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SELENITE REDUCTION AND UPTAKE HYDROGENASE ACTIVITY IN *RHODOSPIRILLUM RUBRUM*

BY

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ABSTRACT

Selenite reduction and uptake hydrogenase activity in *Rhodospirillum rubrum*. - *R. rubrum* grew in the presence of millimolar concentrations of SeO_3^{2-} under anaerobic conditions and in continuous light (10 W/m^2). Selenite affected the growth rate: the end of growing phase was reached already 36 h after inoculation in control cultures, but only after 58 hours in the presence of 0.5, 1 and 2 mM SeO_3^{2-} with generation times of respectively 12.2 h, 13.7 h, 14.2 h and 16.6 h In the 2 latter cultures, a decrease in optical density (A_{650}) occurred 4 to 6 hours before the stationary phase. It coincided with the beginning of the reduction process, the appearance of volatile selenium and of a peak at 420 nm in the absorption spectrum.

The uptake hydrogenase (Hup) activity has been followed in intact *R. rubrum* cells. Generally, an increase in activity was observed 12 h after inoculation in a fresh medium. In the control cultures and in the presence of 0.5 mM selenite, the activity of Hup showed regular fluctuations during the growth and the stationary phases with periods of about 12 h When selenite was present, fluctuating Hup activity stopped with the SeO_3^{2-} reduction, after which it dropped. At 1 and 2 mM SeO_3^{2-}, the low level of Hup was accompanied by a gas overpressure.

The high selenite resistance and conversion capacity of *R. rubrum* could be useful in the perspective of bioremediation of this pollutant.

Key-words: selenium, *Rhodospirillum rubrum*, bioremediation, uptake hydrogenase.

INTRODUCTION

Selenium is a naturally occurring element whose distribution on the earth's surface is likely related to volcanic activity. It is nowadays found in igneous rocks, volcanic sulfur deposits or sedimentary rocks, and in water. Agricultural irrigation or industrial activities such as the production of colored glass, fuel refinery or electronic equipment have furthermore contributed to the release of selenium in water streams and slush. Generally, selenium will enter the soil profile and the atoms will eventually be adsorbed on clay particles, iron hydroxides or organic particles in the form of selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}).

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Selenium is an essential trace micronutrient (< 0.1 mg/kg) in mammal cell metabolism and its deficiency has been linked to several diseases (WILBER, 1980, 1983), whereas accumulation of high concentrations (≥ 5 mg/kg) has disastrous or lethal consequences in animals. Indeed, selenite is the most toxic form of Se (PAINTER, 1941), causing metabolic disorders due to the indiscriminate substitution for sulfur in the amino acids. Around the Kesterson irrigation ponds (California, USA), for instance, adult fowl tissues were found to possess a 100 fold higher selenium concentration than in naturally occurring conditions and their newly hatched chickens presented lethal misformations (FUJII *et al.*, 1989). This dramatic incident contributed to the awareness of chemical or biological remediation of toxic products from the environment. A few microorganisms living in the soil and the sediment have now been found to reduce selenate and selenite into elemental selenium (Se^0) (GARBISU *et al.*, 1995; LOSI & FRANKENBERGER, 1997; MACY *et al.*, 1989; TOMEI *et al.*, 1992), which is not soluble and thus much less toxic (National Research Council, 1976). The reduction of selenate and selenite takes place in anaerobic or micro-aerobic conditions, but the exact biochemical processes involved have not yet been clarified. In *Pseudomonas* species, it was shown that SeO_3^{2-} is used as an electron acceptor in the anaerobic respiration pathway before being released as elemental Se^0 (MACY *et al.*, 1989). The participation of a hydrogenase in the reduction of selenite was furthermore demonstrated in *Clostridium* sp., as it co-purified with the SeO_3^{2-} reductase. In phototrophic non-sulfur bacteria, the reduction of selenite was accompanied by the production of methylated selenium compounds and by the evolution of molecular hydrogen (MOORE & KAPLAN, 1992; VAN FLEET-STALDER *et al.*, 1997). The H_2 evolving nitrogenase complex is believed to be involved in this gas production (LJONES, 1974). In phototrophic bacteria, this enzyme complex is usually associated with the H_2 uptake hydrogenase (Hup), an enzyme that plays an important role in the recycling of H_2 produced by nitrogenase (VIGNAIS & TOUSSAINT, 1994). The biochemical properties of Hup have been well characterized in a broad range of bacteria; its physiological role has, however, barely been studied. In *R. rubrum*, it was recently discovered that this enzyme presents an oscillatory pattern of activity when exposed to either continuous light or darkness (VAN PRAAG *et al.*, 2000). A few inorganic compounds or nutrients were also found to affect the activity of Hup-hydrogenase (GARBISU *et al.*, 1995; GHOSH *et al.*, 1994; KERN *et al.*, 1992), but, to our knowledge, their long-term effects on the activity have not yet been determined.

