Genetic Engineering of D. radiodurans for uranium bioremediation from high radiation environment

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Uranium resources

Primary

High grade ore: 2% U (20,000 ppm)

Low grade ore: 0.1% U (1000 ppm)

[India : 0.03-0.06% U (300-600 ppm)]

Secondary

Rock phosphate: 100-200 ppm

Monazite: 50-200 ppm

Carbonaceous matter: 300 ppm

Uranium in spent fuel

Acidic waste

< 1mM uranyl nitrate, pH 3-7

Alkaline waste

< 1mM uranyl carbonate, pH 7-10

Uranium in sea water

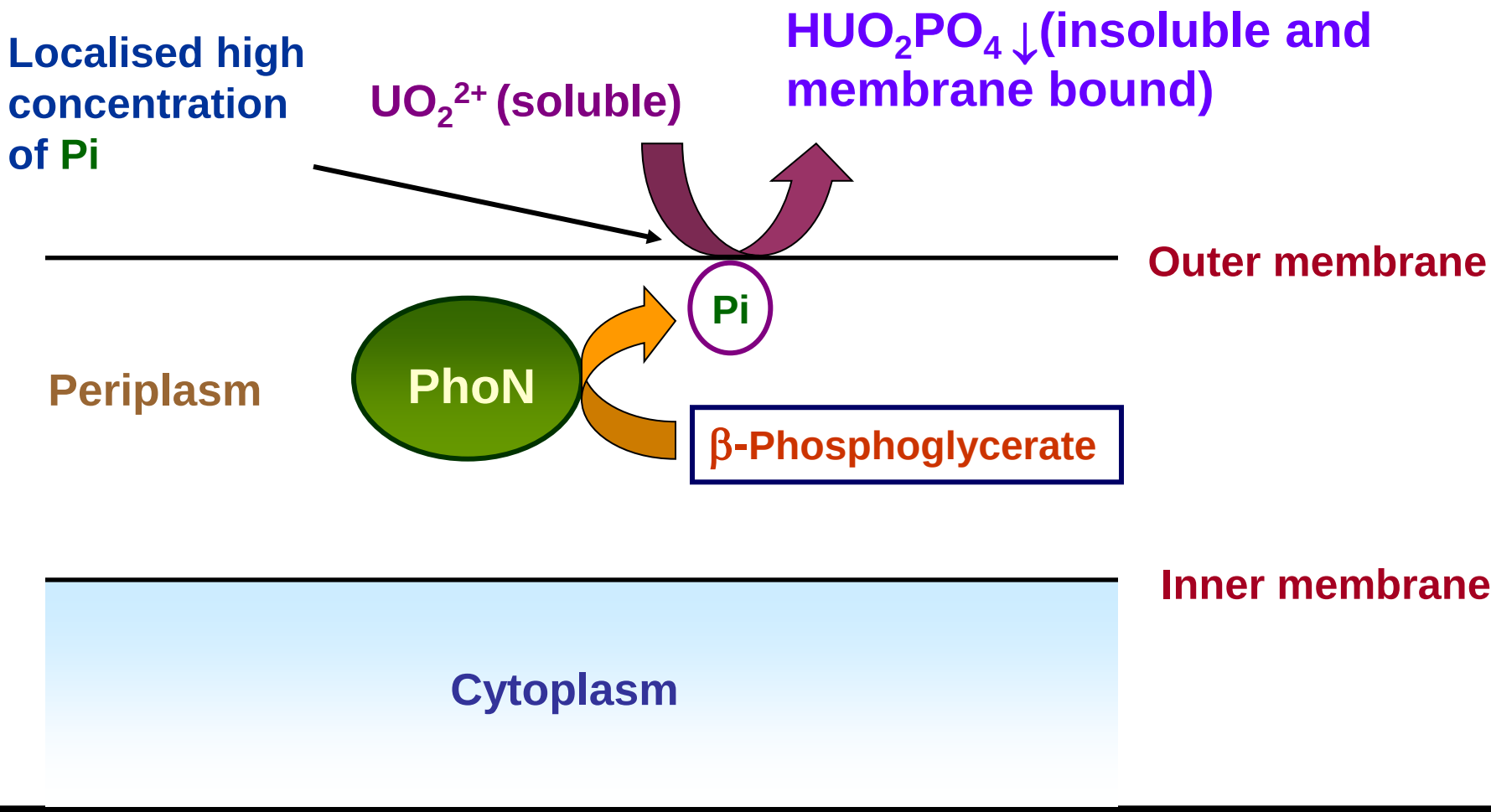
3ppb / 13 nM, pH 7.5-7.8

Di-/Tetravalent uranyl carbonate complex, $[UO_2(CO_3)_3]^{2-}$/ $[UO_2(CO_3)_3]^{4-}$

Total U in sea water: 4.5 billion tonnes (1000 X of terrestial ores)

Dilute solutions with 1- 4 mM uranium at pH 5-10 need to be addressed

Mechanism of metal precipitation by PhoN

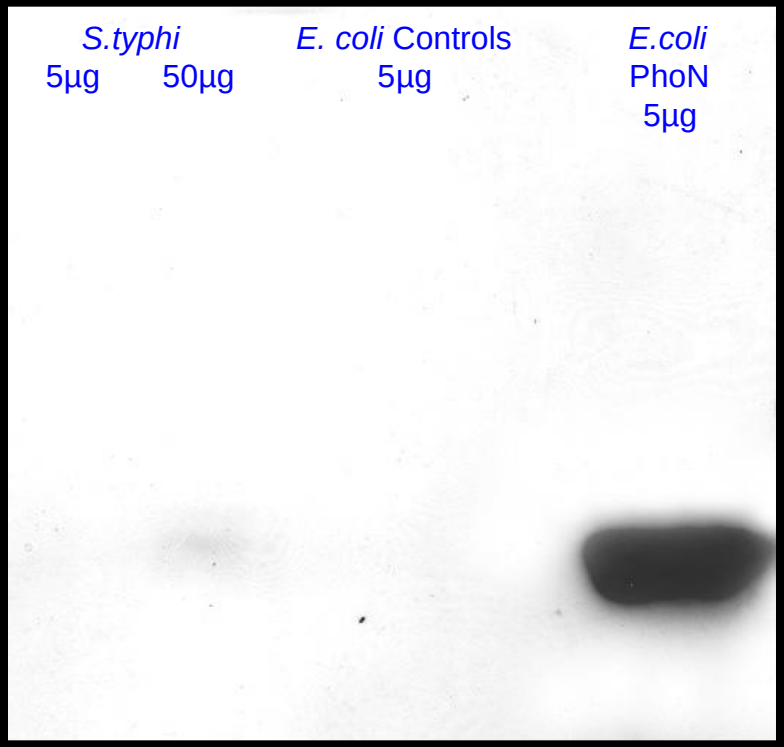


Engineering *E. coli* for PhoN Overexpression

Samonella phoN gene with its native promoter works well in *E. coli*

(Seetharam, Soundarajan, Udas, Rao and Apte, Proc. Biochem. 44: 246-250, 2009)

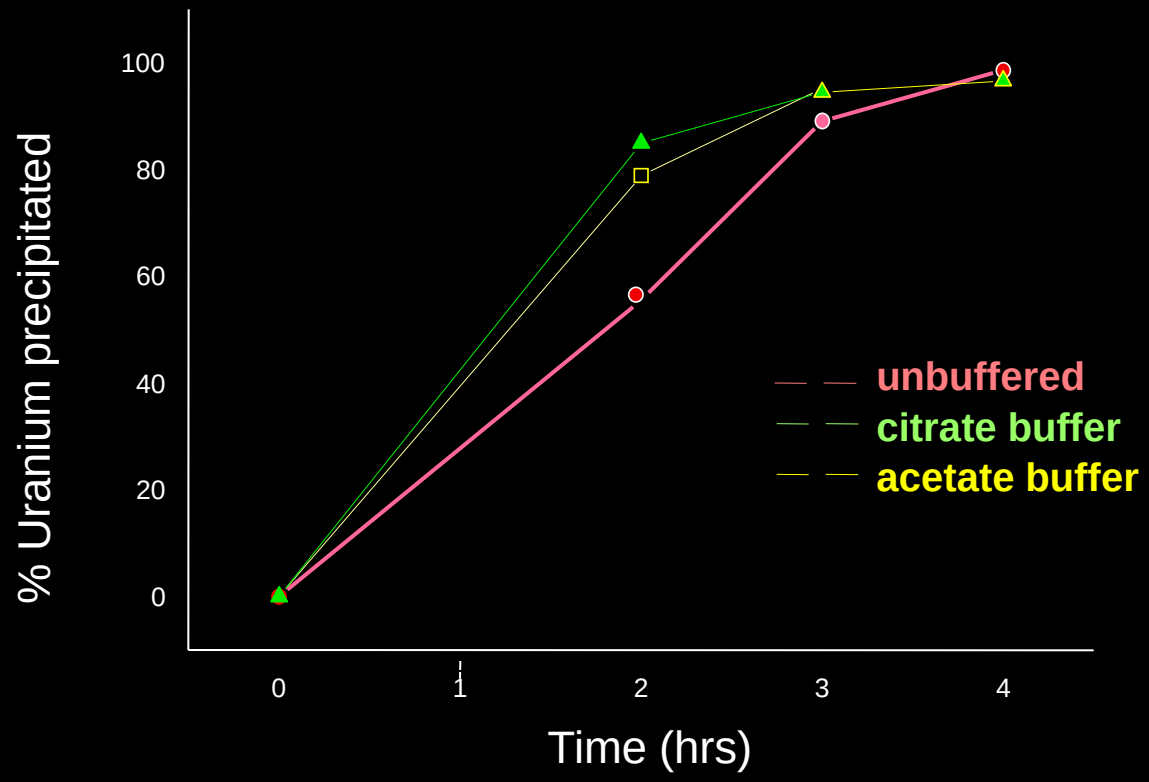
Over expression of PhoN



Multicopy plasmid (pUC19) based PhoN overexpression

URANIUM BIO-PRECIPITATION with BACTERIA

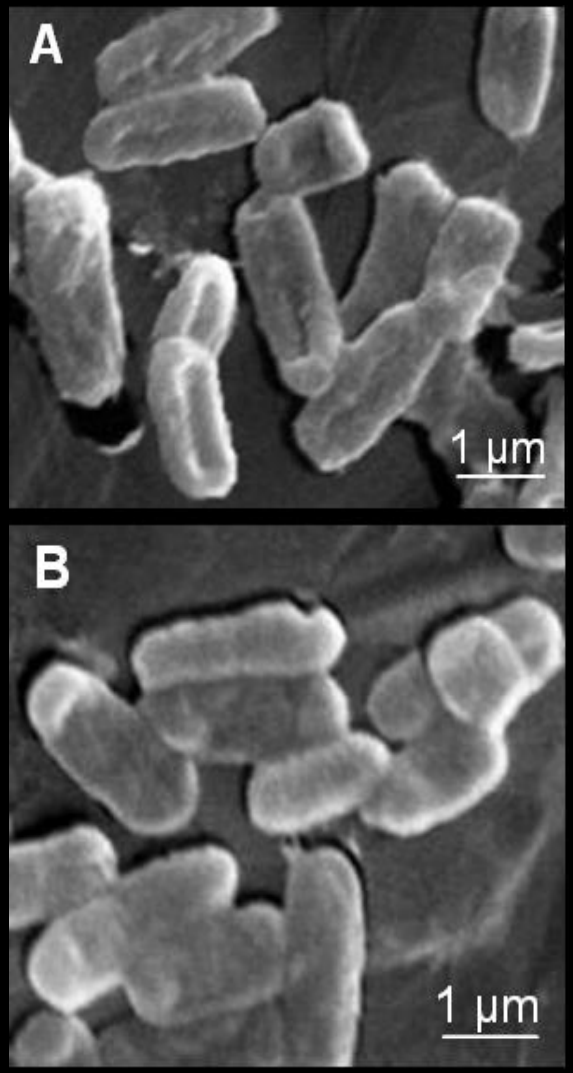
>95% URANIUM (1mM) IS PRECIPITATED
FROM AQUEOUS SOLUTIONS BY GM *E. coli*



VIABLE CELLS ARE NOT NEEDED

Post-lyophilisation Performance of E. coli bearing *phoN*

Cells retained their integrity and activities, but lost viability



System used	PhoN Activity	Metal removed (%)
Resuspended fresh *E. coli* cells bearing *phoN*	932 ± 43	83 ± 5
Lyophilized cells following storage for		
0 month	989 ± 59	83 ± 7
1 month	850 ± 89	ND
3 months	832 ± 16	ND
6 months	781 ± 20	79 ± 4

(Seetharam, Soundarajan, Udas, Rao and Apte, Proc. Biochem. 44: 246-250, 2009)

Column based uranium precipitation by *E. coli-phoN* clones

***E. coli*-pRAD1** ***E. coli*-pPN1**

INPUT SOLUTION

5 mM Uranyl Nitrate with10 mM β-glycerophosphate in 1.5 litres of 2 mM Acetate Buffer

COLUMN

200 mg of lyophilized cells immobilized in 15% polyacrylamide gel.

