ESRF	**Experiment title:** **Resistance of bacteria to selenium oxyanions** **Résistance chez les bactéries aux oxyanions du sélénium**	**Experiment number:** **30-02-626**
Beamline: BM 30B	**Date of experiment:** from: 02/07/2003 to: 08/02/2003	**Date of report:** 15/09/2003
Shifts: 18	**Local contact(s):** Olivier Proux	*Received at ESRF:*

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Introduction:

The bacteria *Ralstonia metallidurans* CH34 is a microorganism characteristic of metal-contaminated biotopes, which can resist to a wide range of metals including Cd^{2+}, Co^{2+}, Zn^{2+}, Tl^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, Hg^{2+}, and CrO_4^{2-}. Recently, we showed that it could also resist to selenite (SeO_3^{2-}) by reducing it into elemental selenium (monoclinic form), which was accumulated inside the cell (ROUX et al., 2001). This bacteria can reduce selenate as well, as shown in a previous experiment (LS 2141). The oxidized forms of Se selenite and selenate (SeO_4^{2-}) are highly soluble and toxic, whereas the reduced form Se(0) is insoluble and much less toxic. This ability to reduce toxic oxyanions into stable forms is the basic principle of bioremediation, a soft remediation method for the cleanup of contaminated soils and effluents. *Ralstonia metallidurans* CH34 is a good candidate for bioremediation thanks to its resistance and bioreduction capacities. It is also a good model to study the genetics of resistance and bioreduction since its genome is entirely sequenced. However, very little is know on the molecular mechanisms of selenite and selenate bioreduction, and this knowledge is required for the development of bioremediation techniques.

A first experiment on the mechanisms of selenite and selenate bioreduction by this bacteria *Ralstonia metallidurans* CH34 was conducted in February 2003 (experiment LS 2141). We showed that selenate was accumulated in much smaller amount than selenite (7 mg g^{-1} Se per g of protein compared to 210 for selenite) and was reduced into selenite and then into organic Se. However, the exact nature of this organic compound was unknown. For selenite bioreduction, no intermediate was detected. For both oxyanions, we showed that the reactions take place in the bacteria.

In the present experiment, the spectra of various reference compounds (alkyl di-Se: $-CH_2-Se-Se-CH_2-$, alkyl Se: $-CH_2-Se-CH_2-$, Se bound by a double bond to C, …) were analyzed in order to better characterize the organic Se compounds associated with the bacteria, and to evaluate the sensitivity of XANES spectroscopy on these molecules. The bioreduction of selenate was studied at long contact time in order to determine whether organic Se is subsequently reduced into Se(0). The bioreduction of selenite was studied at short contact time in order to detect and possibly identify possible organic intermediates. For both oxyanions, the same experiment was repeated at various optical densities (OD) in order to test the influence of the bacterial population and growing stage on the bioreduction.

Experimental

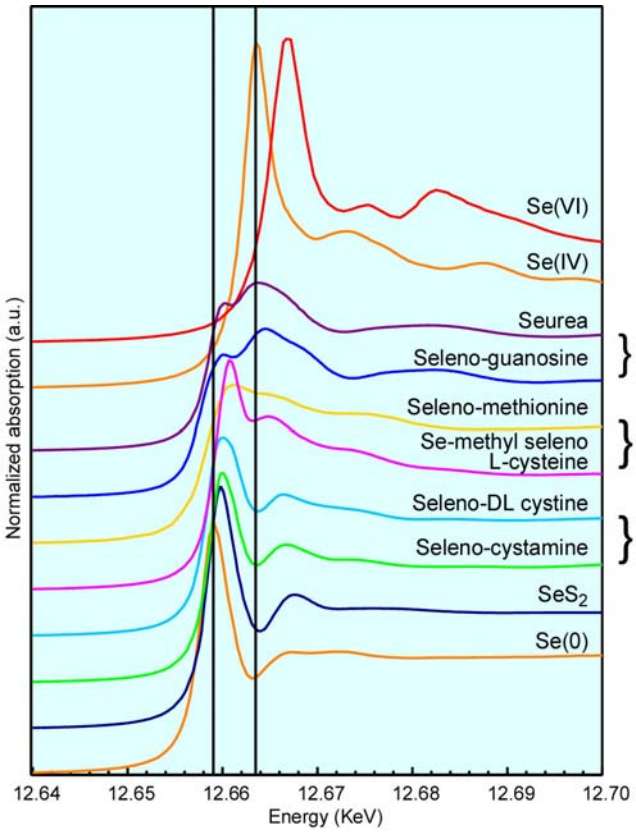
The bacteria were grown in solution. For the selenate experiments, SeO_4^{2-} was added at OD = 0.3 and 1, and aliquots of suspension were sampled every day during 10 days. For the selenite experiment, SeO_3^{2-} was added at OD = 0.3, 1 and 3, and aliquots of suspension were sampled every hour during the first 6 hours, and then every day during 6 days. For each sampling, the bacteria and the solution were separated by centrifugation. Total Se concentrations in the bacteria were measured by ICP-MS.

