NOTES

Remobilization of Heavy Metals Retained as Oxyhydroxides or Silicates by *Bacillus subtilis* Cells

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The stability of heavy metals bound as either silicates or oxyhydroxides by the surfaces of *Bacillus subtilis* with changes in pH and presence of competing cations and EDTA was studied. Wall-bound silicate was very stable. Of all conditions, a pH of 3.0 (by HNO₃) promoted the greatest release of Cd (87% [wt/wt]), Pb (77%), or Cu (54%). Bacterial Pb resisted extraction by EDTA, and Cd that was included in bacterial-silicates proved difficult to remobilize.

Microorganisms can affect heavy metal concentrations in the environment because they exhibit a strong ability for metal removal from solution; this can be through either enzymatic or nonenzymatic mechanisms (8, 15, 18, 20, 25, 29, 31, 32, 38). Bacteria, in particular, are efficient sorbents of heavy metals, although subtle differences can be seen between species and under various physicochemical conditions (for examples, see references 1-3, 5, and 30). Most studies have centered on the ability of microorganisms to retain metals, and only a few have attempted to elucidate the metal-binding mechanisms (5, 6, 10, 30), the ionic species bound (28, 30), the type of complex or mineral formed (4, 6, 12, 13, 19, 26, 33, 37), or the tendency of the immobilization process to be reversed (14, 17, 23, 27). Even though this last factor is rarely addressed, from an environmental viewpoint it is as important as the initial immobilization of metals to ensure that the metals will remain insoluble.

A previous study from our laboratory showed that the gram-positive bacterium *Bacillus subtilis* was able to retain several heavy metals as silicate minerals or as oxyhydroxides at 20 and 4°C under laboratory simulations of natural conditions (36). This research looks at the stability of these wall-bound inorganic complexes when physicochemical conditions of the environment are modified. Twenty-four-week-old mineralized samples from metal-silicate experiments (36) were treated with solutions that simulated possible natural changes in several environmental conditions, i.e., (i) a drop in pH, (ii) the presence of additional complexing agents in solution, and (iii) an increase in ionic strength and competing cations.

B. subtilis cells were exposed to 1 µM Pb, Cd, Cr, Cu, Ni, Zn, Al, and Fe (as nitrates [Fisher and Fluka]) at 4°C (metals cold, or MC system) or at 20°C (ambient metals, or M system) or to the same heavy metals plus silicon (0.15 mM H₄SiO₄, added as Na₂SiO₃ [Fisher]) at 20°C (MSi system) for 24 weeks, resulting in the nucleation and formation of metal oxyhydroxides and silicates on their surfaces (36). The cells were not viable after this period, but since autolysis was inhibited by the heavy metals (35), they remained intact and

no endospores were produced. Three replicates from each system were pelleted by centrifugation (14,000 × g), the supernatants were separated, and the cells were washed once in high-resistance ultrapure deionized water (UDW). Si, Pb, Cr, Cd, Ni, Zn, and Cu were analyzed in the supernatants by graphite furnace atomic absorption spectrometry (AA) by using a Perkin-Elmer 2380 spectrophotometer. Whole mounts of cells were studied by transmission electron microscopy (TEM) in a Philips EM300 microscope operating at 60 kV and by energy-dispersive X-ray spectroscopy (EDS) conducted by using a Link-Analytical eXL/LZ-5 system connected to a Philips EM400T electron microscope operating at 100 kV under standard conditions with a beam current of 10 µA, a beam diameter of 400 nm, and a counting time of 100 s.

Remobilization solutions. Four solutions were employed: (i) UDW that was adjusted to pH 3.0 by using 0.1 M HNO₃ (pH 3 treatment), (ii) a 0.5 mM solution of EDTA (free form) (EDTA treatment), (iii) a 2.0 mM solution of Ca(NO₃)₂ (Ca treatment), and (iv) a 0.5 mM NaOH solution (NaOH treatment). The concentrations of EDTA and Ca(NO₃)₂ solutions were chosen since they showed high extraction efficiency during the study of Flemming and coworkers (14). The pHs of the EDTA, Ca(NO₃)₂, and NaOH solutions were adjusted to 7.0 by using either 0.1 M NaOH or 0.1 M HNO₃.