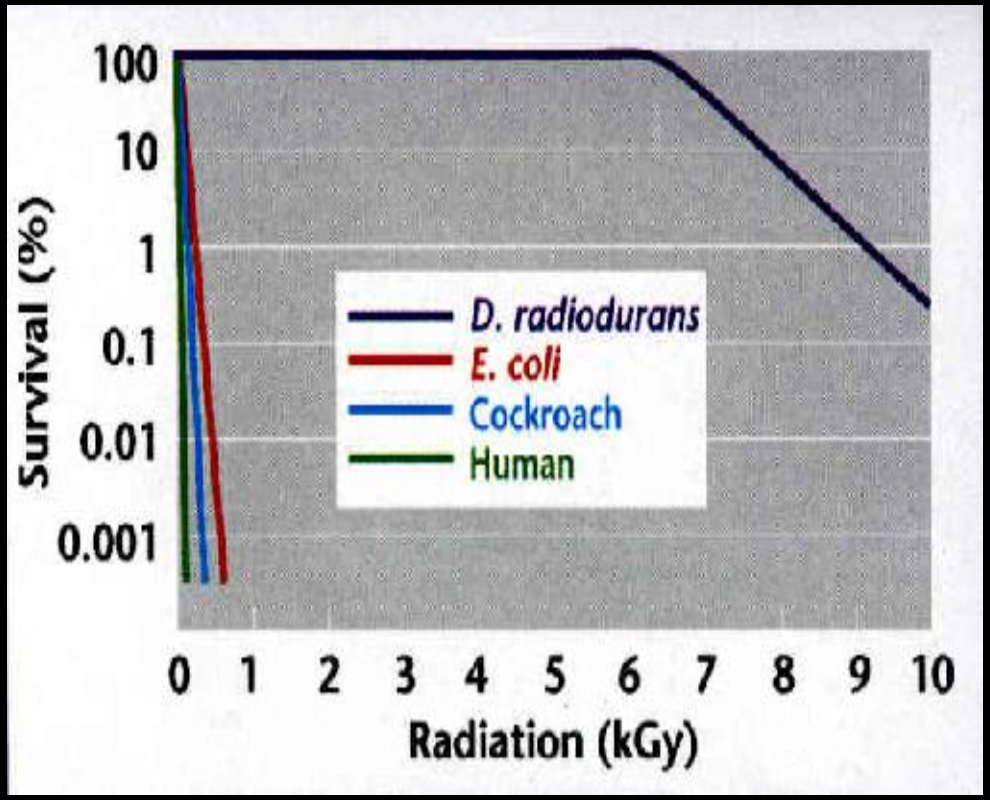
GEL VOLUME : 100 ml

Run time : 56 h

Total Loading on the column

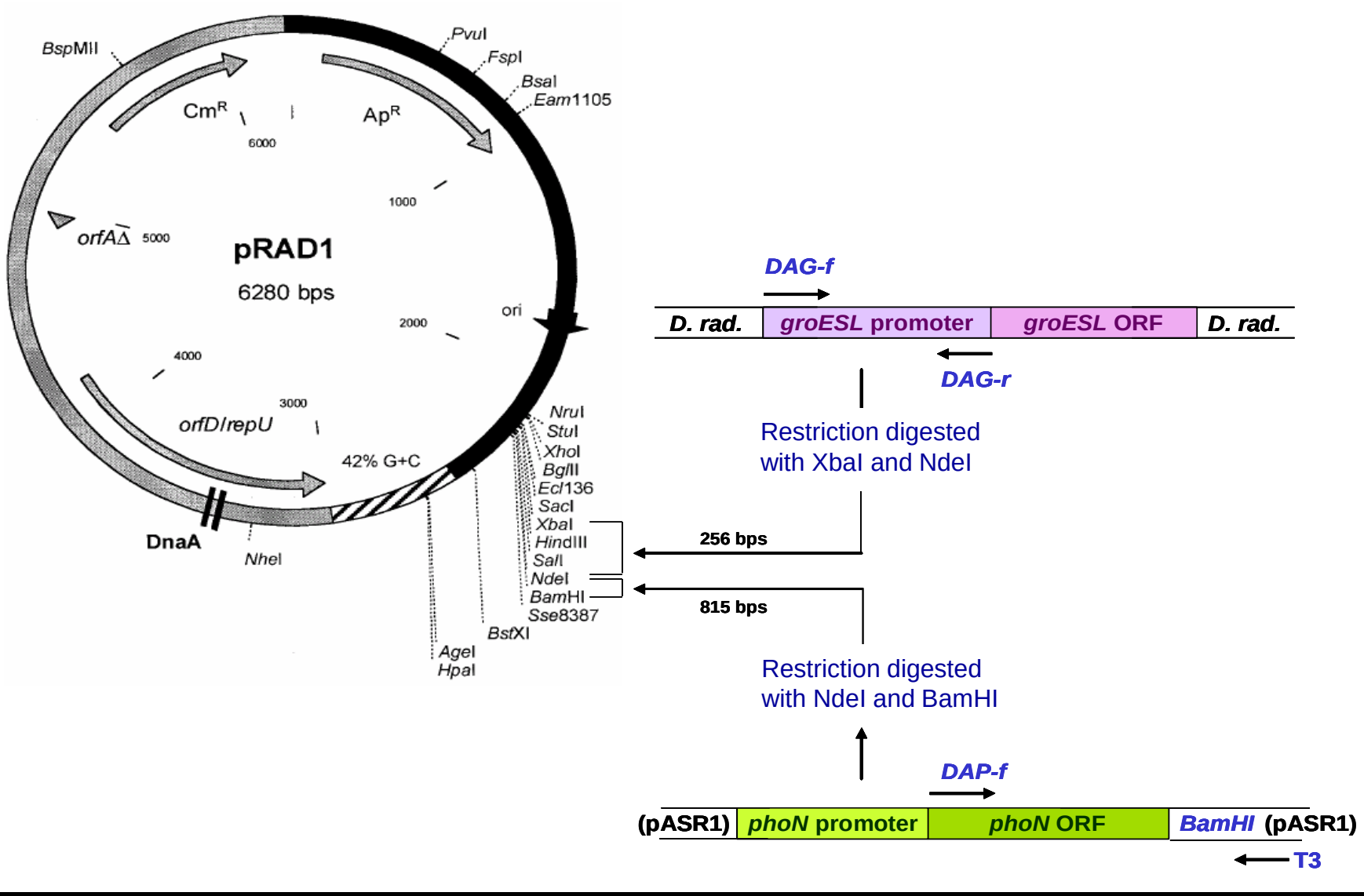
7.6 g of U/g dry wt. of cells

The Extreme Radioresistance of *Deinococcus radiodurans*



Provides opportunities for novel basic research and applications

Engineering *phoN* in *Deinococcus radiodurans*

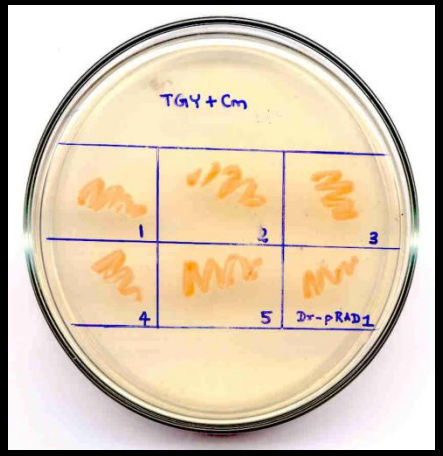


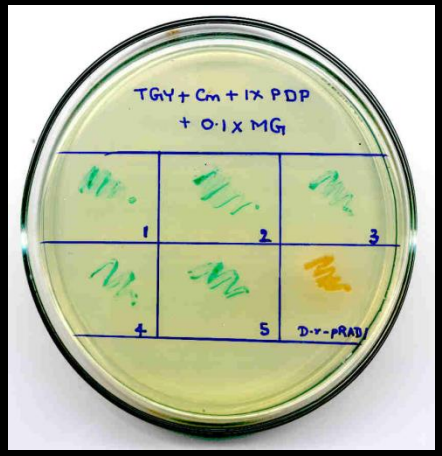
(Appukuttan , Rao & Apte, Appl. Env. Microbiol. 72: 7873-7878, 2006)

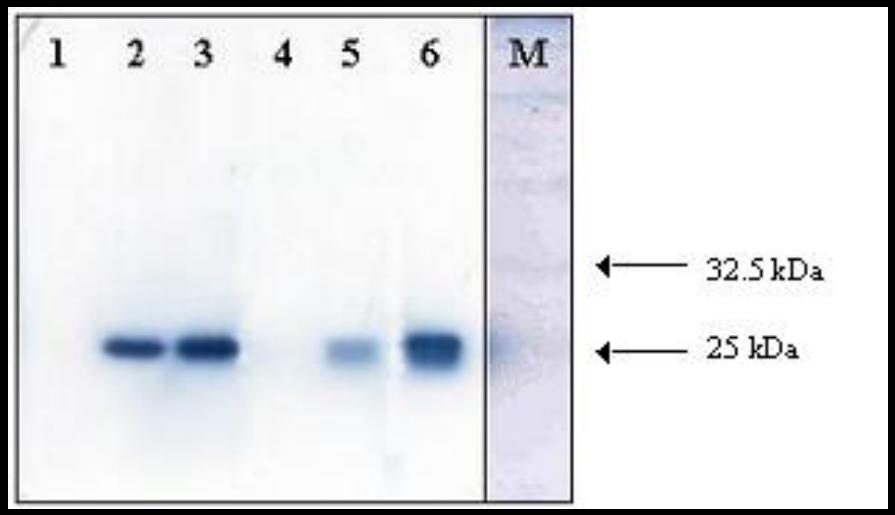
Genetic engineering of *phoN* gene into *D. radiodurans*

Deinococcus radiodurans

Wild type **Engineered**







1 - *E. coli* - pRAD1

2 - *E. coli* - *groESL+phoN* (GN)

3 - *E. coli* - full *phoN* (CL#50)

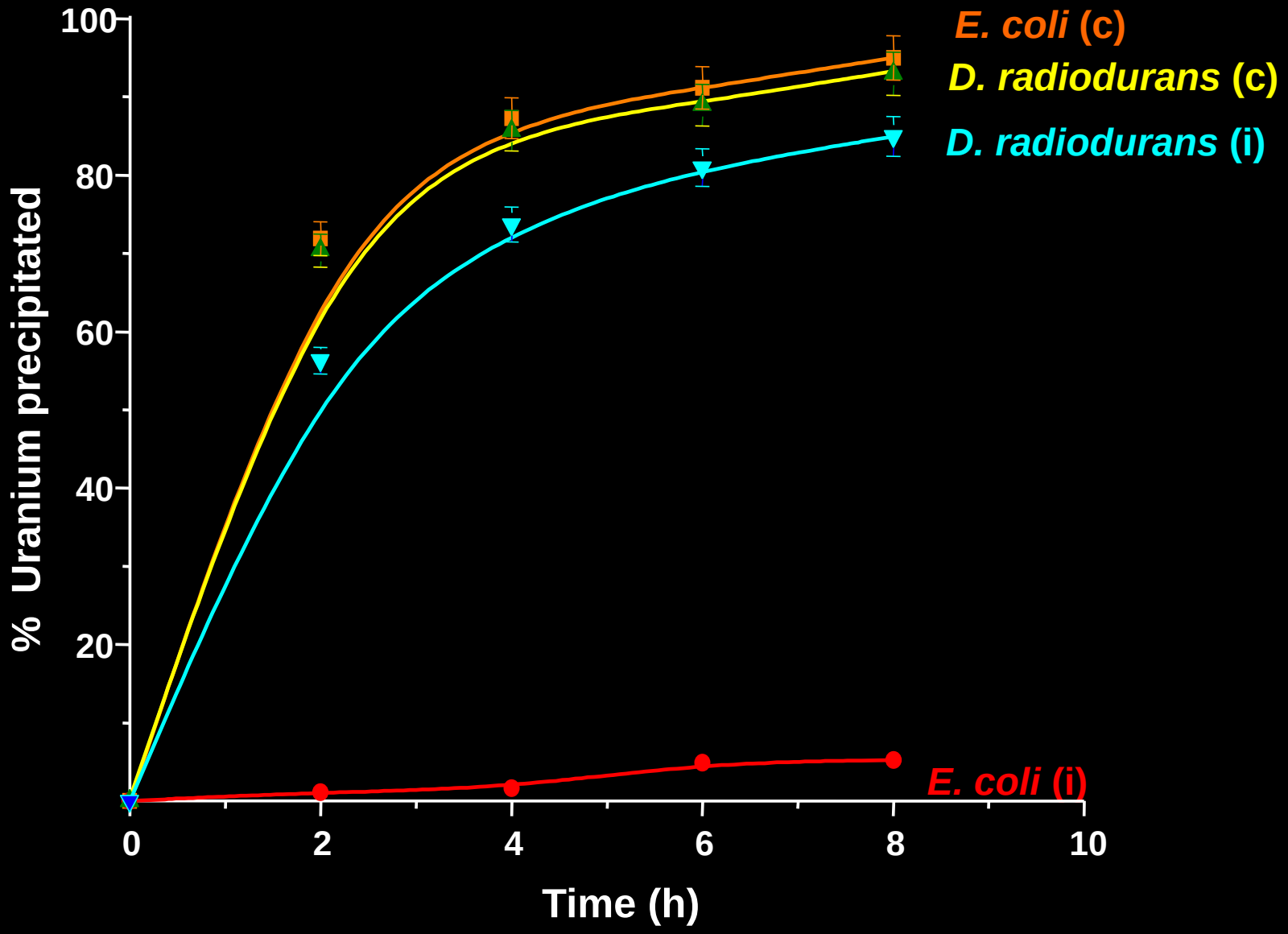
4 - *D. rad* - pRAD1

5 - *D. rad* - full *phoN* (CL#29)

