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# Responses of the marine bacterium *Pseudomonas fluorescens* to an excess of heavy metals: Physiological and biochemical aspects

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## ABSTRACT

A *Pseudomonas fluorescens* strain was isolated from oxic marine sediments obtained from the strand zone of the St Anne Bay (a moderately metal-contaminated site to the west of Cherbourg harbour). The strain, which exhibited a high tolerance to metal contamination when cultivated (minimal inhibitory concentration = 950  $\mu\text{M}$  [62  $\text{mg L}^{-1}$ ] for Zn, 660  $\mu\text{M}$  [42  $\text{mg L}^{-1}$ ] for Cu, and 505  $\mu\text{M}$  [57  $\text{mg L}^{-1}$ ] for Cd), was further characterized by its physiological and biochemical responses to metal additions to the culture medium. Bacterial growth was significantly disturbed by 380  $\mu\text{M}$  Zn (25  $\text{mg L}^{-1}$ ), 315  $\mu\text{M}$  Cu (20  $\text{mg L}^{-1}$ ) and 90  $\mu\text{M}$  Cd (10  $\text{mg L}^{-1}$ ). The Zn-containing alkaline phosphatase was studied as an intoxication biomarker. Its activity was stimulated (+9%) by an excess of Zn, but inhibited by Cd (–55%) and Cu (–10%), these two elements could displace the native Zn or/and disturb the enzyme 3D-structure. Bacterial  $\text{O}_2$  consumption was recorded as a global physiological response to metal stress. This parameter dropped with increasing Cd and Cu contamination (–49% and –45%, respectively, at 20  $\text{mg L}^{-1}$ ). By contrast, Zn increased  $\text{O}_2$  consumption (approximately +40% for the different tested concentrations). The proteomes of bacteria grown in the presence or absence of 20  $\text{mg metal L}^{-1}$  were characterized by 2D-gel electrophoresis. The number of spots exhibiting a difference in intensity between the contaminated sample and the control was 65, 68, and 103, for Zn, Cu and Cd, respectively. Among them, 45, 61 and 82 spots respectively appeared *de novo* or increased in intensity, indicative of metal-stimulated synthesis, particularly for Cu and Cd. In summary, whereas Cd and Cu treatments both stressed cells and slowed down primary metabolism to differing extents, Zn has a stimulating action on several physiological and biochemical parameters.

## Keywords:

*Pseudomonas fluorescens*

Metal stress

Growth

Respiration

Alkaline phosphatase

Proteomics

## 1. Introduction

*Pseudomonas fluorescens* is found in various environments. It is well described in cultivated soils (Silby and Levy, 2004;

Siddiqui et al., 2005) and in sewage sludge (Ciesielski et al., 2006; Takenaka et al., 2007). Many studies indicate its presence in the marine environment, (Newman and Cragg, 2004; Castro-González et al., 2005), but only a few describe strains from the

Abbreviations: AP, alkaline phosphatase; APA, alkaline phosphatase activity; IEF, isoelectric focusing; MIC, minimal inhibitory concentration; pNPP, paranitrophenyl phosphate; pNP, paranitrophenol.

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oxic layer of the strand zone. This zone is very particular in the sense that it is submitted to variations of several parameters such as salinity, temperature, water turbulence, and light. Moreover, during the last decades, increased human activities have resulted in an accumulation of heavy metals, which particularly affect the coastal zone by accumulating in sediments (Zhou et al., 2007a,b). Consequently, the bacteria which live in this specific environment must adapt to numerous stresses (Azam and Malfatti, 2007). *P. fluorescens* species is known for its stress resistance (Stockwell and Loper, 2005). In the marine environment, its capacities of adaptation has been described especially against pollutants like hydrocarbons (Bamforth and Singleton, 2005; Vasudevan et al., 2007) but nothing is known about the metal resistance capacity of strains isolated from the strand zone.

This study reports the characterization of a *P. fluorescens* strain (strain BA3d12) isolated from the strand zone of St Anne Bay at the west of Cherbourg seaport. Previous studies carried out in this bay have reported a metal contamination of the sediments, probably due to seaport activities, urban effluents and industrial wastes which flow into this local marine area (confidential data). We report the ability of this strain to respond to an excess of metals through physiological and biochemical measurements. The metals tested were zinc, copper and cadmium, these elements being currently found in contaminated sites. Zinc and copper are essential elements, required for normal growth, development and survival of all living systems, whereas cadmium is generally toxic because it has no known biological role (Ron et al., 1992). Among the physiological responses tested, we have focussed on the minimal inhibitory concentration (MIC), growth, rate of O<sub>2</sub> uptake and alkaline phosphatase activity (APA). The respiration rate is generally considered to be a reliable index of microorganism metabolic processes, and toxicity of metals can be evaluated by modification of respiratory activity (King and Painter, 1986). Alkaline phosphatase (EC 1.3.1.3) was selected as it represents an intoxication biomarker in ecotoxicological studies, due to its high sensitivity to metal salts (Boge et al., 1988). By precipitating metal ions with phosphates, this enzyme is moreover involved in mechanisms of detoxification (Macaskie et al., 1992). When organisms are submitted to a metal ion stress, particular metabolic pathways generally arise (Silver 2003; Mergeay et al., 2003) involving stress proteins. Currently, the only publication that presents proteomics of metallic-stressed *P. fluorescens* (Sharma et al., 2006) does not concern bacteria isolated from a marine environment. Therefore, we have decided to study the proteome response to metal stress in the marine strain BA3d12 by comparing the proteomes of non-contaminated and contaminated cells using two-dimensional gel electrophoresis.

## 2. Materials and methods

### 2.1. Bacterial strain isolation and growth conditions

*P. fluorescens* (strain BA3d12) was isolated from the top 2-cm layer of oxic marine sediments collected on the seashore, at low tide in the St Anne Bay (west of Cherbourg harbour: 49°39 N–01°40 W, France). Long-term storage was at –80 °C in 25%

glycerol. During the experimental period, the strain was stored at +4 °C on CFC agar plates and maintained by monthly subcultures. For all experiments, *P. fluorescens* BA3d12 was cultivated at +22 °C in 100 mL of nutrient broth containing 1% (w/v) tryptone, 0.5% (w/v) meat extract, 0.5% (w/v) NaCl (Biokar Diagnostics, Beauvais, France). Cultures were inoculated with 1 mL of bacterial suspension from a 24 h-old preculture.

To study growth kinetics, O<sub>2</sub> consumptions and proteomes, the nutrient broth was supplemented with different Zn, Cu or Cd concentrations (0 to 25 mg L<sup>-1</sup>) using 1 g L<sup>-1</sup> ZnCl<sub>2</sub>, CuCl<sub>2</sub>, and CdCl<sub>2</sub> stock solutions. For measurements of alkaline phosphatase activity, O<sub>2</sub> consumption measurements and 2D-electrophoresis, cultures were harvested at the end of the exponential growth phase, so that the bacterial concentration was always 2.10<sup>8</sup> cells mL<sup>-1</sup>, which corresponds to 0.330 absorbance unit at 600 nm (Spectronic 20 Genesis, Rochester, NY, USA).

