# Can Life use Arsenic instead of Phosphorus ?

**Kanai's Lab. Literature Seminar**

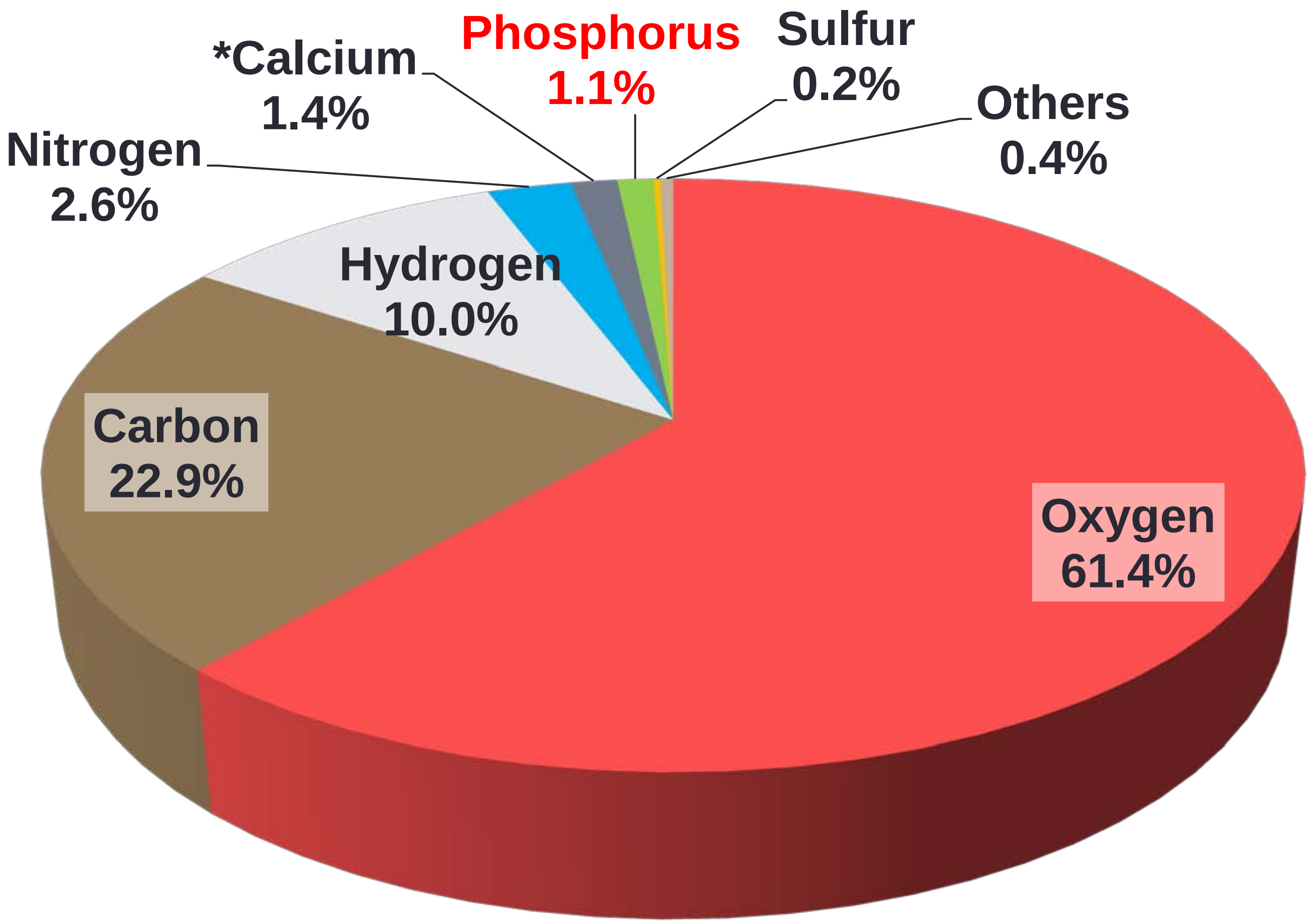
**27th October 2012**

**Junya Kawai (M2)**

# Index

# 1. Phosphorus in Life

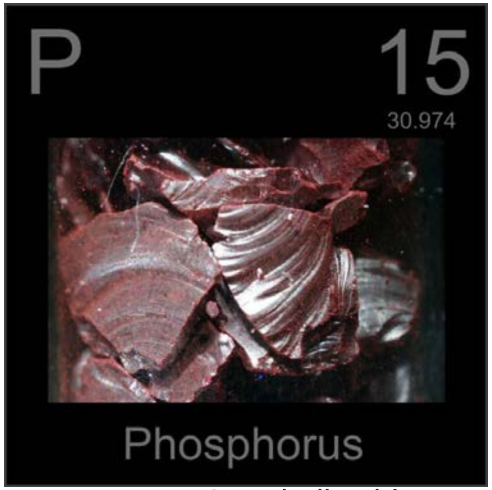
# Six Nutrients Elements of Life




**Elements in human body (w/w %, IAEA, 1972)**

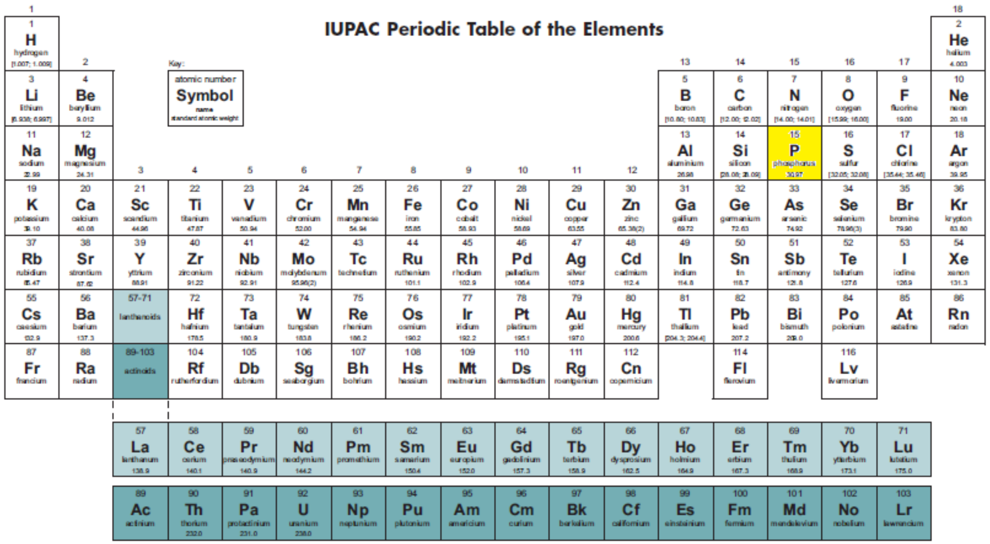
*Calcium is not included in “six nutrients”.

# Phosphorus




© periodictable.com

Atomic number: 15
Atomic weight: 30.973762
Electron configuration: [Ne] $3s^2 3p^3$
Atomic radius: 98 pm
Electronegativity (Pauling): 2.19
Stable isotope: $^{31}P$
Oxidation states: +V ~ -III (+V is most important for life.)
Allotropes: white, red, black, *etc.*

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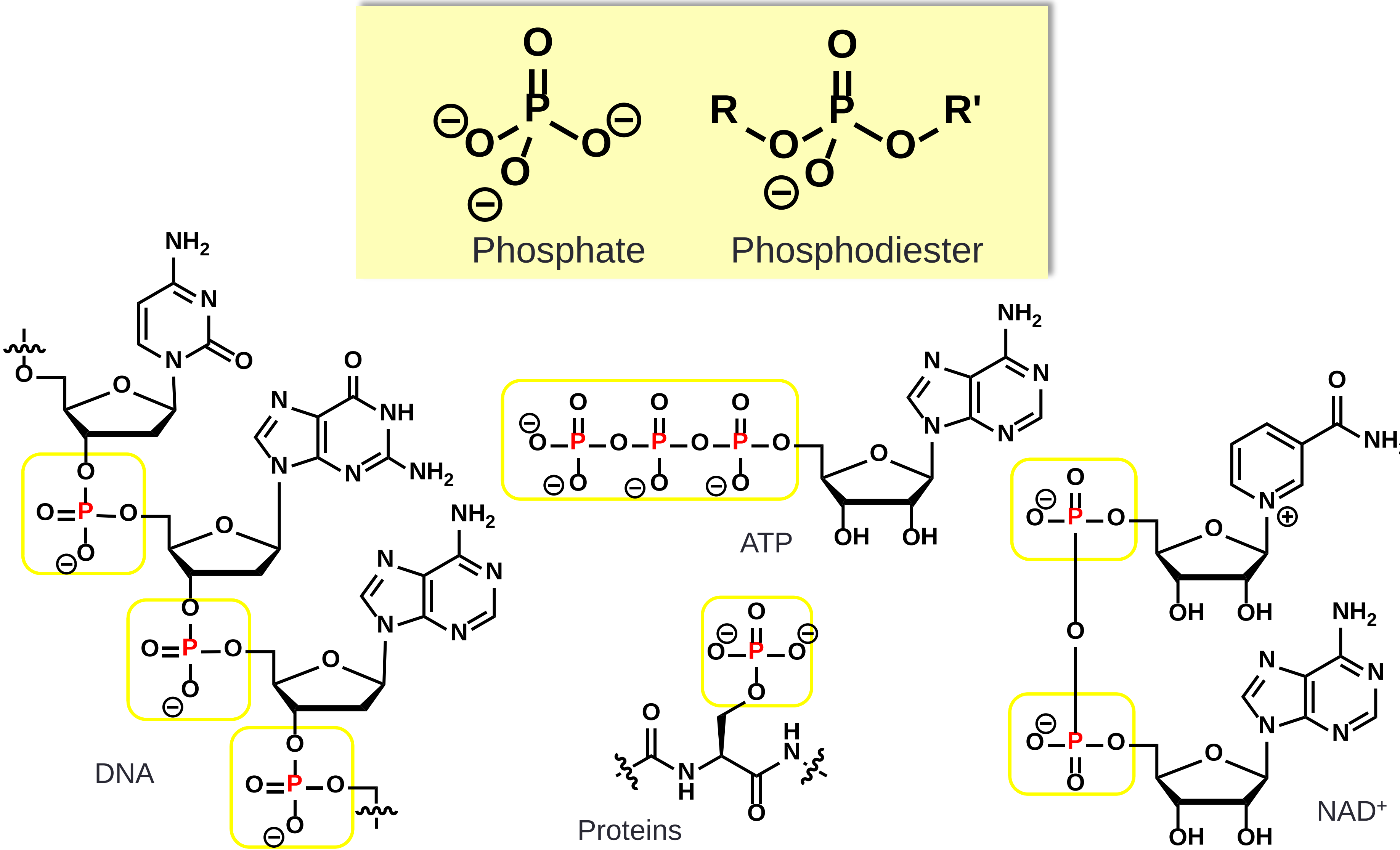
reagent

