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Enhanced Selenate Accumulation in *Cupriavidus metallidurans* CH34 Does Not Trigger a Detoxification Pathway▽

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***Cupriavidus metallidurans* CH34 cells grown under sulfate-limited conditions accumulated up to six times more selenate than cells grown in sulfate-rich medium. The products of selenate reduction detected by X-ray absorption spectroscopy, electron microscopy, and energy-dispersive X-ray analysis did not define this strain as being a good candidate for bioremediation of selenate-contaminated environments.**

Because microbial communities may impact the speciation of selenium, they may have potential applications in remediation of selenium-contaminated soils and waters or in removing selenium from industrial effluents. *Cupriavidus metallidurans* CH34 (formerly *Ralstonia metallidurans* CH34), a soil bacterium characteristic of metal-contaminated biotopes (6, 7), is known to be resistant to selenate and selenite (9, 10). Selenite resistance is usually explained by the ability of this strain to incorporate selenite in the cytoplasm, where it is reduced to red elemental selenium (9). In *C. metallidurans* CH34, selenite may be transformed by two competing reactions (10), an assimilatory pathway leading to selenomethionine and a detoxification pathway initiated after several hours of selenite stress and leading to massive uptake of selenite and quantitative reduction to the insoluble elemental form [Se(0)]. *C. metallidurans* CH34 is very resistant to selenate and takes up only small amounts of selenate (10). In the case of a selenate challenge, Se(0) was detected as a minor species in bacterial cells and seleno-L-methionine was the main reduction product. Sarret et al. (10) hypothesized that the amount of selenium taken up on selenate exposure is insufficient to induce detoxification. It is believed that transport of selenate into the cell occurs nonspecifically through the sulfate permease as shown in *Escherichia coli* (5) and higher plants (11). In this study, we grew the cells in sulfate-limited medium as sulfate deprivation of bacteria causes a derepression of their sulfur assimilation pathway and consequently an increase in the amount of sulfate permease (4). The impact of this growth condition on the uptake of selenate and on its fate was investigated.

C. metallidurans CH34 was grown aerobically at 29°C in Tris-salt mineral medium (TSM) with 2% gluconate as a carbon source as described previously (10). TSM classically contains 3 mM Na_2SO_4 (hereafter called TSM-3). For sulfate deprivation experiments, cells were precultured in TSM-3 until mid-exponential phase and then inoculated in TSM containing only 0.3 mM sodium sulfate (reported as TSM-0.3) at an A_{600} of 0.3. The culture was grown until the beginning of the stationary phase and used to inoculate fresh TSM-0.3. This last procedure was repeated twice. Sodium selenate was prepared as 0.2 M stock solution in ultra-pure water, sterilized by filtration, and added at an A_{600} of 7 to a final concentration of 2 mM. Control cultures without Se were grown in parallel. Aliquots of 5 ml of suspension were sampled at various times during growth, centrifuged, washed twice in 10 mM Tris-HCl (pH 7.2), resuspended in ultra-pure water, and frozen at −20°C until further use. Inductively coupled plasma-mass spectrometry analyses were performed as described previously (10). Transmission electron microscopy (TEM) and energy-dispersive X-ray (EDX) analyses were carried out as previously described (2, 9). X-ray absorption near-edge structure (XANES) spectroscopy experiments performed at the FAME beamline (8) of the European Synchrotron Radiation Facility and treatment of the data were carried out as already described (2, 10).

The selenium content of bacteria grown in TSM-0.3 was compared to that of bacteria grown in TSM-3 for a comparative assessment of selenate uptake. After 5 h of selenate exposure, bacteria cultured in TSM-0.3 had accumulated up to six times more Se than bacteria grown in TSM-3. This result supports the hypothesis that selenate enters cells via the sulfate permease. To check the fate of selenate when incorporated in large amounts by the bacteria, its speciation was determined by

