Paper E-06, in: M. Pellei and A. Porta (Eds.), *Remediation of Contaminated Sediments—2003.* Proceedings of the Second International Conference on Remediation of Contaminated Sediments (Venice, Italy; 30 Sep–3 Oct 2003). ISBN 1-57477-143-4, published by Battelle Press, Columbus, OH, www.battelle.org/bookstore.

TARGETS OF IMPROVEMENT IN BACTERIAL CHROMATE BIOREMEDIATION

M. Keyhan, D. F. Ackerley, and ***A. Matin*** (a.matin@stanford.edu)
(Stanford University School of Medicine, Stanford, California, USA)

ABSTRACT: Cr(VI) (chromate) is a widespread, toxic and soluble environmental contaminant. Bacteria can reduce chromate to insoluble and less toxic Cr(III), and thus chromate bioremediation is of interest. Genetic and protein engineering of suitable enzymes can improve bacterial bioremediation. However, many fundamental parameters that define an organism's capacity to remediate chromate have not previously been characterized. We have measured the innate ability of a wide range of bacteria to reduce chromate. All bacteria that we tested were able to reduce Cr(VI) through to stable Cr(III) end-products. One of these, *Pseudomonas putida* MK1, is a promising candidate for chromate bioremediation, and we show that chromate transformation per mass unit increases in these cells as the environmental chromate concentration rises. However, bacterial growth is inhibited by chromate concentrations above 0.8 mM. Furthermore, although cell extracts show increased chromate reductase activity under slow-growth conditions (mimicking nutrient-limited field conditions), the ability of the whole cells to transform chromate is greatly diminished. Chromate reduction by both whole cells and purified ChrR (a *P. putida* chromate reductase) is also inhibited by co-pollutants of chromate-contaminated sites. Thus, these studies identify several potential areas of improvement for generation of improved chromate-remediating bacteria.

INTRODUCTION

Hexavalent chromium [Cr(VI); chromate)] is generated as a by-product of a large number of industries, for example, welding, paper and pigment production, and chrome plating. This has resulted in large-scale contamination of the environment by chromate. The manufacture of nuclear weapons also produced vast quantities of chromate, which were either discharged directly into the environment or stored in buried canisters. Rusting and leakage of the latter exacerbated the chromate contamination problem at the U.S. Department of Energy (DOE) waste sites with the result that chromate is second only to lead as the most abundant heavy metal contaminant at these sites. There, chromate concentration is reported to be as high as 173 μM in ground water and 76 mM in sediments (Riley, 1992). Since soil water is stored in small capillary spaces, the latter may amount to much higher concentrations. Estimates for the cost of cleanup of the DOE waste sites alone run up to hundred of billion of dollars (McIlwain, 1996).

Chromate is readily taken up by biological cells through the anionic transport systems and is a known toxic and carcinogenic agent. Its stable reduced product Cr(III), on the other hand is not bioavailable, as it is not taken up by the cells. Thus, Cr(III) is much less harmful. Moreover, in contrast to Cr(VI), which is highly soluble, Cr(III) is much less so; thus, while it is very difficult to sequester the former, the latter can readily be confined to discrete regions. For this reason conversion of environmental chromate to Cr(III) offers a solution to the chromate contamination problem.

Certain bacteria can reduce chromate (Cervantes, 2001). This has raised the possibility of remediating chromate through bacterial activity. Such an approach would permit in-situ remediation of chromate and obviate the need for transporting contaminated water and sediments to the surface. The alternative chemical approaches to achieve this are prohibitively expensive. However, so far only scant information is available by which to judge bacterial suitability for in-situ chromate remediation. The capacity for chromate reduction has been shown only for a few bacteria (Cervantes, 2001), and little information is available on the range of chromate concentrations that are toxic to bacteria. Several other issues that determine effectiveness for in-situ bioremediation have not been adequately addressed in the context of chromate. Here we address some of these issues.