http://www.siyaku.com

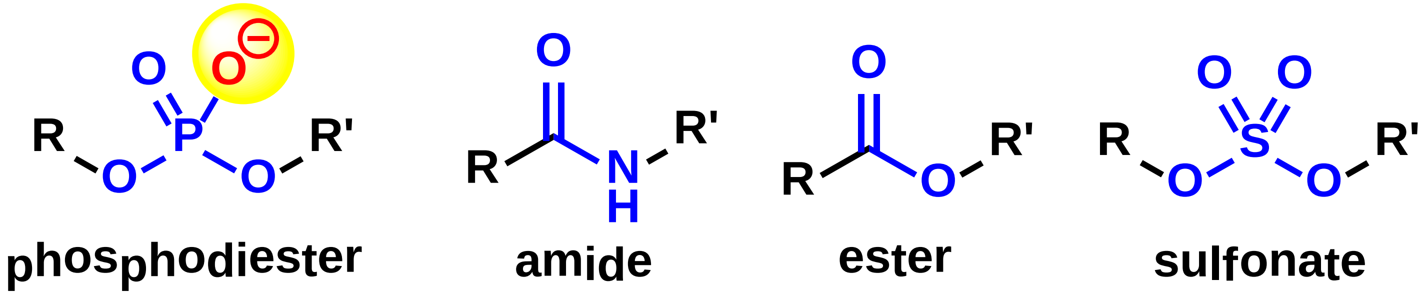

fertilizer

http://www.onoda-kagaku.co.jp

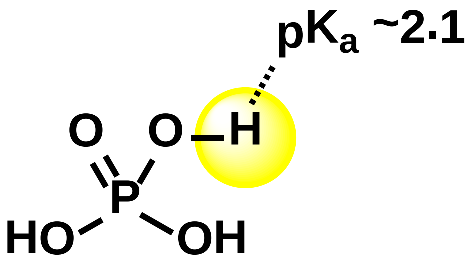
# Phosphorus in Life



# Phosphodiester: an Anionic linker



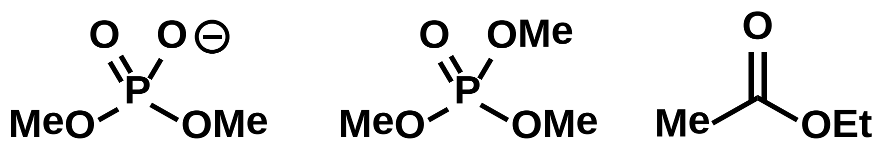

*cf.*




Phosphodiester can link two carbons and still ionize.

# Why DNA use Phosphorus

- Ionized phosphodiester is highly tolerant of hydrolysis.
  - Rates of saponification at 35 °C




| | | | |
|---|---|---|---|
| $k$ ($M^{-1}s^{-1}$) | $2.0 \times 10^{-9}$ | $3.4 \times 10^{-4}$ | $1.0 \times 10^{-2}$ |
| relative rate | 1.0 | $2 \times 10^{5}$ | $5 \times 10^{6}$ |

- Phosphodiester is retained within membrane.
  - Due to the lipophobicity of anion, molecules can be kept inside membrane.

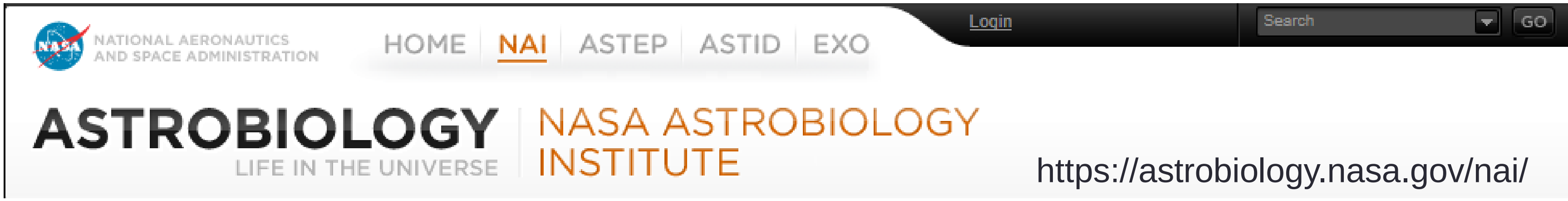
- Molecules can be bound by electrostatic interaction
  - For example, the “packaging” of DNA around histone

- Phosphate is redox inactive in a physiological conditions.
  - It can be reduced to phosphite only at potentials as low as -700 mV.

Westheimer, F. H. *Science* 1987, ***235***, 1173-1178.

# 2. NASA's Report in 2010

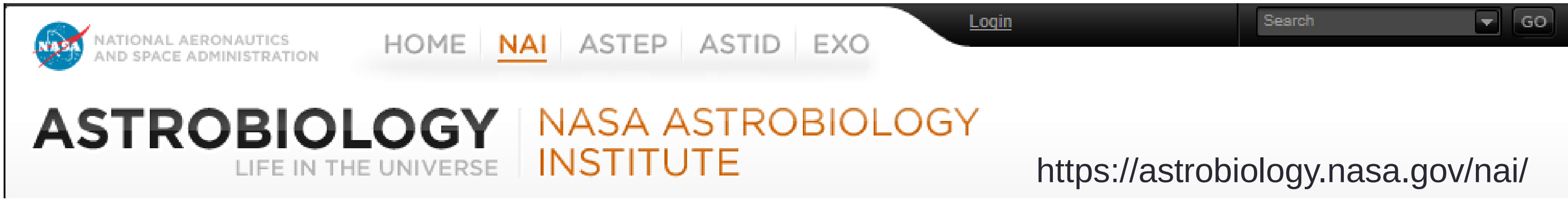
# NASA Astrobiology Institute (NAI)




- Astrobiology

  The study of the origin, evolution, distribution, and future of life in the universe
- Three questions they address
  - How does life begin and evolve?
  - Is there life elsewhere in the universe?
  - What is the future of life on Earth and beyond?
- What they do
  - The search for habitable environments in our Solar System and on planets around other stars
  - The search for evidence of prebiotic chemistry or life on Solar System bodies such as Mars, Jupiter’s moon Europa, and Saturn’s moon Titan
  - Research into the origin, early evolution, and diversity of life on Earth

# NASA’s Press Conference in 2010




- “NASA will hold a news conference at 2 p.m. EST on Thursday, Dec. 2, to discuss an astrobiology finding that will impact the search for evidence of extraterrestrial life.”

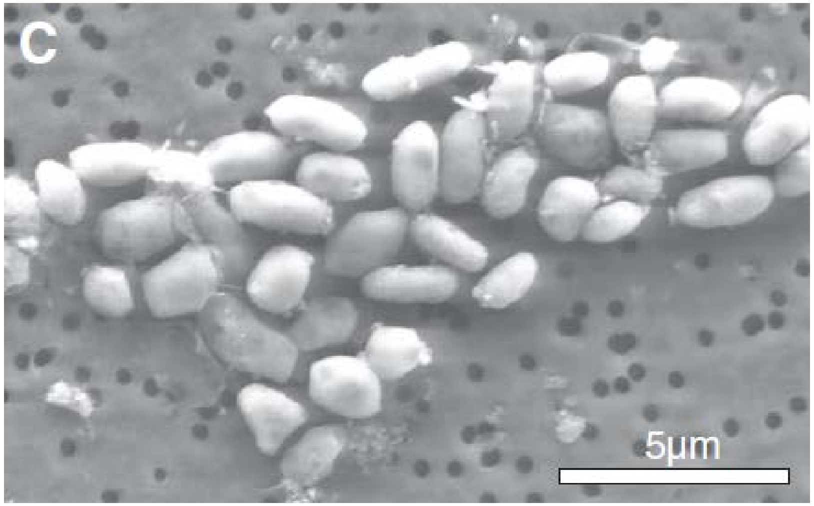
# NASA's Report on *Science*

## A Bacterium That Can Grow by Using Arsenic Instead of Phosphorus

Felisa Wolfe-Simon,[1,2*] Jodi Switzer Blum,[2] Thomas R. Kulp,[2] Gwyneth W. Gordon,[3] Shelley E. Hoeft,[2] Jennifer Pett-Ridge,[4] John F. Stolz,[5] Samuel M. Webb,[6] Peter K. Weber,[4] Paul C. W. Davies,[1,7] Ariel D. Anbar,[1,3,8] Ronald S. Oremland[2]

Wolfe-Simon, F. *et al.* *Science* 2011, ***332***, 1163-1166.