Cells from each replicate were briefly homogenized and resuspended in UDW to the original volume (approximate optical density at 600 nm, 0.2) and were divided into four aliquots and mixed 1:1 with doubly concentrated remobilization solutions. Remobilization of metals or Si was carried out for 48 h at the appropriate temperature. After that time, cells were pelleted by centrifugation (14,000 × g), and the supernatants were kept for AA analysis of all metals and Si. At the same time, whole mounts were prepared for TEM and EDS. On the MSi system, two additional replicates were treated so that supernatants and pellets could be analyzed for Si by AA. The pellets were digested in 2% (vol/vol) HNO₃ before Si determination. Only acid-leached plasticware (soaked in 50% [vol/vol] HNO₃ overnight) was used throughout the experiments to ensure that there was no metal contamination.

Resolubilization in water. Little metal of Si was solubilized

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TABLE 1. Metals and Si solubilized in UDW

System	% of following element ^a :					
	Pb	Si	Cd	Zn	Ni	Cu
MSi	2.8 ± 0.8	5.2 ± 0.7	5.5 ± 1.1	13.7 ± 3.7	1.7 ± 0.5	2.9 ± 1.2
M	2.5 ± 0.4		10.2 ± 4.7	10.4 ± 3.9	5.6 ± 2.8	4.3 ± 2.1
Mc	0.9 ± 0.8		2.9 ± 0.7	7.6 ± 3.0	2.4 ± 0.5	2.3 ± 0.6

^a Expressed as a percentage (wt/wt) of the metal initially retained by bacterial surfaces. Values are means of three replicates per system ± standard deviation.

during the washing procedures before the samples were subjected to the remobilization solutions (this was always less than 13% (wt/wt) of the bacterially immobilized metal [Table 1]). Using the *F* test, there were no statistically significant differences between the replicates in each system or between the individual systems themselves (except for the Pb minerals, which were less soluble in water at 4°C [Mc samples] than at 20°C [MSi and M samples]). Since only small quantities of elements were lost during washing by UDW, a dilution factor by itself will not drastically affect these preformed minerals.

The total amounts of metals and Si that the cells retained after the wash (Table 2) were used to evaluate the effect of the remobilization treatments. Values for both the Si (Table 3) and the metals (Table 4) solubilized by the different remobilization agents are expressed as weight percentages in the tables.

Effect of remobilization solutions on Si. Only small quantities of Si were remobilized (Table 3). Si was not affected by an increase in acidity by HNO₃ (pH 3, MSi system) and remained as fine-grained minerals on the bacterial surfaces as shown by TEM, EDS (Fig. 1A and Table 5), and the AA analysis of the digested cell pellets (data not shown). Solutions of Ca(NO₃)₂ and NaOH also did not remove large quantities of Si (Table 3). In fact, Ca²⁺ ions were actually complexed to the structure of the bacterial silicates (Table 5), which made them quite crystalline (Fig. 1B).

EDTA was the most efficient Si-extracting agent of the group, although with a high standard deviation from the mean (Table 3). Nevertheless, silicates were still associated with the bacterial surfaces, as shown by EDS (Table 5 and Fig. 2) and the AA results of the digested cell pellets (data not shown).

Results showed that, once established, the Si-wall linkage

TABLE 2. Total amounts of elements retained by the bacterial surfaces after a wash in UDW

System and sample replicate	Amt (μg/mg [dry wt] of cells) of following element:					
	Si	Pb	Cd	Zn	Ni	Cu
MSi						
1	16.9	1.10	0.37	0.27	0.20	0.28
2	20.5	1.22	0.41	0.16	0.25	0.36
3	19.5	1.33	0.35	0.21	0.21	0.37
M						
1		1.46	0.31	0.29	0.25	0.23
2		1.45	0.19	0.31	0.22	0.23
3		1.30	0.18	0.22	0.18	0.21
Mc						
1		1.32	0.47	0.26	0.33	0.22
2		1.53	0.52	0.23	0.33	0.25
3		1.37	0.50	0.27	0.33	0.26