6 - *D. rad* - *groESL+phoN* (DN)

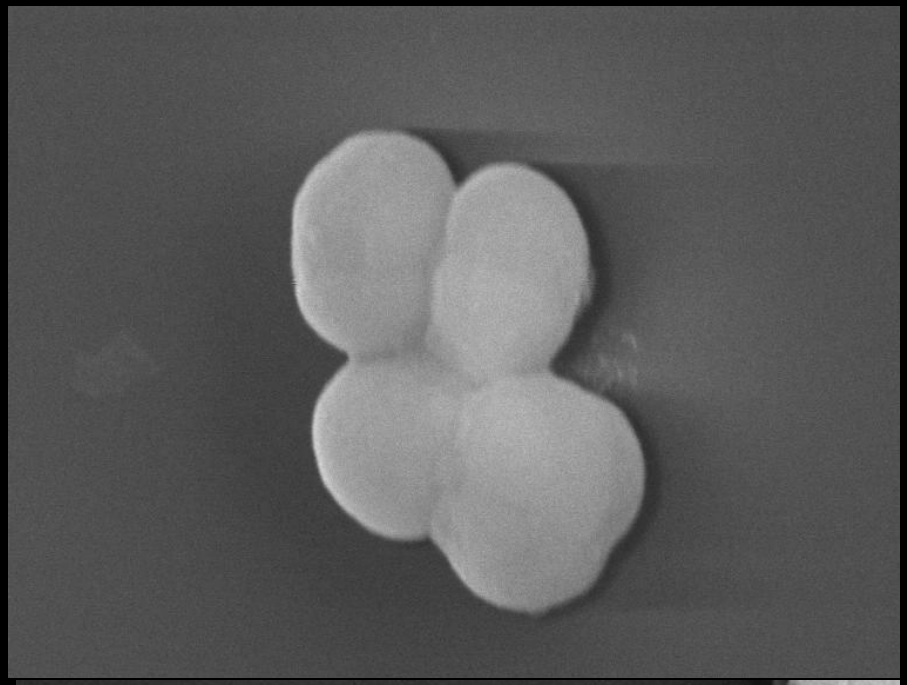
(Appukuttan , Rao & Apte, Appl. Env. Microbiol. 72: 7873-7878, 2006)

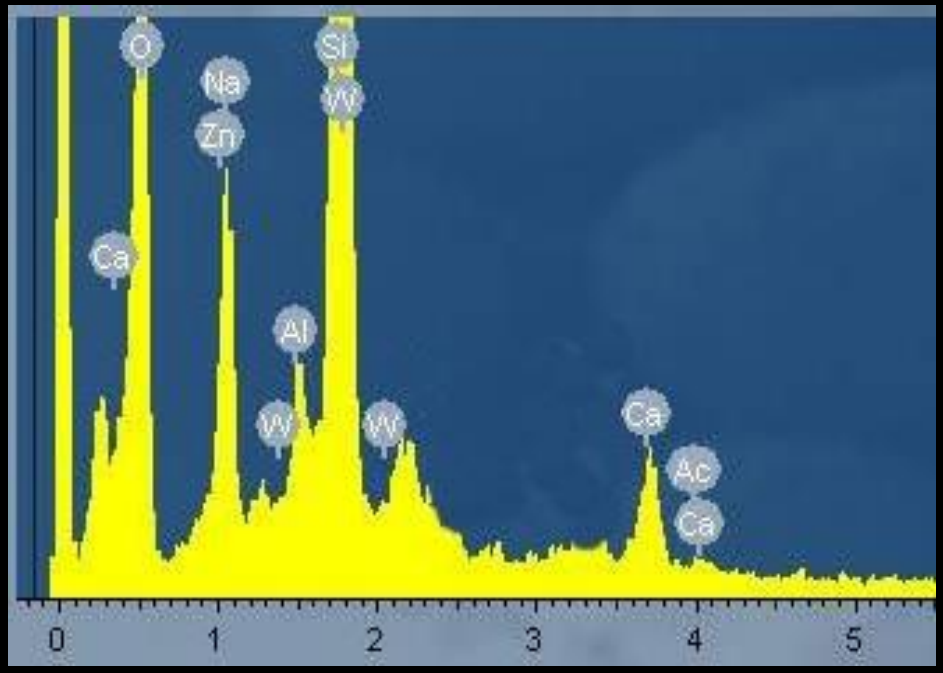
Uranium precipitation by *E. coli* and *Deinococcus* clones under 6kGy dose of irradiation

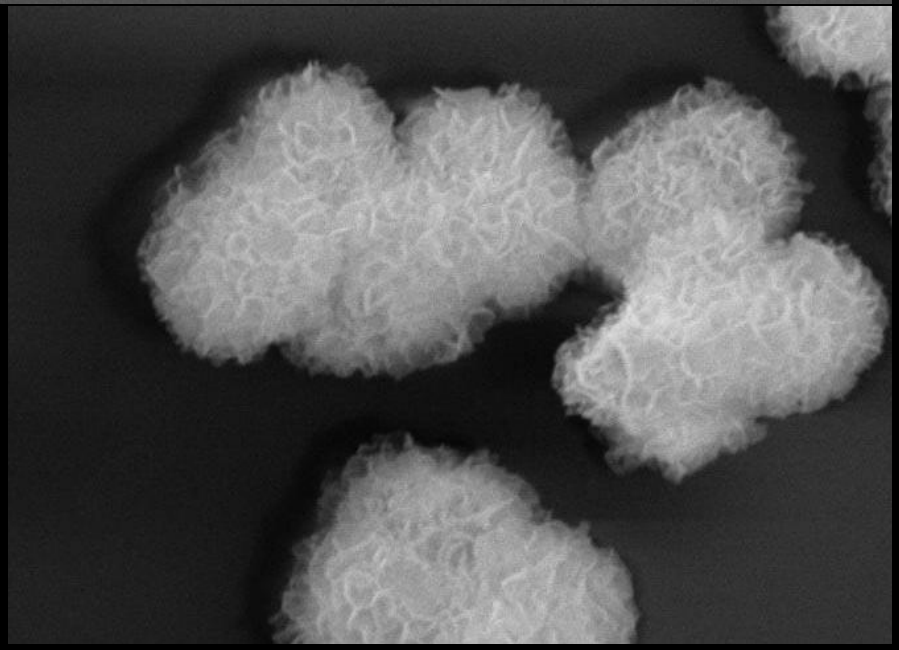


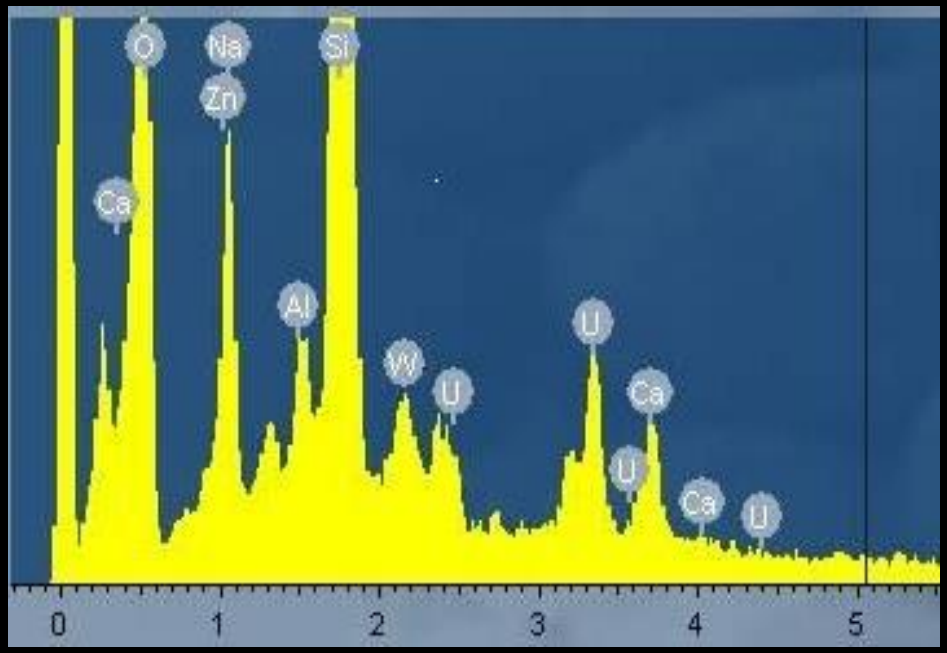
(Appukuttan, Rao & Apte, Appl. Env. Microbiol. 72: 7873-7878, 2006)

Cell-surface bound uranyl phosphate precipitate (SEM)



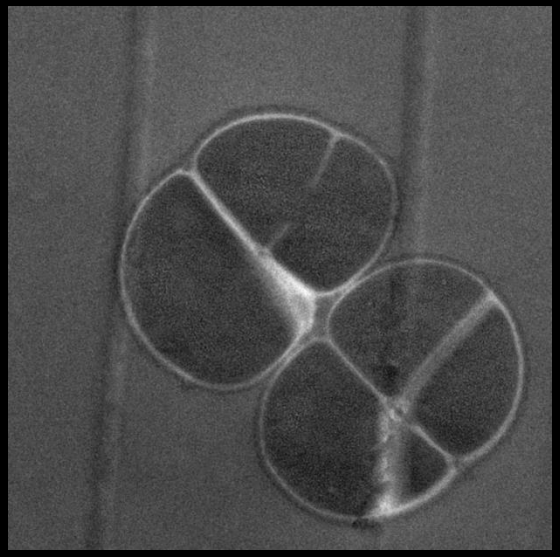


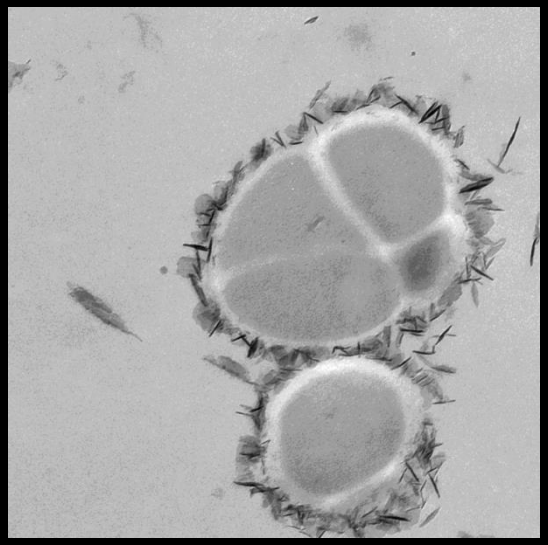


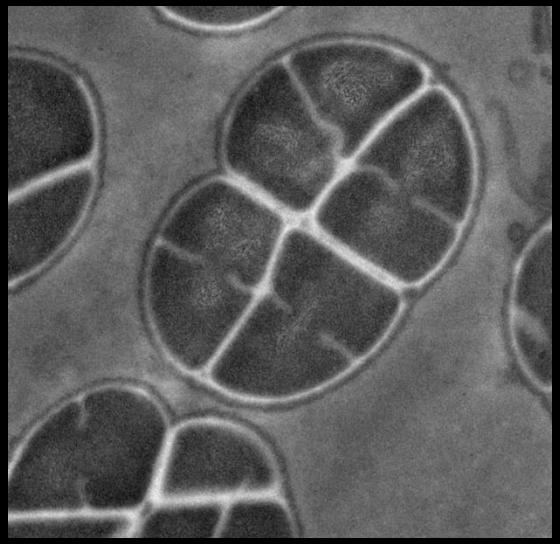


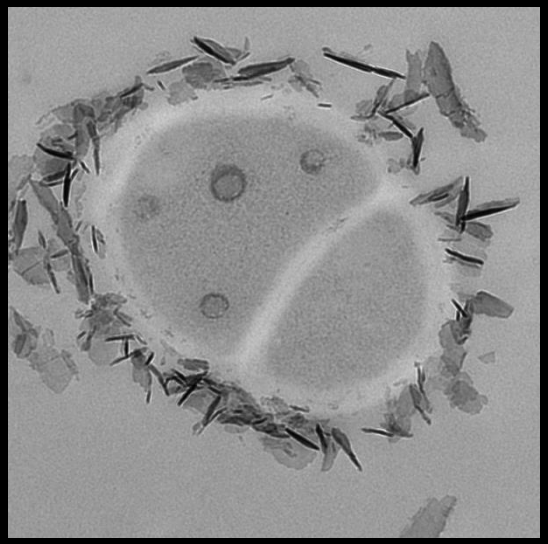
Seeing is believing …….

