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Cloning and Overexpression of Alkaline Phosphatase PhoK from *Sphingomonas* sp. Strain BSAR-1 for Bioprecipitation of Uranium from Alkaline Solutions▽

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Cells of *Sphingomonas* sp. strain BSAR-1 constitutively expressed an alkaline phosphatase, which was also secreted in the extracellular medium. A null mutant lacking this alkaline phosphatase activity was isolated by Tn*5* random mutagenesis. The corresponding gene, designated *phoK*, was cloned and overexpressed in *Escherichia coli* strain BL21(DE3). The resultant *E. coli* strain EK4 overexpressed cellular activity 55 times higher and secreted extracellular PhoK activity 13 times higher than did BSAR-1. The recombinant strain very rapidly precipitated >90% of input uranium in less than 2 h from alkaline solutions (pH, 9 ± 0.2) containing 0.5 to 5 mM of uranyl carbonate, compared to BSAR-1, which precipitated uranium in >7 h. In both strains BSAR-1 and EK4, precipitated uranium remained cell bound. The EK4 cells exhibited a much higher loading capacity of 3.8 g U/g dry weight in <2 h compared to only 1.5 g U/g dry weight in >7 h in BSAR-1. The data demonstrate the potential utility of genetically engineering PhoK for the bioprecipitation of uranium from alkaline solutions.

Environmental metal pollution is a serious problem, and treatment/recovery of desired metals from such wastes is a major challenge. Effective immobilization of radionuclides of metals is critical in order to prevent groundwater contamination (17). Bioremediation of the toxic metal wastes by microbes offers a relatively inexpensive and ecofriendly alternative to commonly used physical and chemical methods (7, 19, 26). In particular, enzymatic bioprecipitation of heavy metals as metal phosphates is very attractive, since it can recover metals from very low concentrations not amenable to chemical techniques (18). Successful bioprecipitation of metals, such as uranium and cadmium, using acid phosphatase from naturally occurring bacteria, such as *Citrobacter* sp. (19), has been reported. The uranium bioprecipitation potentials of *Bacillus* sp., *Rahnella* sp. (5, 20), *Pseudomonas* sp. (22), and *Salmonella* sp. (27) in an acidic-to-neutral pH range have also been explored. Genetic engineering of the radio-resistant bacterium *Deinococcus radiodurans* R1 by using a nonspecific acid phosphatase, PhoN, for the biorecovery of uranium from dilute acidic/neutral wastes was reported by our laboratory recently (2).

Based on the process used, uranium mining and processing generate large quantities of dilute acidic and alkaline nuclear waste containing uranium, which are dumped as mill tailings. Alkaline wastes containing traces of uranium also arise from nuclear reactors and power plants using uranium as fuel. In nature, uranium (VI) forms highly soluble carbonate complexes, such as $[UO_2(CO_3)_2]^{-2}$ and $[UO_2(CO_3)_3]^{-4}$, at alkaline pH levels (9). This leads to increase in mobility and availability of uranium to groundwater and soil from the dumped nuclear wastes, leading to health hazards. Nearly 130 million liters of alkaline nuclear wastes containing uranium carbonate awaits disposition at the Savannah River Site, Aiken, SC, alone (9). In order to extend microbial remediation possibilities to alkaline wastes, we have been exploring efficient microbial alkaline phosphatase sources.

Alkaline phosphatases (EC 3.1.3.1) are ubiquitously distributed and highly conserved in bacteria, archaea, yeast, plants, and mammalian cells (21) and play indispensable roles in microbial ecology, through their involvement in phosphate nutrition, signal transduction (14), and virulence of bacteria (16). Alkaline phosphatases also form key components of molecular techniques, such as enzyme-linked immunosorbent assay, Western blotting, and immunodetection (28). However their utility for metal-phosphate precipitation has not been explored so far. Recently, we isolated a *Sphingomonas* sp. strain, BSAR-1, hereafter referred to as BSAR-1, exhibiting high alkaline phosphatase activity. In this paper, we report cloning of the corresponding alkaline phosphatase gene, designated *phoK*, from BSAR-1, its overexpression in *E. coli*, and application in the bioprecipitation of uranium from alkaline solution.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains, plasmids, primers, and probes used in this study are described in Table 1. BSAR-1 was isolated in our laboratory as a contaminant from histochemical plates used for screening for phosphatases and identified as a *Sphingomonas* sp. by 16S rRNA gene sequencing (GenBank accession no. AY764287). The strain was grown aerobically in TGY (1% Bacto tryptone, 0.1% glucose, and 0.5% yeast extract) medium at 30°C under agitation at 180 rpm and *E. coli* in Luria-Bertani (LB) medium at 37°C under agitation at 180 rpm. Growth was determined as turbidity (absorbance at 600 nm) or CFU on TGY agar plates after 48 h of incubation in the case of *Sphingomonas* sp. BSAR-1 and as that on LB agar plates after 24 h of incubation in the case of *E. coli* strains. The antibiotic concentrations used were 50 μg ml^{-1} streptomycin and 25 μg ml^{-1} kanamycin for BSAR-1 mutants and 100 μg ml^{-1} carbenicillin, 33 μg ml^{-1} chloramphenicol, or 50 μg ml^{-1} kanamycin for various *E. coli* strains, when required.

