MICROBIAL REDUCTION OF PHOSPHATE?

Microbial Diversity project, 1995
by
Alison George,
School of Molecular and Medical Biosciences,
University of Wales Cardiff
Cardiff, UK

Introduction

Phosphorus is found in all parts of the biosphere and occurs most commonly in the form of phosphates in which the P-atom has an oxidation state of +5. Though phosphorus can exist in a range of oxidation states from +5 to -3, it is generally observed that it remains in the +5 state in natural transformations (Fenchel and Blackburn 1979). The biological phosphorus cycle thus appears to be relatively simple, lacking both a gaseous phase and the reduced oxidation states found in the nitrogen and sulphur cycles.

There are, however, numerous reports of trace levels of a reduced form of phosphorus, phosphine (PH3), being present in certain environments. The Will-O' the Wisp observed at night-time over peat bogs, swamps, cemeteries, recent battle fields and stagnant waters may not in fact be due to manifestations of the supernatural, but to the spontaneous ignition of gas containing traces of phosphine which evolved during the decomposition of animals in damp soils (Mellor 1940). In the 1920s a debate raged about whether the biological reduction of phosphate really occurred (Rudakov 1927, Rudakov 1929, Liebert 1927) and this argument has not yet been resolved.

A convincing demonstration of the prevalence of biological phosphine production was given in 1988 (Devai 1988). Examining the gases released from a sewage treatment works he found that 9-20% of phosphorus entering the system was converted to phosphine. He confirmed these observations using bacterial enrichments. Analyzing surface sediments of Hamburg harbour (Gassman and Schorn 1993) phosphine was found in the range of 1.2-56.6ng/kg. However, in 1972 Burford and Bremner failed to find any evidence for evolution of phosphine through microbial reduction of phosphate in waterlogged soils. In summary, it seems clear that trace levels of phosphine are undoubtedly resent in the environment but the mechanism/organism responsible has not been found.

Thermodynamic calculations can be made to predict whether phosphate reduction is an energetically feasible reaction but even these seem to lead to no firm conclusion. Rudakov (1929) and Tsubota (1959) both showed that it was thermodynamically realistic, whereas Liebert (1929) disagreed. Devai (1988) stated that the formation of phosphine "cannot be ruled out or explained by the properties of redox systems".

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There is also evidence that phosphine may play a role in anaerobic steel corrosion. Iverson (1968, 1984) noted that the corrosion product Fe_2P was evolved when phosphate was present in a steel corrosion experiment, and he suggested that this was due to the microbial production of phosphine which then reacts with ferrous iron to from Fe_2P.

Phosphate is a limiting nutrient in many environments due to the fact that it forms many insoluble salts (Ehrlich 1981, Fenchel and Blackburn 1979). It is also a vital nutrient for all biochemical processes. Why then should bacteria employ it as an electron acceptor? In certain environments phosphate will be in great excess. For example, living tissue is a rich source of phosphates in the form of phospholipids, ATP and apatite. (Maybe this could explain why Will-O' the Wisp is seen emerging from graveyards and battlegrounds). There is also evidence of phosphine evolution from blood and gastric juices stored at around 40°C and during the putrification of proteins (Mellor 1940). Sewage sludge also presents a bacteria with a phosphate rich environment, not only from feces but from phosphate added to detergents. In such environments it may give certain bacteria an ecological advantage if it is able to reduce surplus phosphate. There is an extraordinary diversity in microbial diversity in microbial use of inorganic compounds as terminal electron acceptors in anaerobic respiration, from the common Fe(III) (Lovley and Phillips 1988) to the more exotic As(V) (Ahmann et al 1994) and U(VI) (Lovley et al 1991).

The aim of the project is to attempt to observe and quantify phosphate reduction in bacterial enrichments from a variety of environments, and if this is successful, to isolate and characterize the organisms responsible.

