ESRF	**Experiment title:** **Resistance of bacteria to selenium oxyanions** **Résistance chez les bactéries aux oxyanions du sélénium**	**Experiment number**: **30-02-689**
Beamline: BM 30B	**Date of experiment**: from: 04/05/2004 to: 11/05/2004	**Date of report**: 31/08/2004
Shifts: 18	**Local contact(s)**: Olivier Proux	*Received at ESRF:*

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Introduction

The purpose of our study is to improve the understanding of mechanisms of bacterial uptake and subsequent internal reduction of Se oxyanions. *Ralstonia metallidurans* CH34, a soil bacterium characteristic of metal-contaminated biotopes, is known to resist to a wide range of metals as well as the oxyanions selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}). As other soil micro-organisms, its resistance to selenite is based on the reduction of the oxidized form, highly soluble, toxic and bioavailable in the environment, to the elemental form Se(0), extremely insoluble, strongly resistant to oxidation and therefore, less toxic and mobile. In addition, *R. metallidurans* CH34 is a good model for genetic studies since its genome is entirely sequenced.

Microbiology experiments are carried out at the Pierre Süe Laboratory at Saclay to characterize the capacity of resistance and accumulation of the bacteria exposed to both Se oxyanions, and to determine the kinetics of accumulation in various culture conditions.

A first Se K-edge XANES experiment was performed on BM32 in 2001 in which *R. metallidurans* CH34 was shown to resist high selenite concentration and accumulate it after subsequent reduction to elemental selenium in the monoclinic form (ROUX et al., 2001).Two other XANES experiments were carried out on BM30B in 2003. The aims were on one hand to obtain spectra of various references in order to characterize the organic Se compounds synthesized by the bacteria and on the other hand, to focus on the kinetics of selenite and selenate reduction by this bacterial strain (experiments LS2141 and 30-02-626). Our results showed that *R. metallidurans* CH34 reduced selenite more quickly at high cell density (optical density at 600 nm: OD_{600nm}=3). Selenite was present as minor species in the cells from the start of the experiment and a transient organic Se compound was found. This suggest that two reactions with similar kinetics take place: an assimilatory pathway leading to organic Se, and a slow detoxification pathway leading to Se(0). Then, selenite uptake strongly increased and Se(0) was largely predominant, suggesting an activation of selenite transport and bioreduction systems. In addition, *R. metallidurans* CH34 was able to reduce selenate to selenite and then to organic selenium as major product. Elemental Se was detected, but represented less than 25% of total selenium (experiment report 30-02-626 ; Sarret et al., subm. to AEM).

In the present experiment, we studied the kinetics of reduction of both selenite and selenate species by 3 mutant strains, in comparison with the wild-type strain. These mutant strains, selenite-resistant, were developed in the Jacques Covès group in Grenoble. The mutated gene and the corresponding protein putatively involved in selenite transport are being studied by this group.

Materials and methods

Three mutated strains, called RM6, RM7 and RM8 and resistant up to 15 mM of selenite, were cultured in the same conditions as *R. metallidurans* CH34. Two experiments were conducted in parallel in order to test the influence of the bacterial population. For the first experiment, RM7 and RM8 were exposed to 10 mM of selenite added to the culture medium at the beginning of the exponential growth phase (OD_{600nm}=0.3). For the second experiment, RM6, RM7 and RM8 were exposed to 10 mM of selenite or 5 mM of selenate, added to the culture medium at the beginning of the stationary phase (OD_{600nm}=3). The kinetics of the reactions were followed by samplings made at regular interval during 6 days. For each sampling, the bacteria and the culture medium were separated by centrifugation. Total Se concentrations in each fraction were determined by ICP-MS.

Se K-edge XANES spectra were recorded in fluorescence mode using a 7-element solid state Ge detector (Canberra). The monochromator was a Si(220) double crystal. To avoid photoreduction of Se during the measurements, acquisitions were conducted using a cryostream. XANES spectra were calibrated by setting the maximum of the white line of hexagonal (gray) elemental Se 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 reported to the biomass determined by protein content.

Results

I. Reference spectra

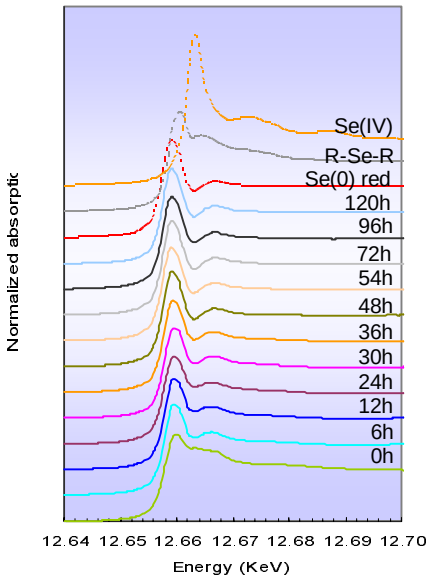
Table 1 details the Se model compounds used to simulate XANES spectra with a linear combination fitting program.