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TABLE 1. Bacterial strains, plasmids, primers, and probes used in this study

Strain, plasmid, primer, or probe	Description[a]	Source
Strains		
BSAR-1	Alkaline phosphatase-producing strain of *Sphingomonas* sp.	Lab isolate
KN20	Alkaline phosphatase-null mutant of *Sphingomonas* sp. BSAR-1 (Kan^r)	This study
E. coli DH5α	F^- *recA41 endA1 gyrA96 thi-1 hsdr17*(r_K^- m_K^+) *supE44 relA*λ *lacU169*	Lab collection
E. coli ANU1041 (pACYC184-Mob)::Tn*5*	*E. coli* ANU1041 strain containing pACYC184-Mob::*Tn5* plasmid (Kan^r)	MTCC, IMTECH, Chandigarh
E. coli BL21(DE3) pLysS	*E. coli* BL21(DE3) strain containing the pLysS plasmid (Cm^r)	Novagen
EK1	*E. coli* DH5α containing a 3-kb SalI fragment from the KN20 mutant cloned in the SalI site of the pBluescriptII SK(+) vector	This study
EK2	*E. coli* DH5α containing a 3-kb PstI-BglII fragment from BSAR-1 in PstI-BamHI sites of the pBluescriptII SK(+) vector	This study
EK4	*E. coli* BL21(DE3) pLysS containing pET29b with the complete *phoK* gene	This study
E. coli pET29b	*E. coli* BL21(DE3) pLysS containing pET29b plasmid	This study
Plasmids		
pBluescriptII SK(+)	Cloning vector (Amp^r)	Stratagene
pET29b	Overexpression vector (Kan^r) containing C-terminal His_6 tag	Novagen
Primers		
$441F_{in}$	5′-CCGAATTCGCCCAGTGCGTGTCGAAGGA-3′	This study
$441R_{in}$	5′-CCGAATTCTTCATGCGGTGGCGTTCGGG-3′	This study
$441F_{out}$	5′-CCGAATTCTCCTTCGACACGCACTGGGC-3′	This study
$441R_{out}$	5′-CCGAATTCCCCGAACGCCACCGCATGAA-3′	This study
F_{ow}	5′-CCAGTTATTGGCGATGATGCC-3′	This study
R_{ow}	5′-GGAGCCTGATCCAGGAAGCG-3′	This study
$M13_{For}$	5′- TCCCAGTCACGACGTCGT-3′	This study
$M13_{Rev}$	5′- GGAAACAGCTATGACCATG-3′	This study
FDNE	5′-GGGAATTCCATATGTTGAAACACGTCGCCGCTGCC-3′	This study
RDXE	5′-CGGAATTCCTCGAGCTGCCCGGCGCAGCTGTCGTCCTTG-3′	This study
DNA probes		
nptII	~800-bp DNA amplified from the *nptII* gene (Kan^r) from Tn*5*	This study
SXEK1	~400-bp SalI-XmnI fragment internal to the *phoK* gene	This study

[a] Underlined bases indicate the nucleotide recognition sequences of NdeI and XhoI incorporated in the primers FDNE and RDXE, respectively.

Tn*5* random mutagenesis of strain BSAR-1. Strain BSAR-1 was conjugated with *E. coli* ANU1041 carrying the plasmid pACYC184-Mob::Tn*5* containing the Tn*5* transposon (Table 1). The exconjugants were plated on histochemical TGY agar plates containing 50 μg ml^{-1} streptomycin (BSAR-1 being inherently resistant to streptomycin) and 25 μg ml^{-1} kanamycin (for selection of Tn*5* transposition events) along with 1 mg ml^{-1} phenolphthalein diphosphate (PDP) and 50 μg ml^{-1} methyl green (MG) for selection of phosphatase-active/-inactive colonies as described earlier (23). On these plates, the wild-type strain showed a bluish-green precipitation zone, while no such zone was observed in the case of an alkaline phosphatase-null mutant. A mutant colony (designated KN20) with an alkaline phosphatase-null phenotype was obtained by screening more than 2,000 Tn*5* random mutants and was confirmed to be of the strain BSAR-1 by partial sequencing of the 16S rRNA gene (GenBank accession no. AY764287). The genomic DNA of mutant KN20 was assessed for the Tn*5* insertion by Southern blotting and hybridization using a digoxigenin (DIG)-labeled nonradioactive Tn*5* probe (3, 24).

DNA sequencing and analysis. DNA sequencing was carried out using various primers (Table 1) by the dideoxy DNA sequencing method of Sanger et al. (25). Similarity searches were carried out by computational analysis using the BLAST algorithms available at http://www.ncbi.nlm.nih.gov/BLAST.

Phosphatase activity. Stationary-phase culture of BSAR-1 or isopropyl-β-D-thiogalactopyranoside (IPTG)-induced culture (induction at 30°C for 4 h) of an *E. coli* clone (EK4) was tested for acid or alkaline phosphatase activity in 100 mM acetate buffer (pH 5.0) or 50 mM Tris-HCl buffer (pH 9.0), respectively, as described earlier (6). The pHs of assay mixtures were varied using the following buffers: 50 mM sodium citrate buffer (pH 3 to 6), 50 mM Tris-HCl buffer (pH 7 to 9), and 100 mM carbonate-bicarbonate buffer (pH 9.2 to 11). The phosphatase activity was expressed as nanomoles *para*-nitrophenol liberated min^{-1} from disodium *para*-nitrophenyl phosphate. In-gel zymogram analysis was carried out with cell extracts obtained by sonication (Branson, Germany) followed by electrophoretic resolution and activity staining, as described earlier (2, 23), except that acetate buffer was replaced by 100 mM Tris–100 mM NaCl buffer (pH 9.5).