Thermodynamic predictions

Calculations were made to see if the reduction of phosphate to phosphine was thermodynamically viable:

$$3H_2PO_4^- + 2C_3H_6O_3 + 3H^+ \rightarrow 3PH_3 + 6CO_2 + 6H_20$$

ΔGfo (kCal/mol)	-243.5	-123.75	0	+3.2	-92.26	-56.69

The standard Gibbs free energy change = **+93.9 kJ/mole**

(ΔGfo values were obtained from the CRC Handbook for Chemistry and Physics).
The reaction does not appear to be a thermodynamically favourable one, and becomes less favourable as the pH increases. However, if the phosphine or carbon dioxide gases are removed from the system by flushing or through a syntrophic association and the pH is reduced then the reaction may be energetically viable.
Calculations could not be made not the energetics of phosphate reduction to orthophosphite or hypophosphite (phosphorus oxidation states +III and +I respectively) because the necessary thermodynamic data ie free energy of formation/electrode potential was not available.

Materials and Methods

The exact biological mechanism (if one exists) of phosphate reduction has not been established. However, the scant literature reports insinuate that phosphine is evolved from anaerobic environments and that the sulphate reducing bacteria *Desulphovibrio desulfuricans* may be able to generate phosphine (Iverson andOlson, 1984). Therefore the general strategy employed to try to enrich for phosphate reducing bacteria was the same as that for the enrichments for sulphate reducing bacteria but with no sulphate ion to act as terminal electron acceptor. Preliminary thermodynamic calculations demonstrated that the reduction of phosphate to phosphine was more likely at low pH so the enrichments were carried out at pH 7 and 4.

Innocula

Six different innocula were used in the initial enrichments.

A: Raw sewage from the inlet pipe of the Falmouth sewage works (pH = 6.7)

B: The sandy black sulphate reducing layer of a microbial mat from the Sippiwissit salt marsh (pH = 6.8)

C: A mixture of the lab SRB enrichments (lactate as the electron donor).

D: A mixture of the lab SRB enrichments (acetate as the election donor).

E: Thiodendren veiled mud from the Sippiwissit salt marsh (pH = 6.8).

F: The black outer surface of a rusty iron nail washed up on Sippiwissit beach.

Enrichment on inorganic phosphate

In this enrichment only phosphate was used as the sole electron acceptor, whilst the electron donor and the pH of the enrichment was varied. The enrichment media was adapted from that used to enrich for sulphate reducing bacteria. The following salts were dissolved in 1 litre of distilled water:

MARINE MEDIUM

NH_4Cl	0.25g
KCl	0.5g
$CaCl_2.2H_2O$	0.06g
NaCl	20.0g
$MgCl_2.6H_2O$	0.62g

This solution was autoclaved and cooled under N_2/CO_2 (80/20) and aliquots of each of the following solutions added:

1M $NaHCO_3$ (under 100% CO2)	30mls
1M Na_2S	1ml
SL-12 six vitamin solution	1ml
Six vitamin solution	1ml
Vitamin B solution	1ml
1M K_2HPO_4	10ml
1M KH_2PO_4	10ml

This solution has a pH value of 7 ± 0.2. Half of this media was then dispensed into serum bottles under N_2/CO_2 (80/20). The pH of the remaining solution was reduced to 4 using sterile HCl and was dispensed as above. Innoculum was added to the medium at a ratio of 1:100. For each innoculum at each pH, lactate, acetate, sodium sulphite or formate were added as possible electron donors to give a final concentration of 10mM. H_2 was also added in gaseous form as a possible electron donor. In the enrichments with H_2 and sulphite as the electron donors the gas phase of the media contained 10% CO_2 to act as a carbon source. The enrichments were incubated at 30°C.

Secondary enrichments were made from the enrichments with the greatest growth. In this case controls were made for each pH with a) no electron donor b) no phosphate c) sterile innoculum (autoclaved sewage).

