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Altered Elemental Composition of the Cell Wall of a *Pseudomonas* Strain Having Inducible Tolerance Mechanism to Chromium(VI)

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Heavy metal pollution of soils and natural waters is a global environmental problem. These metals are used in numerous industrial processes. As a result of this multi-lateral application, heavy metals are present in the effluents and accumulated in the soils and the waters endangering living organisms.

Chromium primarily exists in two valency states in the environment. Chromium(III), the dominant form is relatively immobile at near neutral pH, owing to precipitation as a hydroxide (Rai *et al.* 1989). As a consequence of the immobility this form is less bioavailable and less toxic (Ishibashi *et al.* 1990). The cationic form of the chromium (Cr^{3+}) is an essential element for human metabolism as a glucose tolerance factor (Mertz 1975). Chromium(VI) is the second well known form of the chromium. This is an anionic form (CrO_4^{2-}) which is a strong oxidizing agent in acidic conditions (Palmer and Wittbrodt 1991). In contrast to chromium(III) the chromate is quite mobile in the environment as numerous derivatives of it are water soluble (Katz 1991). Chromium(VI) can easily enter the cells through the cellular membranes acting as a substrate analog of the sulphate transport system (Ohtake *et al.* 1987). In the cell the chromate is reduced to lower oxidation states to chromium(III) (Arslan *et al.* 1987). During this chemical reaction the chromium temporarily exists as chromium(V) and chromium(IV) as well. These forms are thought responsible to direct DNA damages (Wetterhahn and Hamilton 1989).

Different types of microorganisms have been isolated having certain resistance mechanisms to heavy metals including chromium (Silver 1996; Basu *et al.* 1997). The resistance is mainly a plasmid born feature (Mondaca *et al.* 1998). Heavy metal uptake is reduced in the cells having resistance (Tsai *et al.* 1997). This exclusion is described in the case of chromium as well (Ohtake *et al.* 1987). In some cases this mechanism is coupled to an ATP dependent ion pump (Tsai *et al.* 1997; Rosen 1996). The reduction of chromate to a lower oxidation state in the cells is independent of the resistance (Cervantes and Silver 1992). Different membrane-bound and cytoplasmic proteins were isolated and characterised in connection with chromate reduction (Ishibashi *et al.* 1990; Wang *et al.* 1990; Rege *et al.* 1997).

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This paper focuses on (i) the characteristics of the tolerance mechanism of the *Pseudomonas* strain investigated, (ii) the effect of chromium(VI) on cell wall composition and (iii) the altered sensitivity of untreated and chromium(VI) treated cells to ultrasound.

MATERIALS AND METHODS

The *Pseudomonas* strain used for the experiments was isolated from a heavy metal polluted area of Nyíregyháza (Vincze *et al.* 1994). Chemicals were purchased from Sigma Chem. Co. (St Louis, USA) and Serva Fine Biochem. Gmbh. (Heidelberg, Germany). Sodium dichromate was used as hexavalent chromium.

Growth curves were analysed and compared to decide whether chromium(VI)-tolerance is inducible or a constitutive property of the cells investigated. Prior to cultivation, cells were grown in King B medium without added Cr(VI) (uninduced cells, control) or in presence of 100 and 200 ppm Cr(VI) (induced cells, treated), respectively. The cells pregrown under control and chromium(VI) treated conditions were then transferred to Erlenmeyer flasks having King B medium with (the applied concentrations were 50, 100 and 200 ppm) or without chromium(VI). The optical density of the pregrown bacterial suspension was adjusted to 0.5 before inoculation to ensure the comparability of the growth curves obtained. 100 µL of this diluted suspension was used to start cultivation. The optical density of the cell suspension at 540 nm (Perkin Elmer UV/VIS λ2) was measured to monitor the growth rate of the culture. The inoculated broth was grown on a rotary shaker (Lab-line, 26 °C, 130 rpm).