In an attempt to better understand the activity of this enzyme with alternative electron acceptors, we examined the possibility to use selenite instead. Therefore, we present first the viability (growth) of *R. rubrum* in presence of different selenite concentrations. Then, the evidence of active selenite reduction by this organism and, finally, how Hup activity responds to these new oxydo-reducing environmental conditions.

MATERIALS and METHODS

Organism and culture conditions:

The wild type strain, *Rhodospirillum rubrum* S1, was cultivated under anoxic conditions at 31.6 °C ± 0.1 in continuous light (10 W/m^2). A tungsten lamp (Osram, rs1,

60 W) illuminated the different cultures sideways. Batch cultures were grown in 150 ml glass bottles sealed with new rubber septum stoppers and an aluminum lid containing 100 ml of sterile degassed Sistrom medium, modified according to GHOSH *et al.* (1994), under 100% N_2. During growth the cultures were constantly mixed with a magnetic stirrer (± 100 rpm).

For each experiment, two synchronized cultures were inoculated exactly 12 hours apart. In order to verify that the two cultures used for Hup measurements were in phase, samples were taken from both cultures during overlapping hours. To avoid disturbance of the bacterial cultures during sampling, a 10 cm long sterile needle connected to a stopcock was inserted into the bottle through the rubber septum. The samples were immediately assayed for their Hup activity. Each experiment was performed at least twice.

Enzyme assay:

The Hup activity was followed spectrophotometrically in intact cells of *R. rubrum* by following the rate of reduction of oxidized benzyl-viologen in anaerobic cuvettes. The assay medium consisted of potassium phosphate buffer 50 mM, pH 7.5, oxidized benzyl-viologen 4 mM, glucose 25 mM. All chemicals used for the assay and elsewhere were of the purest quality available. The assay mixture was degassed with an aspirator pump before transfer into the assay cuvettes. The cuvettes, sealed with a silicon stopper, were saturated with H_2 by bubbling for 10 min. after which glucose oxydase (10 U) was added. The assay medium was then incubated 15 min. in order to create a fully anoxic environment prior to the addition of the bacteria. Activity was monitored at 30°C by measuring the increase in A_{555} for benzyl-viologen with time.

An absorption coefficient (e) for reduced benzyl-viologen at 555 nm of 8100 $M^{-1}cm^{-1}$ was used (ADAMS & HALL, 1979); 1 U of Hup activity is defined as 1mmol of benzyl-viologen reduced per minute.

The determination of the period of oscillations of the Hup-hydrogenase activity was done with the "peaks and valleys" method and their mean value was used (VAN PRAAG *et al.*, 2000).

Quantitative determinations:

Growth of *R. rubrum* was monitored after sampling by measuring the optical density at 650 nm (Uvikon 860 spectrophotometer) with a 2mm path length cuvette and counting the number of cells with a flow-cytometer (Microcyte) for comparison. Light intensity was determined with the portable Model 730 Radiometer (Optronic Laboratories). The temperature of the growing cultures was followed with a digital thermometer (model 871, Keithley) connected to two sterilized sensors.

The concentration of selenite in the media was determined by the modified colorimetric method of WATKINSON (1966). The mixture consisted of HCl 0.1 M (2.5 ml), EDTA 0.1 M (0.125 ml), NaF 0.1 M (0.125 ml), Na_2-oxalate 0.1 M (0.125 ml), 2,3 di-aminonaphtalene 0.1 % (0.6 ml) and the sample (0.2 ml). The solution was incubated at 40°C during 40 minutes before adding cyclohexane (1.5 ml) in order to extract the selenium-diaminonaphtalene complex. The solution was carefully vortexed and set to rest for 15

minutes before reading the absorbance of the organic phase at 377 nm. All steps were carried out in the dark.