Cell-surface bound uranyl phosphate precipitate (TEM)

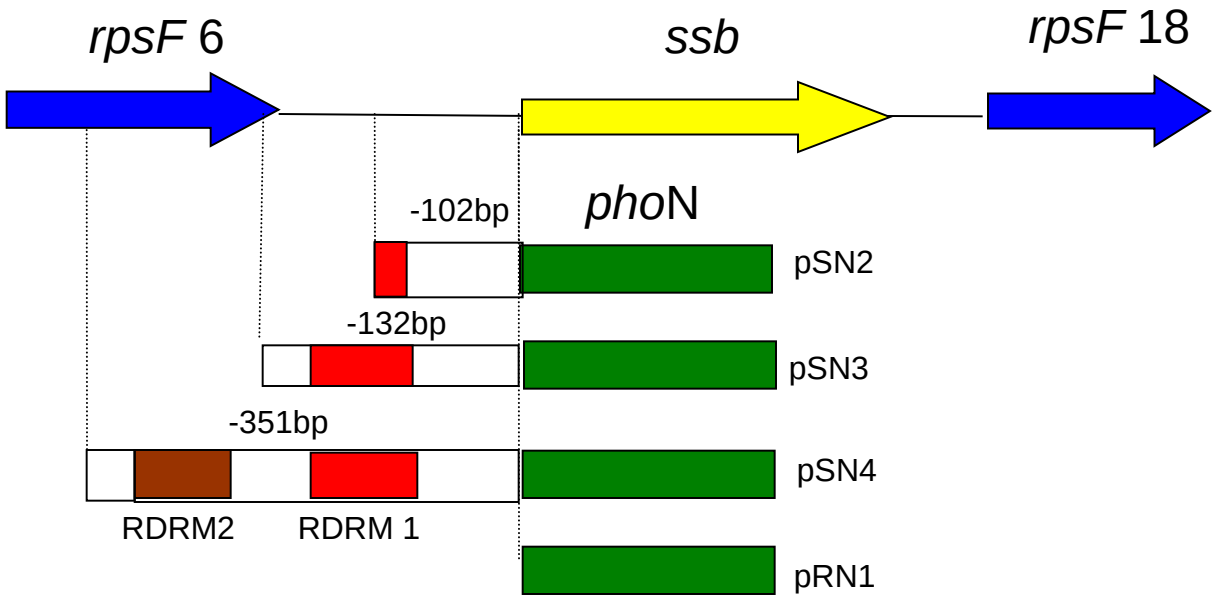


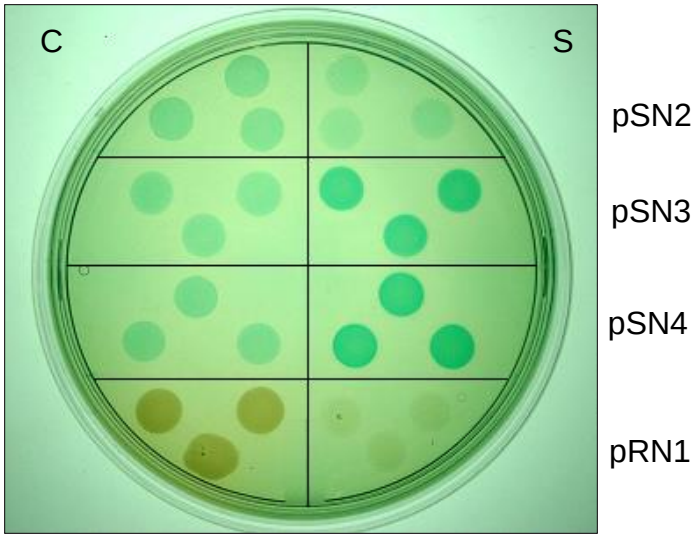




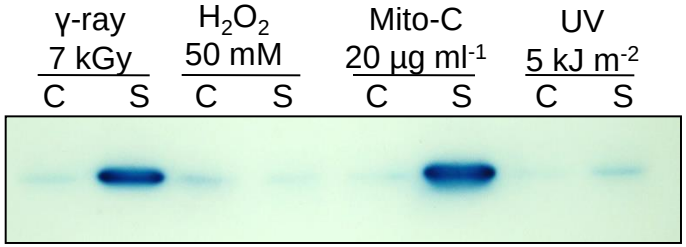


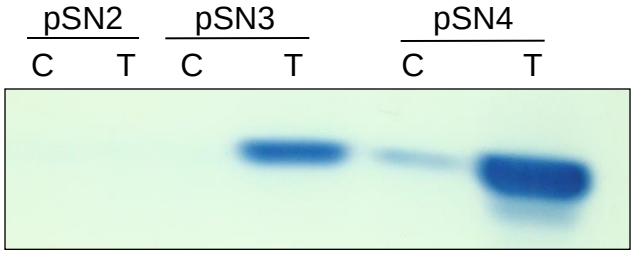
A Radiation responsive Deinococcal Promoter (P_{ssb})





^{60}Co, γ-ray (7 kGy)



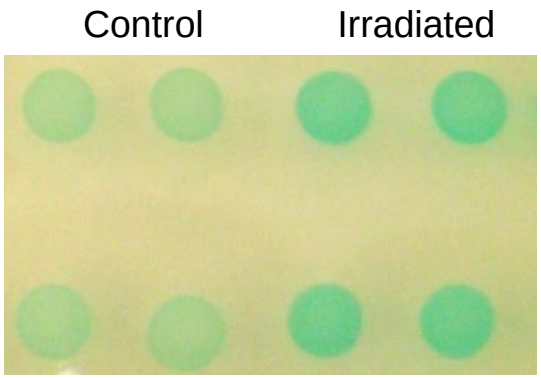


^{60}Co, γ-ray (7 kGy)

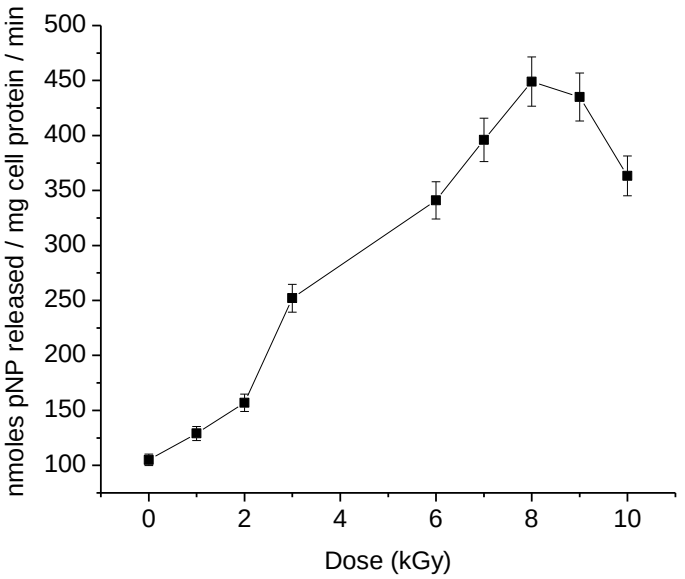
(Ujaoney, Potnis, Dani, Mukhopadhyay & Apte, J.Bacteriol., 2011)

Use of radiation-induced P_{ssb} promoter for U bioprecipitation

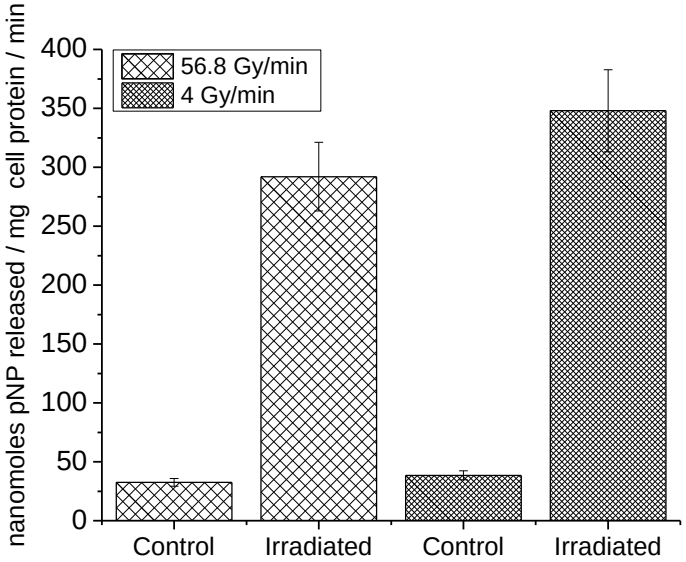
(a)



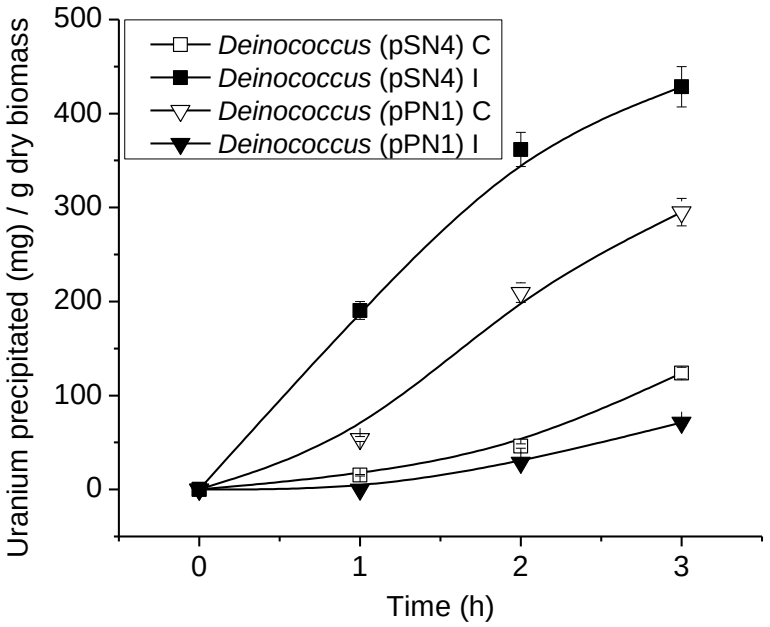
(b)



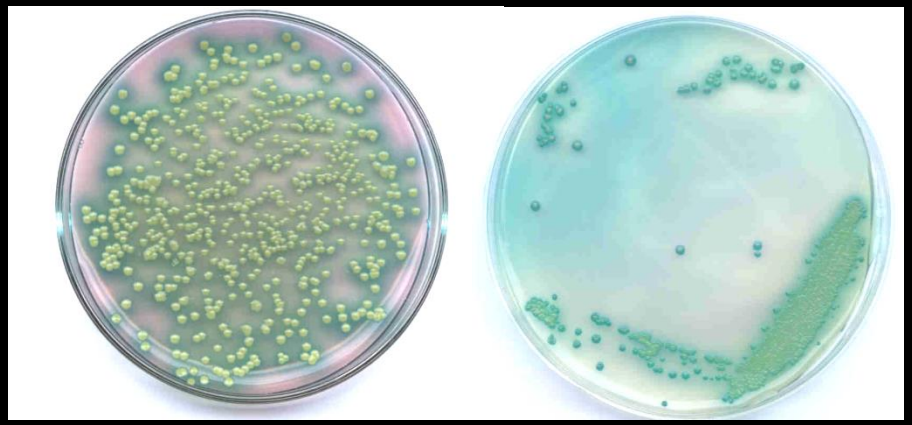
(c)