In situ hybridization

In situ hybridization was carried out on enrichments from black sulphate reducing mud (enrichments B) at pH 7 and 4 that had lactate as the electron donor. The aim of this was to attempt to identify which types of organism had been enriched, and to compare any differences between bacteria observed at pH7 and pH4.

The method followed was that of Bauer (lab exercise). Probes used were:

a) SRB, Flavo, α, β, δ and universal probes for 45°C hybridization temperature.
b) γ (enteric), Archea and universal probes for 37°C hybridization temperature.

Bacterial Isolations

Agar shake series were made according to the method of Jorg Overman for sulphate reducing bacteria but again with no sulphate present and the phosphate present at 20mM (see Marine medium above). Isolations were made from enrichments with the greatest growth, and from both pH7 and pH4 to enable a comparison to be made between bacteria that are capable of growing at these pH conditions. Isolations were made under the same conditions of pH/with the same electron donor as the enrichment from which the innoculum was derived. The tubes were incubated in the dark at 30°C.

HPLC analysis for phosphate and phosphite

Phosphate and phosphite were measured by HPLC (Waters) equipped with an anion exchange (IC PAK A HC) column eluted with gluconate/borate. The eluens was made up as follows: A stock solution X was made by dissolving 16g sodium gluconate, 18g boric acid, 25g sodium tetraborate decahydrate (Borax) and 250ml glycerol in 1litre of ultrapure water. The eluens was made by mixing 20ml stock X, 20ml 1N butanol and 120ml acetonitrile in a total of 1litre ultrapure water. The eluens was filtered through a 0.22μm filter and degassed before use. The flow rate of the HPLC was 2ml/minute. The detector was a 430 Waters conductivity meter running at 35°C.

Samples selected for HPLC phosphate analysis were B enrichment with electron donor source of lactate or acetate and E enrichment with lactate as the electron donor. Of all the enrichments these demonstrated the greatest growth. The 2 negative controls in this experiment were the A enrichment with lactate (which showed very poor growth) and the E enrichment with formate with sustained no growth at all.

Enrichment on organic phosphate

The same method was employed as for enrichment on inorganic phosphate but in this case raw sewage was the only innoculum used and only organic phosphate sources were added to the enrichments. Phytic acid or phosphatidyl choline (dissolved in reagent grade alcohol) were added to the medium to a final concentration of 10mM. The sodium chloride concentration in the medium was reduced to 1g/l NaCl.

The ultimate experiment

Phosphine is "spontaneously flammable in air if there is a trace of P_2H_4 present and burns with a luminous flame. It combines violently with oxygen" (Merck index). Therefore, given that the ideal analytical tool, a GC method to detect phosphine gas, was not available, the ultimate experiment in this project was to attempt to ignite the gases that may have been evolved during the enrichment procedure. This was done by opening the serum bottles and waving the neck across a flame.

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RESULTS AND DISCUSSION

Enrichment on inorganic phosphate

After 8 days the enrichments were analyzed for turbidity. The most turbid enrichments were those derived from innoculum B, the black sulphate reducing layer from Sippiwissitt salt marsh. No growth was observed in the enrichments with the sewage innoculum, and only a black precipitate (with no bacterial growth) was observed in the enrichment from innoculum F, the rusty nail. The enrichments using sulphate-reducing mud sample E showed a little growth. Table 1 below shows the levels of growth observed in the B enrichments.

pH	Electron donor	Growth
7	Lactate	+
7	Acetate	+
7	Hydrogen	-
7	Sulphite	-
7	Formate	-
4	Lactate	++ (spirillum)
4	Acetate	+
4	Hydrogen	-
4	Sulphite	+
4	Formate	+

' + '= growth, ' -' = no growth

Table 1. Growth in primary enrichments for phosphate reducing bacteria with SRB mat black layer as the inoculum.