The monochromator was a Si(220) double crystal recently installed on the beamline. Se K-edge XANES spectra were recorded in fluorescence mode using a 30-element Canberra detector. For some samples, especially the most diluted ones, a photo-oxidation of Se was observed under the beam. In that case, the quick-EXAFS mode was used. XANES spectra were calibrated by setting the maximum of the white line of Se(0) at 12.6592 KeV, and normalized using two polynomial functions. The spectra were then simulated by linear combinations fitting (LCF) using Se reference compound spectra. Given total Se concentration in each sample, the percentages of each Se species were converted into molar concentrations.

Results

I. Model compounds

Figure 1 shows the XANES spectra for various organic and inorganic Se compounds. The peak position varies as a function of the oxidation number, but also as a function of Se chemical environment. Selenium sulfide and alkyl di-Se present approximately the same peak position. There is an energy shift between of about 1eV between alkyl di-Se and alkyl Se compounds, and of about 4 eV between alkyl Se and compounds where Se is double bonded to C. Hence, these species may be identified in unknown samples as far as they do not occur as a complex mixture.



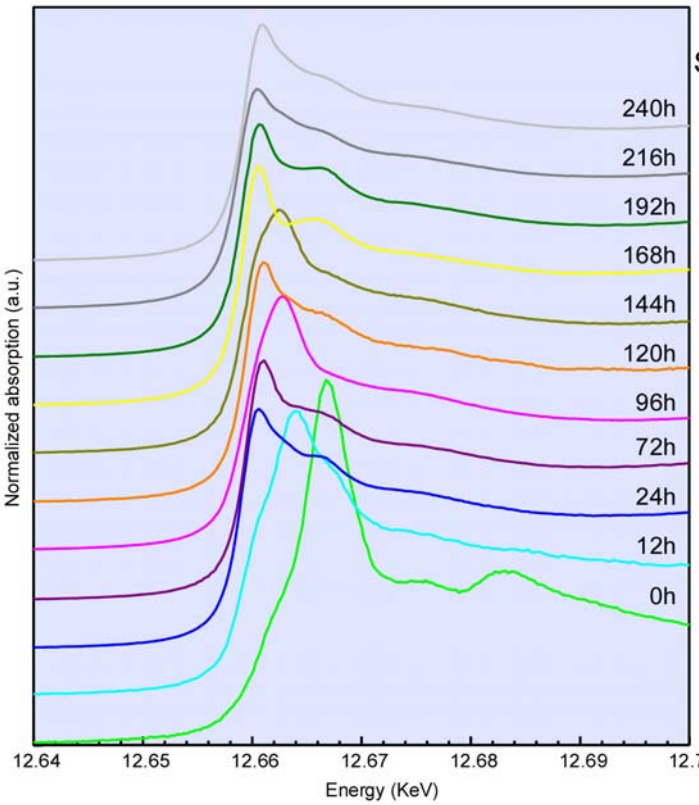
Compound	Max of the white line (KeV)
Se(VI)	12.6670
Se(IV)	12.6638
Se-urea	12.6638
Se guanosine	12.6646
Se-methionine	12.6611
Methyl-Se-L-cysteine	12.6608
Se-DL-cystine	12.6601
Se-cystamine	12.6601
SeS_2	12.6598
Se(0)	12.6592

Figure 1: Se K-edge XANES spectra for reference compounds and position of the maximum of the white line.