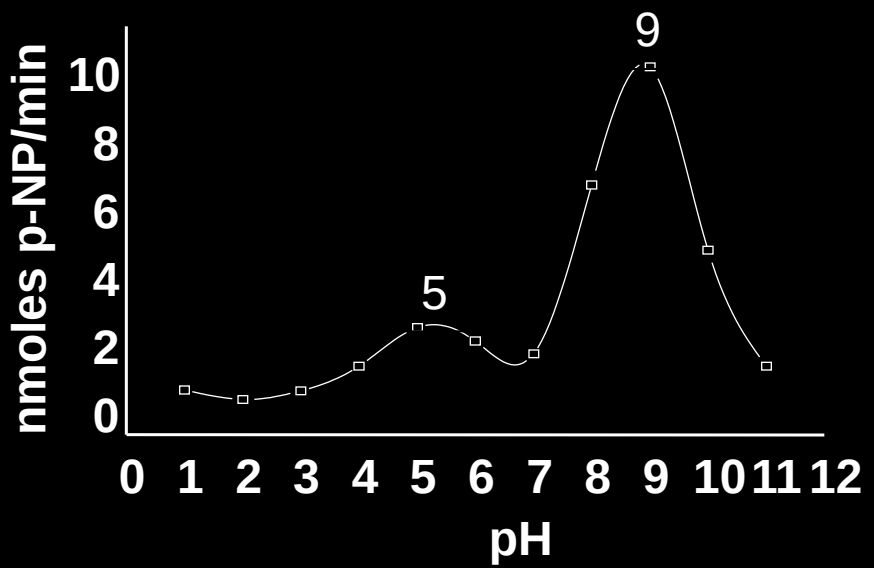
(d)



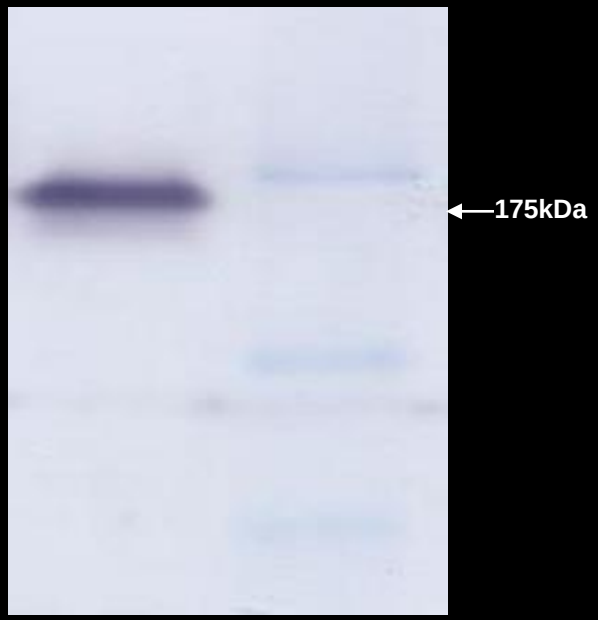
An Alkaline Phosphatase Over-producer Bacterial Isolate



Genetic Basis of this Enzyme Activity was investigated and cloned



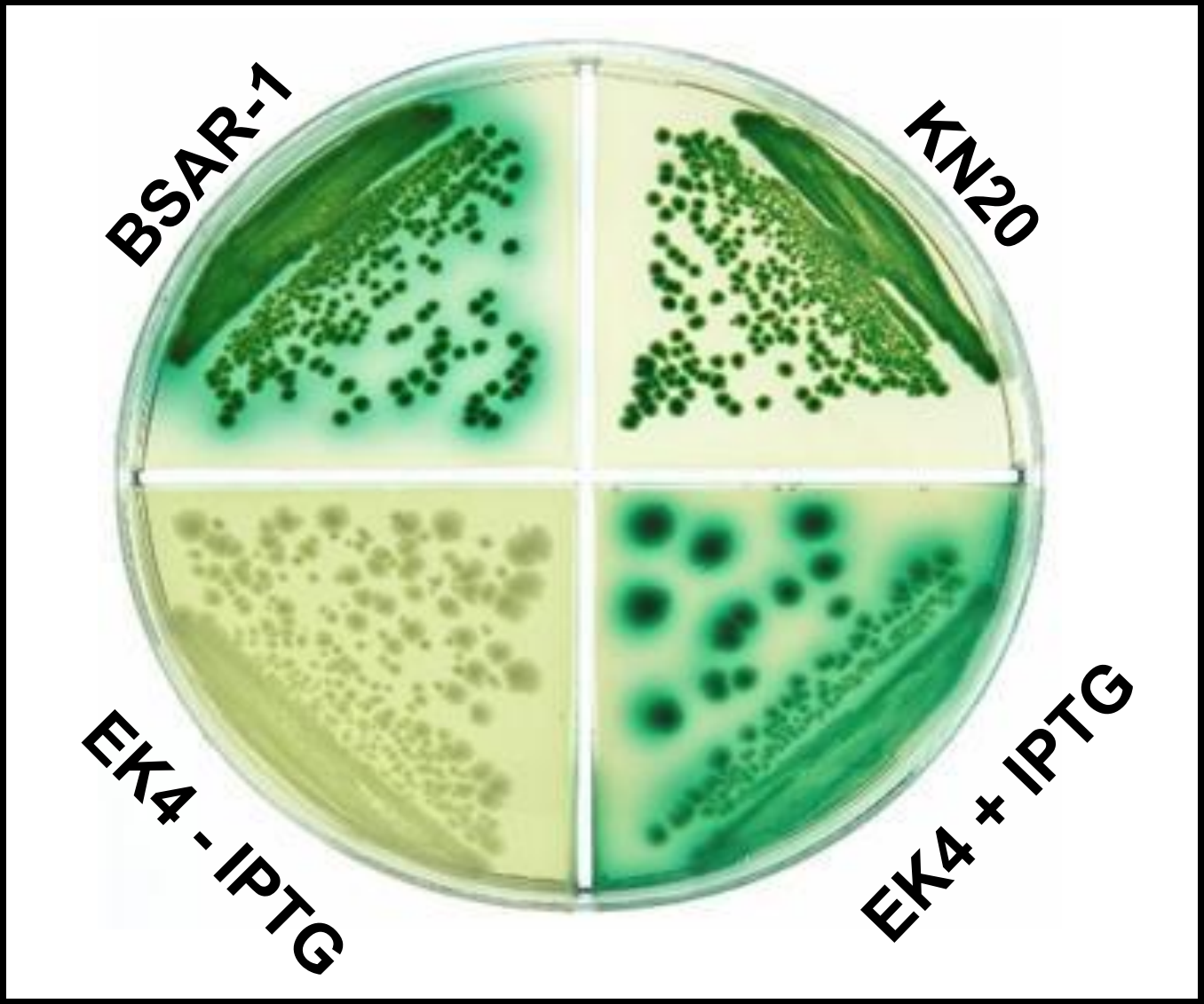
pH optima for acid and alkaline phosphatase of *Novosphingobium sp. BSAR-1*



Zymogram for alkaline phosphatase analysis

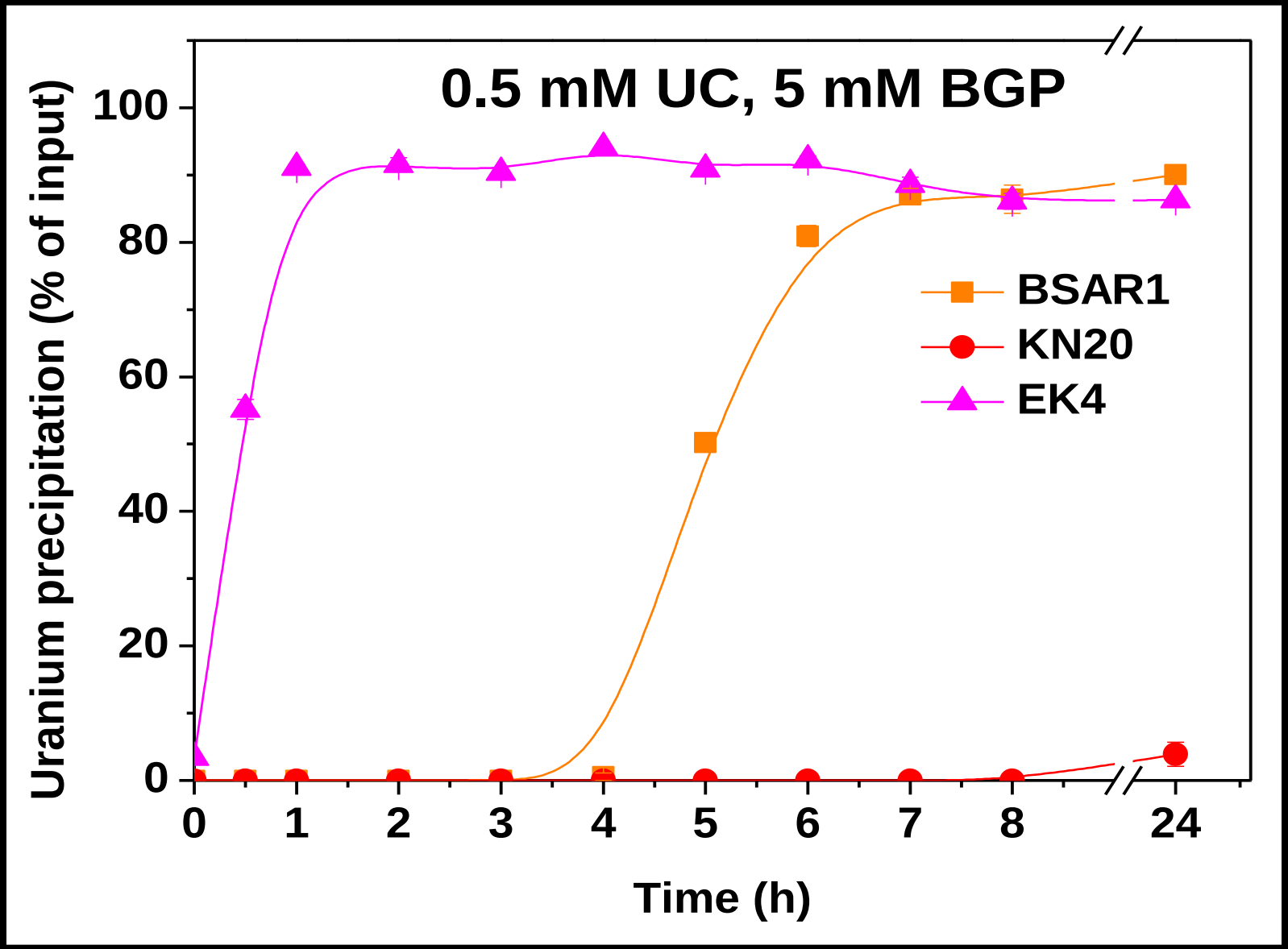
(Nilgiriwala, Alahari, Rao and Apte, Appl. Env. Microbiol. 74: 5516-5523, 2008)

Phenotype of various native and recombinant PhoK *expressing* strains



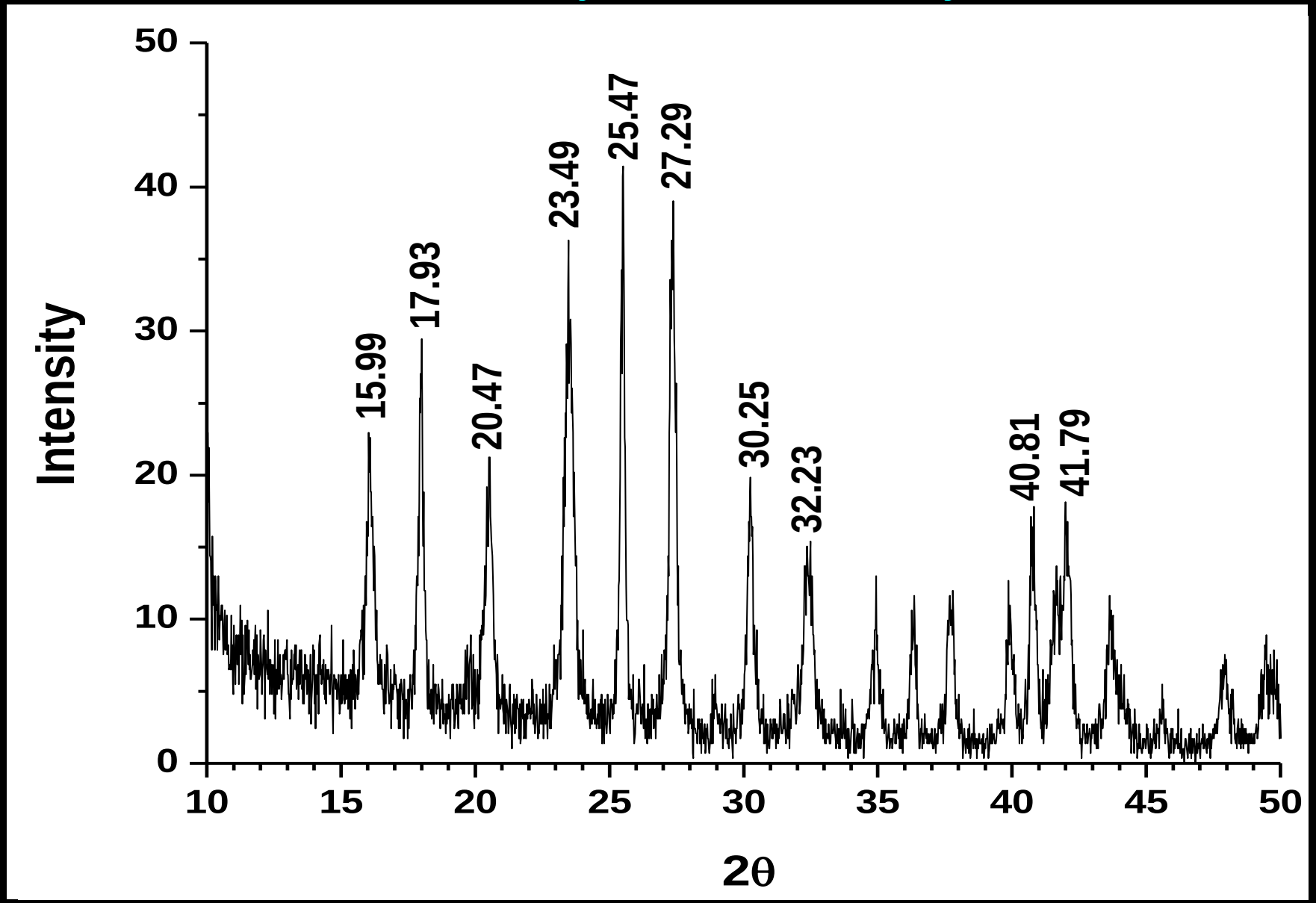
(Nilgiriwala, Alahari, Rao and Apte, Appl. Env. Microbiol. 74: 5516-5523, 2008)