In order to study the effect of chromium(VI) treatment on elemental composition of the cell wall, for the experiment four cultures were chosen: (i) pregrown and cultivated without chromium(VI) as a control, (ii) pregrown on 100 ppm and cultivated in the presence of 50 ppm chromium(VI), (iii) pregrown and cultivated in the presence of 100 ppm chromium(VI), (iv) pregrown and cultivated in medium containing 200 ppm chromium(VI). A part of the bacterial biomass obtained from the cultivation procedure described above was used for elemental analysis. Cells were collected and washed with deionized water twice and centrifuged again (Janetzky 24, 6000 rpm, 10 min). The pellet was resuspended in 10 mL of deionized water, 1 mL of this suspension was centrifuged and the weight of the biomass was determined before elemental analysis (sample 1, cells). A further 4 mL part of the same suspension was subjected to ultrasonic disintegration (44 kHz, 12x1 min treatment with 1 min pause) and used for chemical analysis. Following each 1 min ultrasonic treatment 100 µL of the suspension was centrifuged and 80 µL of the supernatant was added to a tube containing 400 µL 50 m*M* Tris-HCl pH: 7.0 buffer. The rate of cell disruption was checked measuring the optical density of the protein containing solution at 280 nm. The sample taken just before beginning the first ultrasonic treatment was used as a control. The cell wall and the cell membrane were not separated and were considered as one unit during the determination of the elemental composition.

After the ultrasonic treatment 1 mL of the suspension was centrifuged and the pellet (cell wall and cell membrane together) was weighed. This collected material was subjected to chemical analysis (sample 2, cell wall - cell membrane system). The supernatant was considered as cytoplasm (sample 3, cytoplasm) and used to determine the elemental composition. The water of the supernatant was evaporated before digestion. The three prepared samples (cells, cell wall - cell membrane system and cytoplasm) were digested overnight in concentrated HNO_3 and 30% H_2O_2 mixture (12:1 V/V rate) and dried. The mineralized residue was redissolved in 4 mL of 2 *N* HNO_3 solution. The elemental content was determined by a Spectroflame-type inductively coupled plasma atomic emission spectrophotometer (ICP-AES) (Spectro Gmbh, Kleve, Germany with the following parameters: plasma gas 1.6 dm^3 min^{-1}, nebulizer gas 0.6 dm^3 min^{-1}; coolant gas 15 dm^3 min^{-1}, excitation 27 MHz, 1,05 kW, cross flow nebulizer).

The data on ultrasonic disintegration indicated different sensitivity of chromium(VI) treated and untreated cells to ultrasound. In order to clear the relationship between the chromium(VI) treatment and the altered sensitivity to ultrasound, the ultrasonic disintegration was repeated. The absorbtions at 280 nm were plotted and compared as a function of time of the ultrasound treatment.

RESULTS AND DISCUSSION

The optical density of the bacterial cultures was used to monitor the growth rate during the cultivation of the cells. Fig. 1 shows growth curves obtained from the experiments in which induced or non-induced cells were used for the inoculation of chromium free or chromium(VI) containing broth. Analysing the curves it is obvious that the induced or non-induced state of the cells do not affect the growth rate on chromium free medium. Fig. 1A shows that the presence of 100 or 200 ppm chromium(VI) seriously affects the growth rate of cultures inoculated with non-induced cells and causes a great increase of lag-phase. This adaptation period was less than 6 hr under control conditions and more than 36 hr in the chromium(VI) containing cultures. The effect of 50 ppm chromium(VI) is much less compared to the higher concentrations. This inhibition phenomenon is general in heavy metal polluted cell suspensions (Margesin and Schinner 1996). In contrast, comparing the curves of the cultures grown on the medium containing 100 or 200 ppm chromium(VI) there are great differences between the lengths of the lag periods. In the case of non-induced cells (Fig. 1A) more than 36 hr is required for adaptation in presence of both 100 and 200 ppm chromium(VI) concentration. As Fig. 1B and C show, using induced cells the adaptation periods are about 20 and 14 hr, respectively. Evaluating the differences in the growth curves obtained, the tolerance may be said to be inducible. It can be seen that the change in the length of the lag-phase depends on the chromium(VI) concentration. On the other hand, the inducible nature of the resistance could indicate that the expression of some gene could play a central role in the tolerance. As a result of gene expression, proteins may appear which would be parts of certain efflux system mentioned in the literature (Nies et al. 1989; Mondaca *et al.* 1998).