Volatile selenium present in the head-space of the cultures was catched by passing 4 cc of gas through an alkaline-peroxide trap solution (NaOH : H_2O_2 30 % = 4:1). The 2 ml samples were boiled for 20 minutes to remove the residual peroxide, after which the selenite content was determined as described above.

Determination of growth rates, generation times and statistical analysis:

For bacterial growth, Hup-hydrogenase activity and selenite assimilation experiments, all the data points are represented on the different figures, together with an "average" or "trend" curve. Since the data have been sampled with slight non-equidistant points, they have first been linearly interpolated to obtain equidistant and experiment overlapping values (using SigmaPlot for Windows, version 5.0, SPSS Inc.). The obtained points are then averaged and filtered with the Savitzky-Golay smoothing procedure with 5 points, in order to remove noisy signals (GORRY, 1990).

The growth rate of the bacteria has been calculated by linear regression from the differential obtained between two samples during the exponential growth phase. For the determination of the generation time g, values for the turbidity (A_{650}) and the cell number were taken, using the following equation:

$$g = [0.301\ (t - t_0)] / (\log_{10} B_t - \log_{10} B_0)$$

where t_0 represents time zero and t any time after t_0, B_0 = the bacterial turbidity or cell number at time t_0 and B_t = the bacterial turbidity or cell number at time t.

For the SeO_3^{2-} reduction data, regression lines were calculated by the least-square method and a test for the significance of slopes was applied, according to the method of GLANTZ (1997). The statistical comparison of the slopes was effected with a variance F test, as described by LEE & LEE (1982).

RESULTS

Growth of Rhodospirillum rubrum

R. rubrum was able to grow in the presence of millimolar concentrations of SeO_3^{2-}, when exposed to an anaerobic environment, in continuous light and at constant temperature. Growth, however, was slower in the presence of selenite and cell entered the stationary phase only after 58 hours in cultures containing 0.5, 1 and 2 mM SeO_3^{2-} (Figure 1 B, C, D) as compared to 36 h in control cultures (Figure 1 A). The term control will refer to the cultures grown in absence of SeO_3^{2-} throughout this paper. This is also reflected in the generation times of *R. rubrum*, which increased from 12.2 h in the absence of selenite to 13.7 h, 14.2 h and 16.6 h in the cultures containing 0.5, 1 and 2 mM SeO_3^{2-}, respectively (Table 1). A decrease in optical density (A_{650}) was furthermore observed 4 to 6 hours before the stationary phase was reached in cultures grown in the presence of 1 and 2 mM SeO_3^{2-}.