### 2.2. Minimal Inhibitory Concentration (MIC) determination

MICs of the isolated strain BA3d12 were determined in the media described above, supplemented with increasing concentrations (up to 80 mg L<sup>-1</sup>) of a given metal. The cultures were incubated for up to 15 days, and growth was monitored by absorbance measurements at 600 nm. MIC is generally defined as the lowest metal concentration for which the growth is inhibited.

### 2.3. Growth kinetics

Growth kinetics of contaminated and control BA3d12 cultures were monitored until stationary phase, by cell counting with a Thoma cell.

### 2.4. Alkaline phosphatase activities

Each cell culture was first concentrated by centrifugation at 10000 g for 10 min at +22 °C. The pellet was then resuspended in 20 mL of a buffer pH 10.5, containing 0.1 M glycine, 0.5 mM MgCl<sub>2</sub> 6H<sub>2</sub>O, 85 mM NaOH. In order to efficiently extract the phosphatase from the cells, two cell disruption methods have been compared: high pressures delivered by a French Press and ultrasonication using a microtip. The highest APA was consistently found in the French Press extract; as a result, for all APA measurements, the concentrated cell sample was submitted to one passage at 159 MPa through a pre-cooled French Press (French Pressure Cell 40 K, Thermo IEC, Needham Heights, MA, USA). The protein concentration in the enzyme extract was determined according to the Bradford method (Bradford, 1976). All protein measurements were carried out in duplicates. The enzyme extract was immediately exposed to increasing concentrations of substrate (paranitrophenylphosphate = pNPP), contaminated with different concentrations of a given metal. For comparison, a control reaction was performed by incubation of the enzyme with the non-contaminated pNPP. All these reactions were carried out in the 300 µL-wells of a microtitre plate, with a ratio of 100 µL enzyme to 200 µL substrate. The resulting product paranitrophenol (pNP) concentrations were followed by absorbance

measurements at 405 nm using a microplate reader Apollo-1 (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Data acquisition was performed with the MikroWin 2000 4.0 version software (Mikrotek Laborsysteme GmbH, Overath, Germany). Under these conditions, APA was measured at +22 °C for 4 h, at 10 minute intervals. The Michaelis-Menten constants,  $V_{\max}$  (reaction rate when the enzyme is saturated with substrate) and  $K_m$  (half saturation concentration), were calculated with the CurveExpert 1.3 version software (curve fitting system for Windows) as described in [Jean et al. \(2003\)](#).

## 2.5. $O_2$ consumption rates

The  $O_2$  consumption rate of the bacterial cell cultures was measured with a Clark-type electrode maintained at +22 °C with a polystat (Eurosep Instruments, Cergy St Christophe, France). The electrode was first calibrated in air-saturated water (100%  $O_2$ ) and then with a sodium sulfite solution (0%  $O_2$ ). The cell suspensions were constantly stirred (speed 50 rpm), to promote oxygen diffusion through the PTFE membrane. Molecular oxygen was electrolytically reduced by a platinum cathode maintained at 1.5 V. The cathode was then depolarized, allowing a current to flow, proportional to the oxygen activity ([Clark, 1956](#)). Data was acquired using the OxyLab 1.07 version software (Hansatech Instruments, Norfolk, England), which provides the  $O_2$  concentration decrease versus time allowing calculation of the  $O_2$  consumption rate. The results of the first tests carried out in our laboratory indicated that addition of the metal contamination directly to the measurement chamber led to electrode deterioration. For this reason, it was not possible to record the instantaneous effect of an excess of metal on the respiration rate. Therefore, we followed the sample preparation protocol of [Navarro et al. \(1997\)](#), in which the metal was added to the culture broth before bacterial inoculation. The bacteria were cultivated in this contaminated broth until the end of the exponential growth phase. The contaminated cell culture was then centrifuged at 10000 g for 10 min at +22 °C. The pellet was washed in 12 mL of 0.1 M  $K_2HPO_4$  buffer pH 7.5. After removal of the supernatant, the pellet was resuspended in 12 mL of the buffer and distributed into three 4 mL aliquots. These suspensions were centrifuged at 10000 g for 10 min at +22 °C. Finally, each pellet was vortexed in 2 mL of nutrient broth just before analysis using the Clark-type electrode. The three replicates were compared to a control, processed under the same conditions.

## 2.6. 2D-PAGE analysis of soluble proteins

### 2.6.1. Preparation of protein extracts

Contaminated (20 mg  $L^{-1}$ ) and control cultures were centrifuged at 10000 g for 10 min at +22 °C. The pellets were washed twice and resuspended in 10 mL of ultrapure water. Cells were disrupted by three passages at 159 MPa through the pre-cooled French Press. The resulting homogenate was centrifuged for 10 min at 10000 g at +4 °C to pellet down cell debris. Protein concentration in the supernatant was determined according to [Bradford \(1976\)](#). All protein extracts were purified and concentrated five times with a centrifugal concentrator (Amicon

Ultra-4 Millipore, 10 kDa cut-off size, Bedford, MA, USA) and stored at -80 °C until analysis.

### 2.6.2. 2D-electrophoresis

For isoelectric focusing (IEF), 200  $\mu$ g of protein was dissolved in a rehydration buffer containing 8 M urea, 4% (w/v) CHAPS, 20 mM DTT, 0.2% Bio-Lyte 3-10 and 0.001% (w/v) bromophenol blue. After active rehydration at 50 V for 17 h under paraffin oil, 17 cm-long immobilized pH gradient (IPG) strips (pH 3-10, Bio-Rad) were submitted to IEF (Protean IEF Cell, Bio-Rad) with the following voltage/time process: 150 V for 1 h with rapid ramping, 350 V for 15 min with rapid ramping, 750 V for 45 min with rapid ramping, 1500 V for 1 h with rapid ramping and 3500 V for 18 h with rapid ramping (adapted from [Vilain et al., 2004](#)). After IEF separation, strips were consecutively incubated in equilibration buffers A and B for 15 min [50 mM Tris/HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, with 65 mM DTT (solution A) or 135 mM iodoacetamide (solution B)]. In the second dimension, proteins were separated by SDS-PAGE on 17 × 17 cm gels containing 12.5% acrylamide according to [Laemmli \(1970\)](#). The gels were run in a Protean II cell (Bio-Rad) at +4 °C (1 h at 25 V, then at 200 V until the bromophenol blue dye reached 5 mm from the bottom of the gel). Proteins were visualized by gel staining with the Dodeca silver stain kit (Bio-Rad). The stained gels were analysed using the PD-Quest software 7.3.0 version (Bio-Rad).