It should be noted that this is a relative scale and compared to a culture of say E. Coli on nutrient broth, the culture was very thin, with most of the turbidity derived from precipitation of inorganic material. The bacteria present were all small rods or cocci, but spirillum were observed in the enrichment at pH 4 with lactate as the electron donor (indicating that they may not be as fragile as they often appear to be). The enrichment procedure is selective *ie* growth is not observed in every bottle. There is more growth at low pH and acetate or lactate seems to be the preferred electron donors.

Secondary enrichment from B primary enrichments did not have time to fully develop but at the time of writing the only enrichment that had grown at pH 7 was that with acetate as an electron donor. The controls (with either no electron donor, no phosphate or with sterile innoculum) did not sustain growth. Secondary enrichments at pH 4 showed a small amount of growth with lactate and sulphite as electron donors, but growth was observed in the control with no electron donor added.

After incubating for 13 days the other enrichments were studied under the microscope to establish whether the turbidity that was often present was due to precipitation of some the inorganic components of the medium or due to bacterial growth. The following observations were made:

Enrichments A (sewage inoculum)
Small amounts of growth were observed at both pH 4 and 7.

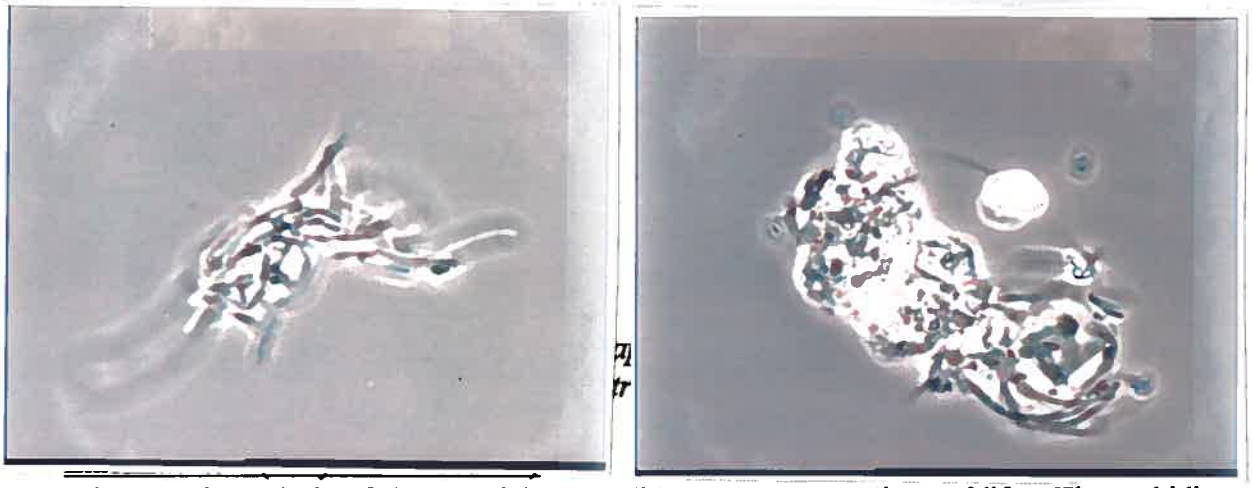
Enrichments C and D (lab collection of SRB enrichments as innoculum)
Trace amounts of growth were observed with lactate as the electron donor, but there was no growth with acetate as the electron donor.

Enrichments E (SRB mud as innoculum)

pH	Electron donor	Observation
4	Lactate	++ Rods and cocci. Long filamentous organisms wrapped around sediment (figure 1)
4	Acetate	-
4	Hydrogen	++ Short rods and cocc.
4	Sulphite	-
4	Formate	-
7	Lactate	++ Rods and cocci
7	Acetate	-
7	Hydrogen	-
7	Sulphite	++ Rods & cocci & the occasional long spirochete
7	Formate	-

Table 2. Growth in enrichments E (with SRB mud as the innoculum).

Please list.



Microscopic analysis of these enrichments did not detect any signs of life. The turbidity present in the flasks was due solely to precipation.

