1201399 Medline
6. P. L. Foster, Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-i (2011). doi:10.1126/science.1201551 Medline
7. S. Oehler, Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-g (2011). doi:10.1126/science.1201381 Medline
8. R. J. Redfield, Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-h (2011). doi:10.1126/science.1201482 Medline
9. B. Schoepp-Cothenet *et al*., Comment on "A bacterium that can grow by using arsenic instead of phosphorus". *Science* **332**, 1149-d (2011).

doi:10.1126/science.1201438 Medline
10. J. Liebig, in Die Grundsätze der Agrikultur-Chemie mit Rücksicht auf die in England angestellten Untersuchungen. (Friedrich Vieweg und Sohn Publ Co., Braunschweig, Germany, 1855).
11. P. Kiefer, N. Delmotte, J. A. Vorholt, Nanoscale ion-pair reversed-phase HPLC-MS for sensitive metabolome analysis. *Anal. Chem.* **83**, 850 (2011). doi:10.1021/ac102445r Medline
12. I. M. Keseler *et al*., EcoCyc: a comprehensive database of Escherichia coli biology. *Nucleic Acids Res.* **39**, (Database issue), D583 (2011). doi:10.1093/nar/gkq1143 Medline
13. S. A. Moore, D. M. Moennich, M. J. Gresser, Synthesis and hydrolysis of ADP-arsenate by beef heart submitochondrial particles. *J. Biol. Chem.* **258**, 6266 (1983). Medline
14. R. Lagunas, Sugar-arsenate esters: thermodynamics and biochemical behavior. *Arch. Biochem. Biophys.* **205**, 67 (1980). doi:10.1016/0003-9861(80)90084-3 Medline
15. D. E. Atkinson, G. M. Walton, Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. *J. Biol. Chem.* **242**, 3239 (1967). Medline
16. G. R. Willsky, M. H. Malamy, Effect of arsenate on inorganic phosphate transport in Escherichia coli. *J. Bacteriol.* **144**, 366 (1980). Medline
17. G. R. Willsky, M. H. Malamy, Characterization of two genetically separable inorganic phosphate transport systems in Escherichia coli. *J. Bacteriol.* **144**, 356 (1980). Medline
18. D. Cánovas, C. Durán, N. Rodríguez, R. Amils, V. de Lorenzo, Testing the limits of biological tolerance to arsenic in a fungus isolated from the River Tinto. *Environ. Microbiol.* **5**, 133 (2003). doi:10.1046/j.1462-2920.2003.00386.x Medline

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