- They claimed that a bacterium GFAJ-1 could sustain its growth by using arsenate instead of phosphate.

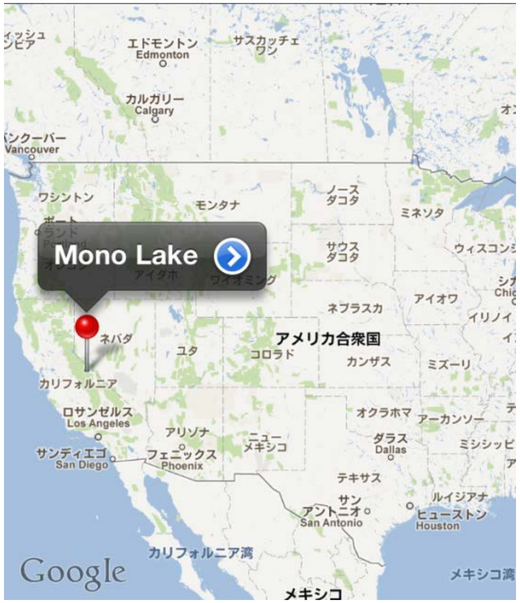



GFAJ-1

- A rod-shaped bacterium
- Isolated from the sediment of Mono lake.
- Belongs to the *Halomonadaceae* family, identified by 16*S* rRNA sequence philogeny.

Figure: SEM image of a strain of GFAJ-1 cultured under arsenic-rich condition

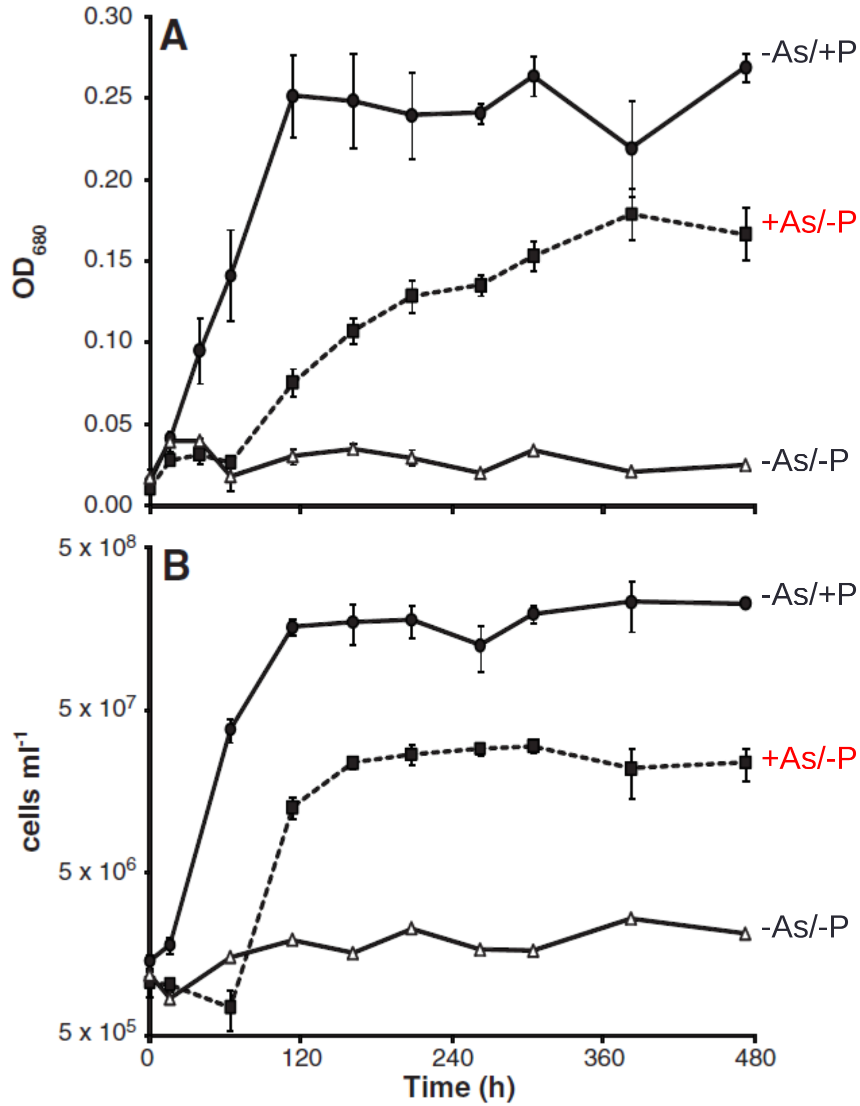
# Mono Lake




© NASA

- Located in eastern California, U.S. (http://www.monolake.org/)
- An example of "extreme environments" on earth
  - Alkaline: pH 9.8
  - Hypersaline: 78 g/L (2.5 times higher than ocean)
  - Arsenic-rich: 200 μM (Highest in the world)
- Some unnatural lives have already been found here.
  As(III)-fueling photosynthesis: Oremland, R. S. *et al. Science* 2008, ***321***, 967-970.

# Growth of strain GFAJ-1



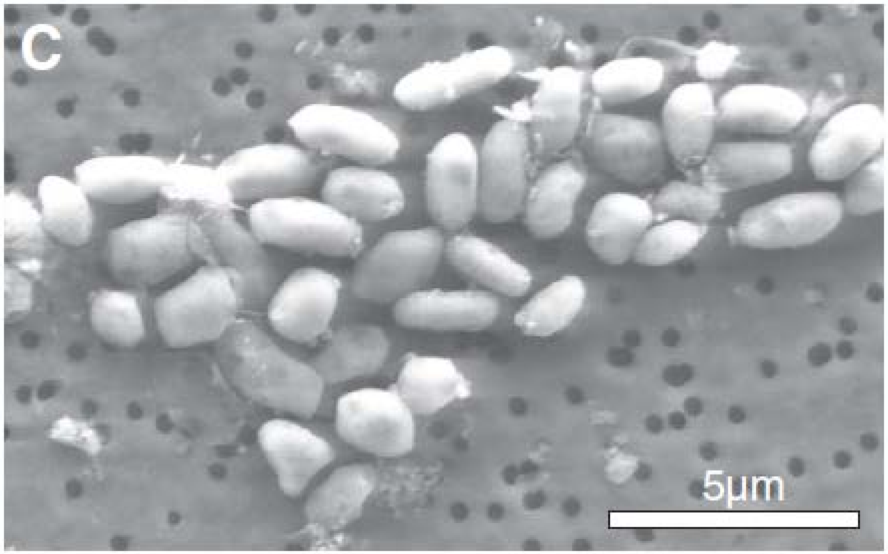

- Cell growth was monitored by both an increase in optical density and cell numbers.
- Cell grew the fastest under –As/+P.
- Also under +As/-P, cell could grow to be 20-fold in cell numbers after 6 days, while it didn't grow under –As/-P.

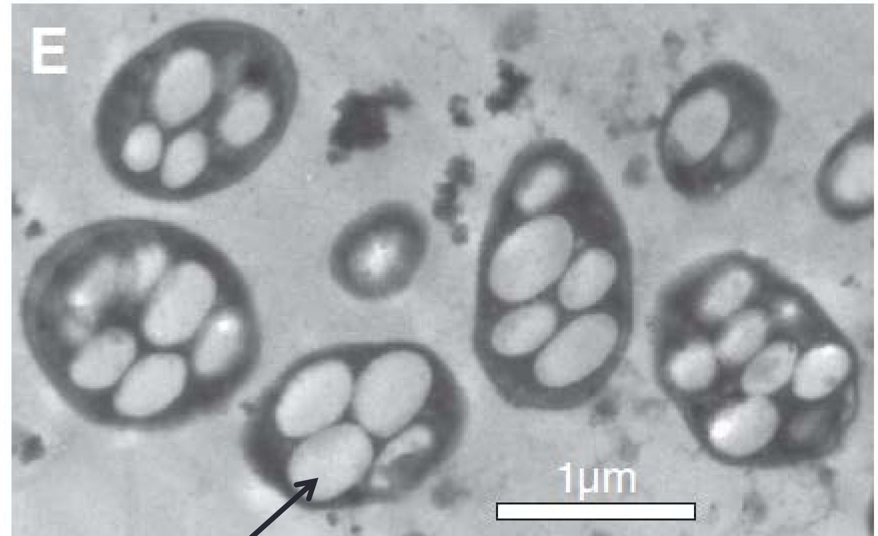
➢ GFAJ-1 seems to use arsenate instead under phosphate-poor condition.

* Artificial medium (pH 9.8) contains 10 mM glucose, other vitamins and minerals required but no phosphate.
**+As: 40 mM $AsO_4^{3-}$
+P: 1.5 mM $PO_4^{3-}$
-P: 3.1 (±0.3) μM $PO_4^{3-}$ (background)

Wolfe-Simon, F. *et al. Science* 2011, ***332***, 1163-1166.

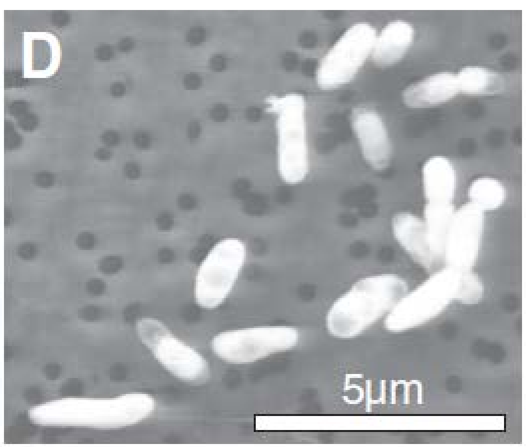
# Electron Microscopy of GFAJ-1




Vacuole-like regions were especially enlarged.