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Bacterial isolations

After an incubation period of 7 days growth could only be observed in medium of pH 7 (from enrichments of pH 7) at dilutions of 10^{-1} to 10^{-3}. The round white colonies were only present at the top centimetre of the agar (Fig.3).

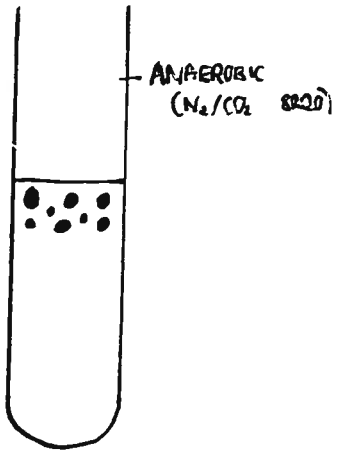

Figure 3. Colony formation in the agar shake tubes at pH 7.

Some colonies were noticibly compact whereas others were more diffuse. The 2 colony morphotypes were analysed under the microscope (Fig. 4).

Compact white colonies:
'Pure' culture of long rods

Diffuse colonies:
Long rods and highly motile cocci

Figure 4. Composition of the colonies derived from the agar shake series.

Time constraints did not allow further analysis of these cultures.

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In situ hybridization

The bacteria present in the enrichment at pH 4 and pH 7 tested positive with only the SRB probe and the universal probes. The bacteria present in these enrichments are predominently sulphate reducing bacteria (this is hardly suprising given that the innoculum for the enrichment was sulphate reducing mud). As can be seen in fig. 2 some cells fluoresced brighter than others but this was also true of the universal probe too. There was no observable difference between the bacteria from the enrichment at pH 4 and pH7. The cells were mostly small rods and cocci which were very skinny compared to the *Rhodospirillum centenum* used as a positive control organism.