Uranium precipitation. Uranium precipitation assays were performed as described previously (19) with certain modifications. Uranyl carbonate stock was freshly prepared by adding saturated ammonium carbonate to 100 mM uranyl nitrate solution (8). Overnight-grown cultures (final optical density at 600 nm [OD_{600}], 1.0) of BSAR-1, KN20, and the induced EK4 clone (induced with 1 mM IPTG at 30°C for 4 h) were recovered from their respective growth media, washed twice with saline, and independently treated with 0.5 to 5 mM uranyl carbonate solution in 2 mM carbonate-bicarbonate buffer, pH 9.2 (final pH of assay was 9.0 ± 0.2), supplemented with 5 mM β-glycerophosphate (β-GP) as the substrate at 30°C under static nongrowing conditions in a final volume of 5 ml. Appropriate abiotic and biotic controls were included to ascertain phosphatase-mediated bioprecipitation. Dead cells, obtained by treatment of cells at 100°C for 15 min that did not disturb their structural integrity (data not included), were also assayed. Aliquots taken at different time intervals were centrifuged (12,000 × *g* for 10 min). Residual uranium in the supernatant or cell pellet was estimated using arsenazo III by the method of Fritz and Bradford (11). The samples (2 to 20 μl) were acidified (in 200 μl of 0.01 N HCl), and then 200 μl of arsenazo III was added to the samples. The resultant purple-colored metal-arsenazo III complex was estimated spectrophotometrically at 655 nm using uranyl nitrate hexahydrate (Merck) as the standard. Metal precipitation was expressed as the percent precipitation of input uranium added initially.

X-ray diffraction analysis. Powder X-ray diffraction (XRD) was performed to identify the nature of bioprecipitated uranium. The cells of strains BSAR-1 and EK4, before and after uranium bioprecipitation, were dried in glass petri dishes in an oven at 60°C for 2 h. The dried pellet was scrapped and crushed to a fine powder, which was subjected to XRD analysis using a high-precision Philips X-ray diffractometer (model PW1071) with Ni-filtered Cu-Kα radiation. An exposure time of 2 h was used. The diffraction pattern was recorded from 10° to

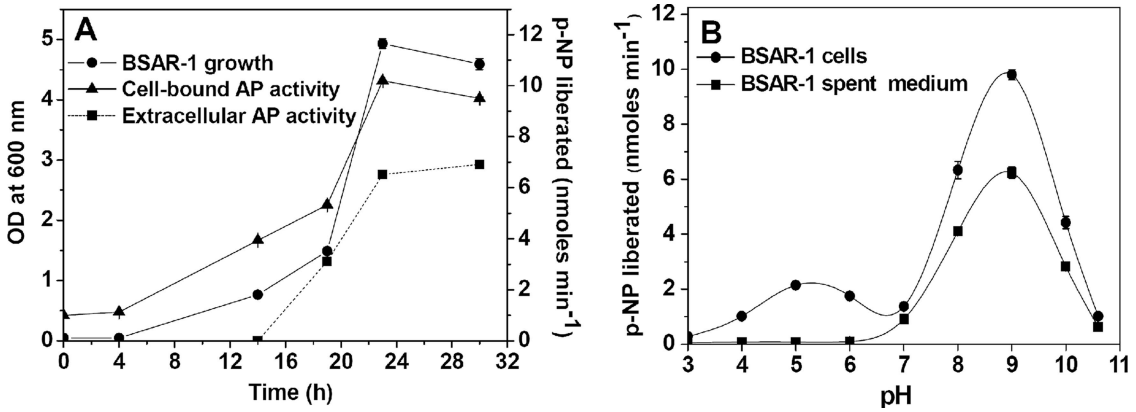

FIG. 1. Constitutive expression and pH dependence of the alkaline phosphatase PhoK. (A) Alkaline phosphatase (AP) activity of BSAR-1 cells (equivalent to 100 μl of cells with OD_{600} of 1) and equivalent spent medium (for extracellular activity) seen at various time points during growth. (B) The pH dependence of the phosphatase activity of BSAR-1. Activity was assayed after 23 h of growth in 100 μl culture aliquots containing BSAR-1 cells with OD_{600} of 1 and equivalent spent medium. All the assays were conducted with three replicates, and the standard errors for the results are shown in the graph. p-NP, *para*-nitrophenol.

50° 2θ, the step length of 2θ being 0.02°. The data fittings were performed by comparing the data peaks with known standards of uranyl phosphate species, as described in the International Centre for Diffraction Data (ICDD) database.

Nucleotide sequence accession number. The complete nucleotide sequence of the alkaline phosphatase gene designated *phoK* has been submitted to GenBank (accession no. EF143994).