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## 3. Results

### 3.1. MICs

The MIC values for the three heavy metals tested are 950  $\mu$ M (62 mg  $L^{-1}$ ) for Zn, 660  $\mu$ M (42 mg  $L^{-1}$ ) for Cu and 505  $\mu$ M (57 mg  $L^{-1}$ ) for Cd. These results show that the order of BA3d12 strain's metal sensitivity was Cd > Cu > Zn.

### 3.2. *Pseudomonas* BA3d12 growth kinetics

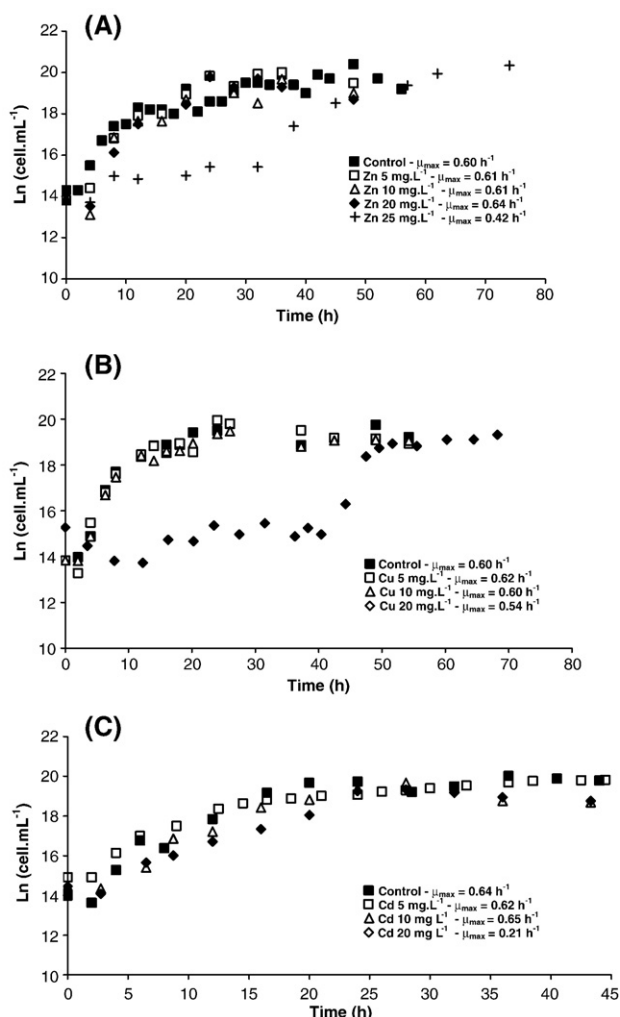
#### 3.2.1. Zinc

BA3d12 growth in the presence of increasing Zn concentrations is presented in [Fig. 1A](#). For zinc concentrations of 0, 5, 10, and 20 mg  $L^{-1}$ , no significant difference was observed in growth kinetics (Wilcoxon test:  $p > 0.01$ ). However, the lag phase for contaminated cultures is slightly longer (4 h instead of 2 h). For 25 mg  $L^{-1}$  Zn (380  $\mu$ M), significant differences occurred in comparison to the control (Wilcoxon test:  $p < 0.01$ ). The lag phase was considerably increased (32 h) and the maximum growth rate was slightly lower than the control (0.42  $h^{-1}$  instead of 0.60  $h^{-1}$ ). Therefore, this strain was highly tolerant to Zn, its growth being significantly affected only by high Zn concentrations (>20 mg  $L^{-1}$  or 305  $\mu$ M).

For the control and all the Zn concentrations tested, the same cell concentration was observed in the stationary phase.

#### 3.2.2. Copper

The results of the evolution of BA3d12 cell concentration in the presence of increasing copper concentrations are shown in [Fig. 1B](#). For 5 mg  $L^{-1}$  Cu, there was no significant change, in either the acclimation phase or the exponential



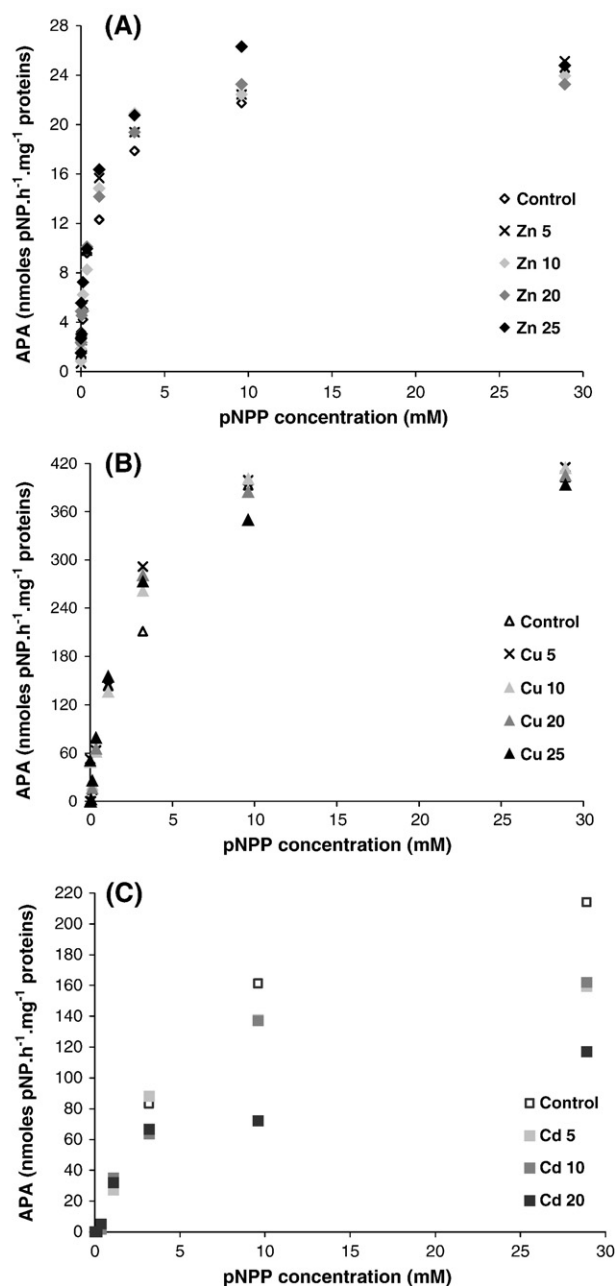
**Fig. 1 – Growth of *Pseudomonas fluorescens* BA3d12 in the presence of different concentrations of Zn (A), Cu (B) and Cd (C).**

phase, compared to the control (Wilcoxon test:  $p > 0.01$ ). For 10  $\text{mg.L}^{-1}$  Cu, the Wilcoxon test indicated that growth kinetics differed ( $p < 0.01$ ), between the control and the contaminated cultures. As indicated by the Wilcoxon test, it is obvious that both kinetics were similar, since no significant difference existed between 5 and 10  $\text{mg.L}^{-1}$  Cu ( $p > 0.01$ ). For the highest Cu concentration (20  $\text{mg.L}^{-1}$  or 315  $\mu\text{M}$ ), growth kinetics were distinct from that of the control (Wilcoxon test:  $p < 0.01$ ). A very long lag phase of 42 h was observed, as well as a slight decrease in the maximum growth rate ( $0.54 \text{ h}^{-1}$  instead of  $0.60 \text{ h}^{-1}$ ). For all the Cu concentrations tested, the cell concentrations at the stationary phase were similar to those of the control.