Compound	monoclinic red elemental Se	seleno diglutathion	seleno-DL-cystin	seleno cystein	methyl seleno L-cystein	selenourea	selenite	selenate
	Se(0)	SeS_2	R-SeH	R-Se-Se-R	R-Se-R	Se=C	Se(IV)	Se(VI)
Max of the white line (keV)	12.6592	12.6598	12.6601	12.6601	12.6608	12.6638	12.6638	12.667

***Table 1**: Se model compounds and the position of the maximum of the white line*

II. Accumulation and bioreduction of selenite by selenite-resistant mutant strains

Interestingly, accumulation experiments showed that the selenite-resistant mutant strains accumulated significantly less selenium added as selenite in the culture medium than the wild-type strain *R. metallidurans* CH34. However, the mechanism of selenium reduction seemed to be unchanged. Figure 1 presents the percentages of the major species obtained after exposure of RM7 to 10 mM of selenite added at OD 0.3: selenite is detected during the first 6 hours of experiment and rapidly reduced to organic Se (as alkyl Se) and elemental Se. Figure 2 presents the same results normalized using total selenium contents in the bacteria and compared to the results obtained with *R. metallidurans* CH34 exposed to 2 mM of selenite added at OD 0.3.



Proportions of Se species (%)

Time, h	Se(0)	SeS_2	R-Se-Se-R	R-SeH	R-Se-R	C=Se	Se(IV)	Se(VI)
0	53	-	-	-	39	-	12	-
6	53	-	-	-	41	-	6	-
12	65	-	-	-	35	-	-	-
24	66	-	-	-	33	-	-	-
30	70	-	-	-	30	-	-	-
36	78	-	-	-	22	-	-	-
48	91	-	-	-	9	-	-	-
54	94	-	-	-	6	-	-	-
72	94	-	-	-	6	-	-	-
96	93	-	-	-	6	-	-	-
120	95	-	-	-	5	-	-	-

***Figure 1**: Se K-edge XANES spectra for mutated strain RM7 exposed to 10 mM selenite added at the beginning of exponential phase (OD 0.3), and proportions of Se species determined by linear combination fitting using reference spectra (tab.1). The uncertainty is estimated at ±10% for organic species, and ±5% for Se(IV) and Se(VI).*

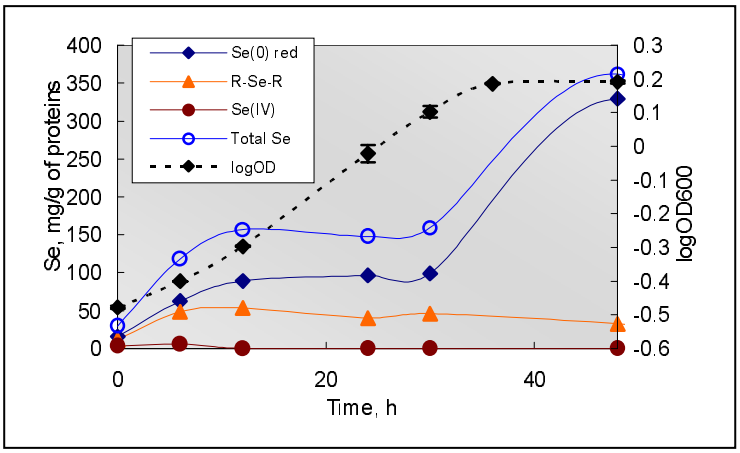
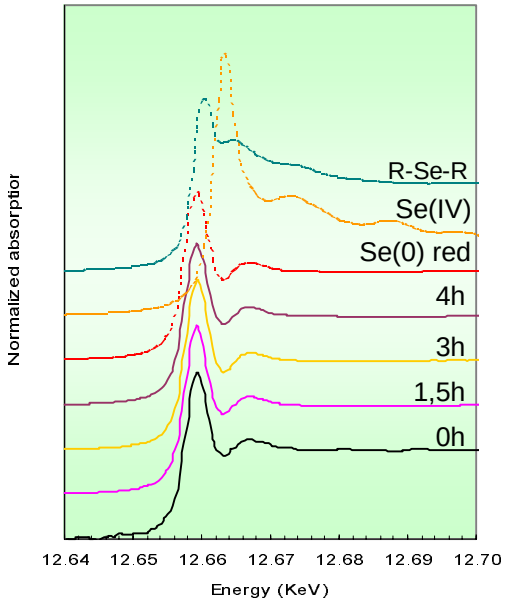


***Figure 2**: Growth curve of the mutated strain RM7 and the concentrations of the various Se species present in the mutated strain exposed to 10 mM selenite added at OD 0.3, calculated from the XANES results and from the total Se concentrations.*

Figure 3 shows the results obtained with RM6 exposed to 10mM of selenite added at OD 3. Like with *R. metallidurans* CH34, at high cell density, the kinetic of selenite reduction was accelerated. The same results were obtained with the other mutated strains.