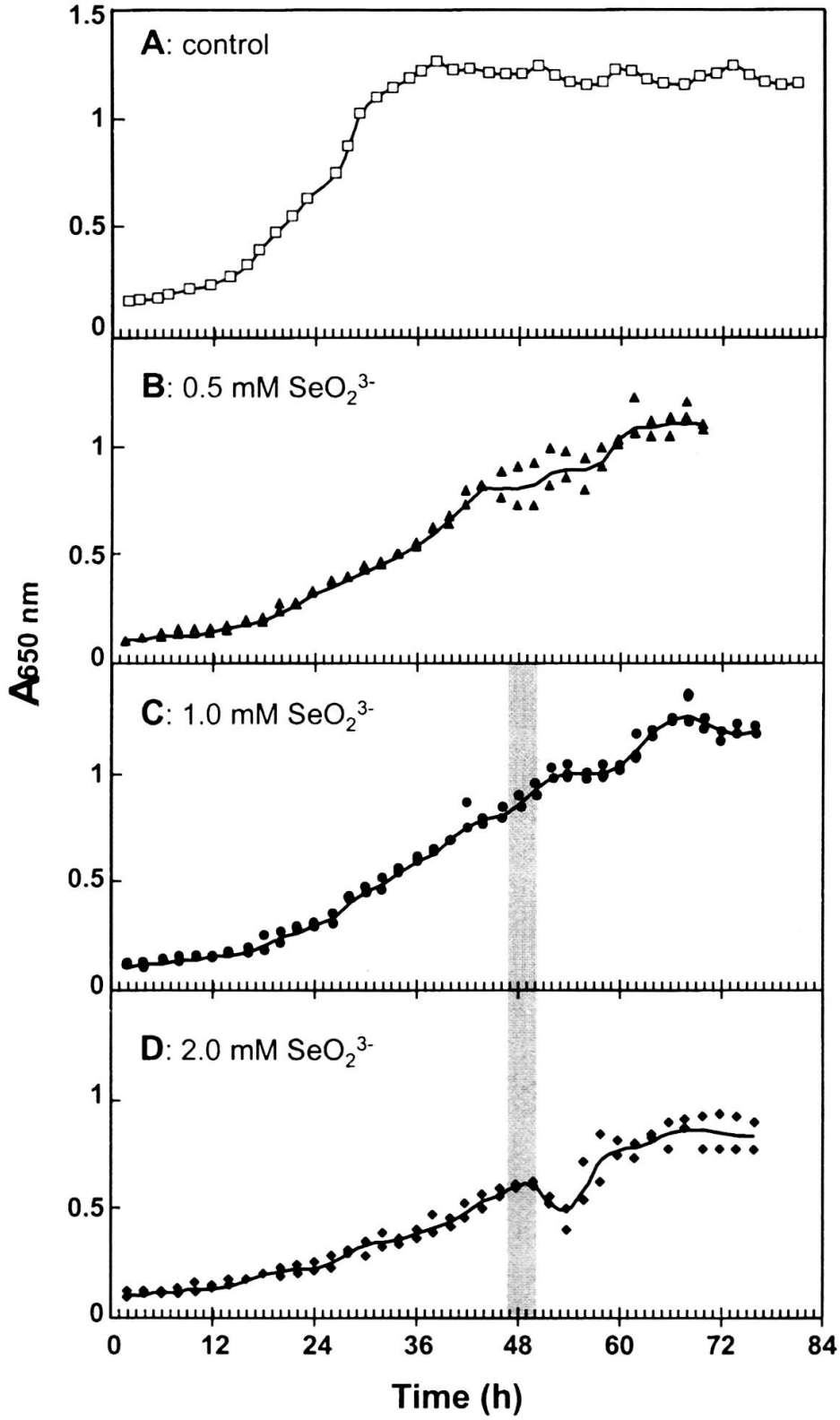

FIG. 1.

Growth of *Rhodospirillum rubrum* in the presence of selenite (SeO_3^{2-}). Cultures were grown in anaerobic conditions under N_2 gas, in continuous light and at 31°C. A: in the absence (control), and the presence of various concentrations of SeO_3^{2-}, B: 0.5 mM; C: 1 mM and D: 2 mM. The dotted grey bar (▒) represents the period where an overpressure of gas is observed by visual inspection in some of the selenite grown cultures.

TABLE 1. Generation time, rate of reduction of selenite and Hup hydrogenase activity in the different cultures of *Rhodospirillum rubrum* growing in the absence or the presence of increasing concentrations of selenite as indicated in the first column. The number (1) or (2) refers to the first or second experiment, respectively.

Selenite	**Generation time (A_{650})** *(h)*	**Generation time 2 (cell number)** *(h)*	**Rate SeO_3^{2-} reduction** *(mM/h)*
0 mM (1)	12.1 ± 1.6	12.4 ± 1.1	-
0 mM (2)	12.3 ± 1.4	12.6 ± 0.9	-
0.5 mM (1)	13.7 ± 1.1	14.1 ± 1.8	31.2
0.5 mM (2)	13.6 ± 1.4	13.9 ± 1.9	31.0
1 mM (1)	14.3 ± 1.4	15.1 ± 1.8	30.3
1 mM (2)	14.1 ± 1.9	14.9 ± 2.1	30.9
2 mM (1)	16.3 ± 1.9	16.9 ± 1.2	30.6
2 mM (2)	16.9 ± 1.5	17.5 ± 2.1	30.4

The dynamics of selenite reduction is shown in Figure 2 A. After an initial phase of decay (~0-18 h), one could observe a steady phase with no significant degradation (2 mM: ~18-54 h; 1 mM: ~18-48 h; 0.5 mM: ~12-60 h), that is confirmed by null slopes statistical test (H_0: null slopes, accepted in each case: 0.5 mM SeO_3^{2-}: $t = -0.4259$, $p = 0.67$, $df = 50$; 1.0 mM SeO_3^{2-}: $t = -0.404$, $p = 0.69$, $df = 30$; 2.0 mM SeO_3^{2-}: $t = -0.86$, $p = 0.40$, $df = 34$). After this, during the third phase an important degradation started (Ho: null slopes, rejected in each case: 0.5 mM SeO_3^{2-}: $t = -13.71$, $p < 0.01$, $df = 14$; 1.0 mM SeO_3^{2-}: $t = -18.85$, $p < 0.01$, $df = 28$; 2.0 mM SeO_3^{2-}: $t = -9.42$, $p < 0.01$, $df = 25$), interestingly at the same rate for all the concentrations (Ho: common slope = -0.03118 mM/h, accepted; $F = 3.23$, $p = 0.046$, $df1 = 2$, $df2 = 67$).