### 3.2.3. Cadmium

The growth kinetics of BA3d12 in the presence of increasing Cd concentrations is presented in Fig. 1C. At 5  $\text{mg.L}^{-1}$ , there was no significant change neither in the acclimation phase, nor in the exponential phase, compared to the control (Wilcoxon test:  $p > 0.01$ ). However, statistical differences were revealed by the Wilcoxon test ( $p < 0.01$ ) between 10  $\text{mg.L}^{-1}$  Cd (90  $\mu\text{M}$ ) and the control. These differences were probably due to the earlier

decline in growth observed for the contaminated culture from 35 h onwards. Indeed, if this decline phase is not taken into account, the statistical test revealed no difference ( $p > 0.01$ ), the lag phase increasing slightly (4 h instead of 2 h), and the maximum growth rates being comparable between the control and the contaminated cultures (respectively  $0.65 \text{ h}^{-1}$  and  $0.64 \text{ h}^{-1}$ ). For the highest Cd concentration (20  $\text{mg.L}^{-1}$  or 180  $\mu\text{M}$ ), a lag phase of 4 h, a major decrease in the maximum growth rate ( $0.21 \text{ h}^{-1}$  instead of  $0.64 \text{ h}^{-1}$ ), and the appearance of a decline phase after 35 h of growth, demonstrated differences between control and 20  $\text{mg.L}^{-1}$  Cd, which were confirmed by the Wilcoxon test ( $p < 0.01$ ). As for Zn and Cu, the cell concentration reached at the stationary phase was the same for control and for Cd-contaminated cultures.



**Fig. 2 – Effects of Zn (A), Cu (B) and Cd (C) on alkaline phosphatase activities of *Pseudomonas fluorescens* BA3d12.**



**Table 1 – Effects of heavy metals (mg L<sup>-1</sup>) on the APA Michaelis-Menten constants V<sub>max</sub> (nmol pNP h<sup>-1</sup> mg<sup>-1</sup> proteins) and K<sub>m</sub> (mM)**

| V <sub>max</sub>      | Zinc | Copper | Cadmium |
|-----------------------|------|--------|---------|
| Control               | 23.4 | 459.0  | 271.9   |
| 5 mg L <sup>-1</sup>  | 24.1 | 463.8  | 189.0   |
| 10 mg L <sup>-1</sup> | 24.0 | 468.4  | 200.4   |
| 20 mg L <sup>-1</sup> | 23.3 | 445.5  | 122.5   |
| 25 mg L <sup>-1</sup> | 25.4 | 416.1  | –       |
| K <sub>m</sub>        | Zinc | Copper | Cadmium |
| Control               | 0.72 | 2.76   | 7.27    |
| 5 mg L <sup>-1</sup>  | 0.53 | 2.11   | 4.28    |
| 10 mg L <sup>-1</sup> | 0.57 | 2.42   | 5.74    |
| 20 mg L <sup>-1</sup> | 0.50 | 1.94   | 3.64    |
| 25 mg L <sup>-1</sup> | 0.48 | 1.73   | –       |

For the three tested metals, the lowest concentration which has affected significantly the bacterial growth was 380 µM for Zn (25 mg L<sup>-1</sup>), 315 µM for Cu (20 mg L<sup>-1</sup>), and 90 µM for Cd (10 mg L<sup>-1</sup>). To conclude, the metal sensitivity of our *Pseudomonas* strain evaluated through its growth can be classified in the same order as for MICs, that is: Cd>Cu>Zn.

### 3.3. APA

The influence of increasing Zn, Cu and Cd concentrations on *P. fluorescens* phosphatase activity is shown in Fig. 2. Michaelis-Menten constants V<sub>max</sub> and K<sub>m</sub> for the different used metal concentrations are included in Table 1. Note that the V<sub>max</sub> and K<sub>m</sub> values greatly differed between the three controls. Indeed, although all the concentrations of the same metal and its control were tested at the same time from the same enzyme extract, the three metals were studied separately, at different days, and from different extracts. Consequently, the V<sub>max</sub> and K<sub>m</sub> values of the control vary from one enzyme extract to another, and this variation can be generated either by the French Press (used to extract the enzyme), or by the storage of the strain at +4 °C during several days between the various experiments (cf Material and methods section). Taking into account our experimental procedure, the influence of the metal concentration on the V<sub>max</sub> and K<sub>m</sub> values was studied metal by metal.

#### 3.3.1. Zinc

Increasing Zn concentrations seemed to increase the *P. fluorescens* phosphatase activity, as shown by the V<sub>max</sub>

values globally higher for the Zn-contaminated sample than for the control. This positive influence of Zn was more pronounced at 25 mg L<sup>-1</sup> (+9%), for which a higher V<sub>max</sub> value (25.4 nmol pNP h<sup>-1</sup> mg<sup>-1</sup>) was recorded.

#### 3.3.2. Copper

The influence of Cu was much less significant, with almost no difference between phosphatase activities of the 5, 10 and 20 mg L<sup>-1</sup> contaminated medium and the control. However, at 25 mg L<sup>-1</sup>, Cu slightly inhibited the APA, as shown by the moderate decrease (–10%) for the contaminated medium (V<sub>max</sub>=416.1 nmol pNP h<sup>-1</sup> mg<sup>-1</sup>) relative to the control (V<sub>max</sub>=459.0 nmol pNP h<sup>-1</sup> mg<sup>-1</sup>).

#### 3.3.3. Cadmium

For pNPP concentrations higher than 4 mM, the enzyme kinetics in the presence of Cd greatly differed from the control. Cd strongly inhibited the APA at all concentrations tested, as confirmed by the lower V<sub>max</sub> when enzyme and substrate were exposed to the toxic metal. For example, the minimal V<sub>max</sub> (122.5 nmol pNP h<sup>-1</sup> mg<sup>-1</sup>) observed at the highest Cd concentration (20 mg L<sup>-1</sup>), represented only 45% of the control V<sub>max</sub> (271.9 nmol pNP h<sup>-1</sup> mg<sup>-1</sup>).

Finally, among the three metals tested, Cd induced the more drastic differences compared to the control. In contrast, whatever the metal tested, the phosphatase affinity for pNPP, expressed as K<sub>m</sub> values, was always greater than for the control. Higher affinities (lower K<sub>m</sub> values) were recorded at the highest metal concentrations (0.48 mM pNPP for 25 mg L<sup>-1</sup> Zn, 1.73 mM pNPP for 25 mg L<sup>-1</sup> Cu and 3.64 mM pNPP for 20 mg L<sup>-1</sup> Cd).