II. Accumulation and bioreduction of selenate by *R. metallidurans* CH34

Right after the introduction of selenate in the growing medium, a distribution of Se is observed : 79% of Se(VI), 12% of Se(IV) and 12% of organic Se (Fig. 2). At t = 12h, selenate is still present as a minor species (10%), but it is not detected at longer contact times. At t > 24h, the predominant species is organic Se. The LCFs show that alkyl Se is the predominant species. However, there is a variability in the XANES spectra recorded between 24h and 240h, which indicates that different organic Se species are formed. Moreover, some spectra such as t = 96h (Fig. 2), were not correctly simulated using our library of reference compounds. No

elemental Se was detected. Consequently, selenite detected at the beginning of the reaction is not reduced into elemental Se, as it is observed in cultures exposed to selenite. A possible interpretation of this phenomenon is the inhibition of the reaction "organic Se => Se(0)" by selenate.



Proportions of Se species

Se(0)	SeS2 or Alkyl di-Se	Alkyl Se	Se=C	Se(IV)	Se(VI)
0	0	101	0	0	0
0	38	56	0	7	0
0	10	62	29	0	0
0	16	84	0	0	0
0	0	62	22	16	0
0	0	82	12	7	0
0	0	58	19	22	0
0	0	100	0	0	0
0	19	66	10	5	0
0	0	49	0	43	10
0	0	12	0	12	79

Figure 2: Se K-edge XANES spectra for *R. metallidurans* CH34 exposed to 2 mM selenate introduced at OD=0.3, and proportions of Se species determined by linear combination fitting using reference spectra. The uncertainty is estimated at ± 10% for organic species, and ± 5% for Se(IV) and Se(VI).

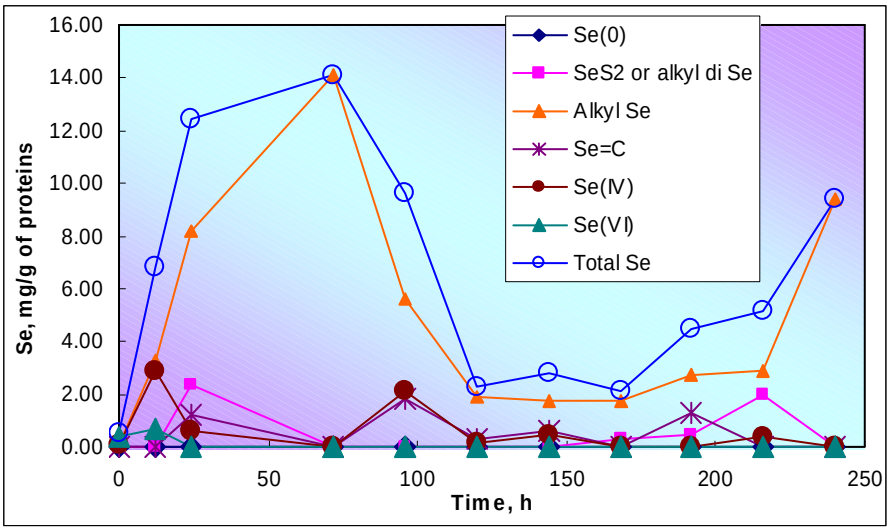


Figure 3: Concentrations of the various Se species present in the bacteria *R. metallidurans* CH34 exposed to 2 mM selenate, calculated from the XANES results (Fig. 2) and from the total Se concentrations.

Figure 3 shows that Se concentration reaches a maximum 72h after the introduction of the contaminant, decreases until 170h and then re-increases. A similar profile was observed when selenate is introduced at OD = 1. It suggests an efflux of Se after 170h.
The comparison of the two OD showed that the bioreduction is faster at high OD(not shown).

III. Accumulation and bioreduction of selenite by *R. metallidurans* CH34

Unlike selenate, selenite is accumulated up to very high concentrations. Selenite is detected in the bacteria during the first hour (Fig. 4). The bacteria reduces it into organic Se (alkyl Se, alkyl di Se and possibly SeS_2)

and elemental Se (major species, Fig. 5). As for selenate, the kinetics of the bioreduction is accelerated at high OD (not shown).