RESULTS

Alkaline phosphatase activity in BSAR-1. The strain BSAR-1 showed a growth-dependent expression of the alkaline phosphatase with highest activity during the stationary phase of

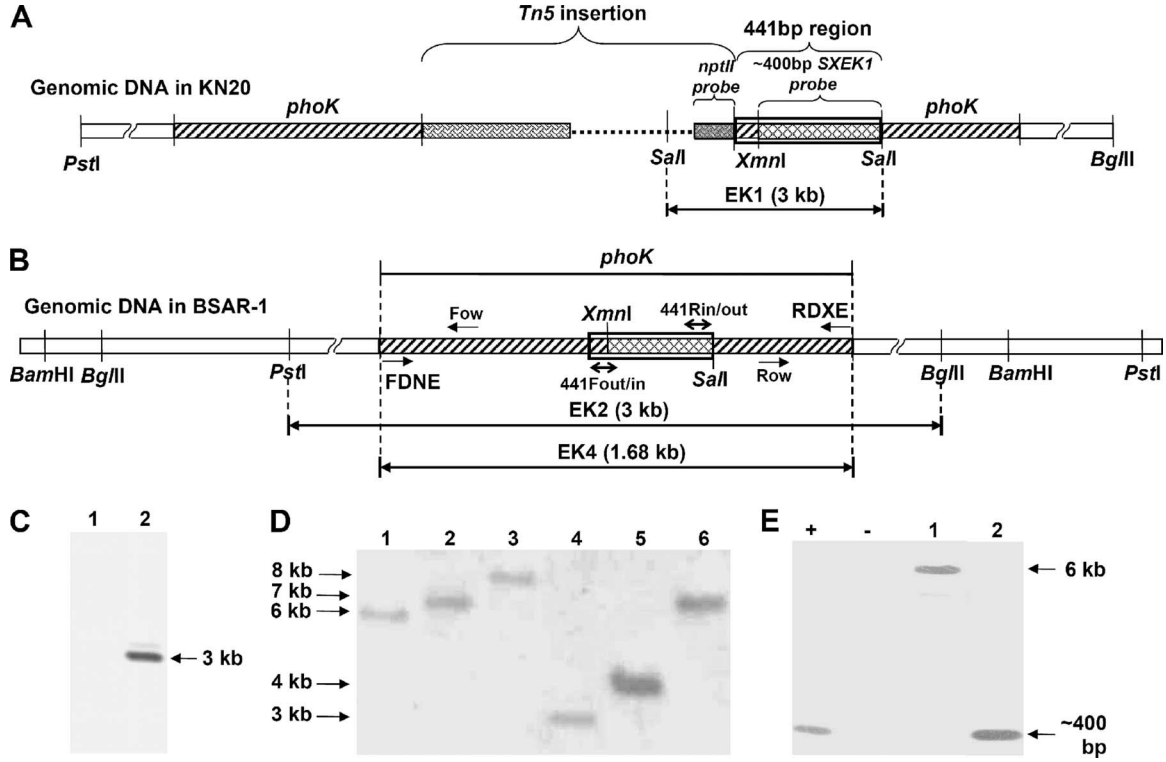

FIG. 2. Mutagenesis and cloning of *phoK* from BSAR-1. (A) Schematic representation (not to the scale) of the Tn*5* insertion in KN20 showing the clone EK1 and the DNA probes used. (B) The *phoK* gene in BSAR-1 and clones obtained therefrom. (C to E) Southern blotting and hybridization. (C) SalI-digested genomic DNA of BSAR-1 (lane 1) and of the KN20 mutant (lane 2) hybridized to the *nptII* probe. (D) Genomic DNA of BSAR-1 hybridized to the SXEK1 probe. The DNA was digested with PstI (lane 1), BglII (lane 2), BamHI (lane 3), PstI-BglII (lane 4), PstI-BamHI (lane 5), or BglII-BamHI (lane 6). (E) Analysis of the clone EK2 with SXEK1 probe. The lanes marked + and − contain the SXEK1 probe and an empty pBluescriptII SK(+) vector, respectively, and are used as the positive and negative controls, respectively. The recombinant plasmid in EK2 was linearized with PstI (lane 1) or digested with SalI-XmnI (lane 2).