Uranium bioprecipitation at pH 9.0 using PhoK



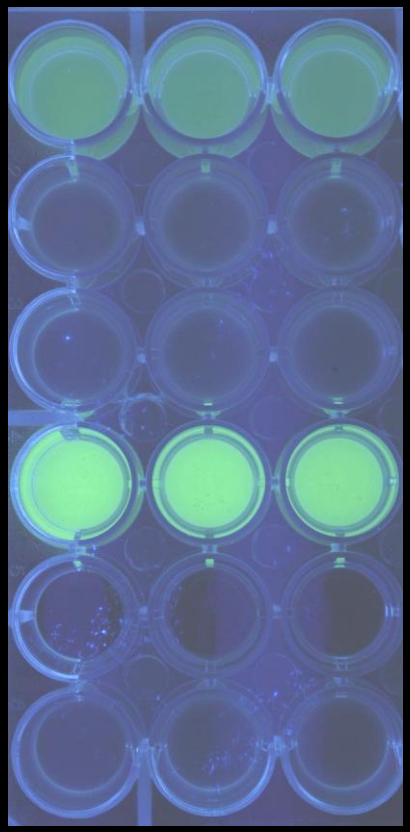
(Nilgiriwala, Alahari, Rao and Apte, Appl. Env. Microbiol. 74: 5516-5523, 2008)

Precipitate identified as $H_2(UO_2)_2(PO_4)_2.8H_2O$, metaautunite or chernikovite by Powder-XRD analysis



(Nilgiriwala, Alahari, Rao and Apte, Appl. Env. Microbiol. 74: 5516-5523, 2008)

Uranium precipitation at pH 9.0 using PhoK alkaline phosphatase



BSAR-1

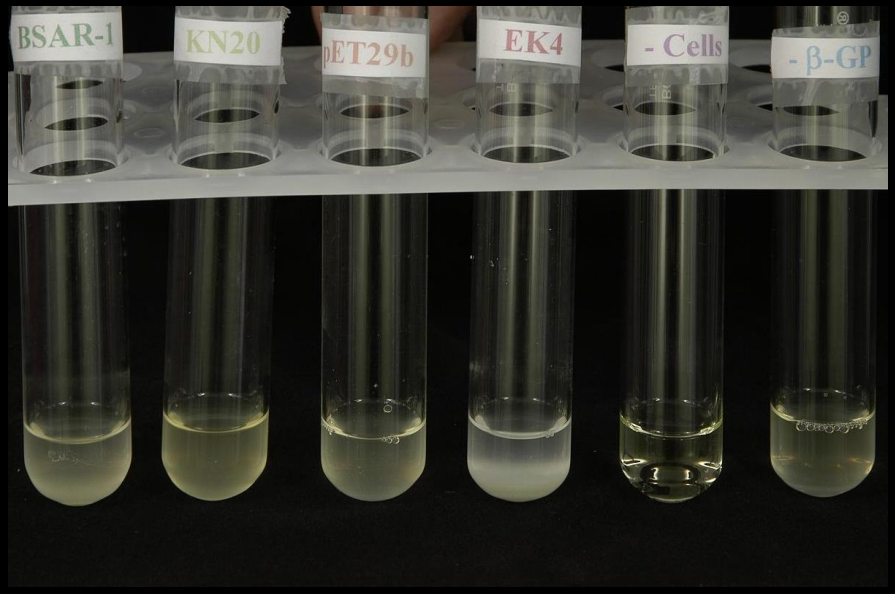
KN20

pET29b

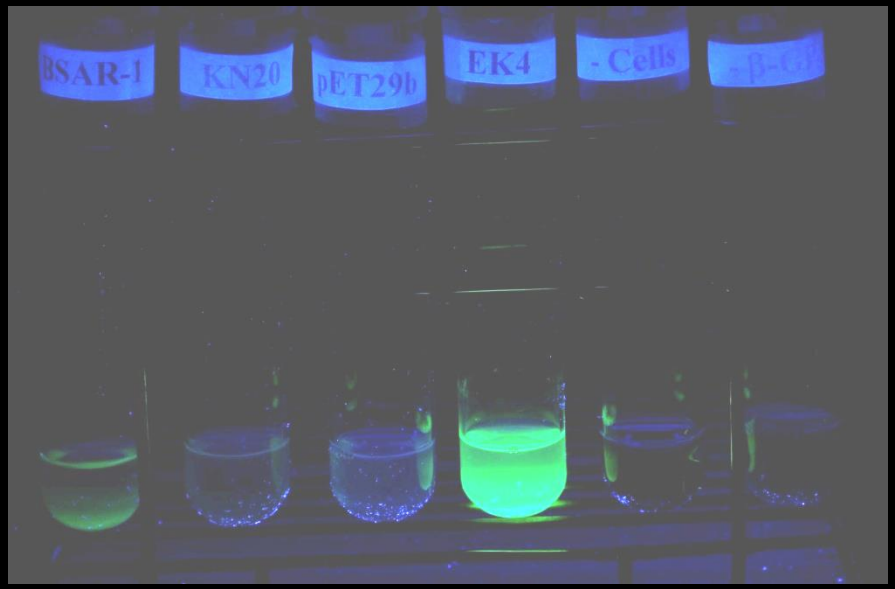
EK4

- cells

- βGP



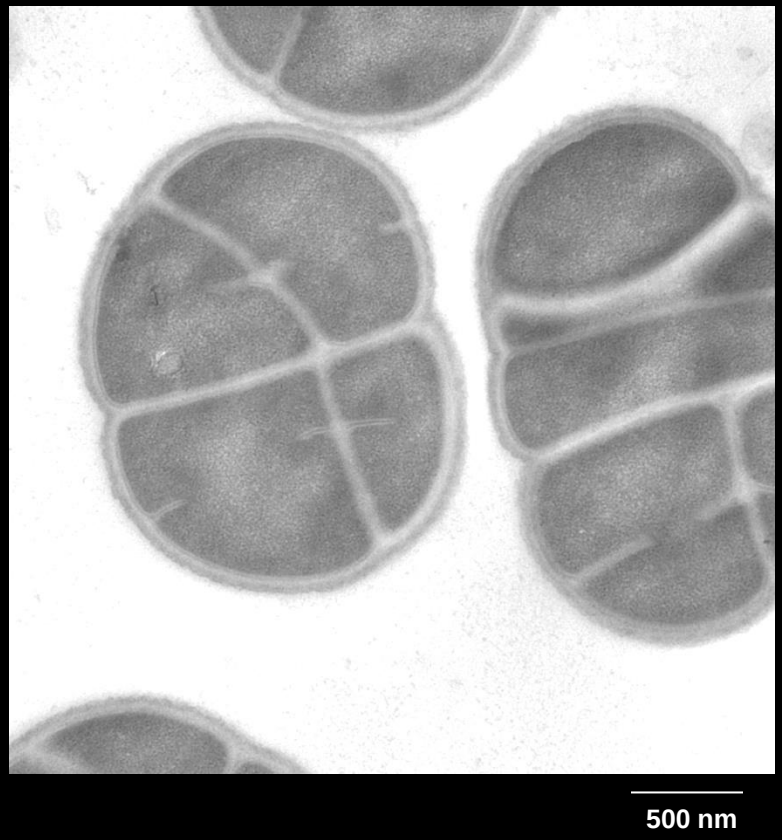
White light



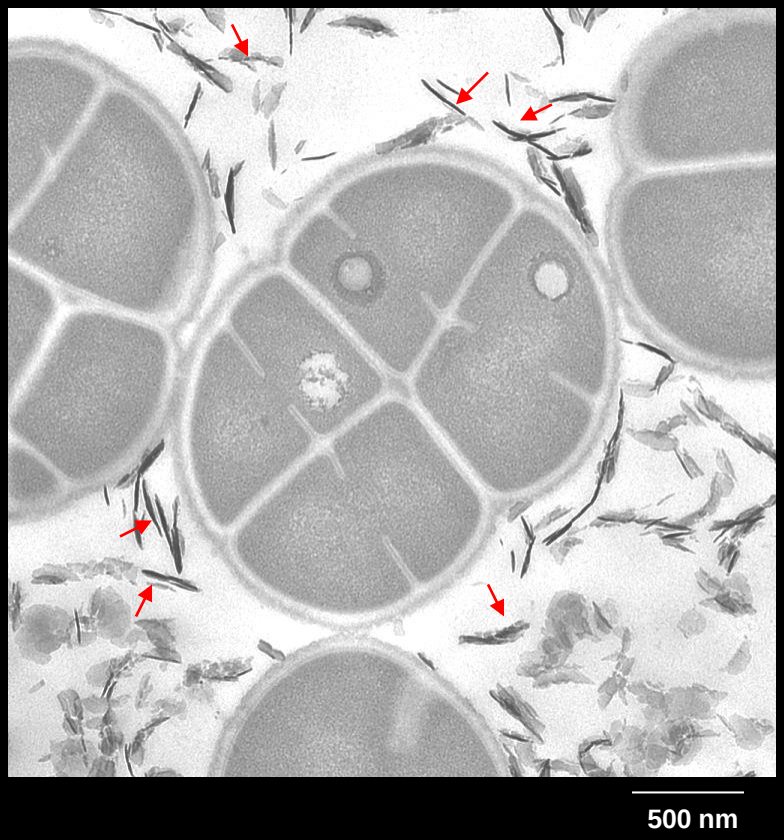
UV light

Seeing is believing …….