and the Si minerals were quite stable. Only EDTA remobilized this element, probably by extracting multivalent metal cations that were involved with the cation bridging of the silicate anions to the cell surface through outer sphere complexes, as has been postulated (35). Since Si-rich crystallites remained on the surfaces of EDTA-treated cells, it is also possible that only the poorly ordered or less mature phases of silicate were remobilized and only the better-structured crystallites remained after treatment. Cation bridging also explains the stabilization effect that the Ca solution had on the bacterial silicates. Ca²⁺ would help stabilize existing silicate complexes with additional cation bridges to electronegative sites on the walls. As well, the Ca²⁺ should also intercalate into less-organized domains of crystallites to better order the silicate into crystalline lattices.

Effect of remobilization solutions on metals. Metals showed quite a variable behavior. Remobilization of bacterially retained Pb fluctuated greatly. The ability to release Pb increased in the order NaOH ≤ Ca < EDTA < pH 3 (Table

TABLE 3. Si extracted by the remobilization solutions in the MSi system^a

Agent and replicate	Si retained	
	%	Mean %
pH 3		0 ± 0
1	0	
2	0	
3	0	
4	0	
5	0	
EDTA		27 ± 21
1	69	
2	27	
3	16	
4	13	
5	13	
Ca		6 ± 4
1	8	
2	0	
3	5	
4	12	
5	5	
NaOH		5 ± 7
1	4	
2	1	
3	2	
4	19	
5	0	

^a Expressed as a percentage (wt/wt) of the Si retained by cells. Values are averages of three aliquots per replicate. For more details of the systems, see Table 2.

TABLE 4. Metals extracted by the remobilization solutions in the three systems^a

System and agent	% of following element retained by cells				
	Pb	Cd	Zn	Cu	Ni
MSi					
pH 3	79 ± 15	73 ± 19	15 ± 4	58 ± 27	13 ± 8
EDTA	36 ± 10	21 ± 8	20 ± 12	20 ± 12	6 ± 2
Ca	6 ± 1	31 ± 12	10 ± 1	0 ± 0	5 ± 4
NaOH	1 ± 0.5	12 ± 3	11 ± 11	2 ± 3	5 ± 3
M					
pH 3	72 ± 16	100 ± 0	9 ± 12	67 ± 27	1 ± 2
EDTA	39 ± 7	37 ± 4	0 ± 0	21 ± 10	1 ± 2
Ca	4 ± 1	64 ± 26	12 ± 9	0 ± 0	4 ± 0
NaOH	2 ± 1	23 ± 2	3 ± 5	4 ± 0.5	4 ± 0
Mc					
pH 3	79 ± 16	89 ± 11	63 ± 24	36 ± 15	17 ± 4
EDTA	57 ± 14	50 ± 12	16 ± 13	58 ± 10	9 ± 2
Ca	8 ± 2	62 ± 2	74 ± 15	5 ± 4	6 ± 0
NaOH	1 ± 0.5	10 ± 1	0 ± 0	0 ± 0	0 ± 0

^a Values are means of three replicates, and there were three aliquots per replicate. For more details of the systems, see Table 2.

4; the results between EDTA and Ca and those for pH 3 and EDTA are significant at the 99% confidence level, using the least significant difference test). The type of extraction agent was the crucial factor to explain sample variance (99% confidence level, *F* test) since there were no significant differences due to the conditions for cell mineralization or system-agent statistical interactions. The pH 3 nitric acid treatment remobilized most ($\approx 77\%$) of the adsorbed Pb. TEM of those samples showed a complete disappearance of the typical Pb minerals on the cells, and EDS confirmed that only residual Pb was left in the cells (Fig. 1A; Table 5). Only a small fraction of the total cellular Pb was incorporated into these siliceous forms (36). Since metal oxyhydroxides dissolve at low pHs, it is obvious that this is the cause of Pb remobilization by the nitric acid treatment. High-level Pb removal by strong acidity has also been observed in soils (11, 16, 21, 22).