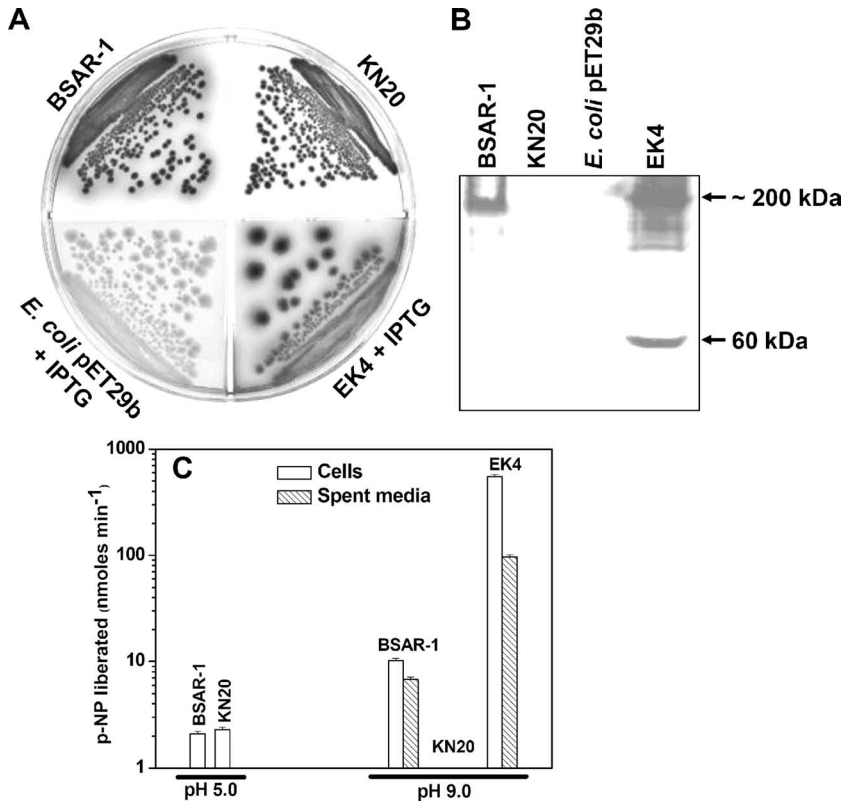

FIG. 3. Phenotypic selection of strains for alkaline phosphatase. (A) Bacterial strains were grown on TGY agar (quadrants 1 and 2) or LB agar (quadrants 3 and 4) containing PDP and MG with the appropriate antibiotics at 30°C for 48 h. (B) Zymogram analysis of cell lysates from BSAR-1 and KN20 grown at 30°C for 23 h, *E. coli* pET29b cells, and EK4 cells induced with 1 mM IPTG at 30°C for 4 h. (C) Phosphatase activities of cells and corresponding spent media in cultures of BSAR-1, KN20, and IPTG-induced EK4 at pHs 5.0 and 9.0. All the assays were conducted with three replicates, and the standard errors for the results are shown in the graph. p-NP, *para*-nitrophenol.

growth (23 h of growth [OD_{600}, 1]) (Fig. 1A). Stationary-phase cells exhibited minor acid phosphatase activity (pH 5.0) and major alkaline phosphatase activity (pH 9.0) (Fig. 1B). Nearly 40% of the total alkaline phosphatase was found secreted in the medium after 23 h of growth.

Cloning of the *phoK* gene. A Tn*5* random mutagenesis as described in Materials and Methods, followed by a chromosome walking-based cloning approach, was used. Screening of more than 2,000 Tn*5* random mutants of BSAR-1 yielded a single colony having an alkaline phosphatase-null phenotype (designated KN20). The site of the Tn*5* insertion in KN20 mutant was determined, its flanking region was subcloned and sequenced, and then the complete *phoK* gene was cloned, as illustrated in Fig. 2A and B. Initial hybridization experiments were conducted with an *nptII* probe from Tn*5*, and the smallest 3-kb SalI fragment was cloned in the pBluescriptII SK(+) vector in *E. coli* DH5α and was designated EK1 (Fig. 2A and C). The 3-kb insert in the plasmid in EK1 was sequenced using M13 universal primers (Table 1), which confirmed the presence of a DNA (441 bp) flanking the Tn*5* and homologous to an internal stretch of a predicted alkaline phosphodiesterase/nucleotide pyrophosphatase of *Sphingomonas* sp. strain SKA58. Primers $441F_{in}$, $441R_{in}$, $441F_{out}$, and $441R_{out}$ (Table 1; Fig. 2B) were used to sequence the DNA flanking either side of the 441-bp DNA fragment subcloned from EK1. A new ~400-bp SXEK1 probe (Table 1; Fig. 2A) was constructed by digesting the plasmid from clone EK1 with SalI-XmnI and was used to screen the genomic-DNA digests of BSAR-1 (Fig. 2B). The smallest DNA fragment (3-kb PstI-BglII) was subcloned in the pBluescriptII SK(+) vector and designated EK2 (Fig. 2B and D). Restriction digestion of EK2 (Fig. 2E) showed that it contained the complete *phoK* gene (Fig. 2B). EK2 exhibited an alkaline phosphatase-positive phenotype on selective histochemical plates containing PDP and MG (data not shown). Primers F_{ow} and R_{ow} were used for primer walking on the gene in the clone EK2 (Fig. 2B) to obtain the complete nucleotide sequence of the alkaline phosphatase gene, designated *phoK*, which has been submitted to GenBank (accession no. EF143994).

Overexpression of alkaline phosphatase. The complete *phoK* gene was PCR amplified from the genomic DNA of BSAR-1 by using the primers FDNE and RDXE (Table 1; Fig. 2B), restriction digested with NdeI-XhoI, and cloned in the pET29b overexpression vector containing a C-terminal His_6 tag. The recombinant plasmid was transformed in *E. coli* BL21(DE3) pLysS cells to obtain strain EK4. The EK4 cells were grown to an OD_{600} of 0.8 and induced for 4 h at 30°C with 1 mM IPTG. A comparison of alkaline phosphatase activities of colonies of BSAR-1, KN20, induced *E. coli* pET29b, and EK4 on histochemical plates containing their respective antibiotics is shown in Fig. 3A. KN20 showed green colonies due to the presence of acid phosphatase but lacked the bluish-green