Immediately after the reduction took place, appearance of volatile selenium in the gas phase was also observed (Figure 2 B). This phenomenon is concomitant to selenite reduction (Figure 2 A). Moreover, although measurements are more scattered, the rate of appearance of the volatile element does not differ for the different selenite concentrations (Figure 2 B). This is in agreement with the rate of reduction (Figure 2A).

When absorption spectra of the bacteria grown in the presence of millimolar concentrations of SeO_3^{2-} were recorded, a peak with a maximal absorbance at 420 nm arises 36 hours after inoculation. This absorbance increased with time and culminated about 50 hours after inoculation, after which the peak gradually disappeared again (Figure 3). After 55 h, the presence of elemental selenium in the cultures (appearance of a characteristic red color) increased the turbidity in the SeO_3^{2-} containing cultures, which strongly interfered with the optical measurements.

Hup activity at increasing selenite concentrations

The Hup activity has been followed in whole *R. rubrum* cells, with measurements starting immediately after inoculation in a new medium. No activity was detected in autoclaved controls. In the control and the selenite containing cultures (Figure 4 A-D), a

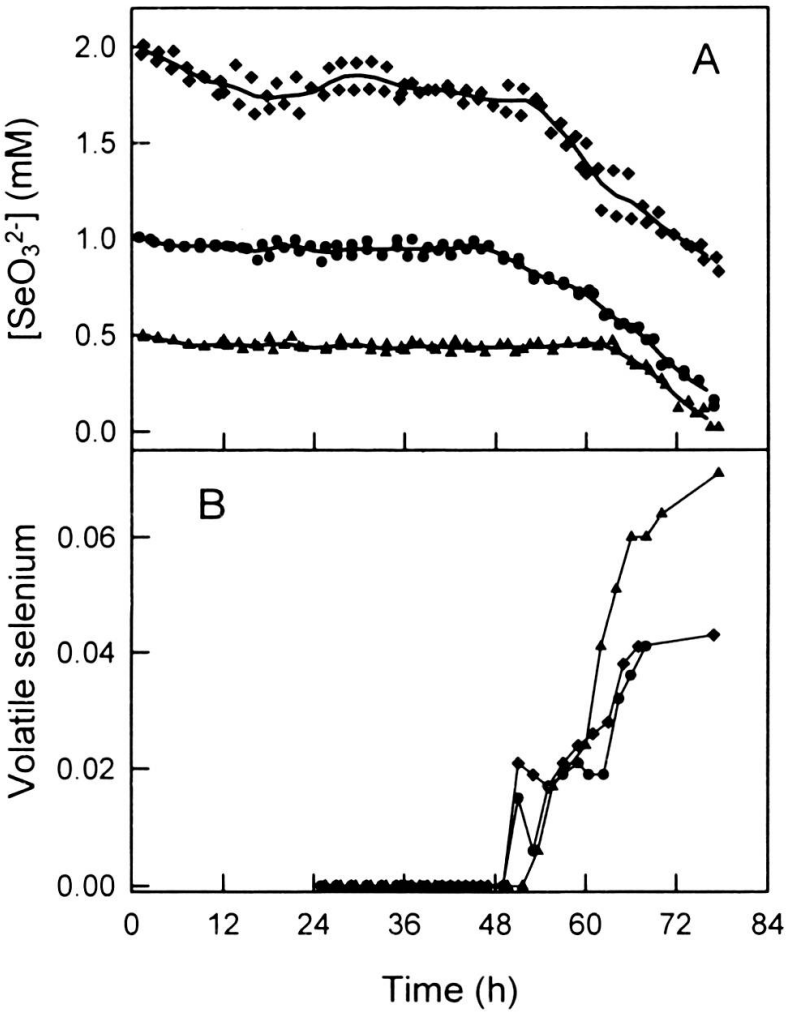

FIG. 2.