MATERIALS AND METHODS

Bacterial Strains. Luria-Bertani (LB) liquid medium was used for bacterial cultures unless otherwise specified. The following bacterial strains were employed in this study: *Pseudomonas putida* KT2440, *Pseudomonas putida* MK1, *Pseudomonas syringae, Pseudomonas fluorescens, Bacillus subtilis, Vibrio harveyi, Escherichia coli* AB117, *Shewanella putrefaciens, Deinococcus radiodurans, Staphylococcus aureus,* and *Acinetobacter* sp. strain ADP1 (Table 1). *P. putida* MK1 is a rifampicin resistant derivative of ATCC strain 12633 (Kim, 1995). Unless otherwise specified, *P. putida* in this paper refers to strain MK1.

Growth Conditions and Assays. Cell growth was followed by A_{660} measurements. In cultures with precipitate, A_{660} measurements of growth were made after permitting the precipitate to settle. Protein concentrations were determined with the Bio-Rad Dc protein assay kit, using bovine serum albumin as standard. Chromate reduction rates were quantified in growing cultures or cell suspensions, as specified. Residual chromate was measured using the diphenyl carbazide method (Park, 2002) after removing the cells by microcentrifugation. Cell suspensions were prepared from overnight cultures, which had been washed and resuspended (A_{660} 4 – 6; 1.3-1.9 $mg.ml^{-1}$ of total protein) in fresh LB medium containing the specified concentration of potassium chromate. The suspensions were incubated with shaking at 37°C (*B. subtilis* and *E. coli*) or 30°C (all others) and sampled at appropriate intervals to measure residual chromate. At these cell concentrations, little change occurred in culture density during the experiment. Cell-free extract preparation and chromate reductase assays were as described previously (Greenberg, 1981; Park, 2000). Cr(III) was detected by XANES analysis as described in Park (2000). Chemostat cultivation was performed as described in Zgurskaya (1997). Unless otherwise stated, all growth and chromate transformation data are the mean of at least three measurements with the standard error of the mean <5%.

Sediment from a contaminated DOE site was analyzed as follows. Ten grams of sediment sample were crushed in a mortar and pestle, then thoroughly mixed with 25 ml deionized water, and incubated at room temperature overnight with shaking. Following centrifugation (7,000 × g) for 15 min, the clear supernatant was aspirated and filter sterilized. 0.947 ml of the supernatant was used per ml enzyme reaction mixture; for growth studies, the supernatant was used to dissolve the LB solids. Assays were run for enzyme activity using 25 µl of 50 $\mu g.ml^{-1}$ purified chromate reductase enzyme (Park, 2002).

Electron Microscopy. Formvar carbon-coated copper grids were placed on drops of diluted (A660 of 0.5) 6-day cultures and incubated for 3 min to allow cell adherence. After three washings in distilled water, the grids were negatively stained (2 min) in 1% uranyl acetate and washed again. A Philips CM12 transmission electron microscope operating at 80 kV was used.

RESULTS AND DISCUSSION

The Capacity to Reduce Chromate is Widespread in Bacteria. The initial rates of chromate transformation were quantified for several bacteria belonging to different taxonomic groups, as described in Materials and Methods. Each bacterium examined possessed this capacity (Table 1). Different bacteria differed markedly in this capacity, ranging between ca. 8 nanomoles chromate converted.mg protein^{-1}·h^{-1} (*P. putida* KT2440) and 193 nmol.mg^{-1}·h^{-1} (*Shewanella putrefaciens*). Among the *Pseudomonas* species examined, *P. syringae* exhibited highest activity. Except for *Escherichia coli*, in which overnight growth in the presence of 0.4 mM chromate decreased the chromate transforming activity, pre-exposure to chromate enhanced the transforming activity two- to three-fold (Table 1). An energy source was required for chromate transformation in all cases; little transformation was seen in 0.1 M phosphate buffer (pH 7.0). Cells suspended in LB broth showed more rapid transformation than those suspended in glucose-M9 medium. The requirement for an energy source strongly suggests that chromate transformation in the suspensions resulted from its uptake into the cells and subsequent reduction, and not merely from the adsorption of chromate to the cell surface.