TABLE 2. Bioprecipitation of uranium from 2 mM uranyl carbonate solution

Time (h)	Mean uranium loss (% of input)[a] using:									
	Abiotic control treated in solution:		Biotic control				Test sample			
	Without β-GP	With β-GP	BSAR-1	KN20	*E. coli* BL21 + pET29b	EK4	BSAR-1	KN20	*E. coli* BL21 + pET29b	EK4
0	1.11	1.10	4.03	4.48	3.34	3.17	0.11	0.00	0.16	6.88
1	1.11	1.11	4.59	5.85	5.13	6.57	3.02	0.42	0.27	85.73
6	1.09	1.12	5.87	6.58	6.58	7.20	90.66	1.07	0.73	91.15
24	1.11	1.12	6.71	7.00	6.94	7.39	92.89	5.65	3.59	93.57

[a] The values are averages of three replicates wherein variation was less than 15%. Abiotic control, no added cells; biotic control, heat-treated cells; test sample, live cells.

halo of secreted alkaline phosphatase in BSAR-1, which was enhanced by the IPTG induction of EK4. The induction of the PhoK alkaline phosphatase (an ~60-kDa monomer on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [data not shown]) was at its maximum level after 4 h at 30°C. Zymogram analysis under mild denaturing conditions showed significant enhancement in the alkaline phosphatase activity of EK4 visualized as a major high-molecular-mass protein (Fig. 3B), suggesting that active PhoK may be a multimeric protein. The EK4 lysate additionally showed a minor 60-kDa activity band, probably due to the presence of excess protein monomer due to overexpression. Size exclusion chromatography using Sephacryl S400 also indicated that the active enzyme is a multimer (~200 kDa) (data not shown). The PhoK activities of the IPTG-induced EK4 cells were 55 and 13 times higher in cells and in external medium, respectively, relative to those of BSAR-1 cells (Fig. 3C). No significant difference was observed in their acid phosphatase activity, as expected.

Uranium bioprecipitation. Strains BSAR-1 and KN20 and clone EK4 induced with IPTG were evaluated for their uranium precipitation abilities with 5 mM β-GP as the substrate (Table 2; Fig. 4A to C). BSAR-1 cells showed a lag of 4 h at a