A: Reduction of selenite (SeO_3^{2-}) by *R. rubrum* in the presence of 0.5 mM (▲), 1 mM (●) and 2 mM (◆) selenite. B: appearance of volatile selenium in the gas phase of the same cultures (symbols as in A).

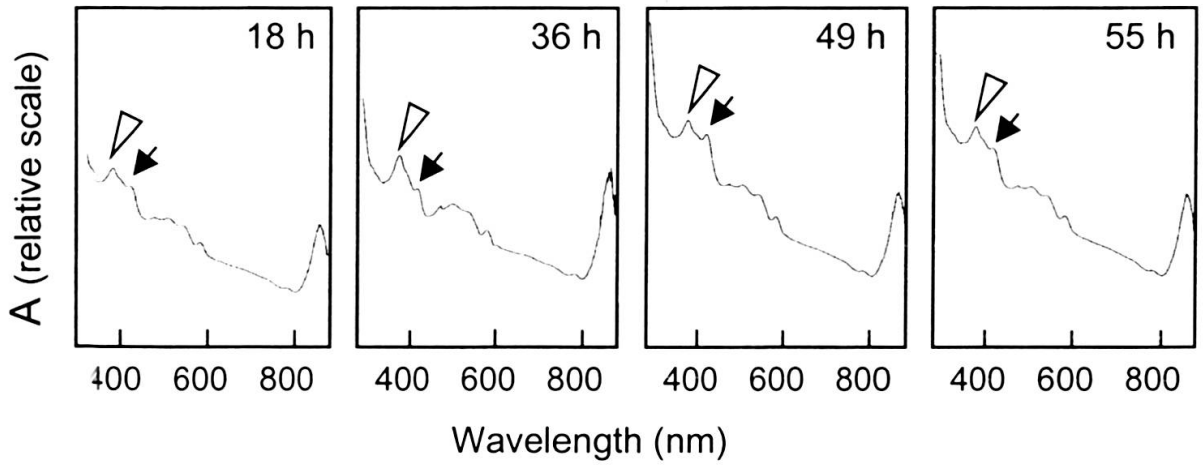

FIG. 3.

Absorption spectra of the selenite (SeO_3^{2-}, 1 mM) grown culture of *R. rubrum*, with the peak at 420 nm (▶) and the peak of Bacteriochlorophyll at 375 nm (▷).