EDTA also extracted a considerable amount of Pb from the cells (44% [average of the three systems]). Theoretically, EDTA affinity for Pb cations is high ($\log K_m = 18.9$ [24]), and indeed, studies conducted with inorganic sorbents (kaolinite, illite, and montmorillonite) showed greater Pb extraction by EDTA than by acid pH (11). The reason that EDTA did not extract the Pb from our systems as effectively as it did in the Farrah and Pickering clay study (11) is presumably because of the bacterial surfaces; these surfaces possess numerous carboxylate groups (from the peptidoglycan) and phosphate groups (from the teichoic acid) which will compete effectively with the three carboxylate groups of EDTA (34). Since in both cases COO^- are the active groups, bacterial peptidoglycan and EDTA should have similar affinities for Pb. Also, some of the Pb was retained intracellularly (36), and this fraction would not be extracted by EDTA, since this reagent cannot easily enter the cytoplasm (9). Bacterially bound Pb also resisted increases in ionic strength and ion exchange (Table 4, Ca and NaOH treatments). Size differences in hydrated radii between Ca^{2+} or Na^+ and Pb^{2+} ions could explain the lack of exchangeability. Similar results were found in soils, where Ca and Na solutions had little Pb-extractive ability (11).

The greatest remobilization in this study occurred with Cd (Table 4), increasing in the order $\text{NaOH} < \text{EDTA} < \text{Ca} <$

pH 3; these differences were all statistically significant at a 99% confidence level. Cd in silicate structures was more resilient than Cd in oxyhydroxide forms (only 34% of Cd was extracted from bacterial silicates, on average). EDS showed residual Cd bound to the bacterial surfaces after each treatment (Table 5). Ca^{2+} exchange with bound Cd^{2+} was also important for remobilization; the two cations have similar charges and an ionic size which favors homoionic exchange (7). Na^{2+} did not have the same effect because it is a much bigger ion.

The remobilization solutions had different effects on wall-bound Zn (Table 4). The greatest remobilization occurred at 4°C, a condition under which Zn was easily extracted from the cells, especially by competing Ca^{2+} ions and acidic pH (99% confidence level). Exchange of Zn by Ca was also found for metal adsorbed to kaolinite, illite, or montmorillonite (11). At 20°C, EDS (Table 5) confirmed that Zn was relatively more abundant after the treatments than Cd, Cr, or Ni, showing that Zn had not been leached from the cells to the same extent as the other metals, although statistical analysis was not conducted on this set of data.

The stability of the wall-bound Cu during the remobilization treatments varied with the conditions under which metals were bound to the cell surfaces (statistical significance was at a 97% level of confidence). At 20°C (MSi and M systems), the extraction efficiency increased in the order $\text{Ca} \leq \text{NaOH} \leq \text{EDTA} < \text{pH 3}$ (at a 99% confidence level, using the least significant difference test), but at 4°C it was $\text{NaOH} \leq \text{Ca} \leq \text{pH 3} \leq \text{EDTA}$ (Table 4). Cu could not be quantified by EDS, because copper grids were used for TEM.

Again, strong acidity (by nitric acid) and organic complexing ligands (EDTA) were the strongest Cu-remobilizing agents, and as for Zn, remobilization occurred most easily at low temperatures. It was unaffected by the Ca and Na treatments. Cu extractability by EDTA was slightly reduced when the metal formed part of the silicate structure (although differences did not reach statistical significance by the least significant difference test, at a 99% confidence level). This reagent was less efficient at extracting Cu than expected on the basis of its affinity constant ($\log K_m = 19.7$ [24]). Our results showed that Cu was more resilient during EDTA and Ca treatments than when bound only to isolated

FIG. 1. (A) EDS spectrum of the bacterial surfaces in the MSi system after the pH 3 treatment; (B) TEM micrograph of a whole mount preparation of MSi cells after Ca treatment. Bar, 200 nm. Cu peaks in the EDS spectra correspond to the Cu grid for TEM, and P peaks are from the cell wall.