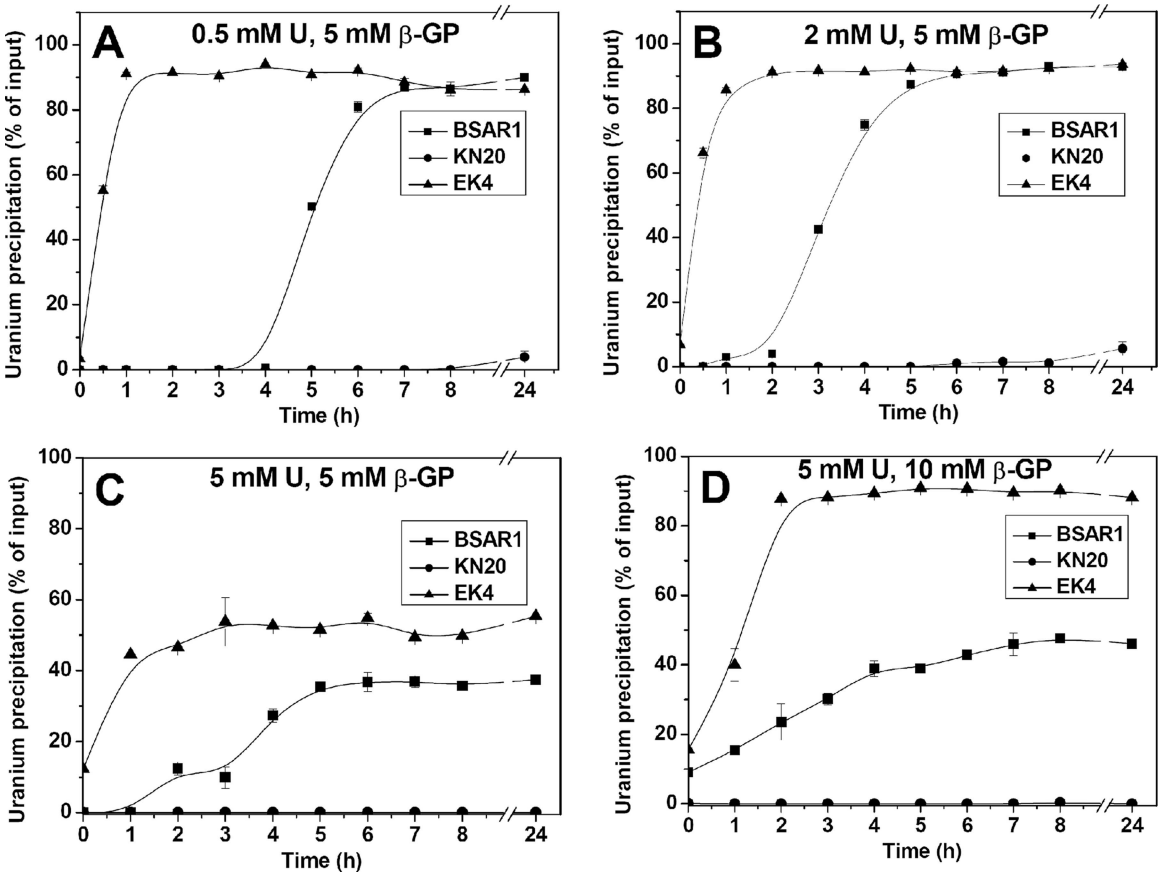

FIG. 4. Uranium bioprecipitation by various strains. The bioprecipitation assays were conducted with cells (OD_{600}, 1) of BSAR-1 and KN20 and strain EK4 induced with IPTG at 30°C in the presence of 5 mM β-GP (A to C) or 10 mM β-GP (D) as the substrate. Uranium was added to the assays as uranyl carbonate at a concentration of 0.5 mM (A), 2 mM (B), or 5 mM (C and D).

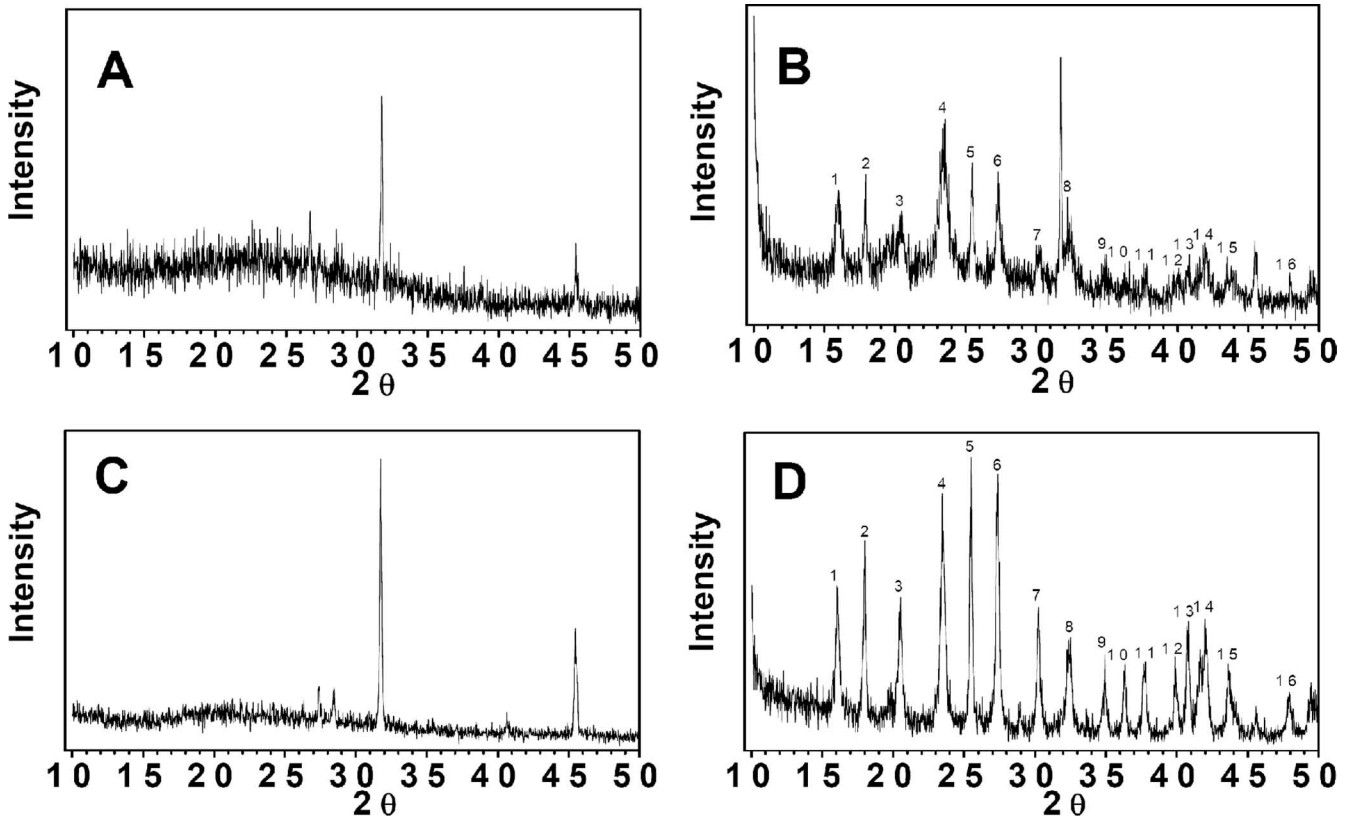

FIG. 5. Identity of bioprecipitated uranium. XRD spectra of BSAR-1 cells before exposure to uranium (A) and after 8 h of exposure to 2 mM uranyl carbonate solution (B) were compared with XRD spectra of EK4 cells before exposure to uranium (C) and after 3 h of exposure to 2 mM uranyl carbonate solution (D).