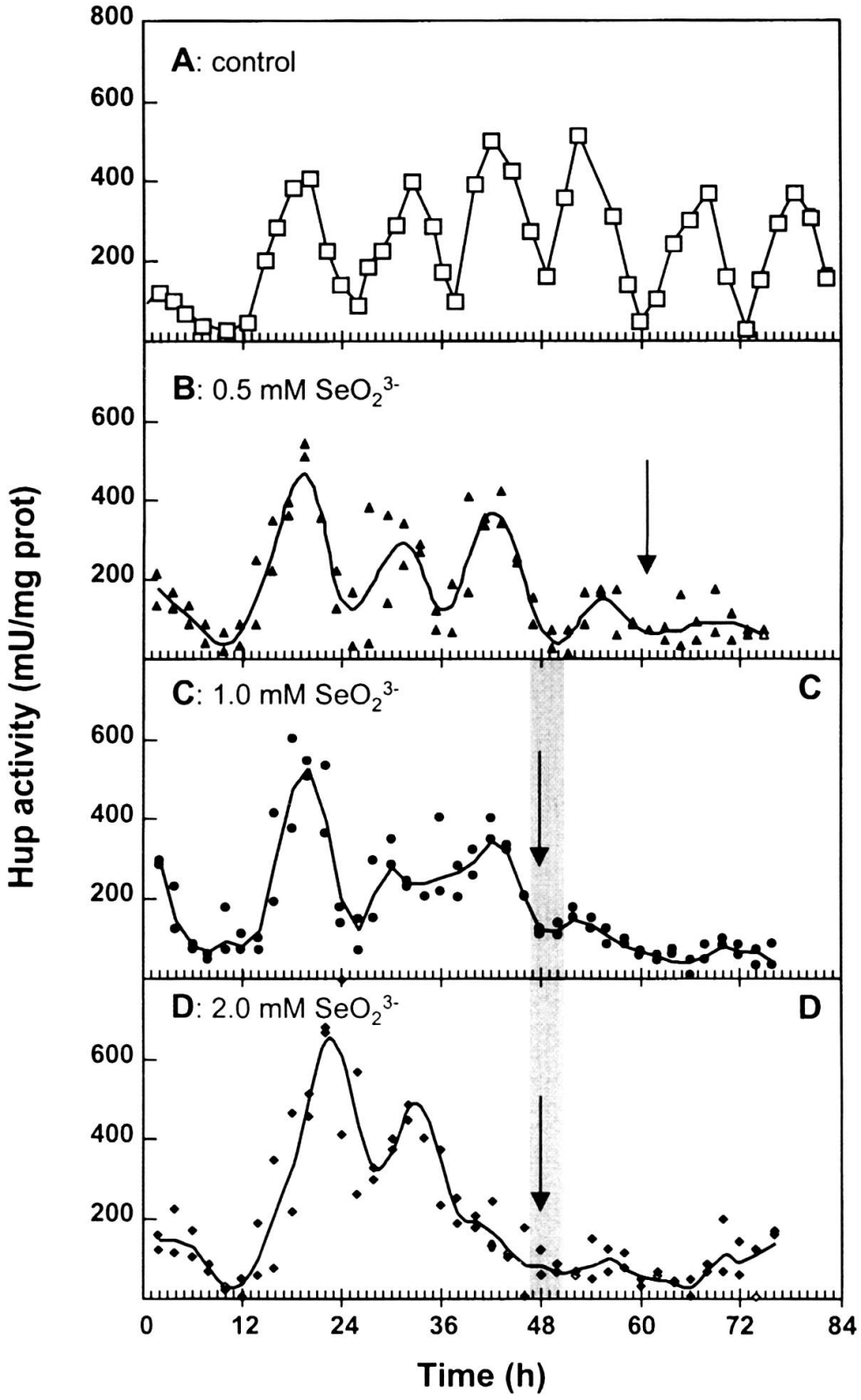

FIG. 4.

Activity of uptake-hydrogenase (Hup) in *R. rubrum* and selenite (SeO_3^{2-}) reduction. A: *R. rubrum* cells grown in the absence (control) of SeO_3^{2-}, and in the presence of various concentrations of SeO_3^{2-}, B: 0.5 mM; C: 1 mM and D: 2 mM. The dotted grey bar ( ) represents the period where an overpressure of gas is observed by visual inspection in some of the selenite grown cultures. The arrows represent the time when SeO_3^{2-} begins to be reduced (see Figure 2 A).

lag period of about 12 hours preceded the increase of Hup activity. In control cultures, the Hup activity rose rapidly 12 hours after inoculation in the new medium and gave rise to an oscillating pattern during the bacterial growth and the stationary phase. The period of Hup activity, determined with the "peaks and valleys" method, is 12.1 ± 1.1 h In bacteria growing in the presence of 0.5 mM SeO_3^{2-}, oscillations of the Hup activity were observed, with a period of 12.3 ± 1.3 h, but they stopped with the onset of SeO_3^{2-} reduction (Figure 4 B-D). In the presence of 1 or 2 mM SeO_3^{2-}, Hup activity showed the same pattern during the first 24 h. But, subsequently, the oscillations became irregular. At the beginning of the SeO_3^{2-} reduction (48 h after inoculation), a severe drop of activity was observed, after which it remained very low (Figure 4 C-D). At this time, an over-pressure of gas was simultaneously observed, lasting for about 4 hours (Figure 4 C-D).

DISCUSSION

Our results demonstrate that the purple non-sulfur bacterium *R. rubrum* is highly resistant towards selenite and its growth is only slightly affected despite the toxicity of that compound. Most of the selenite present in the medium was reduced into amorphous, elemental selenium once the bacterial cultures reached the end of the growing phase, indicating that its reduction is not a resistance mechanism (TOMEI *et al.*, 1992). The actual level of selenium oxyanions removed from the media reached 95% and their reduction rate proved to be independent of the selenite concentration present in the media, with a mean rate of 30.7 mM selenite per hour. An increasing production of volatile selenium compounds was furthermore detected at the end of the growing phase and during stationary phase. This observation is similar to results obtained in *Rhodobacter* sp., where an increasing production of volatile selenium-containing molecules was observed during the stationary phase (VAN FLEET-STALDER *et al.*, 1997).