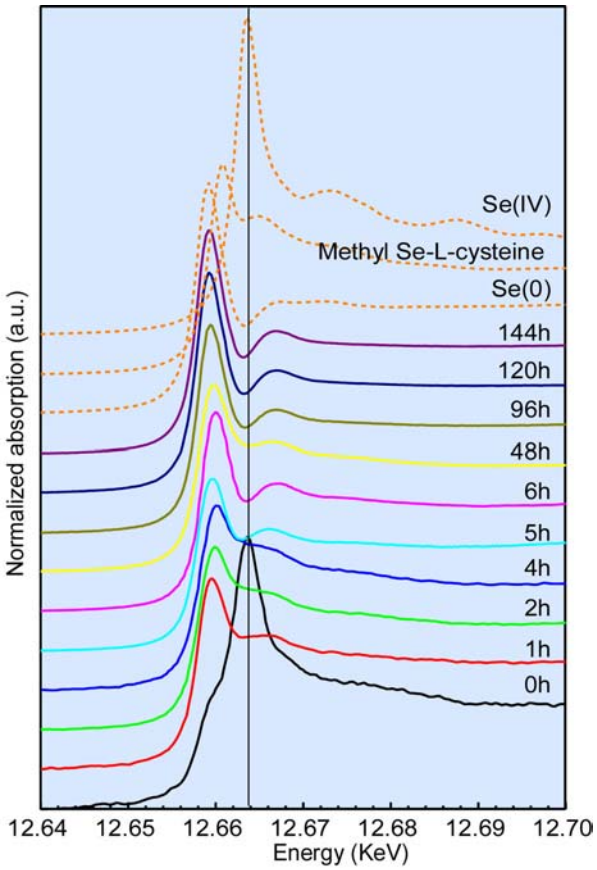


Figure 4: Se K-edge XANES spectra for *R. metallidurans* CH34 exposed to 2 mM selenite, introduced at OD = 0.3.

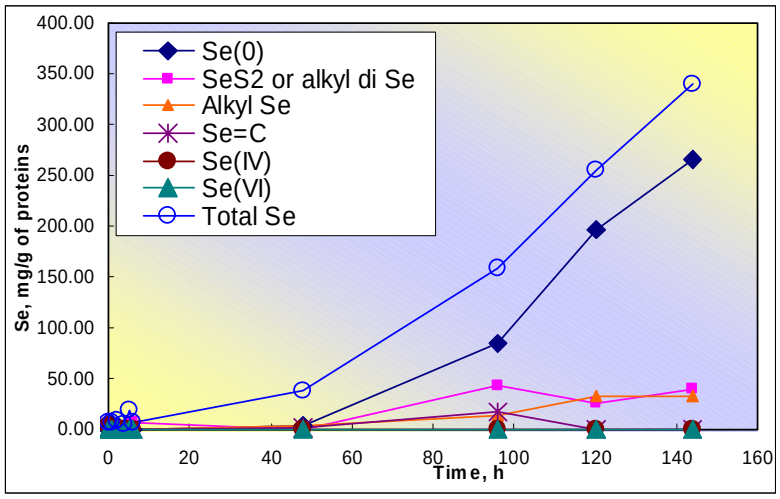


Figure 5: Concentrations of the various Se species present in the bacteria *R. metallidurans* CH34 exposed to 2 mM selenite, calculated from the XANES results (Fig. 4) and from the total Se concentrations.

Conclusions and perspectives

BM30B proved particularly adapted to study diluted samples containing several hundreds of ppm of Se. This experiment showed the great sensitivity of XANES spectroscopy for organic and inorganic Se compounds, and provided new insights on the mechanisms of selenite and selenate bioreduction by the bacteria *R. metallidurans* CH34. An article presenting these results is in preparation.

The results showed that alkyl Se are the major organic Se species synthesized by the bacteria. However, other organic transient species could not be identified. It is planned to record other Se reference compound spectra in order to better characterize these species. In addition, we plan to expose the bacteria to both selenite and selenate in order to test the hypothesis of the inhibition of the reaction "organic Se=> Se(0)" by selenate. Se compounds released in the nutrient solution after 150h by the bacteria will be studied.

Bibliography

Roux M., Sarret G., Pignot-Paintrand I., Fontecave M., and Covès J. (2001) Mobilization of Selenite by *Ralstonia metallidurans* CH34. *Appl. Environ. Microbiol.* **67**(2), 769-773.