E: TEM** image of +As/-P
** Transmission Electron Microscopy




Cells became 1.5-fold larger.

C: SEM* image of +As/-P
D: SEM* image of -As/+P
* Scanning Electron Microscopy

***+As: 40 mM $AsO_4^{3-}$
+P: 1.5 mM $PO_4^{3-}$
-P: 3.1 ($\pm$0.3) μM $PO_4^{3-}$ (background)

Wolfe-Simon, F. *et al.* *Science* 2011, ***332***, 1163-1166.

# ICP-MS Analysis of Intracellular Elements

ICP-MS: Inductively Coupled Plasma Mass Spectrometry

Possible to detect the quantity of each containing element (or its isotope)

(http://www.chem-agilent.com/contents.php?id=513)

**Table 1.** Bulk intracellular elemental profile of strain GFAJ-1. Cells were grown and prepared with trace metal clean techniques (*11*). Numbers in parentheses indicate replicate samples analyzed. As:P ratios were calculated based on all samples analyzed (*11*). Units are percent dry weight.

| Condition | As | P | As:P |
|---|---|---|---|
| +As/–P (8) | 0.19 ± 0.25 | 0.019 ± 0.009 | 7.3 |
| –As/+P (4) | 0.001 ± 0.0005 | 0.54 ± 0.21 | 0.002 |

They claimed followings:

- Higher intracellular As & lower P were detected for the +As/-P cells.
- Intracellular P grown +As/-P is 96.5% less than that of –As/+P, which is far below the required amount to support growth (1-3 % P by dry weight).

Wolfe-Simon, F. *et al. Science* 2011, ***332***, 1163-1166.

# Elemental Distribution

- Radiolabeled $^{73}AsO_4^{3-}$ was employed to check the intracellular distribution.

**Table 2.** Intracellular radiolabeled $^{73}AsO_4^{-}$ arsenate distribution. All major cellular subfractions contained radiolabel after cell-washing procedures. Small molecular weight (s.m.w.) metabolites potentially include arsenylated analogs of ATP, NADH, acetyl-CoA, and others (*11*). SE is shown.

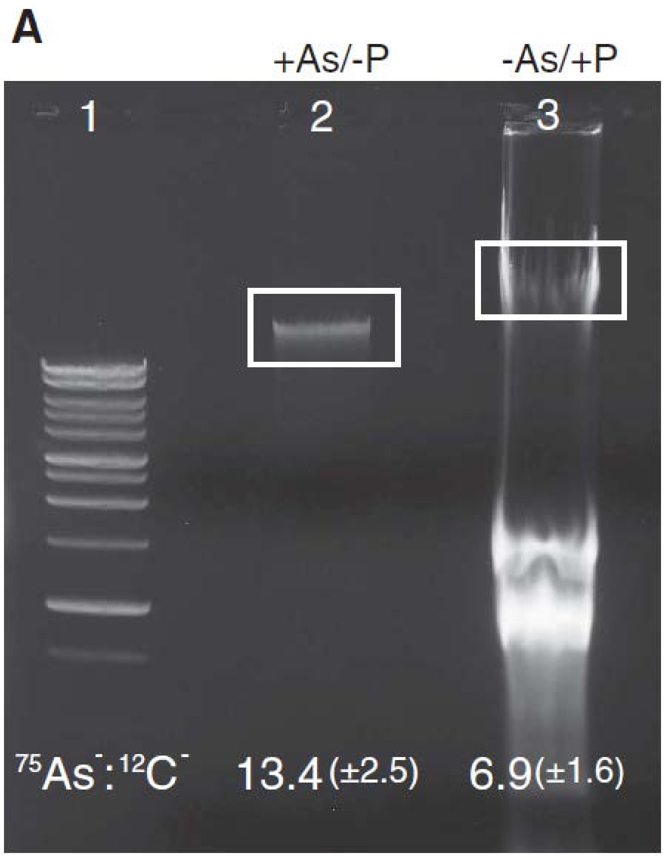
| Solvent (subcellular fraction) | Cellular radiolabel recovered (percent of total) |
|---|---|
| Phenol (protein + s.m.w. metabolites) | 80.3 ± 1.7 |
| Phenol:Chloroform (proteins + lipids) | 5.1 ± 4.1 |
| Chloroform (lipids) | 1.5 ± 0.8 |
| Final aqueous fraction (DNA/RNA) | 11.0 ± 0.1 |

DNA may contain $^{73}As$.

Proteins & small molecule weight metabolites (NADH, ATP, acetyl-CoA *etc.*) may be arsenylated.

Wolfe-Simon, F. *et al. Science* 2011, ***332***, 1163-1166.

# NanoSIMS Analysis of DNA




- The extracted DNA/RNA fractions were loaded on agarose gel and the bands indicated were analyzed.
- $^{75}As$ ion ratio of +As/-P relative to $^{12}C$ was twice as much as that of –As/+P.
  (The value of –As/+P is almost same as blank.)
- $^{31}P$:$^{12}C$ ratio was 3.4 times less than –As/+P.

➢ Purified DNA from +As/-P seems to contain As.

**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 (lane 2) +As/–P and (lane 3) –As/+P as compared with (lane 1) a DNA standard. Genomic bands were excised as indicated and analyzed with NanoSIMS. Ion ratios of $^{75}As^{-}$:$^{12}C^{-}$ of excised gel bands are indicated below with 2σ error shown (all values multiplied by $10^{-6}$).

Wolfe-Simon, F. *et al. Science* 2011, ***332***, 1163-1166.

# XANES & EXAFS Analysis

- XANES : X-ray Absorption Near Edge Structure
  Electron status or symmetry can be detected.

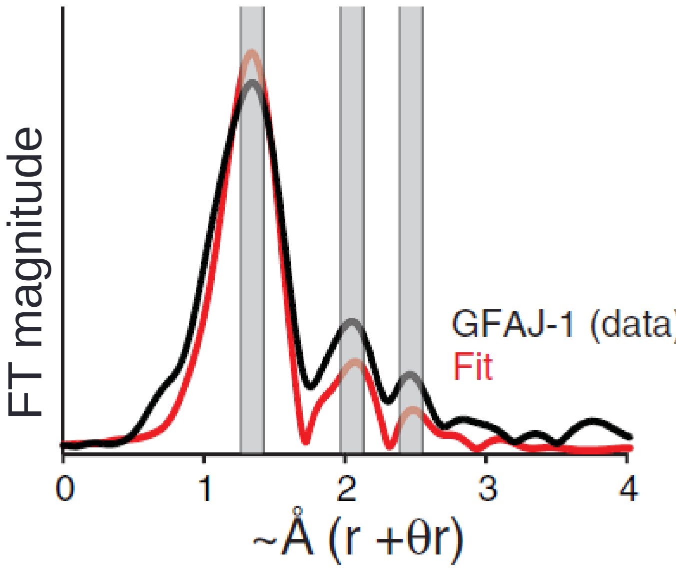
- As(V) coordination was detected.

- EXAFS : Extended X-ray Absorption Fine Structure
  Coordination number and atomic distance can be obtained.

(http://support.spring8.or.jp/Doc_lecture/PDF_090127/xafs_4.pdf)

- The first neighbor shell around As consisted of 4 oxygen ligands and had a second shell, which is inconsistent with previous data.

- But authors said its spectrum matched with that of the model structure of arsenylated DNA (red), metabolites, or proteins.

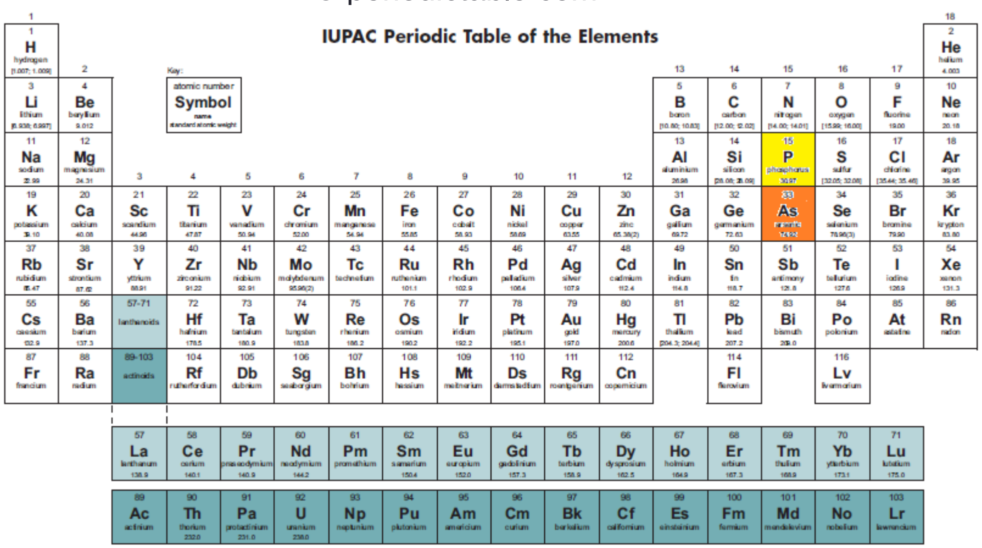



Wolfe-Simon, F. *et al. Science* 2011, ***332***, 1163-1166.