Many different mechanisms have been suggested for selenite reduction, since elemental Se^0 was found outside the bacterial cell (HARRISON *et al.*, 1980), in the periplasmic space (GERRARD *et al.*, 1974) or in the cytoplasm (TOMEI *et al.*, 1992). Reduction of selenite could even be a non-enzymatic process in Gram-negative bacteria (SCHMID & KONETZKA, 1986), with a chemical reaction between glutathion and SeO_3^{2-}. The fact that the absorption spectra of whole cells of *R. rubrum* reveal modifications which are specific to the addition of SeO_3^{2-} in the culture favors the idea that metabolic processes are involved. Indeed, few hours before the begin of the reduction process, an absorption peak at 420 nm appears and only remains during the time of selenite reduction. In both *Thauera selenatis* (SCHRÖDER *et al.*, 1997) and *Geospirillum barnesii* (STOLZ *et al.*, 1997), a cytochrome specific to bacteria grown on selenate was demonstrated. Preliminary results indicate that this absorption peak is related to the presence of a hydrophobic protein in the chromatophores and in the cell walls (VASSEROT, 1998). The failure to detect a covalently bound heme suggests that the absorption peak is related to the presence of a b-type cytochrome (STOLZ *et al.*, 1997; VASSEROT, 1998). In *T. selenatis*, a *b*-type cytochrome was shown to be associated with the selenate reductase (SCHRÖDER *et al.*, 1997). In *R. rubrum*, appearance of the absorbance at 420 nm coincides with the steady decline of Hup activity. Furthermore, the temporal activity of Hup showed clear

differences between the control and selenite containing cultures. As previously reported, a clear rhythmic activity was observed in the control (VAN PRAAG *et al.*, 2000) and in the 0.5 mM selenite-containing cultures during the growing and the stationary phases with a regular period of activity that seems closely related to the generation time of *R. rubrum*. When a higher concentration of selenite is present, a rhythmic activity of Hup is observed till the onset of selenite reduction; later on the enzyme activity can barely be detected. These results suggest that Hup-hydrogenase is not involved in the supply of electrons needed for the reduction of selenite into elemental selenium and reduced volatile selenium compounds (VAN FLEET-STALDER *et al.*, 1997). This role could be taken over by the reversible hydrogenase, as in *Clostridium pasteurianum*, where a hydrogenase functions as selenite reductase in the hydrogen uptake reaction (YANKE *et al.*, 1995).

Finally, these results suggest that the high resistance of *R. rubrum* to selenite could help to prevent and remediate selenite pollution in the aquatic environment.

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RÉSUMÉ

RÉDUCTION DU SÉLÉNITE ET ACTIVITÉ DE L'UPTAKE HYDROGÉNASE CHEZ *RHODOSPIRILLUM RUBRUM*

R. rubrum croît en présence de concentrations millimolaires de SeO_3^{2-}, dans des conditions anaérobies et en présence de lumière continue. La présence du sélénite influence le taux de croissance des bactéries; alors que la fin de la phase de croissance est atteinte 36 heures après inoculation dans les cultures de contrôle, il faut 58 h en présence de 0.5, 1 et 2 mM de sélénite. Les temps de génération sont 12.2 h en absence de sélénite et 13.7 h, 14.2 h et 16.6 h dans les cultures contenant 0.5, 1 et 2 mM SeO_3^{2-}, respectivement. Chez ces 2 dernières, une diminution de la densité optique (A_{650}) a lieu 4 à 6 heures avant le début de la phase stationnaire. Cette chute coïncide avec le début du processus de réduction du sélénite par la bactérie, l'apparition de sélénium volatile et d'un pic à 420 nm dans les spectres d'absorption des bactéries cultivées en présence de sélénite.

L'activité de l'uptake hydrogenase (Hup) a été suivie *in vivo* dans les bactéries. Généralement une augmentation de l'activité est observée 12 h après inoculation. Dans les cultures de contrôle, l'activité de Hup montre des oscillations régulières (période de 12 h) durant la phase de croissance et la phase stationnaire. En présence de 0.5, 1 ou 2 mM SeO_3^{2-}, les fluctuations de l'activité de Hup s'arrêtent avec le début de la réduction du sélénite, puis l'activité décroît. A 1 ou 2 mM, on observe une surpression temporaire de gaz dans les cultures.

La grande résistance au sélénite montrée par *R. rubrum*, ainsi que ces propriétés de conversion du sélénite peuvent être utiles dans la perspective de son utilisation comme agent de bioremédiation pour lutter contre la pollution par ce composé.

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