Chromate efflux systems may be present in bacteria (Cervantes, 2001). One such system, which may be similar to the ArsB transport system of *E. coli* (responsible for arsenite extrusion) has been documented in *P. aeruginosa* (Alvarez, 1999). This mechanism is inhibited by sulfate. The difference in the chromate conversion rates of the bacteria examined could have been a reflection of their different capacity to extrude chromate. If so, the presence of sulfate might diminish chromate conversion. To our surprise, however, addition of $CuSO_4$ to the cell suspensions of several of the bacteria increased the chromate conversion rate by 2.5- to 11-fold (Table 1). To test whether the effect was due to sulfate, we repeated these measurements in the presence of Na_2SO_4. No stimulation was observed. On the other hand, addition of $CuCl_2$ caused stimulation comparable to that observed with $CuSO_4$. Why Cu^{2+} stimulates chromate conversion is currently under investigation.

TABLE 1. Chromate conversion rates of different bacteria[a.]

Organism	Activity (nmol/mg protein/h)		
	Grown without chromate	Grown with 0.4 mM chromate	Assayed in presence of 10 mM $CuSO_4$[b.]
P. putida KT2440	**8.4**	**27.6**	**52.8**
P. putida MKI	**13.2**	**42.6**	**72.0**
P. syringae	**151.8**	**N.D.**[c.]	**N.D.**
P. fluorescens	**114.6**	**N.D.**	**N.D.**
B. subtilis	**15.6**	**33.0**	**40.8**
V. harveyi	**132.0**	**264.0**	**1440.0**
E. coli AB117	**49.8**	**32.4**	**156.0**
S. putrefaciens	**193.2**	**N.D.**	**N.D.**
D. radiodurans	**186.6**	**N.D.**	**N.D.**
S. aureus	**139.2**	**N.D.**	**N.D.**
Acinetobacter	**133.8**	**N.D.**	**N.D.**

a. As cell suspensions in Luria Broth
b. Cells were pre-grown without chromate
c. Not Determined

End Product of Chromate Conversion. The end product of chromate reduction was determined for three of the organisms tested: *P. putida* MK1, *B. subtilis*, and *E. coli* AB117, as described previously (Park, 2000). Six day old cultures of the three bacteria, grown in LB broth supplemented with 0.4 mM chromate, contained Cr(III); no Cr(VI) could be detected. The time course of chromate disappearance with growth in LB + 0.4 mM chromate were determined for *P. putida* MK1; it was coincident with growth (Fig. 1).

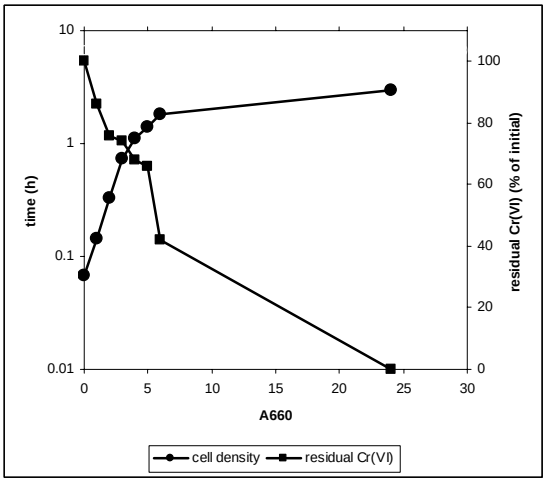

Figure 1. Growth and chromate-transformation curves for *P. putida* MK1 cells grown in LB containing 0.4 mM K_2CrO_4.