# Arsenic



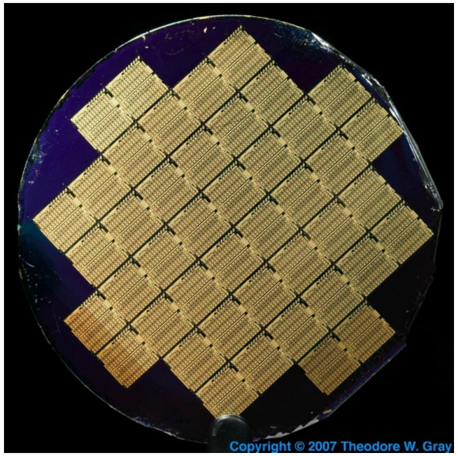

Atomic number: 33
Atomic weight: 74.92160
Electron configuration: [Ar] $4s^2\,3d^{10}\,4p^3$
Atomic radius: 114 pm
Electronegativity (Pauling): 2.18
Stable isotope: $^{75}As$
Oxidation states: +V ~ -III
Allotropes: grey, yellow, black






Salvarsan (drug)






Semiconductor (Gallium arsenide)

# Arsenate vs Phosphate

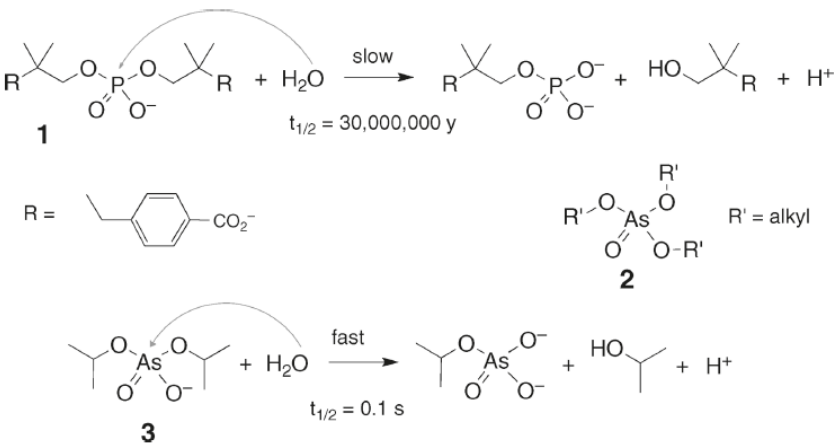
## ➢ Similarities

- Same electron configuration ($s^2p^3$ for unclosed shell)
- Nearly identical $pK_a$ (As: 2.2, 6.97, 11.53 / P: 2.1, 7.2, 12.7)
- Thermochemical radii (only 4% different)

## ➢ Differences

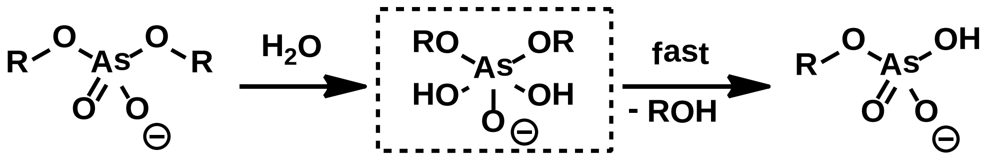
- Less tolerance for hydrolysis
  - Kinetic stability of arsenate in $H_2O$ is far below that of phosphate.

  Edwards, J. O. *et al. Inorg. Chem.* 1980, ***20***, 907.



Gates, K. S. *et al. ACS Chem. Bio.* 2011, ***6***, 127.

- Easily reduced to As(III)



# Summary of NASA’s Claim

- Cells grew under +As/-P, although they didn’t under -As/-P.
- Higher intracellular As & lower P were detected (than –As/-P).
- P value was less than required for its growth (1-3 % P by dry weight).
- Based on the purification by extraction, proteins, s.m.w. metabolites and DNA might contain arsenic.
- NanoSIMS analysis of DNA also supported the fact above.
- XANES & EXAFS suggested the arsenate was incorporated in DNA or other microorganisms.

# 3. Objections to NASA's Report

# Eight Technical Comments have come !!

TECHNICAL COMMENT

## Comment on "A Bacterium That Can Grow by Using Arsenic Instead of Phosphorus"

Stefan Oehler

Wolfe-Simon *et al.* (Research Articles, 3 June 2011, p. 1163; published online 2 December 2010) reported that a naturally occurring bacterium, strain GFAJ-1, can substitute arsenic for phosphorus in its biomolecules. However, straightforward experiments to support this claim, including density gradient centrifugation of DNA assumed to contain arsenic, were either not performed or not presented. As a result, the authors' conclusions remain uncertain.

The title of the research article by Wolfe-Simon *et al.* (*1*) asserts that a bacterium, strain GFAJ-1, can grow by using arsenic instead of phosphorus. However, their study presents only preliminary results, not the confirmatory experiments one would expect to find in support of this claim.

The tolerance to and possible conditional dependence on arsenic of the bacterial isolate is very interesting. However, the main claim that "strain GFAJ-1...can vary the elemental composition of its basic biomolecules by substituting As for P" does not appear to be sufficiently supported by the data. Straightforward experiments that could verify incorporation of arsenic into biological macromolecules were either not performed or not reported. These could, for example, be density gradient centrifugation of DNA assumed to contain arsenic or autoradiography of electrophoretically separated proteins and restriction fragments of DNA from cells grown in the presence of radioactive arsenic. A comparison of the hydrolysis rates of DNA from bacteria grown in arsenate medium versus DNA from bacteria grown in phosphate medium could also have been easily done. Without these data, the authors' claim that the bacterium they described can grow by using arsenic instead of phosphorus remains unconvincing.

Biomedical Sciences Research Center Alexander Fleming, 16672 Vari, Greece. E-mail: oehler@fleming.gr

References

1. F. Wolfe-Simon *et al.*, *Science* **332**, 1163 (2011); published online 2 December 2010; 10.1126/science.1197258.

7 December 2010; accepted 17 May 2011
Published in *Science* Express 27 May 2011;
10.1126/science.1201381

All doubted whether GFAJ-1 was truly using arsenic in its DNA.

# 1. Unsolved Matters

- Stability against hydrolysis of arsenate diester
  - Arsenic ester can be hydrolyzed in water much faster than phosphate.
  - If the arsenylated DNA was stabilized by association with another molecule, it would be required to remain associated with DNA through extraction and gel electrophoresis processes.

- High redox potential of As(V)
  - As(V) is reduced into As(III) in the physiological range of redox potential.
  - The resulting structural change & oxidation of other molecules impair the metabolic processes.

- Relatively lower intracellular As concentration

  Medium: As/P = *ca.* 10000

  Cell: As/P = only <10

# 2. Inadequate Data & Analyzing Methods

- No direct evidence of incorporation of As in DNA

- Insufficient purification method of DNA
  - Aqueous DNA/RNA fraction they used directly is typically contaminated.
  - It wasn't purified from the agarose gel which may contain some elements.

- High phosphate concentration on background (*ca*. 3 μM)
- Unknown purity of arsenic
- Incorrect statistical calculation and large estimated errors

| Condition | As | P | As:P |
|---|---|---|---|
| +As/−P (8) | 0.19 ± 0.25 | 0.019 ± 0.009 | 7.3 |
| −As/+P (4) | 0.001 ± 0.0005 | 0.54 ± 0.21 | 0.002 |

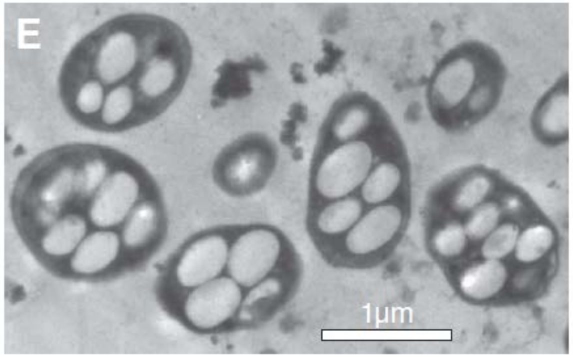
Error is larger than value itself !

Calculation of average is not correct, in fact !

# 3. Alternative Interpretations

- Induced Pst system under +As/-P condition
  - Many bacteria have two systems to assimilate phosphorus:
  (i) phosphate inorganic transport (Pit): Low affinity for P and always active
  (ii) the phosphate-specific transport (Pst): High affinity for P and active when phosphate levels are low
  - Arsenate is known to poison the Pit system and to accelerate Pst system.
  Malamy, M. H. *et al. J. Bacteriol.* 1980, ***144***, 366.
  - Growth under +As/-P is due to the increased uptake of P by Pst system ?

- Cell growth in volume perhaps means the storage of toxic molecules.
  *Nature* 2010, ***468***, 741.




- Some bacteria only need much less than 1 % P by dry weight.