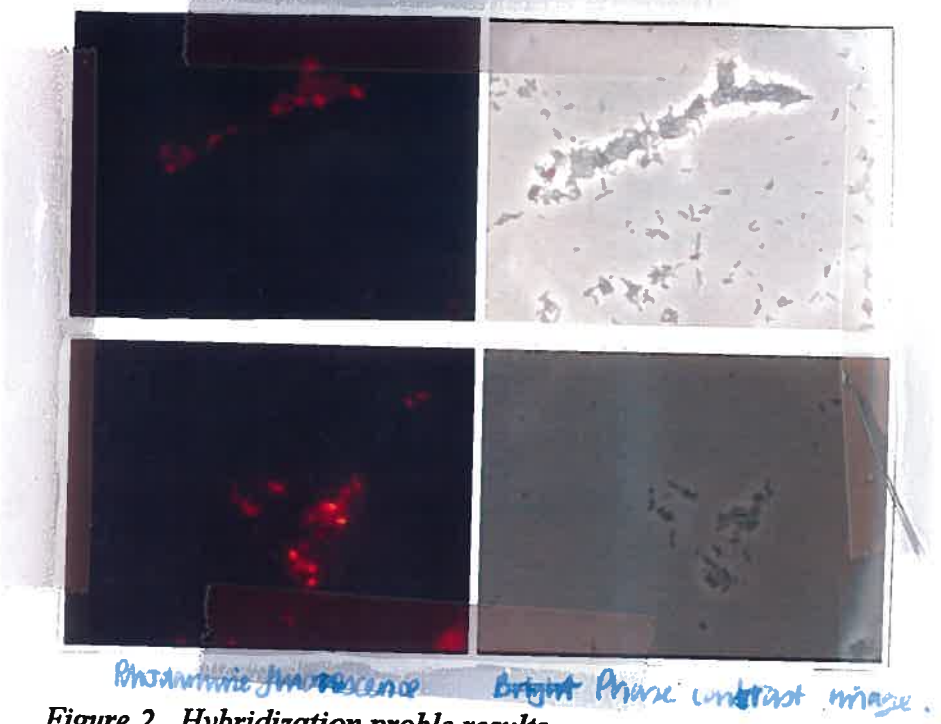

Figure 2. Hybridization proble results

Enrichment on organic phosphate

With phytic acid as a phosphate source growth was observed in all the enrichments, including, unfortunately, the controls (except for the one with sterile innoculum). It thus appeared that organisms within these enrichments were capable of growing at the expense of substrates other than those intended. Again, significant growth was observed at pH 4 and 7, and spirochetes were observed in the enrichment at pH7 with lactate as the electron donor.

Phospholipid, when added to enrichment medium, formed a milky white immulsion. It was therefore difficult to assess by eye whether bacterial growth had occured in these enrichments. Microscopic analysis revealed that any turbidity was due to lipid emulsion not to bacterial growth.

HPLC analysis for phosphate and phosphite

Phosphate (in the form of NaH_2PO_4 pH7) was easily and accurately determined by this method. However no hypophosph,ite could be detected (I used standards of low concentration up to100μM because I predicted that if any phosphate had been reducted, at this early stage in the enrichment only low levels of the reduced form would be found). Levels of phosphate measured in certain enrichments are shown below in Table 3:

Sample	Growth?	[Phosphate] mM
B lactate pH 7 enrichment	+	19.46
B acetate pH 7 enrichment	+	22.68
E lactate pH 7 enrichment	+	22.26
A lactate pH 7 enrichment	trace	22.33
E formate pH 7 enrichment	-	15.54

Table 3. Phosphate concentrations of selected enrichments after X days on incubation.

Phosphate levels do not decrease in proportion to the ammount of growth observed. The large discrepancies in the concentration of phosphate measured can be explained in terms of the amount of phosphate-containing precipite forming in a given enrichment. Phosphate readily forms insoluble precipitates with calcium and magnesium even when these ions are present in very low levels. This precipitation renders the phosphate undetectable in the aqueous phase of the enrichment from which the sample for HPLC analysis is derived. Given the small amount of bacterial growth in the enrichments, it is likely that the dominant factor governing the amount of phosphate measured is precipitation of the phosphate rather than biological processing.

The value of a measuring the amounts of phosphate used up in the enrichments as an indication of phosphate reduction is limited because it is difficult to measure of how much phosphate the cells would be using in 'normal daily life', not as an electron acceptor. Ideally the method would be applied to the secondary enrichments which would have had less carry-over of nutrients and for which suitable controls were available for comparison. However, not enough growth was observed in these enrichments to be able to observe significants decreases in the concentration of phosphate.

The ultimate experiment

Unfortunately (or fortunately for some) this experiment did not yield explosive results, or even one little luminous flame! 3 cultures seemed to make the flame go "phut" a tiny bit, but that could have just been due to air currents as the gas phase emerged from the serum flask or wishful thinking on my part.

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Conclusions

The results shown above neither confirm or rule out the possibility of biological phosphate reduction. They do however nicely demonstrate that bacteria were surviving/growing under very stringent conditions in terms of their choice of electron donor/acceptor and in terms of the pH in which they were placed. Given that estuarine bacteria require up to a week to switch on the necessary enzymic mechanisms and to grow suffiently to cause a significant decrease in levels of the highly biodegradable surfactant SDS, it seems very unlikely that I would have observed a significant reduction in the levels of phosphate in the enrichments given the limited time scale of the project.

It is quite reassuring to read that Devai (1988) had to wait 56 days before he could observe significant reductions in the phosphate levels in his *in vitro* experiments to detect phosphate reduction. However, the lack of futher publications from him on this topic would seem to indicate that maybe his enrichments for phosphate-reducing bacteria were not so sucessful after all.

Though phosphine is undoubtedly present in trace quantities in certain environments, the mechanism for its production remains a mystery. The secret of Will-O'the Wisp lives on.

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