TEM images of *Deino-PhoK* cells



Deino-PhoK without uranium treatment

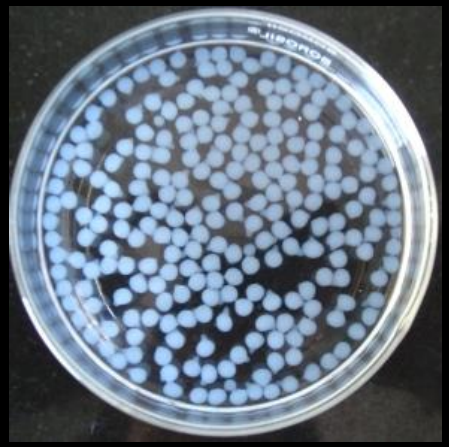


Deino-PhoK with uranium treatment

Needle shaped crystals of uranyl phosphate seen in uranium treated samples

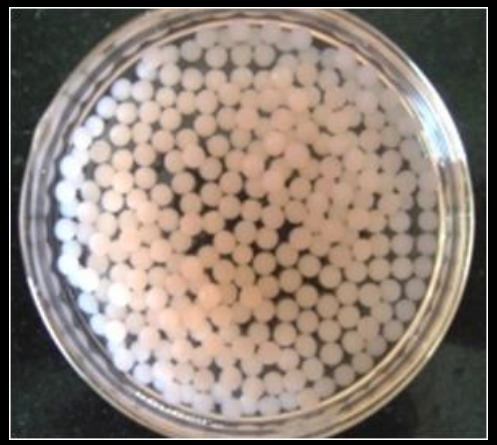
Easy recovery of precipitated uranyl phosphate through beads