Effect of Varying Chromate Concentration on *P. putida*. Chromate concentration varies through a wide range in the polluted environments. We thus determined the concentration range of chromate that could be effectively transformed by *P. putida* MK1; this bacterium is ideal for in-situ bioremediation (Timmis, 1999). The growth yield of *P. putida* in LB medium was not affected by chromate up to a concentration of 0.8 mM. Above this concentration, it declined progressively with increasing chromate (Table 2). There was complete disappearance of chromate in cultures containing up to 0.4 mM chromate, but only partial transformation above this concentration. Thus, after 24 h of growth, 9.4 mM chromate remained untransformed in medium initially amended with 10 mM chromate; at 30 mM, the 24 h concentration was 26.5 mM. Nonetheless, the amount of chromate transformed per unit A_{660} of growth increased with increasing chromate concentration: it was 0.79 at 10 mM chromate and 20.6 at 30 mM chromate. This suggests that concentration dependent diffusion of chromate into the cells may have a role in chromate transport, and that chromate transport is a limiting factor in its transformation by this bacterium.

Despite the fact that the end product of chromate transformation was Cr(III), no precipitate was seen in cultures containing up to 0.8 mM chromate. At higher

TABLE 2. Effect of growth in the presence of different chromate concentrations on *P. putida*

Initial K_2CrO_4 (mM)	A_{660} [a.]	K_2CrO_4 transformed (mM)	Residual K_2CrO_4 (mM)	K_2CrO_4 transformed per A_{660} of growth	Precipitate formed[b.]
0.0	2.59	-	-	-	-
0.2	2.60	0.2	0.0	.08	none
0.4	2.40	0.4	0.0	.17	none
0.8	2.55	0.48	0.32	.12	none
10	0.76	0.60	9.4	.9	yellow (+)
15	0.48	2.50	12.5	5.2	red-yellow (+++)
20	0.28	5.10	14.9	18.2	red-yellow (++)
30	0.17	3.50	26.5	20.6	red-yellow (++)

a. After 24 h incubation; starting A_{660} ca. 0.1
b. plus signs indicate relative intensity

concentrations, a yellow to rusty-yellow precipitate was observed (Fig. 2). It is not known why Cr(III) does not precipitate at lower concentrations. One possibility is that it interacts with some component(s) of the LB medium. However, it has been reported that the ultimate reduction product of Cr(VI) by the *E. coli* Fre protein is soluble because it is complexed with NAD (Puzon, 2002). If formation of such soluble Cr(III) products are a general phenomenon at lower chromate concentrations, bacterial bioremediation of chromate in low chromate environments would fail to attain the objective of localizing chromate to discrete sites. Taken together, these observations suggest that the wild type *P. putida* may have limited usefulness in chromate bioremediation. At low chromate concentrations, while it is effective in transforming chromate to Cr(III), this Cr(III) may remain in a soluble form; at higher chromate concentrations, although the Cr(III) formed is precipitated, the proportion of chromate transformed is minimal.

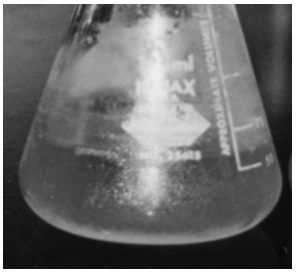

Figure 2. Sediment in a 24 h *P. putida* culture grown in LB + 15 mM K_2CrO_4.

Effect of Chromate on Cellular Characteristics. Chromate generated marked changes in cell size (Fig. 3). With progressive increase in chromate concentration up to 15 mM, the cells became both longer and wider (Table 3). However, further increase in chromate led to a decrease in cell size: cells grown with 30 mM chromate were smaller than the cells grown without chromate. The enlarged forms observed at 15 mM chromate also exhibited hyperflagellation, with the flagella wound around the cells (Fig. 3B). Cells grown with 30 mM chromate exhibited surface bulges (Fig. 3C). Thus, growth in the presence of chromate affects *P. putida* at many levels. These effects apparently did not involve mutations, since normal morphology was recovered upon cultivation of the cells in LB medium.