### 3.4. O<sub>2</sub> consumption

The oxygen consumption rates for contaminated and control *P. fluorescens* cultures, are presented in Table 2. The table indicates also the percentages of metal activation (+) or inhibition (–) on oxygen consumption.

#### 3.4.1. Zinc

As observed for the enzyme activities, Zn was the only metal that activated O<sub>2</sub> consumption by *P. fluorescens*. For any Zn concentration tested, O<sub>2</sub> consumption rates were always higher than those of the control. These differences were statistically significant (T test: p<0.05). Compared to the control, the increase in O<sub>2</sub> consumption (+30%) was similar for 5, 20 and 25 mg L<sup>-1</sup> Zn, whereas it was maximal (+44%) at 10 mg L<sup>-1</sup> Zn.

**Table 2 – Effects of heavy metals (mg L<sup>-1</sup>) on the oxygen consumption rates (R, in µmol O<sub>2</sub> mL<sup>-1</sup> min<sup>-1</sup>) of *Pseudomonas fluorescens* BA3d12. Metal activation (+) or inhibition (–) on oxygen consumption is expressed by P, in %**

| Metals                | Zinc         |      | Copper       |      | Cadmium      |      |
|-----------------------|--------------|------|--------------|------|--------------|------|
|                       | R            | P    | R            | P    | R            | P    |
| Control               | –0.479±0.039 | –    | –0.841±0.027 | –    | –0.684±0.077 | –    |
| 5 mg L <sup>-1</sup>  | –0.614±0.003 | +28% | –0.791±0.005 | –6%  | –0.578±0.024 | –15% |
| 10 mg L <sup>-1</sup> | –0.692±0.031 | +44% | –0.636±0.041 | –24% | –0.387±0.006 | –43% |
| 20 mg L <sup>-1</sup> | –0.638±0.007 | +33% | –0.461±0.025 | –45% | –0.350±0.011 | –49% |
| 25 mg L <sup>-1</sup> | –0.651±0.016 | +36% | –            | –    | –            | –    |

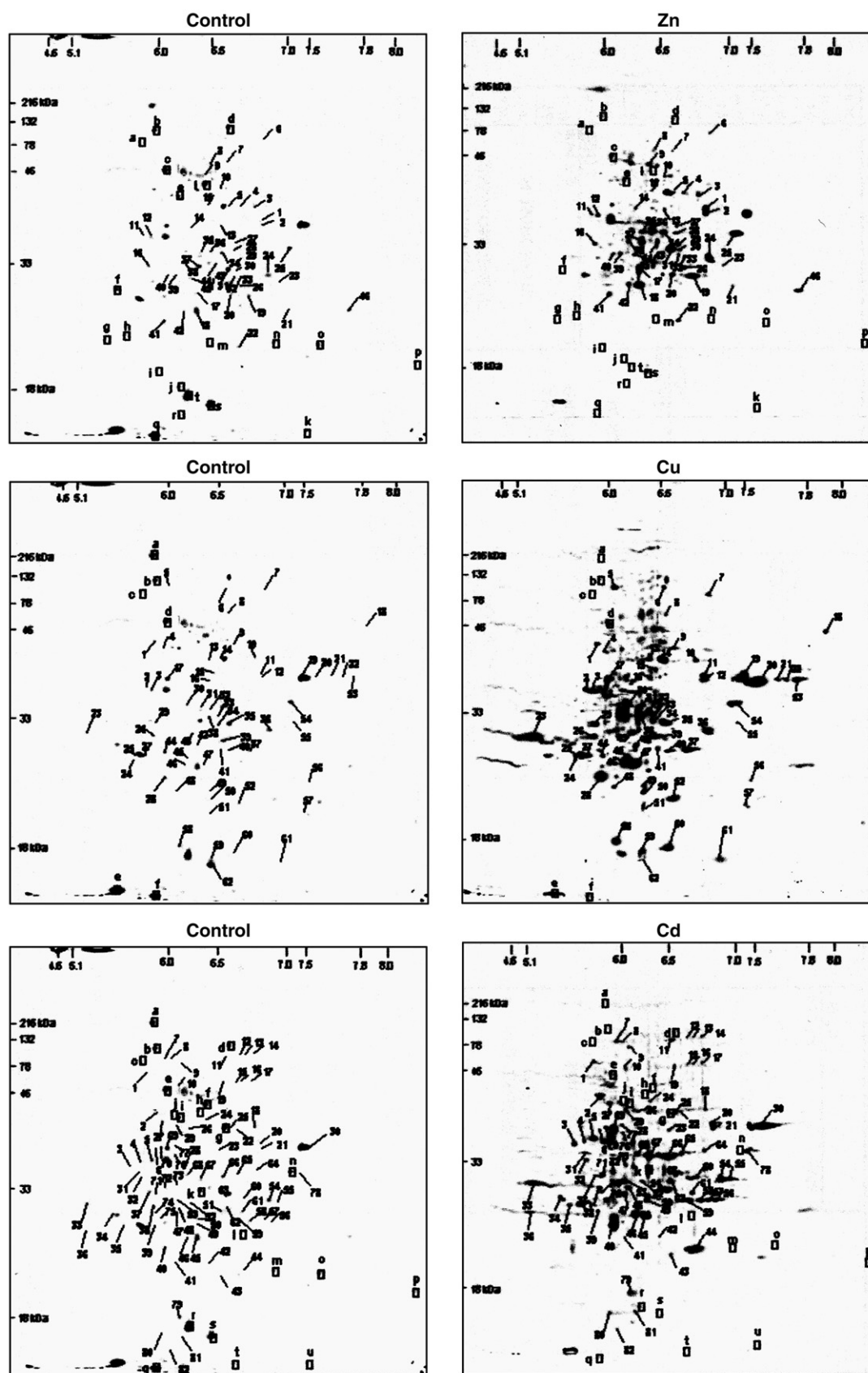


Fig. 3–2D-electrophoresis gels of *Pseudomonas fluorescens* BA3d12 proteome when grown without metal, in the presence of  $20 \text{ mg L}^{-1}$  Zn, Cu or Cd. Proteins were stained with silver nitrate. Squares and lines indicate less and more intense spots, respectively, after metal treatments.

**Table 3 – Differential protein spots visualized when *Pseudomonas fluorescens* BA3d12 was exposed to 20 mg L<sup>-1</sup> of Zn, Cu or Cd**

| Metal   | Overexpressed or appeared proteins | Underexpressed or disappeared proteins | Total differential expressed proteins |
|---------|------------------------------------|--|---------------------------------------|
| Zinc    | 45                                 | 20                                     | 65                                    |
| Copper  | 62                                 | 6                                      | 68                                    |
| Cadmium | 82                                 | 21                                     | 103                                   |

### 3.4.2. Copper

The presence of Cu reduced O<sub>2</sub> uptake rates, as shown by differences between the contaminated sample and control. Whereas at 5 mg L<sup>-1</sup> Cu, O<sub>2</sub> uptake differed little from the control (–0.791 and –0.841 μmol mL<sup>-1</sup> min<sup>-1</sup>, respectively), modifications were more significant (T test:  $p < 0.05$ ) at higher metal concentrations, particularly at 20 mg L<sup>-1</sup> Cu. At this latter concentration, a clear reduction (–45%) in O<sub>2</sub> uptake rate was observed in comparison to the control.