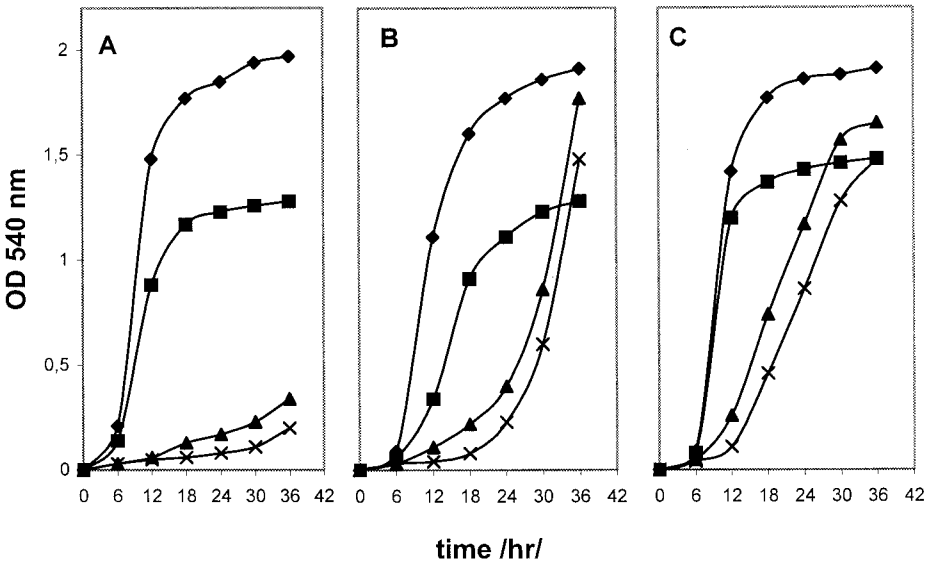

Figure 1. Growth of uninduced (A) and induced (B, C pregrown on medium containing 100 and 200 ppm chromium (VI), respectively) cells of *Pseudomonas* strain without (◆) or in the presence of 50 ppm (■), 100 ppm (▲) and 200 ppm (×) chromium(VI).

Concentrations of magnesium, calcium, iron, chromium, manganase and copper were determined. The amount of manganase and copper were under the detection limit in the samples. The concentrations of metals are presented in Table 1. Data on chromium show that the cells of investigated bacterial strain can bind and accumulate chromium mainly in the cell wall - cell membrane system (cell wall and cell membrane were not separated in our experiments). The high rate accumulation of chromium in the cells is obvious, comparing the data belonging to control and treated cells: the chromium content of the control cells is less than 1 ppm, meanwhile the same values in the case of treated cells exceed several hundreds of ppm.

The experimental data show that the changes in concentrations of the metals measured are considerable. The concentrations of chromium and calcium show parallel changes with each other. An increasing chromium content causes a higher calcium concentration both in whole cells and the cell wall - cell membrane system respectively. The increased calcium concentration is a surprising result, because an ion exchange would rather be expected instead. Concentrations of magnesium and iron change in a parallel way to each other, but in an opposite way to those of chromium and calcium. A considerable decrease was detectable in both iron and magnesium. The bulk of accumulated chromium is in the cell wall - cell

Table 1. Elemental composition of the cells grown without and in the presence of 50, 100 and 200 ppm chromium(VI). The values are given in ppm. nd: not detectable