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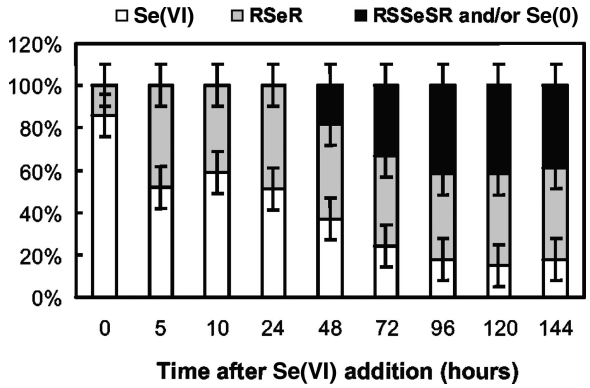
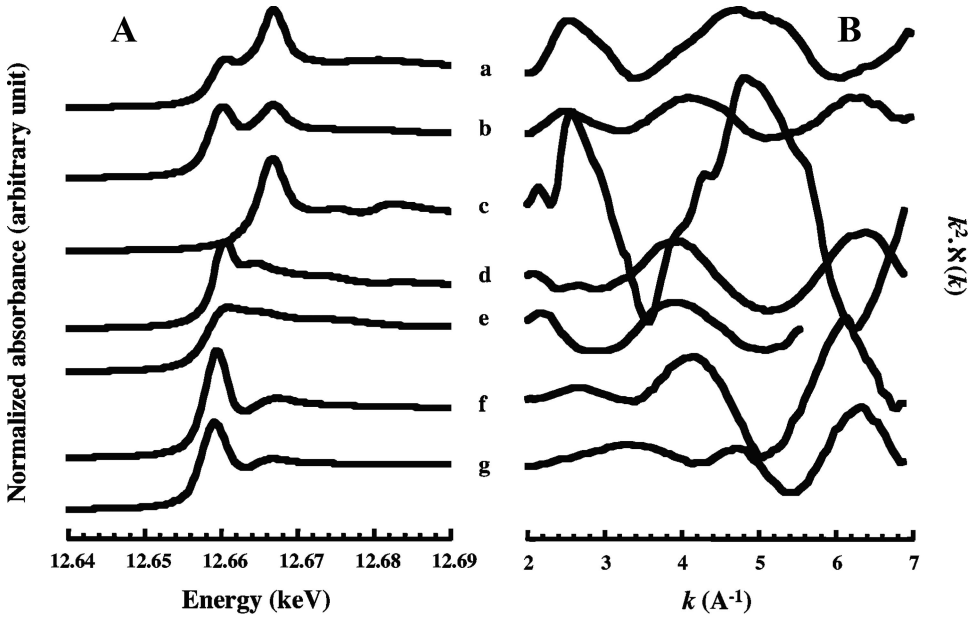


FIG. 1. Distribution of selenium species obtained at different times of exposure of *C. metallidurans* CH34 cells to 2 mM selenate added in TSM medium containing 0.3 mM sulfate. Selenate was added in the culture medium at the end of the exponential growth phase. Distribution of selenium species was determined from Se K-edge XANES spectra simulated by linear combination from reference spectra of crystallized sodium selenate [Se(VI)] and either the selenodiglutathione organic species, RSSeSR, or red elemental selenium, Se(0).

XANES spectroscopy after varying lengths of exposure to selenate in TSM-0.3. Principal component analysis and linear combination fits (LCFs) were used to treat the set of spectra obtained. A mixture of three components was proposed (eigenvalues [eV] of 58.8, 5.3, and 1.2) (Fig. 1). Two were identified as selenate and alkyl selenide (normalized sum-squares residual values of 1.5×10^{-2} and 1.2×10^{-3}, respectively). The alkyl selenide likely corresponded to the previously identified seleno-L-methionine (RSeR) (1), but XANES spectroscopy did not permit its direct identification. A good fit of the experimental signal was obtained with either selenodiglutathione (RSSeSR) or Se(0) (normalized sum-squares residual values of 1.1×10^{-3} and 3.0×10^{-3}, respectively) as the third compound of the three-component mixture. Because this third species was in the minority and the RSSeSR or Se(0) spectrum was shifted by less than 1 eV, we could not distinguish these species by using XANES (Fig. 2A). Just after selenate introduction, bacteria contained mainly selenate (86%) with a small fraction of alkyl selenide (14%). After 5 h, about 50% of the selenate was transformed to alkyl selenide. This proportion remained stable for up to 24 h. For longer exposure times, the percentage of RSeR remained stable while the selenate proportion decreased concomitantly with an increase in the