### 3.4.3. Cadmium

The Cd-contaminated samples exhibited the greatest reduction in O<sub>2</sub> consumption rate (Table 2). O<sub>2</sub> consumption rates quickly decreased with increasing Cd contamination, ranging from –0.578 μmol mL<sup>-1</sup> min<sup>-1</sup> at 5 mg L<sup>-1</sup> to only –0.350 μmol mL<sup>-1</sup> min<sup>-1</sup> at 20 mg L<sup>-1</sup>. Compared with the control, this reduction in O<sub>2</sub> consumption was first moderate at 5 mg L<sup>-1</sup> (–15%), to finally reach significant values (T test:  $p < 0.05$ ) at 10 and 20 mg L<sup>-1</sup> (respectively –43 and –49%).

Among the three tested metals, whereas Cu and Cd uniformly reduced O<sub>2</sub> consumption, Zn always stimulated its consumption.

## 3.5. *Pseudomonas* BA3d12 protein maps

The proteins from control and contaminated bacteria, separated on 2D-gels, are shown in Fig. 3. Whatever the metal added, the protein maps differed greatly from that of the control; the number of spots exhibiting a difference in intensity was 65, 68, and 103, for Zn, Cu and Cd, respectively (Table 3). Most of them appeared *de novo* or increased in intensity (Table 3).

## 4. Discussion

### 4.1. MICs

*P. fluorescens* BA3d12 has shown a high level of resistance to all the metals tested, since continued growth was observed in the presence of high metal concentrations. The MIC values indicated that this strain was sensitive to heavy metals in the following order: Cd > Cu > Zn. A relatively diverse range of *Pseudomonas* MICs have been reported for Cu, Cd or Zn. For Cu, Teitzel and Parsek (2003) found a MIC of 127 mg L<sup>-1</sup> for *Pseudomonas aeruginosa*, and Chen et al. (2006) 190 mg L<sup>-1</sup> for *Pseudomonas putida*, whereas Bender and Cooksey (1986) obtained a value of 6.35 mg L<sup>-1</sup> for *Pseudomonas syringae*, and

Tom-Petersen et al. (2001) 3 mg L<sup>-1</sup> for *P. fluorescens*. For Zn, MICs range between 50 mg L<sup>-1</sup> for *Pseudomonas* sp. (Malik and Jaiswal, 2000) and 3139 mg L<sup>-1</sup> for *P. aeruginosa* (De Vincente et al., 1990). For Cd, some *Pseudomonas* strains were incapable of growth at Cd concentrations above 1.1 mg L<sup>-1</sup> (Zawadzka et al., 2007), whereas other strains could tolerate concentrations as high as 1000 mg L<sup>-1</sup> (*Pseudomonas* sp.: Malik and Jaiswal, 2000; *P. putida*: Hu and Zhao, 2007). By comparing our results with those quoted previously, it appears that our *Pseudomonas* strain presents a high metal resistance.

Many publications have shown that MICs depend on the contamination of the study site from which strains have been isolated. In a general way, the absence of metal in the bacterial environment noticeably reduces its degree of resistance (Timoney et al., 1987; Ryan et al., 2005). By contrast, harbours are known to be metal-contaminated and for this reason accommodate resistant species (Ford et al., 2005). Previous studies have reported a metal contamination of St Anne's Bay (confidential data), which could explain the presence at this site of metal-tolerant *Pseudomonas* strains. According to Teitzel and Parsek (2003), MICs depend on the culture medium used. Generally, the highest values are obtained with a complex-rich growth medium, probably on account of the high complexation of the metal ions by growth medium components. Metal speciation is crucial to an understanding quantitative aspects of metal toxicity. Diffusion, complexation and availability of metals differ in solid culture media in comparison to liquid media. In our study, we have worked in liquid medium, since in these conditions, the free toxic metal ions evoke lower inhibitory concentrations than in solid medium where these ions are less available (Hassen et al., 1998a,b; Yilmaz, 2003). Moreover, liquid media better permit an evaluation of metal toxicity in polluted environments (Hassen et al., 1998a). As the MIC values depend on several parameters, it is difficult to compare data from different authors and experimental conditions.

### 4.2. Effects of heavy metals on *Pseudomonas* BA3d12 growth kinetics

Although many studies address the effects of metals on growth kinetics, it is difficult to make an accurate and meaningful comparison of our results with the literature, due to the very diverse growth media and incubation conditions. However, it is generally accepted that addition of metals in the culture medium implies modifications in *Pseudomonas* growth kinetics. The most frequently mentioned effects are: an increase in the lag phase (Cabrero et al., 1998; Teitzel and Parsek, 2003; Kassab and Roane, 2006; Chen et al., 2006), a decrease in the  $\mu_{max}$  (Appanna and Whitmore, 1995; Appanna and St Pierre, 1996; Cabrero et al., 1998; Yilmaz, 2003; Kassab and Roane, 2006; Tsai et al., 2006), mortality at the beginning of metal exposure (Kassab and Roane, 2006), decrease in biomass production (Appanna and Whitmore, 1995; Cabrero et al., 1998; Benka-Coker and Ekundayo, 1998; Tsai et al., 2006), stimulation of biomass production (Chen et al., 2006), or an early decline phase (Chen et al., 2006). In our study, a major increase in lag phase was observed for only high metal concentrations: 20 mg L<sup>-1</sup> Cu (315 μM) and 25 mg L<sup>-1</sup> Zn (380 μM). During this longer lag phase, cells could acquire a



metal resistant phenotype involving protein overproduction (Cooksey, 1993; Appanna et al., 1995; Hantke, 2001; Valls et al., 2000; Sharma et al., 2006; Kassab and Roane, 2006), or an increased exocellular metabolite production in order to complex metal ions and reduce bioavailability (Chan and Dean, 1988; Appanna and Whitmore, 1995; Appanna et al., 1995; Kassab and Roane, 2006). For BA3d12, no Cd concentration tested induced a longer lag phase. This observation suggests that this strain rapidly adapted to high Cd contamination (20 mg L<sup>-1</sup> or 180 µM). In the presence of this metal, the moderate increase in lag phase, the decrease in  $\mu_{\max}$  and the early decline phase agreed well with the results obtained by Higham et al. (1986). These authors have shown that the resistance mechanisms of *P. putida* to Cd, involved a complexation of this metal in polyphosphate granules, changes in the structure of cell membrane and the induction of three cysteine-rich low molecular weight proteins. For similar concentrations (20 mg L<sup>-1</sup> = 315 µM for Cu and 305 µM for Zn), Zn had the least effect on growth kinetics, whereas Cu showed the strongest effect, which was in good agreement with several studies (Hassen et al., 1998a; Cabrero et al., 1998; Yilmaz, 2003; Chen et al., 2006). Indeed, Zn is an essential trace element and *Pseudomonas* is known to have many Zn homeostasis regulatory systems (Appanna et al., 1995; Hassan et al., 1999; Hantke, 2001). Cu is also essential but can be toxic at low concentrations (Wayne Outten et al., 2001). In this case, non-inducible systems (efflux, biosorption...) cannot be sufficient to control Cu homeostasis, resulting in a perturbed bacterial growth. As a result, it may become necessary for tolerant microorganisms to use inducible metal resistance mechanisms, including ATP-dependent efflux pumps and intracellular sequestration (Cooksey, 1993; Saxena et al., 2002; Chen et al., 2006; Zhang and Rainey, 2007).