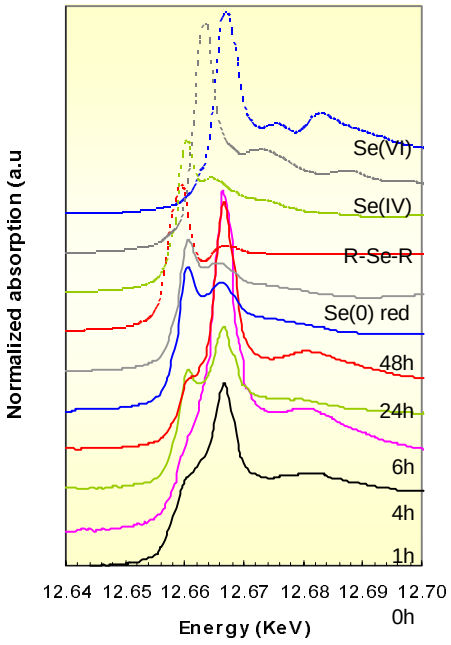


Time, h	Proportions of Se							
	Se(	Se	R-S R	R-S	R-Se-R	Se=C	Se(IV)	Se(VI)
0	88	-	12	-	-	-	-	-
1.5	100	-	-	-	-	-	-	-
3	100	-	-	-	-	-	-	-
4	100	-	-	-	-	-	-	-

***Figure 3**: Se K-edge XANES spectra for mutated strain RM6 exposed to 10 mM selenite added at the start of stationary phase (OD 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).*

III. Accumulation and bioreduction of selenate by selenite-resistant mutant strain

Results obtained after exposure of RM8 to 5 mM of selenate added at OD 3 are presented figure 4 and figure 5. The accumulation of selenium by the mutated strain was quite similar as the wild-type strain (<2%). It can be noticed that in these conditions, right after the addition of selenate in the culture medium, selenate is present in the bacteria in the same proportions as organic Se. Selenite is present as a minor species during the first 6 hours of exposure. Then, organic Se becomes the predominant species and selenate proportions decrease. Elemental Se is only detected at 24h. A similar mechanism of selenate reduction had been observed with the wild-type strain CH34. For the other mutated strains RM6 and RM7 exposed to selenate, data are still in process.



Proportions of Se

Time, h	Se(0)	Se S_2	R-Se-Se-R	R-SeH	R-Se-R	Se=C	Se(IV)	Se(VI)
0	-	-	-	-	51	-	9	45
1	-	-	-	-	17	-	27	91
4	-	-	-	-	69	-	-	31
6	-	-	-	-	24	-	-	83
24	17	-	-	-	73	-	-	9
48	-	-	14	-	59	27	-	-

***Figure 4*: Se K-edge XANES spectra for mutated strain RM8 exposed to 5 mM selenate added at the beginning of stationary phase, 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).**

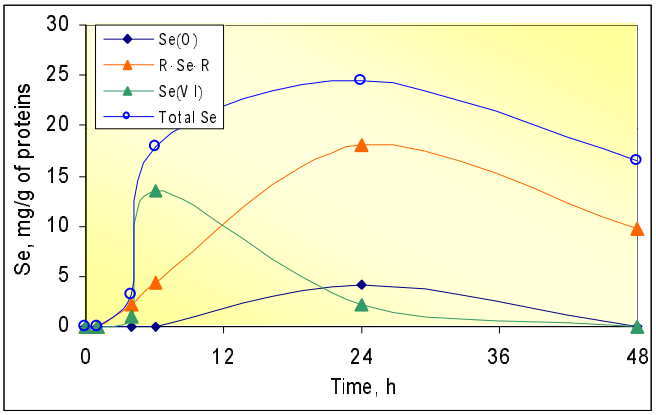


***Figure 5*: The concentrations of the various Se species present in the mutated strain RM8 exposed to 5 mM selenate, calculated from the XANES results (Fig. 4) and from the total Se concentrations**

Conclusions and perspectives

These results show that the sensitivity of the beamline BM30B allowed us to probe even the lowest Se concentration samples. BM30B proved to be particularly well suited to the study of diluted samples containing Se concentrations in the micromolar range. This experiment proved as well that even if the mutated strains of *R. metallidurans* CH34 accumulated far less selenium added as selenite in the culture medium than the wild-type strain (2% of the selenite added at zero time compared to 100%, respectively), the mechanism of selenium reduction seemed to be the same. For experiments with selenate, the major species was organic Se after an entrance of selenate during the first hours. Like for the wild-type strain, we noted the presence of selenite and elemental Se in the bacteria. The mutated protein seems to be highly implicated in selenite transport through the bacterium.

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.
- Sarret G, Avoscan L, Carrière M, Collins R, Geoffroy N, Covès J, Gouget B (submitted to Appl. Environ. Microbiol.)