	Whole cell				Cell wall - cell membrane				Cytoplasm			
	Cr	Ca	Mg	Fe	Cr	Ca	Mg	Fe	Cr	Ca	Mg	Fe
Control	0.9±0.3	14.1±1	2695±63	27.4±0.4	0.6±0.3	12.7±0.7	1656±127	21.7±0.8	nd	0.4±0.1	10±1.3	0.3
50 ppm	563±12	49.3±0.5	984±24	9.6±0.5	617±44	46.4±2.3	1124±40	11.2±0.9	2.5±0.4	0.9±0.2	6.9±1.8	nd
100 ppm	747±6	63.8±0.8	766±9	3.1±0.2	915±22	78.4±5.2	928±21	3.8±0.2	8.4±1.2	1.2±0.1	3.8±0.9	nd
200 ppm	766±20	70.1±3	633±22	3.4±0.3	386±46	38.1±6.2	147±32	4.7±0.8	17.7±0.8	1.8±0.1	16.8±1.1	nd

Values represent the mean ± SD of one experiment in triplicate

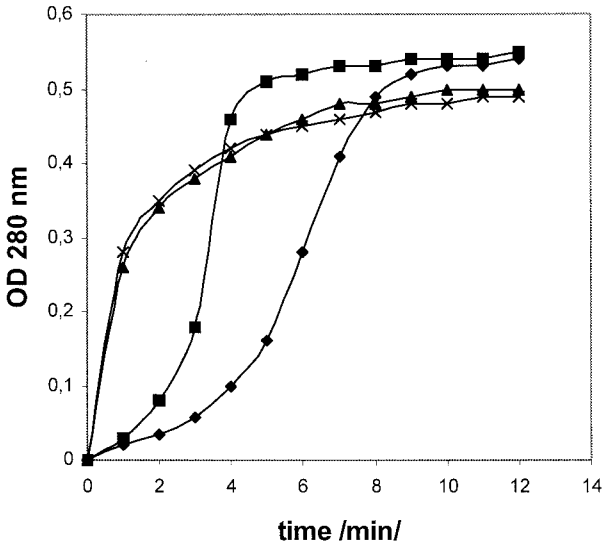

Figure 2. The sensitivity to ultrasound of the cells of the *Pseudomonas* strain grown without (◆) or in the presence of 50 ppm (■), 100 ppm (▲) and 200 ppm (×) chromium(VI).

membrane system and only a smaller part of the chromium is detectable in the cytoplasm. These results agree with those reported in the literature, which suggests that metal accumulation is decreased by a plasmid-encoded efflux system in the resistant cells (Cervantes and Silver 1992; Mondaca *et al.* 1998). Comparing the data concerning cell wall - cell membrane system and cytoplasm, similar tendencies can be seen in the changes in concentrations of the metals measured. Chromium and calcium concentrations increased while magnesium and iron concentrations decreased.

Bacterial cultures grown under different conditions were used to study the sensitivity of cells to ultrasonic treatment. The results are presented in the Fig 2. The effectiveness of ultrasound treatment, that is the rate of cell disruption, was monitored by measuring the optical density of the solution at 280 nm as an indicator of the protein content.

The bacterial cells grown on the medium containing chromium(VI) are more sensitive to ultrasound than those grown under control conditions. Comparing the time required to achieve 50 % disruption of the cells represented by the half maximum protein content of the solutions, it can be seen, that the control culture needs about 6 min while the chromium(VI) treated cells need less then 1 min. It means that the cell wall became more rigid and as a consequence more fragile during the chromium(VI) treatment. Changes in the sensitivity of cells to

ultrasonic treatment could be related to an altered composition of the cell wall. In cells treated with other heavy metals similar alterations in the cell wall composition were described (Venkateswerlu and Stotzky 1986). It is not clear at present whether the changes in the inorganic or in the organic compounds of the cell wall are responsible for the increased sensitivity.

Comparing these changes in the elemental composition of the cell wall - cell membrane system with the increased sensitivity of cells to the ultrasonic treatment, it seems that these alterations affect the cell stability. It is not clear at present whether the increased sensitivity of cells to ultrasound is the direct consequence of accumulation of reduced chromium in the cell wall or an indirect effect of chromium decreasing magnesium and iron content of the cell wall or due to some other factors not investigated here.

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