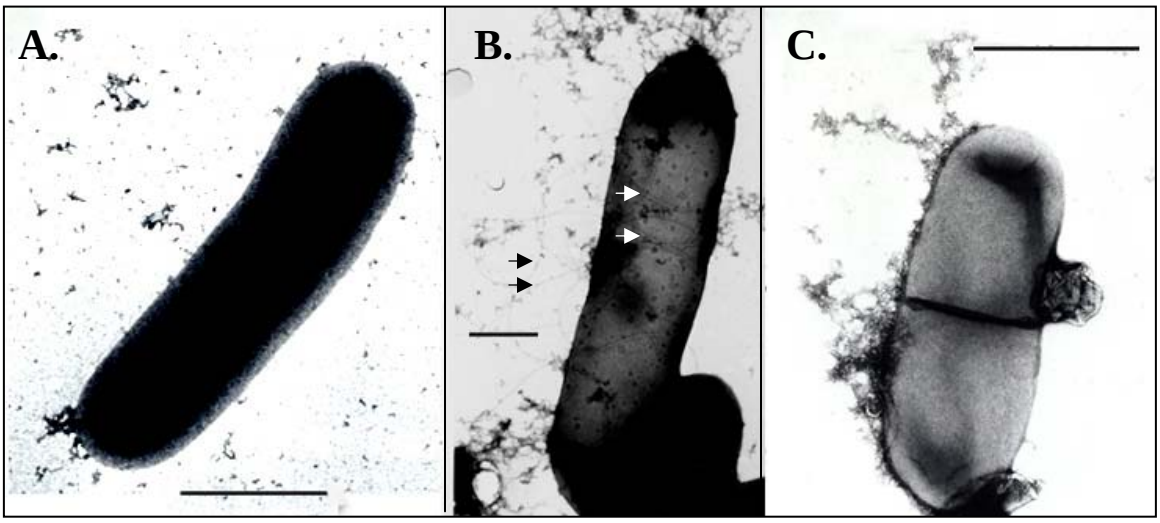

Figure 3. Electron micrographs showing the morphology of *P. putida* after incubation in LB for six days without (**A**), with 15 mM (**B**), or with 30 mM (**C**) chromate. In each frame the scale bar represents 1 μm. Examples of the flagella in (**B**) are indicated by arrows.

Effect of Growth Rate on Bulk Chromate Reductase Activity and Chromate Transformation. One of the effective means of in-situ bioremediation is biostimulation, which entails adding of nutrients to the environment in order to stimulate the growth of bacteria. Although enhanced growth promotes bioremediation, the resulting large amount of biomass can clog subsurface pores and confine effective remediation to a narrow zone around the feeding port (McCarty, 1994). This problem can be minimized by ensuring high level expression of a desired activity in slowly growing cells (Matin, 1994; Matin, 1995; Matin, 2001).

TABLE 3. Effect of chromate on *P. putida* MK1 cell dimensions[a.]

Cr(VI) concentration (mM)	Cell length[b.] (µM)	Cell width[b.] (µM)	Number of cells measured
0	4.60[c.]	1.58[c.]	50
10	7.01	2.20	31
15	14.86	2.72	38
20	6.26	3.12	17
25	4.31	2.27	50
30	3.52	1.19	50

a. After six days incubation in LB at specified chromate concentrations
b. Mean cell measurements (sem <1%)

We therefore determined the pattern of expression of bulk chromate reductase activity in *P. putida* and its chromate transforming ability at low growth rates. A chemostat was used to obtain a series of steady-state submaximal growth rates. Bulk chromate reductase activity was quantified in extracts of cells obtained after growth at different rates; chromate transforming ability was measured in whole cells by maintaining 0.4 mM chromate concentration in the chemostat vessel. Cells grown at different dilution (growth) rates, showed increased chromate reductase activity in cell-free extracts at low growth rates, attaining highest levels in stationary phase (Fig. 4). However, even though the bulk enzyme activity increased, the cells transformed less chromate with decreasing growth rate. This presumably results from non-availability of reducing equivalents at low growth rates (Matin, 1976).