TABLE 5. EDS elemental analysis of individual crystallites on the bacterial surfaces after the remobilization treatments^a

System and agent	% of following element:									Description	
	Fe	Si	Al	P	Cd	Ni	Cr	Zn	Pb		Ca
MSi											
Before treatment	5.4	31.8	0.0	52.3	0.0	1.6	5.7	1.9	1.0		Surface Fussiform Granular
	6.7	0.2	0.6	30.9	3.1	2.6	1.0	5.3	48.5		
	7.0	78.3	0.0	9.1	0.0	2.8	0.9	1.8	0.0		
pH 3	50.2	2.6	0.0	3.2	2.4	0.0	3.1	37.9	0.5		Surface
	12.9	0.0	0.0	81.3	0.0	1.8	0.5	3.4	0.0		Surface
	5.9	40.6	1.9	43.6	2.8	0.9	0.8	2.7	0.5		Granular
EDTA	10.0	10.0	1.9	64.5	4.8	2.2	0.7	5.9	0.0		Granular
	2.4	4.8	0.0	29.1	7.4	1.3	0.6	7.6	46.6		Granular/octagonal
	9.1	12.1	2.1	40.8	18.4	9.3	2.2	5.6	0.2		Granular
Ca	6.7	67.0	0.2	18.2	0.9	0.1	0.0	4.9	2.0		Granular
	5.2	43.7	1.2	40.8	1.7	2.0	0.0	5.4	0.0		Fossilized cell
	3.7	91.2	1.0	1.1	0.5	0.6	0.7	1.2	0.0		Granular
NaOH	12.2	38.0	2.1	39.6	0.0	0.0	2.0	5.8	0.0		Granular
	9.1	16.9	1.1	24.0	2.0	0.4	0.8	3.3	42.2		Granular/octagonal
	9.2	13.7	1.0	18.4	1.7	0.9	0.6	4.0	50.3		Fusiform/granular
	4.5	0.0	0.6	30.3	2.9	0.0	0.3	4.6	56.6		Granular/fusiform
	7.4	74.8	2.6	4.5	0.0	0.3	2.9	6.4	0.9		Granular
	47.1	9.6	10.5	1.1	8.1	0.0	0.0	23.5	0.0		Granular
M											
Before treatment	8.5		2.2	33.2	3.8	0.0	6.3	11.6	34.3		Octagonal Granular Rounded
	10.1		0.4	40.3	0.0	2.3	6.5	8.2	32.0		
	9.2		0.6	45.0	1.4	1.7	1.6	4.4	35.9		
pH 3	16.3		1.0	36.4	6.5	0.0	2.5	16.5	9.8	10.9	Octagonal
EDTA	24.3		0.0	54.8	0.0	0.0	1.1	17.2	0.0	2.6	Surface
	7.3		2.1	83.9	0.3	1.4	0.0	3.3	0.0	1.6	Surface
Ca	25.3		0.0	17.8	0.0	2.3	0.2	4.1	0.0	50.2	Granular
NaOH	5.1		0.2	21.9	3.6	0.3	1.1	1.1	47.6	19.0	Fusiform
	3.4		0.0	15.9	3.7	1.0	0.0	1.5	72.3	2.0	Fusiform
	12.2		0.0	69.9	7.1	0.0	3.2	6.2	0.0	1.2	Surface
	4.8		0.0	20.5	5.9	0.0	0.0	8.9	57.3	2.4	Fusiform
Mc											
Ca	8.8		0.2	33.1	4.3	0.0	2.2	1.8	2.8	46.7	Granular
	3.6		0.0	22.2	0.0	0.0	1.0	2.0	1.1	70.0	Surface
NaOH	5.8		0.3	50.0	4.3	3.3	2.7	6.5	14.5	12.5	Pseudocubic
	14.0		0.0	22.7	0.0	0.0	7.2	12.4	38.1	5.5	Granular

^a In boldface are representative examples of the compositions of different types of precipitates before the treatments (36).

walls ([Ca released approximately 60% of the Cu bound in these isolated walls [14]). Contrary to this, diluted nitric acid at pH 3 extracted higher proportions of Cu than it did from isolated bacterial walls (14). It seems that the inclusion of Cu in silicate minerals or the presence of other metals has subtly changed the character of the Cu-wall linkage found in isolated walls.