# Two Definitive Reports in 2012

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

Tobias J. Erb,[1]*† Patrick Kiefer,[1]* Bodo Hattendorf,[2] Detlef Günther,[2] Julia A. Vorholt[1]†

The bacterial isolate GFAJ-1 has 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 lacks 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 arsenate, but further experiments showed they

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 (e.g., nucleotides and sugar phosphates) 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, cytidine trisphosphate (CTP), guanosine trisphosphate (GTP), and uridine trisphosphate (UTP)], as a measure for cellular energy status,

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

Marshall Louis Reaves,[1,2] Sunita Sinha,[3] Joshua D. Rabinowitz,[1,4] Leonid Kruglyak,[1,5,6] Rosemary J. Redfield[3]*

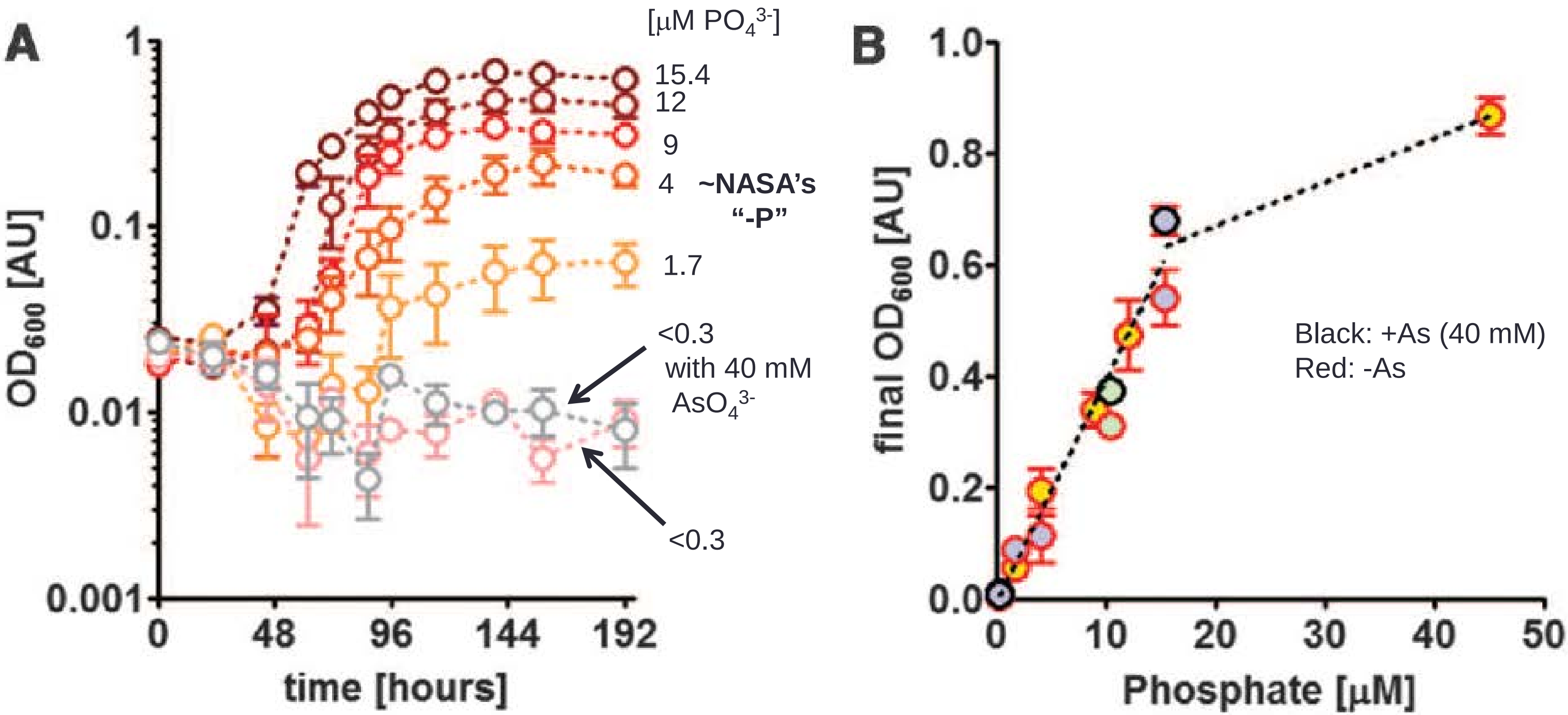
A strain of *Halomonas* bacteria, GFAJ-1, has been claimed to be able to use arsenate as a nutrient when phosphate is limiting and to specifically incorporate arsenic into its DNA in place of phosphorus. However, we have found that arsenate does not contribute to growth of GFAJ-1 when phosphate is limiting 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.

ever, 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 16*S* 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

Erb, T. J. *et al. Science* 2012, ***337***, 467-470.; Redfield, R. J. *et al. Science* 2012, ***337***, 470-473

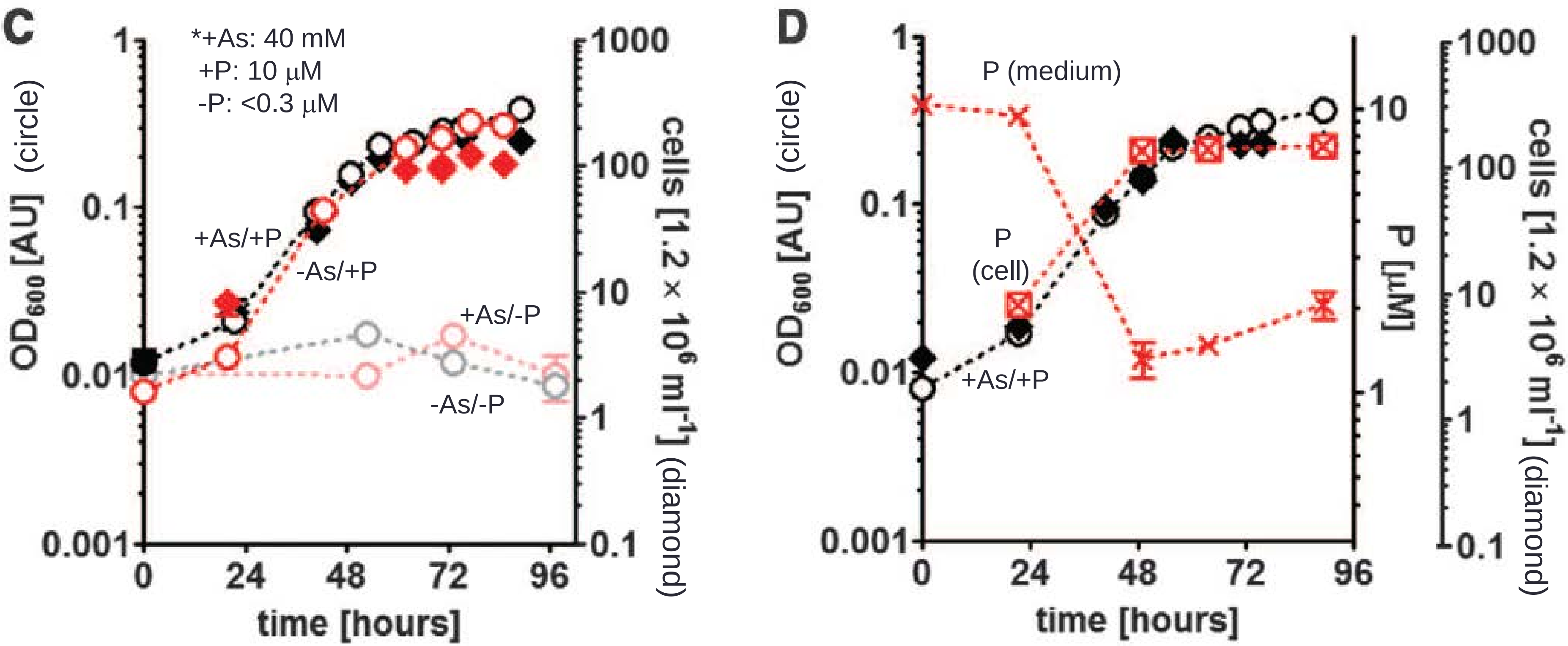
# "As-Resistant", but still P-Dependent




- Growth correlated with the amount of phosphate, and 1.7 μM is sufficient.
- +As/-P (<0.3 μM): No growth occurred.
- Final growth didn't change whether or not arsenate was added.

Erb, T. J. *et al. Science* 2012, ***337***, 467-470.

# "As-Resistant", but still P-Dependent




- Growth correlated with the amount of phosphate, not with that of arsenate.
- During growth, phosphorus in the medium decreased and became enriched in the cellular fraction.

➢ During growth, GFAJ-1 is obviously using phosphorus in the medium.

Erb, T. J. *et al. Science* 2012, ***337***, 467-470.

# Metabolomic Analysis by HRMS

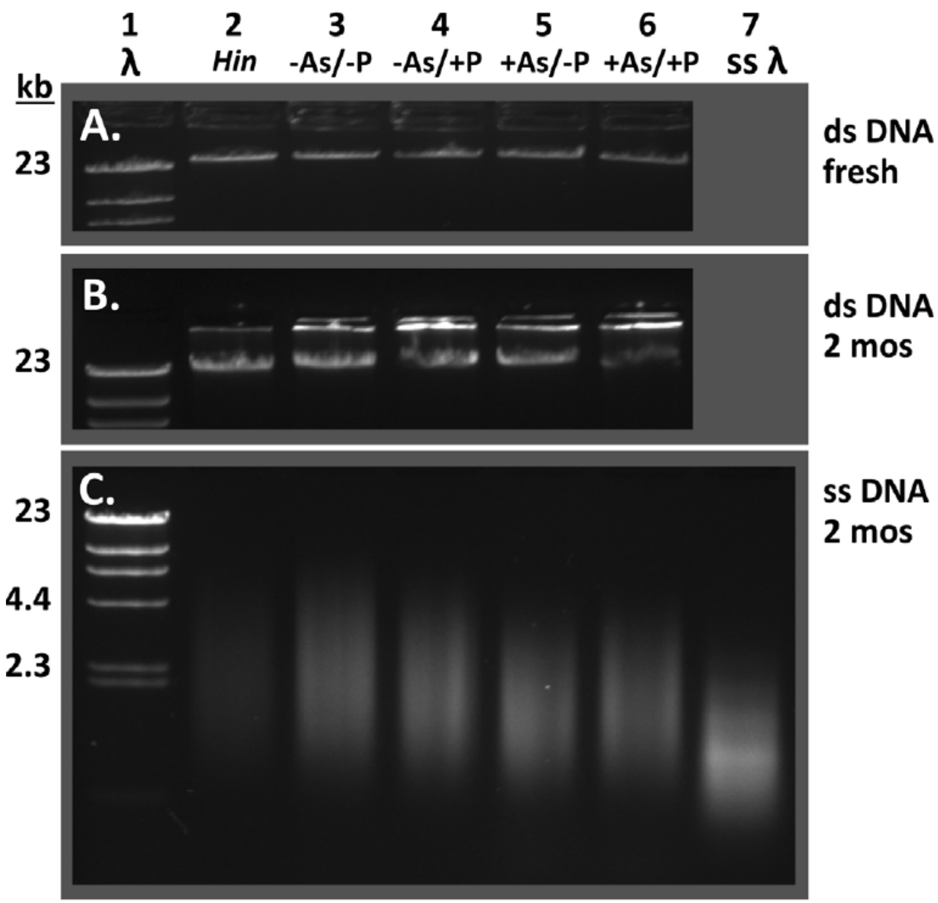
- Cells were cultured in almost the same medium as reported.
  (As: 40 mM(+) or 0 mM(-), P: 9 μM, glucose: 10 mM)