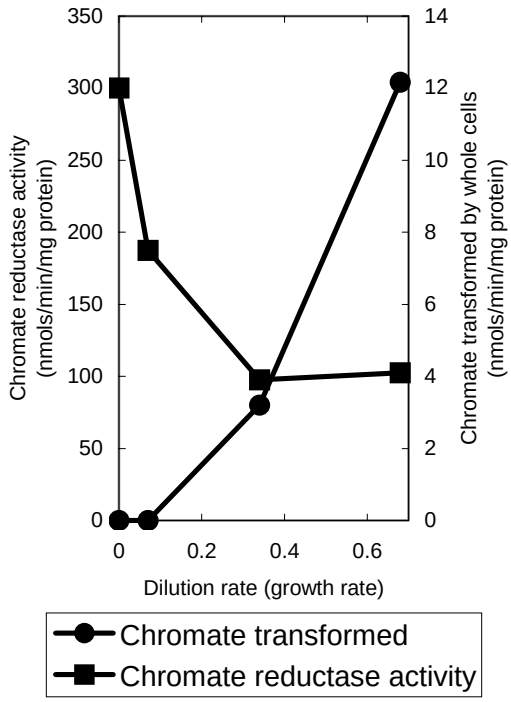

Figure 4. Chromate reductase activity in cell extracts and chromate transformation by *P. putida* cells grown at different dilution (growth) rates in a chemostat; the inflow medium was LB broth. Zero growth rate data pertain to batch culture stationary phase cells.

Effect of DOE Waste Site Materials on Chromate Reductase Activity and *P. putida* Growth. In-situ capacity to remediate necessitates the ability of a remediating organism and its key enzymes to function in the presence of mixed waste, since most polluted environments, especially the DOE sites, contain multiple pollutants. To examine the suitability of *P. putida* in this respect, we determined the effect of DOE contaminants on a chromate reductase enzyme that we have purified to homogeneity (Park, 2002), and on the growth of the bacterium itself. Sediment from a DOE site was extracted as described in Materials and Methods. The extracted contaminants, even after dilution, markedly inhibited the enzyme activity (Fig. 5). *P. putida* grown with the extract showed growth inhibition; after overnight incubation, the A_{660} in the amended cultures was 0.45 vs. ca. 1 in the un-amended culture; both cultures possessed 400 μM chromate.

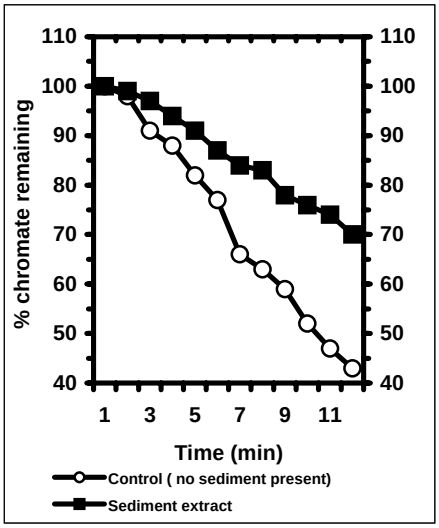

Figure 5. Inhibition of chromate reductase activity by DOE sediment extract. 1.25 μg of enzyme were incubated with 400 μM Cr(VI) with and without DOE sediment extract. Residual Cr(VI) was assayed every minute.

CONCLUSION

While bacterial remediation of chromate appears to be a promising approach, the above results reveal several areas in which a key bacterium (*P. putida* MK1) can be improved to make this a realistic possibility. These include measures to: a) enhance Cr(III) precipitation at low concentrations; b) decrease chromate toxicity to the bacterium; c) enhance the effectiveness of chromate reducing enzymes at low growth rates; and d) decrease enzyme and bacterial sensitivity to polluted site co-contaminants. Our ongoing work concerning chromate reductase genes we have cloned from several bacteria (Park, 2002) and enhancing their activity through approaches such as DNA shuffling (Stemmer, 1994) is aimed at attempting these improvements.

ACKNOWLEDGMENTS

This research was funded by grant DE-FG03-97ER-624940 to A.M. from the Natural and Accelerated Bioremediation (NABIR) program, Biological and Environmental Research (EBER), and U.S. Department of Energy (DOE). DA was supported by fellowship STAX0101 from FRST New Zealand. We thank Nafisa Ghori for her help in taking the electron micrographs and Scott Fendorf for the XANES analysis.