C

Species (%) determined from EXAFS simulations

	Se(0)	RSSeSR	RSeR	Se(VI)
Option RSSeSR = 0	13	0	79	8
Option Se(0) = 0	0	36	52	12

FIG. 2. Se K-edge XANES (A) and EXAFS (B) spectra for bacteria sampled at the beginning (a) and end (b) of exposure to 2 mM selenate in TSM medium containing 0.3 mM sulfate. $k^2 \cdot \chi(k)$, EXAFS function weighted by k^2, with k being the wave vector in inverse Angstroms (A^{-1}). Se references are also shown as follows: c, selenate [Se(VI)]; d, methyl-seleno-L-cysteine (RSeR); e, seleno-L-methionine (RSeR); f, selenodiglutathione (RSSeSR); and g, red elemental selenium [Se(0)]. (C) Species distribution obtained with bacteria exposed for 6 days to 2 mM selenate in TSM medium containing 0.3 mM sulfate. Simulations of EXAFS spectra were performed by an LCF of three components: Se(VI), RSeR and RSSeSR, or Se(0). Values below 10% (in italics) were considered not significant.

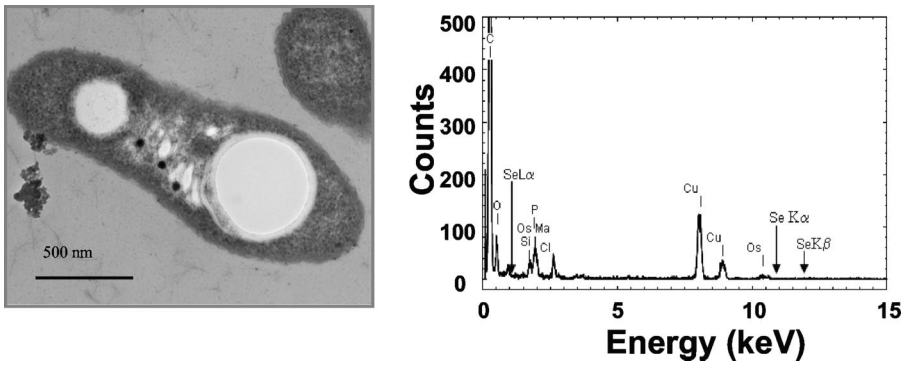


FIG. 3. (Left) TEM images of *C. metallidurans* CH34 cells after 49 h of exposure to 2 mM selenate in sulfate-limited medium. (Right) EDX spectrum of the electron-dense particles observed in the cytoplasm of the bacteria (X-ray emission lines for selenium would have been at 1.37, 11.22, and 12.49 keV [Lα, Kα, and Kβ rays, respectively]).

RSSeSR and/or Se(0) fraction. This latter reaction was almost complete after 96 h. Thus, the evolution of the distribution of Se species differed from what was observed under normal sulfate conditions (10).

The first extended X-ray absorption fine-structure (EXAFS) oscillations were also informative on Se speciation. A comparison of the spectra for the bacteria exposed to selenate for 5 and 144 h in TSM-0.3 and for reference compounds is shown in Fig. 2B. The simulation of the spectra by LCF gave a species distribution quite comparable to that deduced from XANES analysis, considering that a fraction of elemental selenium of less than 10% was not significant (Fig. 2C). The cultures grown under these conditions never showed the red color characteristic of Se(0). Based on the XANES results and the total Se content, the Se(0) content in the bacteria could not have exceeded 17 mg/g of proteins. The putative presence of Se(0) was checked by TEM-EDX (Fig. 3). Large white inclusion bodies probably corresponding to polyhydroxybutyrate granules and electron-dense granules were observed. However, EDX microanalyses of the latter granules did not indicate the presence of selenium, strongly suggesting the absence of Se(0) under these conditions.

In conclusion, although our results indicate increased selenate incorporation under sulfate-limited conditions, the total selenium level in the cells appeared to remain too low to trigger the detoxification pathway observed during selenite challenge (10). Twenty-four hours after the addition of selenate, half of the oxyanions was transformed to an alkyl selenide (RSeR) likely corresponding to seleno-L-methionine (1). Then, while the quantity of RSeR remained nearly constant, selenate was slowly reduced to that of an RSSeSR species. This species could be selenodiglutathione, since glutathione was shown to play a role in selenite reduction in other strains (3, 12). It should be noted that under these conditions, selenite was not detected even as a transient species. As the organoselenium species detected after the selenate challenge remain mobile and bioavailable, we conclude that *C. metallidurans* CH34 is not a suitable strain to target selenate-contaminated environments.

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