- In both media, only phosphorylated metabolites were detected.
  - Only nucleotide bisphosphates (ADP *etc.*) appeared elevated in the cell cultured +As, which might result from a higher energy demand when grown +As.
    (Due to ATP-dependent detoxification or induced Pst system ?)

- Although hexose arsenate was detected in MS, it was found this is abiotically formed glucose arsenate.

Erb, T. J. *et al.* *Science* 2012, ***337***, 467-470.

# Analysis of Well-Purified DNA




- Independent of As and P, similar-sized fragments are obtained in both double- & single-stranded DNA .
- Hydrolysis or other stabilizing molecules were not observed.

- According to the elemental analysis of purified DNA, no As was detected.

**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 ± 1800 | 17,400 ± 1900 |
| Phosphorus in nucleic acids (ng) | 934 ± 13 | 1043 ± 8 |
| Arsenic in nucleic acids (ng) | <1* | <1* |
| As/P molar ratio | <0.001 | <0.001 |

*Below detection limit.

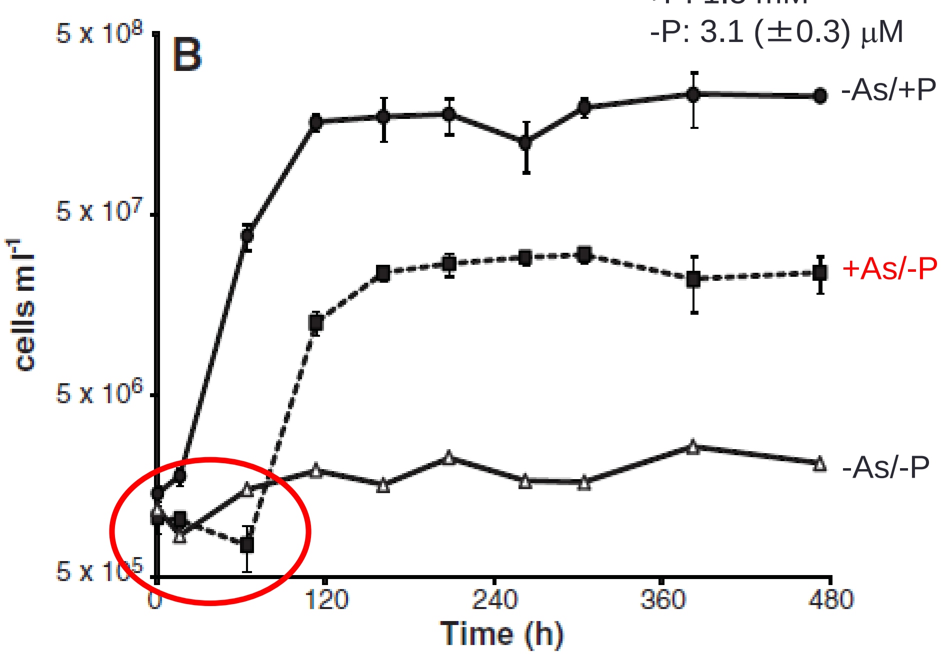
A. Soon after purification
B. 2 months later @ 4 °C
C. 2 months @ 4 °C, 10 min. @ 95 °C

Redfield, R. J. *et al. Science* 2012, ***337***, 470-473
Erb, T. J. *et al. Science* 2012, ***337***, 467-470.

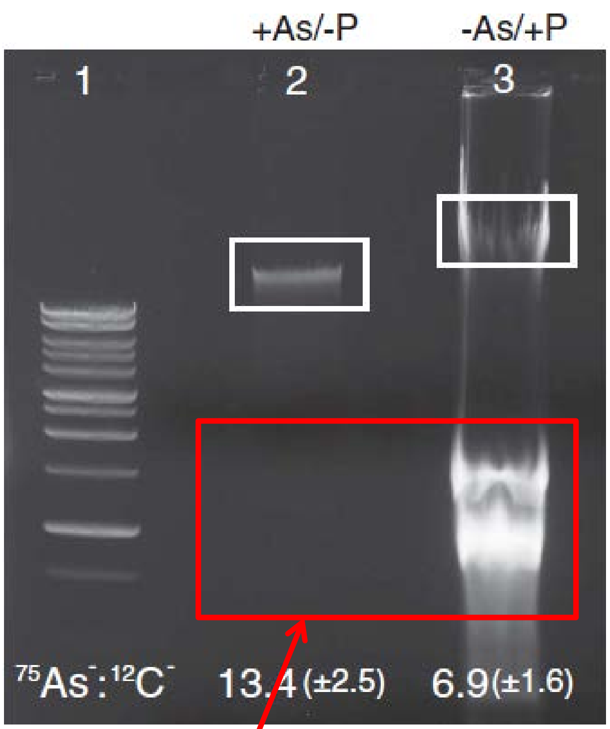
# As-induced Massive Ribosome Breakdown

- One reason why arsenate stimulated the growth of GFAJ-1 might be that arsenate induces massive ribosome degradation, providing a source of phosphate.

*+P: 1.5 mM
-P: 3.1 (±0.3) μM




Long lag period
prior to start of growth




Two intensive bands disappeared in +As/-P. (23S and 16S rRNA ?)

Wolfe-Simon, F. *et al. Science* 2011, ***332***, 1163-1166.

Deutscher, M. P. *et al. J. Biol. Chem.* 2012, ***287***, 28816.

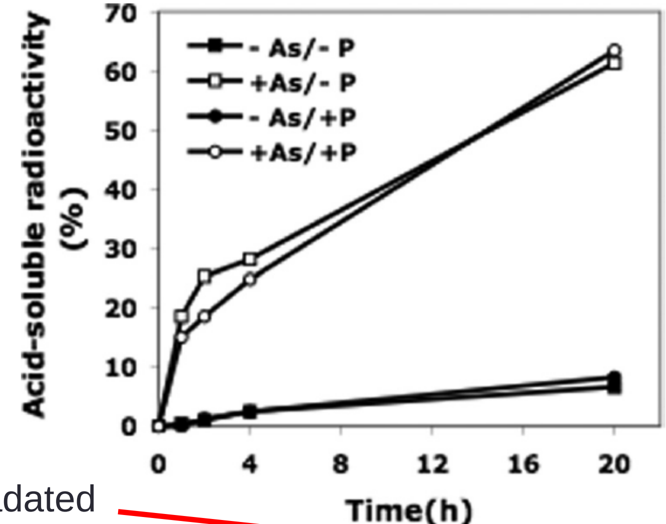
# As-induced Massive Ribosome Breakdown

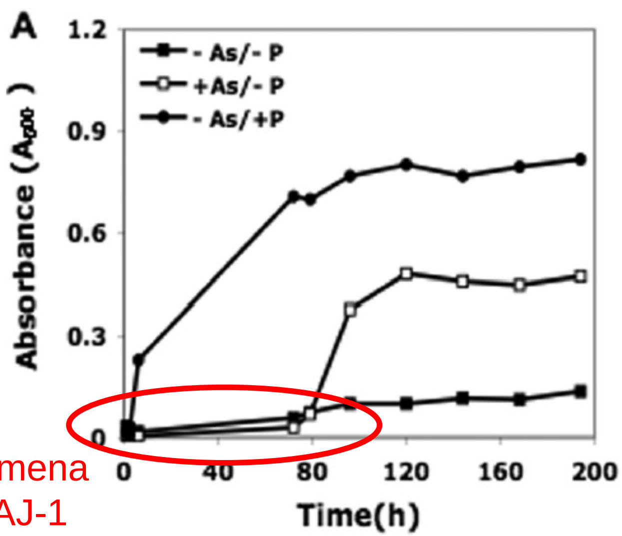
_E. coli_ : known to be As-resistant to some extent
*E. coli* growing in 0.1 mM phosphate medium
was prelabeled with [$^3$H]-uridine. (ribosome label)

After washing, cultured under various media

Measured release of acid-soluble radioactive products.