REFERENCES

Alvarez A.H., R. Moreno-Sanchez, and C. Cervantes. 1999. Chromate efflux by means of the ChrA chromate resistance protein from *Pseudomonas aeruginosa*. *J. Bacteriol. 181*:7398-400.

Cervantes, C., J. Campos-Garcia, S. Devars, F. Gutierrez-Corona, H. Loza-Tavera, J.C. Torres-Guzman, and R. Moreno-Sanchez. 2001. Interactions of chromium with microorganisms and plants. *FEMS Microbiol. Rev. 25*:335-47.

Greenberg, A.E., J.J. Connors, D. Jenkins, and M.A. Franson (ed.). 1981. Standard methods for the examination of water and wastewater, 15th ed., pp.187–90. American Public Health Association, Washington, DC.

Kim, Y., L.S. Watrud, and A. Matin. (1995) A carbon starvation survival gene of *Pseudomonas putida* is regulated by σ^{54}. *J. Bacteriol. 177*: 1850-9.

Matin, A. 1994. Starvation promoters of *Escherichia coli*, their function, regulation, and use in bioprocessing and bioremediation. *Ann. N. Y. Acad. Sci. 721*:277-91.

Matin, A. 2001. Stress response in bacteria. *Encyclopedia of Environmental Microbiology*, John Wiley and Sons: New York. Volume 6, pp 3034-6.

Matin, A., and J.C. Gottschal. 1976. Influence of dilution rate on NAD(P) and NAD(P)H concentrations and ratios in a *Pseudomonas* sp. grown in continuous culture. *J. Gen. Microbiol. 94*:333-41.

Matin, A., C.D. Little, C.D. Fraley, and M. Keyhan. 1995. Use of starvation promoters to limit growth and selectively enrich expression of trichloroethylene- and phenol-transforming activity in recombinant *Escherichia coli* [corrected]. [erratum appears in *Appl. Environ. Microbiol. 61*:4140.] *Appl Environ. Microbiol. 61*:3323-8.

McIlwain, C. 1996. Science seeks weapons clean-up role. *Nature 383*:375-379.

McCarty, P.L. 1994. Ground-water treatment for chlorinated solvents. In *Handbook of Bioremediation*, J.E. Matthews, ed. (Ann Arbor, Mich.: Lewis Publishers), pp. 87-116.

Pandza, S., M. Baetens, C.H. Park, T. Au, M. Keyhan, and A. Matin. 2000. The G-protein FlhF has a role in polar flagellar placement and general stress response induction in *Pseudomonas putida*. *Mol. Microbiol. 36*:414-23.

Park, C.H., C.F. Gonzalez, D.F. Ackerley, M. Keyhan, and A. Matin. 2002. Molecular engineering of soluble bacterial proteins with chromate reductase activity. *Volume III of Proceedings of the First International Conference on Remediation of Contaminated Sediments*, Venice, Italy, Hinchee et al. (eds.), Battelle Press, pp. 103-111.

Park, C.H., M. Keyhan, B. Wielinga, S. Fendorf, and A. Matin. 2000. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl. Environ. Microbiol. 66*:1788-95.

Puzon, G.J., J.N. Petersen, A.G. Roberts, D.M. Kramer, and L. Xun. 2002. A bacterial flavin reductase system reduces chromate to a soluble chromium(III)-NAD(+) complex. *Biochem. Biophys. Res. Commun. 294*:76-81.

Riley, R.G., J.M. Zachara, and F. J. Wobber. 1992. Chemical contaminants on DOE lands and selection of contaminants mixtures for subsurface science research. *Report DOE/ER-0547T*. U.S. Department of Energy, Washington, DC.

Stemmer, W.P. 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA. 91*:10747-51.

Timmis, K.N., and D.H. Pieper. 1999. Bacteria designed for bioremediation. *Trends Biotechnol. 17*:200-4.

Zgurskaya, H., M. Keyhan, and A. Matin. 1997. The σ^{s} level in starving *Escherichia coli* cells increases solely as a result of its increased stability, despite decreased synthesis. *Mol. Microbiol. 24*:643-51.