#### 4.3. Effects of heavy metals on APA

A clear inhibition of APA occurred at low Cd concentrations (5 mg L<sup>-1</sup>), whereas a moderate decrease in APA was observed only at a high Cu concentration (25 mg L<sup>-1</sup>). Many authors have reported an inhibition of phosphatase activities in the presence of heavy metals (Tyler, 1974; Roy et al., 2004). It is accepted that Cd and Cu are more toxic than other metals (Hiroki, 1992; Blum and Schwedt, 1998). Our APA results are in agreement with those of Chan and Dean (1988), who found that Cd inhibited APA of *Pseudomonas marina* at all tested concentrations, with an inhibitory effect proportional to the concentration tested. Similarly, Acosta-Martinez and Tabatabai (2001), who examined the effect of 25 trace elements on enzyme activities in soils, showed that Cd was one of the most effective inhibitors (>55%), whereas Cu was a little less inhibitory (between 35 and 53%). The activity of extracellular phosphatases will be affected by trace metals that induce changes in the three-dimensional enzyme conformation (Juma and Tabatabai, 1977; Geiger et al., 1998). These changes could be partly explained by metal sulfide formation at the cysteine residus (Roy et al., 2004). Other hypotheses to explain APA inhibition by Cd and Cu, include metal interaction with the enzyme-substrate complex, or a metal complexation with the substrate alone (Eivazi and Tabatabai, 1990; Nannipieri, 1995). Alkaline phosphatase is a metalloenzyme composed

of two monomers, requiring Zn and/or Mg to give the dimer responsible for enzymatic activity (Coleman, 1998). As a result, a possible mechanism to explain heavy metal toxicity on phosphatase activities could be the substitution of Zn by another element in the enzyme structure (Renella et al., 2003). Taking into account the chemical properties of Zn, Cu and Cd, Cd – better than Cu – could substitute Zn within the enzyme. Other studies have mentioned inhibition of APA by Zn beyond a certain threshold concentration (Gary and Santiago, 1977; Mazorra et al., 2002). Indeed, the excess of Zn would replace Mg at the Mg-binding sites in the AP, thus inducing inhibition of APA (Yan and Chen, 1986; Lan et al., 1995).

Metals can inhibit the APA, but they do stimulate it, too. Indeed, in the presence of metal, high enzyme affinities for the substrate (expressed as low  $K_m$  values) were recorded in our study, which suggests some efficient interactions between the enzyme and its substrate, due to the metal. We also observed a positive influence of Zn on APA, through the  $V_{\max}$  values. Similar observations have been reported by Wong et al. (1995), who found a positive correlation between APA and total Zn content in the marine sediments of a polluted Hong-Kong harbour. Lan et al. (1995) have also demonstrated that APA was significantly increased by 0.1 mg L<sup>-1</sup> Zn in the red sea bream *Chrysophrys major*. As described by Blum and Schwedt (1998), other analogous enzyme activities can be activated by this metal, such as that of phosphodiesterase I. Similarly, several studies have shown that the loss of enzymatic activity could be restored by addition of Zn (Olsen et al., 1991; Blum and Schwedt, 1998). The best explanation for the stimulation by this element is probably that the phosphatase (a Zn-enzyme) works optimally when it has enough Zn (Blasco et al., 1993). Finally, the optimal APA seems to be ensured by an optimal – but not excessive – Zn concentration.

#### 4.4. Effects of heavy metals on O<sub>2</sub> consumption

In the present study, O<sub>2</sub> consumption by *P. fluorescens* was clearly decreased in the presence of Cd. In most cases, inhibition of respiration by toxic metals is expected, with a gradual decrease in O<sub>2</sub> consumption in response to an increase in metal concentration. In their study on the effects of Cd on respiration, Chan and Dean (1988) also found that O<sub>2</sub> uptake by the marine bacterium *P. marina* was inhibited at all Cd concentrations. According to Smith and Giller (1992), Cd is expected to cause the most adverse effects on the biomass activity, which agrees well with our results. Inhibition of respiration by Cd is attributed to the Cd transport into the bacterial cells via the energy-dependent Mn<sup>2+</sup> transport system, and additionally, to the binding of Cd ions to the –SH groups of the respiratory enzymes (Perry and Silver, 1982).

Our results also indicated an inhibition of oxygen consumption by Cu at high concentrations (20 mg L<sup>-1</sup> or 315 µM). The toxic influence of this metal on bacterial metabolism is often reported, as shown by Tsai and Tuovinen (1985) who obtained an almost complete repression of O<sub>2</sub> uptake by *Nitrobacter* spp. in the presence of 17 mM Cu. In our study, Cu inhibition seemed less severe than that observed for Cd, whereas Codina et al. (1994) showed a similar effect of Cu or Cd on *P. fluorescens*. Riis et al. (2002) reported a higher toxicity of Cu compared to Cd on a soil bacterium population, and Renella

et al. (2002) reported a variable relative toxicity of the two elements on a soil microbial biomass taking into account the metal incubation time. Taken together, these results underline once more that comparisons between data are difficult as parameters are rarely comparable from one study to another.