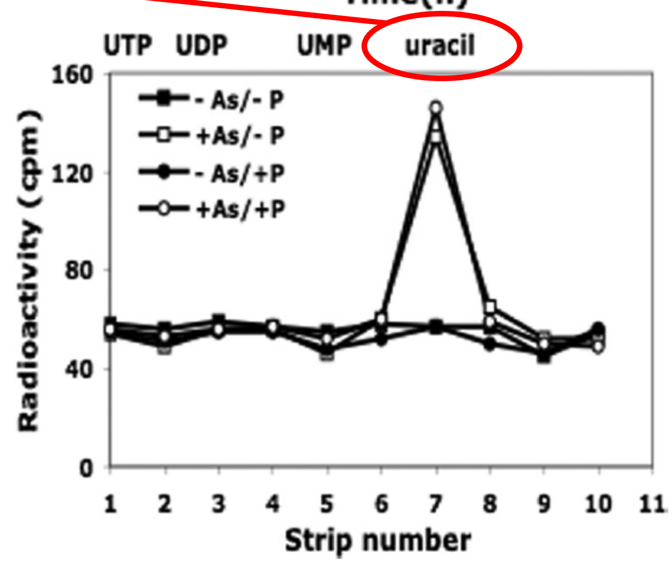
Deutscher, M. P. *et al. RNA* 2011, ***17***, 338.




Same phenomena as GFAJ-1

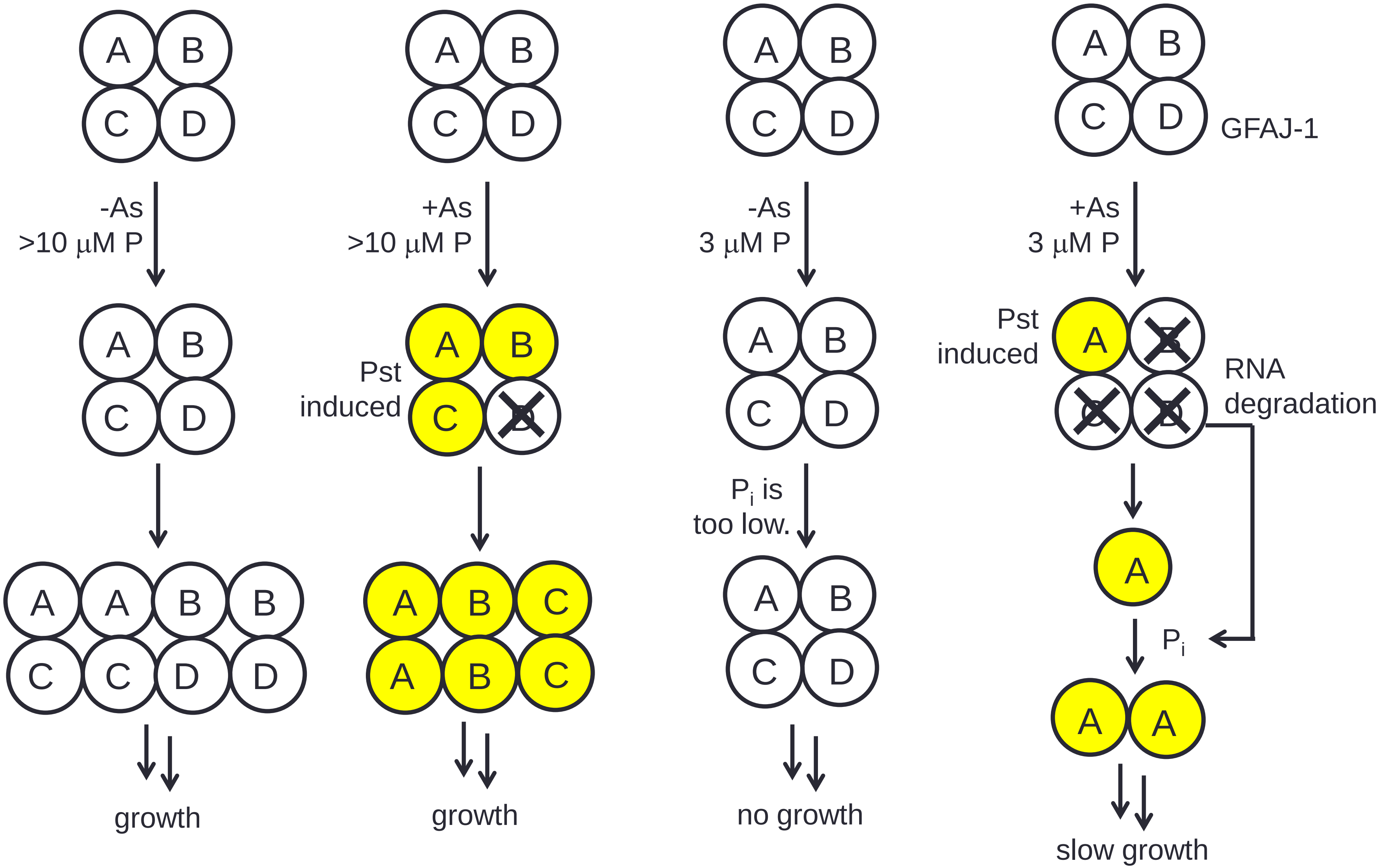
rRNA was degraded to free base, providing Pi.




+As/-P: After 80 h, small numbers of As-torelant cells appeared and then started to increase.

Deutscher, M. P. *et al. J. Biol. Chem.* 2012, ***287***, 28816.

# Supposed Conclusion



# Summary of Objections

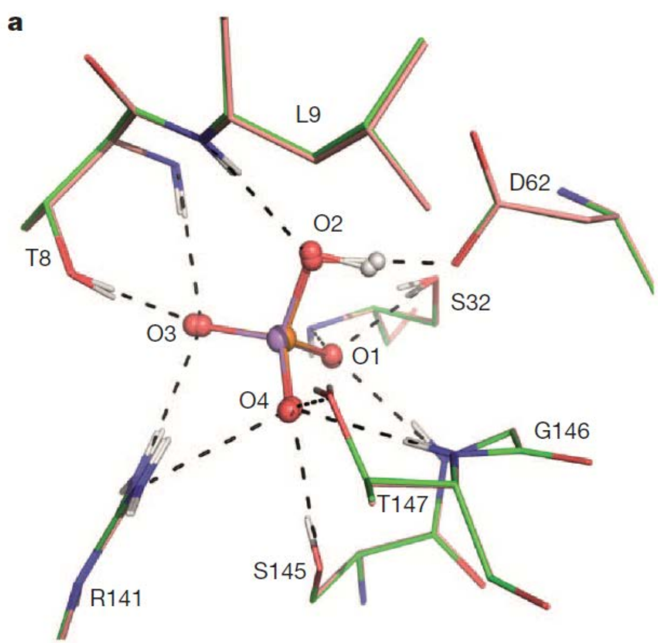
- GFAJ-1 used contaminated phosphate in medium for growth.
- Higher intracellular As is due to the inadequate purification of DNA.
- Metabolomics & elemental analysis showed no incorporation of As into DNA or other microorganisms.
- The reason arsenate stimulated the growth of GFAJ-1 might be either massive ribosome breakdown or induced Pst system or both.

➢ GFAJ-1 is still phosphate-dependent.

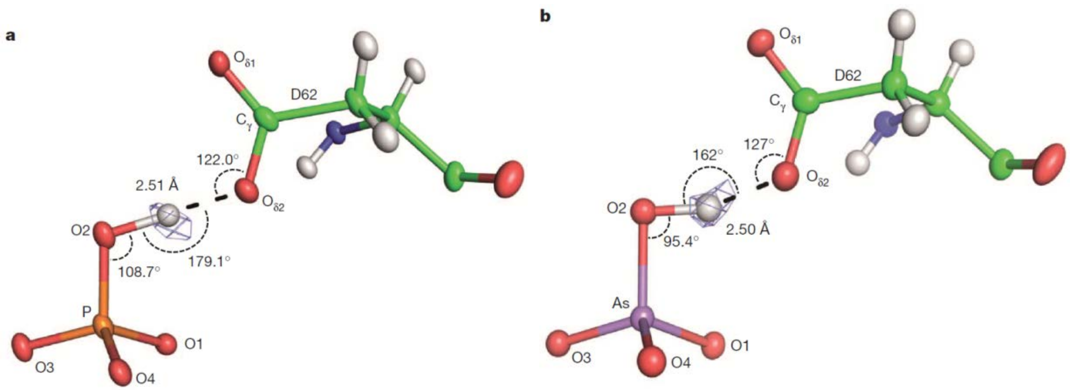
- Although As was found to be inessential for that strain to grow, still we cannot deny the possibility that GFAJ-1 is using As for some purposes.

# Appendix: How Life differentiate P & As

- PBP (Phosphate Binding Proteins):
  - The important unit for recognition of phosphate in Pst system (more selective one)
  - Discrimination of phosphate from arsenate is generally *ca.* 500-fold.
  - GFAJ-1 has two types of PBPs, one of which has >4500-fold discrimination ability.




PBP of *P. fluorescens* (pH 8.5)
As (red & violet sphere), As-bound (green)
P (orange & red sphere), P-bound (pink)




phosphate    Arsenate

- The only difference is the direction of a high-energy hydrogen bond between O2-H & Asp62 anion.
- Asp62 enables phosphate to bind more selectively than arsenate.

Elias, M. *et al. Nature*, 2012 (doi:10.1038/nature11517)

# 4. Conclusion

# Conclusion

- The answer to the title question,
  "Can life use arsenic instead of phosphorus ?" --- is still unclear.

- At least, GFAJ-1 is not arsenic-dependent bacterium. It still needs a small amount of phosphorus to grow.

- It is very important also for us
  to select the proper methods to make the things confirmed,
  to think whether alternative explanations for results are impossible,
  and to conduct control experiments strictly.

- And, it seems that life system is much mightier than I thought.