In general, the Ni extraction level was low (Table 4). The remobilization efficiency increased in the order NaOH < Ca = EDTA < pH 3. Ni retention by bacterial surfaces was highest at 20°C if only metals were present (M system), although amounts extracted in all systems were always less than 10%. Ni content in individual crystallites (Table 5) was generally low, at least with the sensitivity provided by the EDS technique.

In a previous publication on a different system, acidic pH was the most efficient agent in removing Cr, Ag, and Cu from bacterial walls, envelopes, and clay minerals and their composites (14). Overall, our present results agree that strong acidity by nitric acid (pH 3.0) promotes the greatest release of metals (Table 6), especially Cd and Pb. Wall-

bound Si was essentially stable, and these results fit well with our proposed cation bridging mechanism for silicate binding to bacterial walls (35).

In nature, if soluble Si and heavy metals are available to bacteria, a number of mineral phases will be formed in time. The metals will complex to available organic sites on the bacterial surface and will, eventually, form mineral crystallites which are driven by the abundant counter ions to the mineralized metals in the surrounding fluid phase (e.g., sulfates, carbonates, silicates, and oxyhydroxides). As silicates develop on the bacterium, more heavy metals will be incorporated into the Si-metal fabric as well. Our present experimentation reveals that these neoformed bacterial Si-metal minerals are generally more resistant to metal remobilization than the wall-metal variety. A previous study (14) suggests that the wall-metal minerals are more resilient than metals adsorbed to preformed silicates such as smectite and kaolinite. Overall, metal remobilization seems to follow the order preformed silicates > wall-metal crystallites > bacterial Si-metal minerals. It is apparent that the metals are more

TABLE 6. Summary of conditions that would promote remobilization in this study (higher than 50% of the amount of element retained)

Element and system	Summary of extraction efficiencies ^a	Process ^b	Environmental conditions that will promote remobilization ^c
Pb	pH 3 > EDTA ≥ Ca ≥ NaOH 77 44 6 1	Dissolution	Strongly acidic pH
Cd	pH 3 > Ca > EDTA > NaOH 87 53 36 15	Ionic exchange with Ca ²⁺ and H ⁺	Strongly acidic pH or the presence of Ca ²⁺ ions in solution, especially at low temperature (4°C)
Cu			
MSi, M	pH 3 > EDTA ≥ NaOH > Ca 63 20 3 0	Dissolution	Strongly acidic pH
Mc	EDTA ≥ pH 3 > Ca > NaOH 58 36 5 0	Complexation	Presence of complexing agents at low temperature
Zn			
MSi	EDTA > pH 3 ≥ NaOH = Ca 35 15 11 10	Complexation	
M	Ca ≥ pH 3 ≥ NaOH ≥ EDTA 12 9 9 0		
Mc	Ca ≥ pH 3 > EDTA > NaOH 74 63 16 0	Ionic exchange with Ca ²⁺ and H ⁺	Presence of Ca ²⁺ at low temperature or a strongly acidic pH
Si	EDTA ≥ Ca = NaOH > pH 3 37 4 2 0		

^a Extraction efficiencies are expressed as percentages (wt/wt) of bacterially retained element.^b See reference 14 for an extended explanation of these processes.^c When the element is retained by bacterial cells.

FIG. 2. EDS spectrum of granular precipitates on cell surfaces from the MSi system after EDTA treatment. Cu peaks correspond to the Cu grid for TEM.

strongly retained in bacterially derived crystallites than in their inorganic counterparts.

It is especially interesting that the metals bound to bacteria were quite resistant to extraction by organic ligands, since the abundance of organic compounds in soils and waters makes them the most probable metal-remobilizing agents; many metal-organic complexes are water soluble, and as such, they will be transported in the environment. Confined systems are rare in nature; most commonly, transfers of water and matter occur between soils, surface waters, groundwaters, and organisms. Efficient ways to retain contaminants of any kind in the nonliving solid phase then have to be promoted. On the basis of the most recent results in different fields of research, bacteria seem quite adequate agents to do so.

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