In the present study, the results on O<sub>2</sub> uptake obtained with bacteria contaminated with Zn are surprisingly quite different from those described in the presence of Cd and Cu. Whatever the concentration added in the medium, Zn always activated O<sub>2</sub> uptake, by up to 44%. Although Wong et al. (1997) and Rajapaksha et al. (2004) found an inhibition of respiration in the presence of Zn, our results are in agreement with other studies. Indeed, Brookes and McGrath (1984) report a specific respiration rate in contaminated soils two-fold higher than in uncontaminated soils. Similarly, Khan and Scullion (2000, 2002) have shown that the Zn addition to sewage sludges increased microbial respiration rates. Respiration responses to metal inputs are determined by a balance of reductions in microbial biomass and stress-induced increases in metabolic quotients (Fließbach et al., 1994). In our experiments, we have always measured O<sub>2</sub> consumption in samples with the same bacterial densities. As a result, differences in biomass cannot explain the activation of respiration by Zn, nor its inhibition by Cu and Cd. For Zn, our results suggest rather that the metabolic responses to stress are important, making Zn less toxic than Cu and Cd. This is in agreement with the study of Khan and Scullion (2000). By virtue of its strong Lewis acid properties, zinc can polarize, coordinate and activate substrates, which enables this heavy metal to participate in various regulatory processes (Appanna and Whitmore, 1995). It is a component or activator of some enzymes; it is a stabilizer of macromolecules and biological membranes (Chvapil, 1973) and it maintains the integrity of the cell wall of gram-negative bacteria (Eagon et al., 1965). Zinc could also stimulate the activity of constitutive enzymes, as for example, the oxygen-dependent glucose dehydrogenase (EC 1.1.99.17) whose activity is enhanced by 5 mM Zn in *Erwinia* sp. (Marcinkeviciene et al., 1999). As this enzyme is also present in *P. fluorescens* (Matsushita and Ameyama, 1982), it is possible that such a stimulation exists also for this species. Another enzyme capable of being activated by an excess of Zn may be the Zn enzyme carbonic anhydrase (EC 4.2.1.1). This enzyme is known to catalyse transformation of CO<sub>2</sub> produced by bacterial metabolism to HCO<sub>3</sub><sup>-</sup> anions (Pelmont, 1988). The *P. fluorescens* O<sub>2</sub> uptake activation by Zn could result in a

carbonic anhydrase stimulation or increased synthesis. Various studies have shown that elevated Zn concentrations induce siderophore biosynthesis in *Pseudomonas* (*P. fluorescens*: Rossbach et al., 2000; *P. aeruginosa*: Hoefte et al., 1994). Pyoverdine, the main siderophore synthesized by *P. fluorescens*, is an iron-scavenging molecule facilitating Fe<sup>3+</sup> uptake into bacterial cells (Linget et al., 1992). As a result, this pyoverdine overproduction could induce higher iron concentrations in the intracellular compartment, resulting in a possible stimulation of enzymes involved in the bacterial respiratory chain, such as cytochrome oxidases.

#### 4.5. Effects of heavy metals on *Pseudomonas* BA3d12 protein map

After silver staining of our gels, we have visualized 65 differential protein spots for Zn, 68 for Cu, and 103 for Cd. Sharma et al. (2006) is the sole previous report of the impact of metals on *P. fluorescens* using proteomics; and among the metals tested by these authors, Cu is the only element in common with our study. For this metal, no more than 18 differentially expressed protein spots appeared, but the authors have used the Coomassie Brilliant Blue staining which is known to be less sensitive than the silver staining (Switzer et al., 1979). Our results, and those of Sharma et al. (2006), indicate that *P. fluorescens* responds to the stress due to metal contamination, by metabolic changes. Our results demonstrate that the main *Pseudomonas* response to metal contamination is the synthesis of new proteins and/or the increased synthesis of constitutive proteins (Table 3). These proteins could allow the bacteria to cope with metal stress through diverse strategies, such as biotransformation, active efflux, intra- or extracellular sequestration and metal transport (Appanna and Whitmore, 1995; Appanna et al., 1996; Saxena et al., 2002; Chen et al., 2006; Zhang and Rainey, 2007). Our results are in agreement with those of Hassen et al. (1998b) who observed that Zn stimulated protein synthesis by *P. aeruginosa*, this bacterium producing an amount of proteins two-fold higher than the control. In our case, when *P. fluorescens* was exposed to 20 mg L<sup>-1</sup> Zn, we observed several more intense spots in the map area at approximately 30 kDa and pI 6 (spots 16, 39 and 40 Fig. 4). Among them, carbonic anhydrase (MW 29 kDa and pI 6) is a possible candidate for over-expression. As explained previously, the O<sub>2</sub> uptake activation observed after *P. fluorescens* exposure to Zn, could

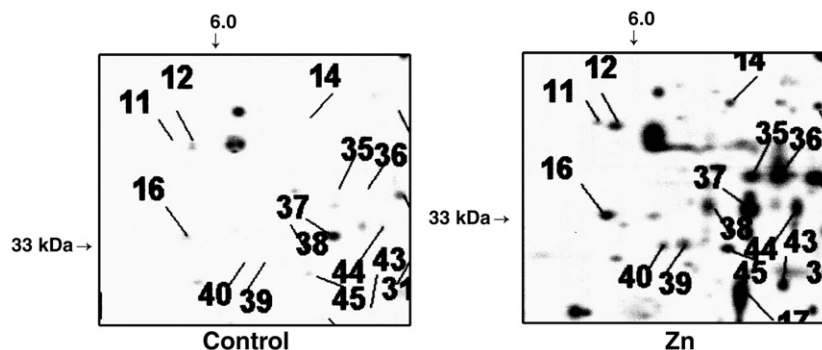


Fig. 4 – Zooms of protein maps of *Pseudomonas fluorescens* BA3d12 for control and 20 mg L<sup>-1</sup> Zn, at approximately 30 kDa and pI 6.

be followed by the carbonic anhydrase over-synthesis in order to eliminate the excessive CO<sub>2</sub> production resulting from the respiratory activity. To confirm this hypothesis, the protein spots 16, 39 and 40 should be identified by mass spectrometry. In contrast, although Zn has activated APA, no spot at approximately 40 kDa and pI 4.5, which characterises the alkaline phosphatase (Plisova et al., 2005; Yamada and Tanoue, 2006), was observed. This suggests that this membrane enzyme may have been eliminated during preparation of the hydrosoluble protein extract, no protein spot corresponding to alkaline phosphatase being detected on the control 2D-gel. A stimulation of protein synthesis by heavy metals has already been observed by some authors. For instance, in response to oxidative stress induced in the presence of Cu, Sharma et al. (2006) reported an increased level of the enzyme enolase. Cd is known to lead to newly synthesized proteins, called pseudothioneins and described previously (Higham et al., 1986). Finally, the results concerning the differential protein spots were in accordance with growth kinetics in the presence of metals. Indeed, we have observed only few modifications in growth kinetics with 20 mg L<sup>-1</sup> Zn, which is also the metal that induced the lowest number of differential protein spots. In contrast, with 20 mg L<sup>-1</sup> Cd or Cu, the strong modifications during bacterial growth could be linked with the important changes in the protein maps. This observation suggests that the protein over-synthesis probably occurred at the cost of the bacterial growth.

## 5. Conclusions

This study showed that *P. fluorescens* BA3d12, isolated from the strand zone of the St Anne Bay, is tolerant to high levels of metals, confirming that its site of origin is relatively polluted. Although living in a hostile environment, when this strain is cultivated in the laboratory, it is able to develop adaptation strategies to protect against heavy metal toxicity. The resistance that we observed is probably associated with newly synthesized stress-linked proteins or with elevated synthesis of constitutive proteins. In further studies, we will aim to identify by mass spectrometry analysis some of the differentially expressed proteins induced when the strain *P. fluorescens* BA3d12 is exposed to heavy metals.

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