uranyl carbonate concentration of 0.5 mM and a lag of 2 h at a 2 mM concentration of uranyl carbonate before commencement of uranium precipitation. The induced *phoK*-overexpressing clone EK4 showed more than 90% uranium precipitation within 1 h of exposure to 0.5 to 2 mM uranyl carbonate, whereas strain BSAR-1 achieved such precipitation after 6 to 8 h of exposure to the uranyl carbonate solution (Fig. 4A and B). At a 5 mM uranium concentration, uranium bioprecipitation was limited by the available substrate concentration (Fig. 4C), and EK4 and BSAR-1 cells showed only 55% and 37% precipitations, respectively. When a higher substrate concentration (10 mM β-GP) was used, EK4 showed ~90% bioprecipitation in 2 h while BSAR-1 still showed only 47% precipitation before reaching its loading saturation in 7 h (Fig. 4D) since it contains ~55 times less PhoK than does EK4. The KN20 mutant completely lacked uranium precipitation ability in all the assays (Table 2; Fig. 4A and B). The abiotic controls with and without β-GP did not show any spontaneous precipitation, while the dead-cell biotic controls and *E. coli* BL21 containing pET29b showed less than 8% biosorption/bioprecipitation even after prolonged exposure (Table 2). Uranium-loading capacities of BSAR-1 and EK4 cells were found to be 1.533 and 3.825 g uranium per g dry weight, respectively.

Identity of the bioprecipitated uranium. The XRD patterns of the uranium-loaded cells of BSAR-1 and EK4 (Fig. 5) confirmed the presence of uranyl hydrogen phosphate hydrate $H_2(UO_2)_2(PO_4)_2 \cdot 8H_2O$, also known as chernikovite or hydrogen meta-autunite, by data fitting in which at least 16 data peaks showed a match with the identified chemical species of uranium (ICDD CAS no. 08-0296). Interestingly, the XRD patterns of uranium-loaded BSAR-1 cells and engineered EK4 cells were quite identical.

DISCUSSION

A large number of *Sphingomonas* sp. strains have been isolated from different environments as novel species capable of biodegradation of organic pollutants (10, 12). A related organism, *Caulobacter crescentus*, may hold promise to precipitate toxic metals (13). However, bioremediation of metals involving phosphatases from the genus *Sphingomonas* has not yet been explored. A *Sphingomonas* sp. strain, BSAR-1, was isolated in our laboratory and shown to possess very high alkaline phosphatase activity. The corresponding gene, designated *phoK*, was cloned first by its functional inactivation by Tn*5* random mutagenesis, followed by cloning of the native gene by chromosomal walking using Tn*5*-targeted DNA as a probe. The recombinant PhoK protein has 559 amino acids and an estimated molecular mass of 59,982 Da. The enzyme was released extracellularly and showed optimum activity at pH 9.

This study evaluated the precipitation of uranium from alkaline solutions using PhoK alkaline phosphatase. A recombinant *E. coli* strain, EK4, was constructed to overexpress PhoK.

TABLE 3. Inorganic phosphate (P_i) released in bioprecipitation assays

Strain	Time (h)	P_i released (mM)[a]	$\log(PO_4^{-3}/CO_3^{-2})$[b]
BSAR-1	0.25	0.03	−2.60
	1	0.90	−1.05
	6	2.85	−0.55
EK4	0.25	3.62	−0.44
	1	4.95	−0.31
	6	4.98	−0.30

[a] The values were estimated in the presence of 5 mM β-GP without addition of uranyl carbonate.
[b] The values were calculated in the presence of 5 mM β-GP and 2 mM uranyl carbonate solution.

The strain bioprecipitated uranium very rapidly and removed >90% of input uranium in less than 2 h from 0.5 to 5 mM concentrations, compared to the native BSAR-1 strain, which precipitated uranium in >7 h (Fig. 4 and 5). It is rather difficult to precipitate uranium as phosphate in the presence of excess carbonate at pH 9.0. However, earlier studies (29) have shown that it is feasible only at $\log(PO_4^{-3}/CO_3^{-2})$ values of >-3. We determined the amount of P_i released by our strains in the absence of uranium and calculated the $\log(PO_4^{-3}/CO_3^{-2})$ value in assays containing 0.5 to 5 mM uranium concentrations. Data obtained at 2 mM uranyl carbonate concentration is shown in Table 3. The required ratio was attained in all cases, thereby facilitating extracellular precipitation of uranium as phosphate at pH 9. The precipitated uranium remained cell bound, leading to high loading of uranium up to 1.5 g U/g dry weight for BSAR-1 and 3.8 g U/g dry weight for EK4 at 5 mM uranyl carbonate at pH 9.0 in 2 h and 6 h, respectively.

Bioprecipitation of heavy metals, including uranium, has been shown to be successful using bacterial strains producing acid phosphatases (2, 4, 5, 15, 19, 20). Treatment of alkaline wastes containing uranium and other heavy metals, though desirable, has been somewhat neglected (9). As mentioned earlier, in alkaline mill tailings and other nuclear wastes, migration of uranium into groundwater is facilitated by formation of stable uranyl carbonate complexes in nature and can have a catastrophic effect on the ecosystems (1). Recovery of uranium from such wastes is desirable not only to protect the environment but also to recover traces of valuable uranium, which is not possible by physicochemical processes. To the best of our knowledge, this is the first instance of bioprecipitation of uranium from alkaline solutions by using an alkaline phosphatase. The data presented demonstrate that engineering alkaline phosphatases for bioprecipitation of heavy metals holds promise for the development of appropriate technologies for the recovery of uranium from alkaline nuclear wastes.

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