A



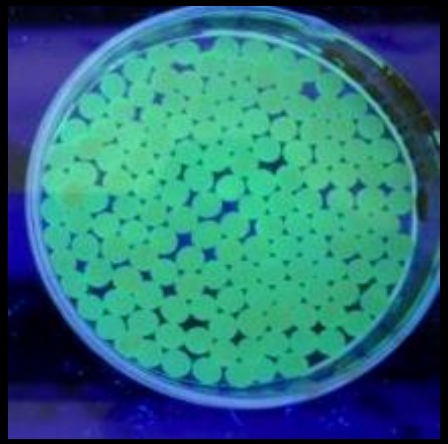
Empty beads

B

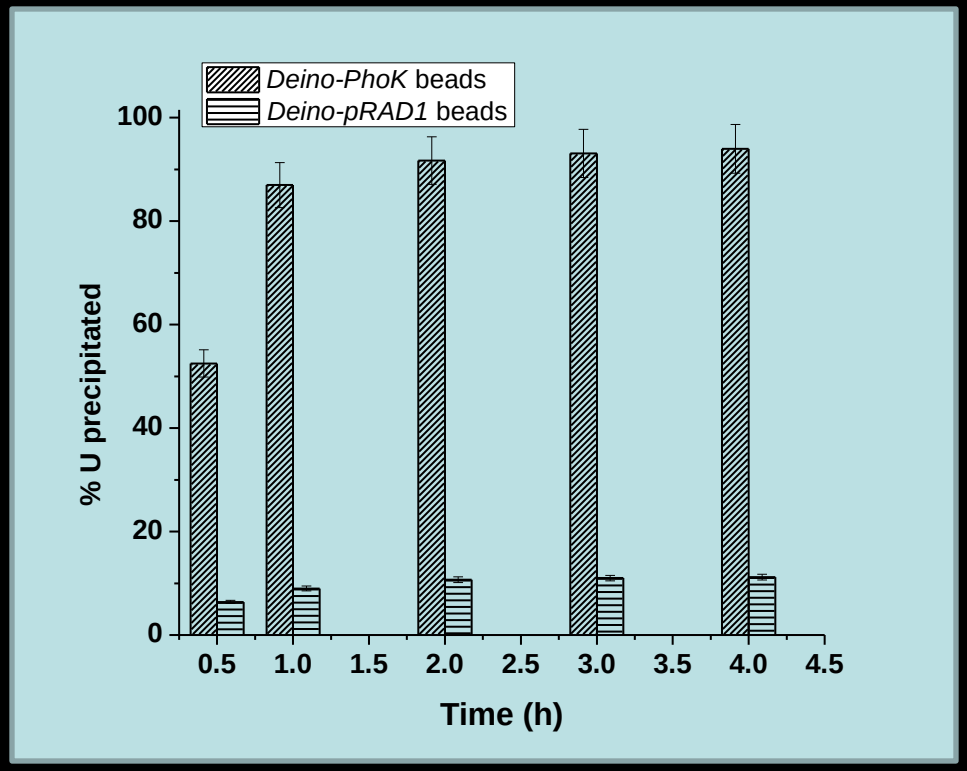


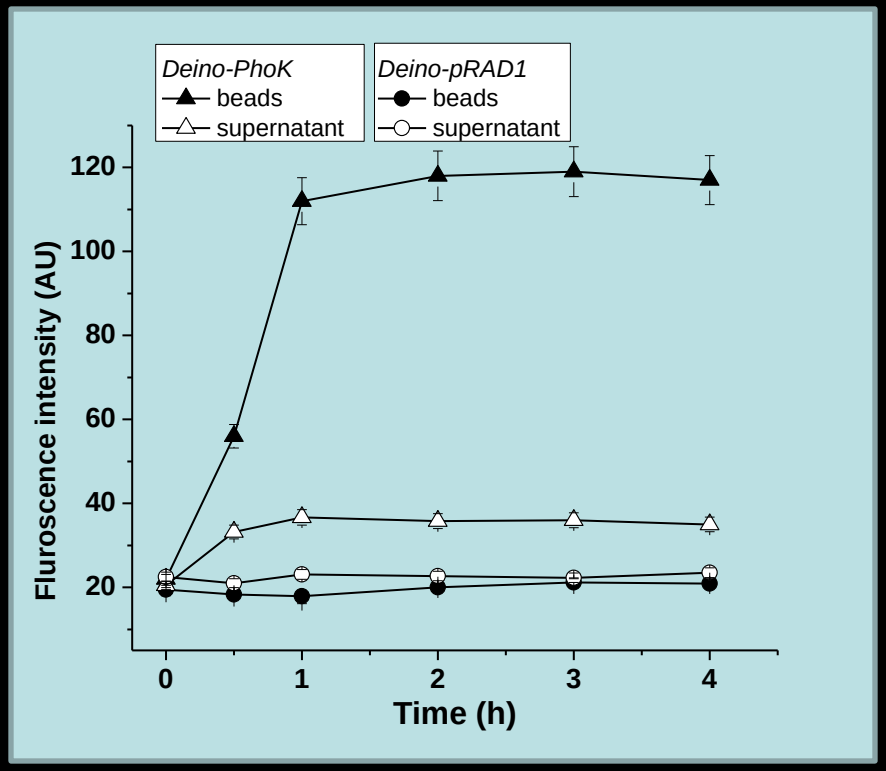
***Deino-PhoK* (-U)**

C



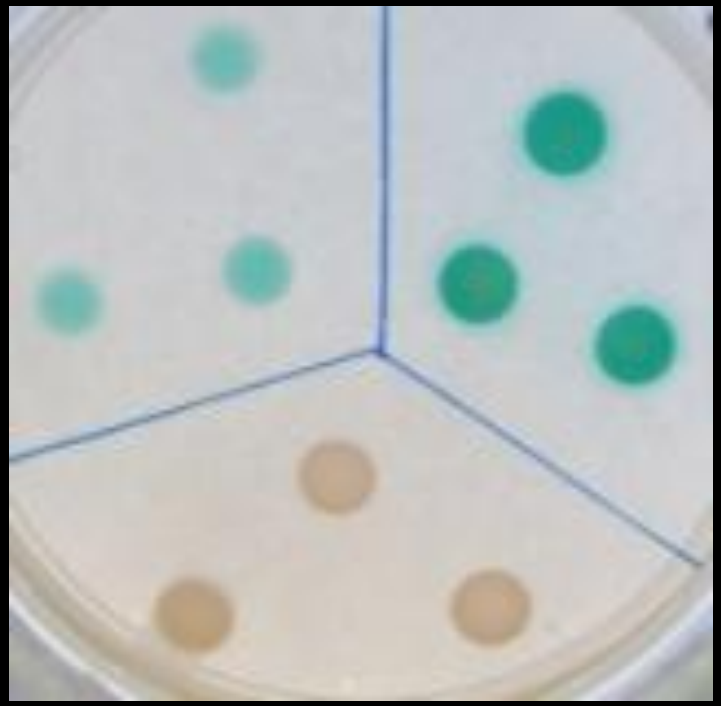
***Deino-PhoK* (+U)**





Comparison of *Deino-PhoN* and *Deino-PhoK* strains

Recombinant *Deinococcus strains* on PDP-MG plate



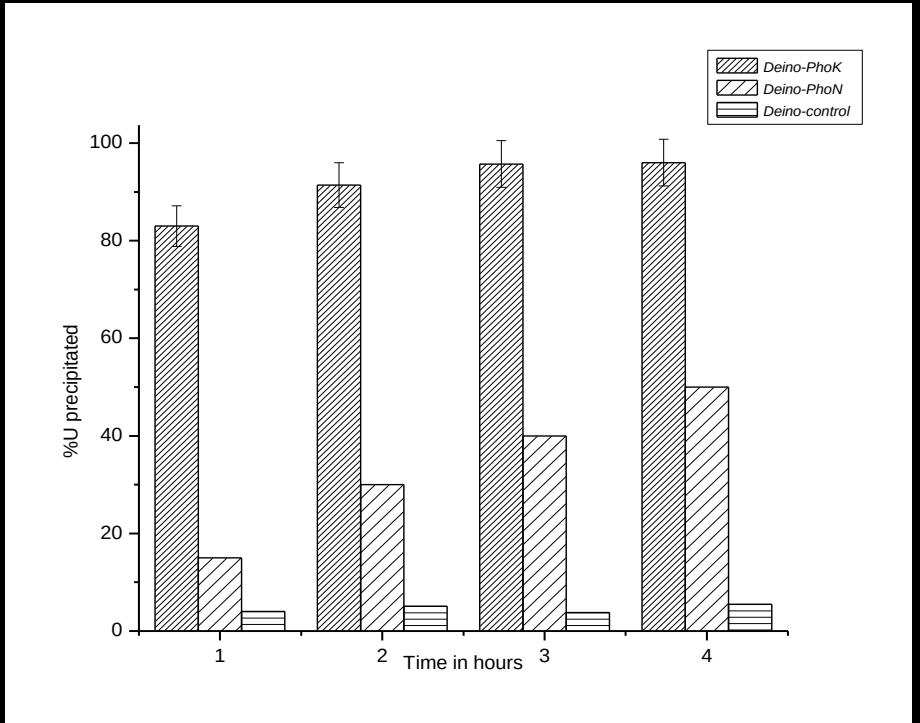
1 *Deino-pRAD1*

2 *Deino-PhoK*

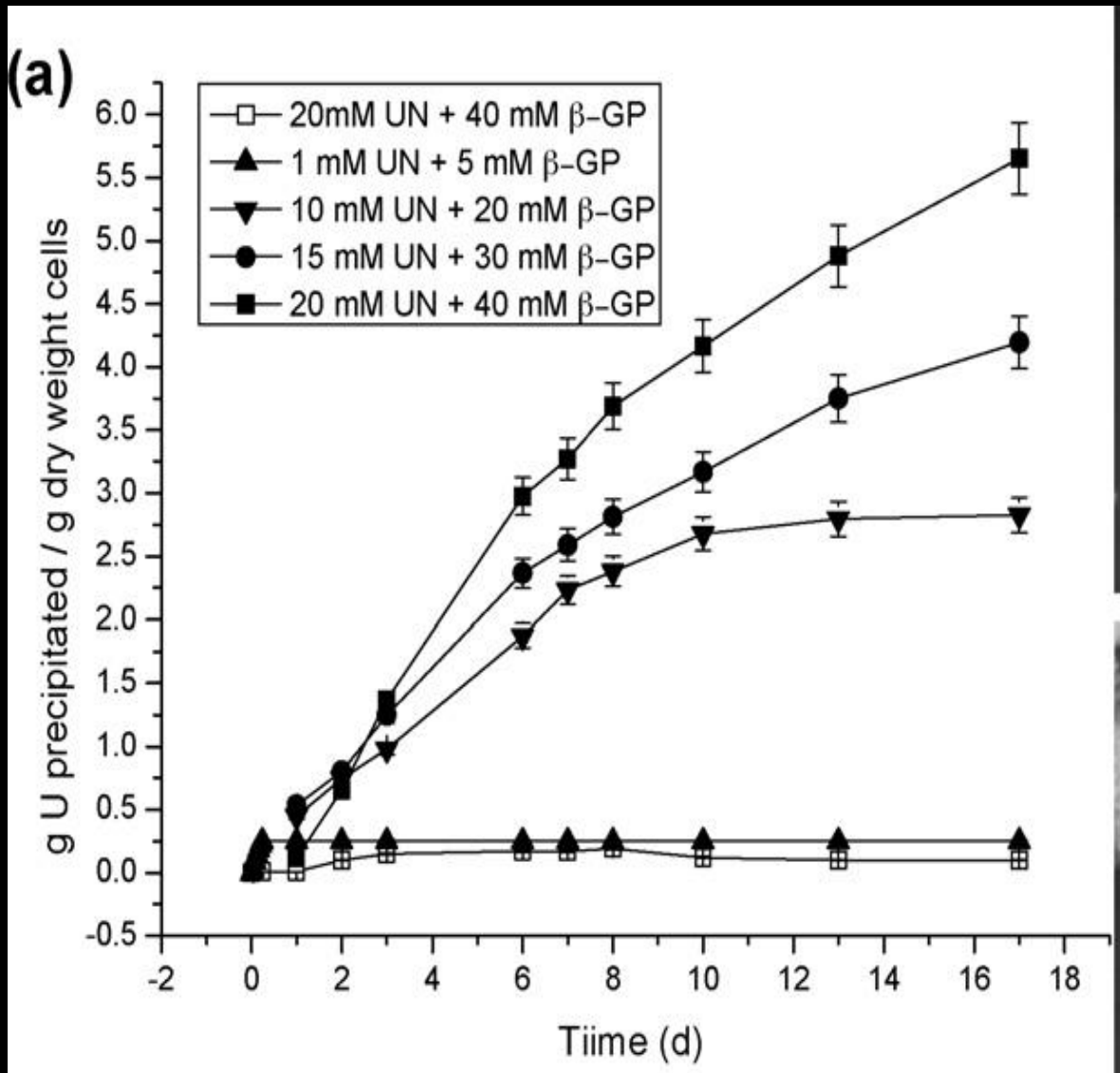
3 *Deino-PhoN*

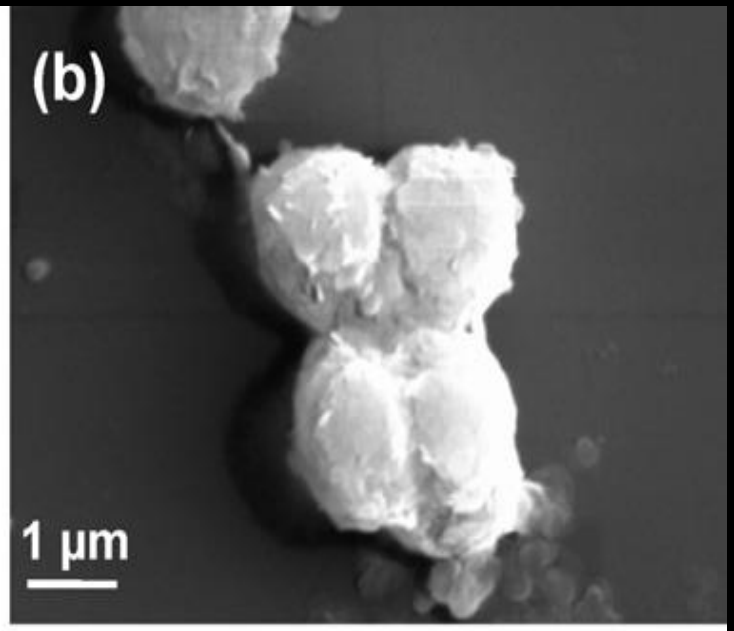
Phosphatase activity of recombinant strains

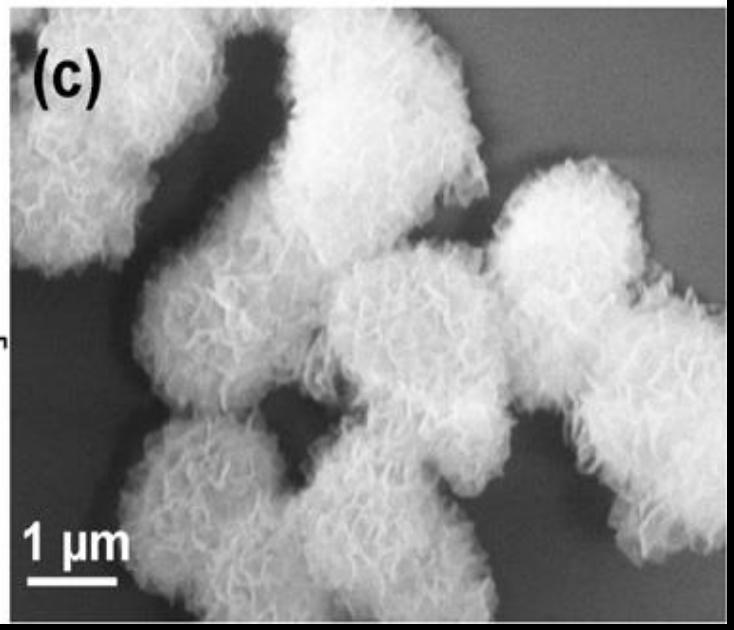
Clones	Specific Activity (nmoles of p-NP liberated/min/mg of total cellular protein)
Deino- pRAD1	18 ± 5
Deino-PhoK	7000 ± 1000
Deino-PhoN	200 ± 10



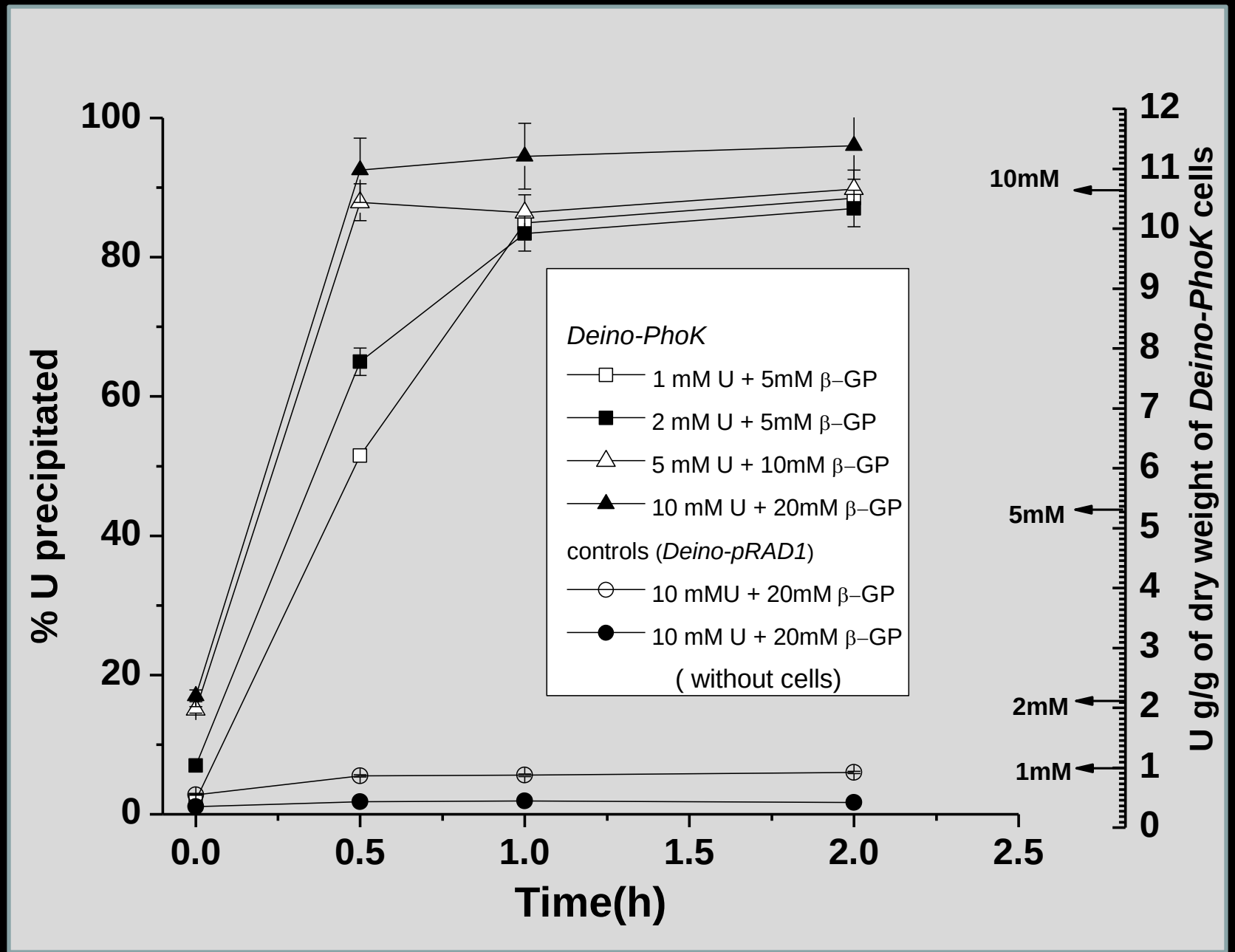
Maximum loading possible with *Deino-PhoN*







Maximum loading possible with *Deino-PhoK*



SUMMARY

Metal precipitation using phosphatases is an old story.

Novelty of the present work :

- ❖ Use of heavy metal tolerant enzymes
- ❖ Cloning/characterization of a very active alkaline phosphatase (PhoK)
- ❖ Extension of metal bioremediation to alkaline solutions
- ❖ Recombinant radioresistant microbes to biorecover uranium from acidic/alkaline solutions in high radiation environments.
- ❖ Lyophilization to extend shelf-life while retaining precipitation ability
- ❖ Volume reduction, high U loading, easy recovery

Related Publications

- Appukuttan, D, Rao, A. S. and Apte, S. K. (2006) Appl. Env. Microbiol. 72 : 7873-7878.
- Nilgiriwala, K., Alahari, A., Rao, A. S. and Apte, S. K. (2008) Appl. Env. Microbiol. 1784 : 1256-1264.
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- Appukuttan, D., Seetharam, C., Padma, N., Rao, A. S. and Apte, S. K. (2011) J. Biotechnol. 154 : 285-290.
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- Misra, C.S., Mukhopadhyaya, R. and Apte, S. K. (2014) J. Biotechnol. 189 : 88–93.

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Useful Vectors	*Mary Lidstrom*
PhoN for U/Cd bioprecipitation	*Deepti Appukuttan, Chitra Seetharam & A.S. Rao*
PhoK for U bioprecipitatio	*Kayzad Nilgiriwala, Anuradha Alahari & A. S. Rao, Sayali Kulkarni,*
SEM-EDX	*Shovit Bhattacharya & N. Padma (TPPED, BARC)*
TEM	*Anand Ballal, Alka Gupta*
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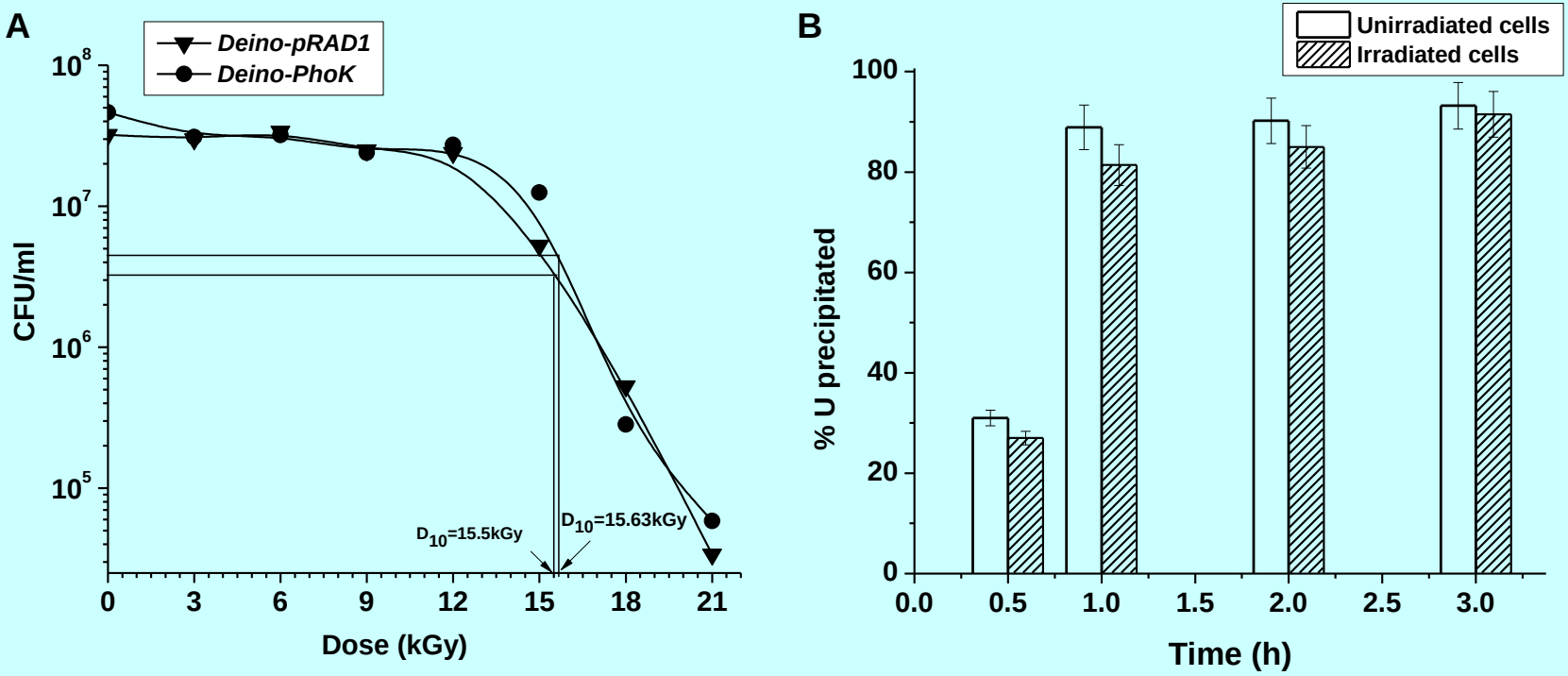
Recombinant strain functions well in high radiation environment



Addition of PhoK does not compromise or alter radioresistance

Irradiation (6 kGy, ^{60}Co -rays) does not influence bioprecipitation