Aquatic Toxicology 128–129 (2013) 215–232

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Strategies developed by the marine bacterium *Pseudomonas fluorescens* BA3SM1 to resist metals: A proteome analysis

Isabelle Poirier[a,*], Philippe Hammann[b], Lauriane Kuhn[b], Martine Bertrand[a]

[a] *Microorganismes Métaux et Toxicité, Institut National des Sciences et Techniques de la Mer, Conservatoire National des Arts et Métiers, BP324, 50103 Cherbourg-Octeville Cedex, France*

[b] *Plateforme Protéomique Strasbourg Esplanade, CNRS FRC1589, Institut de Biologie Moléculaire et Cellulaire, 15 rue Descartes, 67084 Strasbourg Cedex, France*

ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form 5 December 2012
Accepted 9 December 2012

Keywords:
Pseudomonas fluorescens
Marine bacteria
Cadmium
Zinc
Copper
Resistance mechanisms
Proteomics

ABSTRACT

A global proteomic evaluation of the response of the marine bacterium *Pseudomonas fluorescens* BA3SM1 to Cd, Zn and Cu was performed by two dimensional gel electrophoresis followed by mass spectrometry. When stressed with Cd, the most toxic metal for *P. fluorescens* BA3SM1, cell growth is rapidly affected and the number of proteins up-regulated (sixteen for 0.4 mM Cd) remains low in comparison with results obtained for Zn and Cu (twenty eight for 1.5 mM Zn and forty four for 1.5 mM Cu). The changes in protein expression indicate that the cell adapts to metals by inducing essentially seven defense mechanisms: cell aggregation/biofilm formation (Zn = Cu > Cd); modification of envelope properties to increase the extracellular metal biosorption and/or control the uptake of metal (Cu > Zn); metal export (Cd = Zn and probably Cu); responses to oxidative stress (Cu > Zn > Cd); intracellular metal sequestration (Zn = Cu and probably Cd); hydrolysis of abnormally folded proteins (Cd = Cu), and the over-synthesis of proteins inhibited by metal (Cd > Cu > Zn). To the best of our knowledge, this is the first report showing that a marine *P. fluorescens* is able to acquire a metal-resistant phenotype, making the strain BA3SM1 a promising agent for bioremediation processes.

1. Introduction

In the marine environment, the tidal flat area is naturally submitted to wide variations of many parameters such as salinity, temperature, water turbulence and light. In recent decades, many tidal flats have been impacted by metal pollution resulting from the increase of human activities (Zhou et al., 2007). While various physico-chemical processes have been developed for treating these pollutants, these approaches are often prohibitively expensive, non-specific, disruptive to the sites affected, and not suitable to vast ecosystems such as tidal flats (Srivastava and Majumder, 2008).

In this specific environment, bioremediation with microorganisms seems to be an attractive alternative to conventional techniques. The application of microorganisms for the remediation of metals is a recent field of research in environmental engineering and several studies have demonstrated the efficiency of metal removal by bacteria (Ansari et al., 2011; Cristani et al., 2012; Yu et al., 2011). Among the bacteria capable of major adaptation strategies for surviving in polluted habitats is the genus *Pseudomonas*. The pseudomonads are frequently found in contaminated areas and are known for their stress resistance (Hu and Zhao, 2007; Kiliç et al., 2010; Sharma et al., 2006; Wu et al., 2011). To tolerate metals, *Pseudomonas* species exhibit numerous survival mechanisms such as export of metal ions outside the cell (Hu and Zhao, 2007; Miller et al., 2009; Wu et al., 2011), energy-dependent efflux of metal ions (Hu and Zhao, 2007; Miller et al., 2009; Wu et al., 2011), enzymatic detoxification/reduction (Andreazza et al., 2010; Chang et al., 2008; Wu et al., 2011), extracellular biosorption (Andreazza et al., 2010; Kiliç et al., 2010), cell aggregation/biofilm formation (Chen et al., 2011; Kotrba et al., 2011), resistance to oxidative stress (Sharma et al., 2006; Thompson et al., 2010), intra- and extracellular sequestration (Miller et al., 2009), and preservation of Fe acquisition and homeostasis (Hu and Zhao, 2007; Thompson et al., 2010).

Many strains of the species *Pseudomonas fluorescens* (*P. fluorescens*) possess excellent capacity for colonizing plant roots, promoting plant growth in soils contaminated with toxic metals (Shilev et al., 2007). Many studies indicate the presence of this species in the marine environment (Castro-González et al., 2005; Poirier et al., 2008, 2009), but little is known of the abilities of strains living in the tidal flat sediments to combat toxic effects of metals. We have previously isolated several *P. fluorescens* strains from tidal flat sediments collected in St. Anne Bay, a moderately metal-contaminated site to the west of Cherbourg seaport (France). Of particular interest among these strains was the BA3SM1 strain, which is highly resistant to metals and to cell disruption (Poirier et al., 2009). Here, we have examined differences in the proteome

* Corresponding author. Tel.: +33 2 33 88 73 34; fax: +33 2 33 88 73 39.
E-mail address: isabelle.poirier@cnam.fr (I. Poirier).

0166-445X/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquatox.2012.12.006

of *P. fluorescens* BA3SM1 subjected to Cd, Zn and Cu. This work is essential to an understanding of the physiological and biochemical basis of bacterial resistance to metals, which for *P. fluorescens* BA3SM1 is an important component of its potential as a bioremediation agent.

2. Materials and methods

2.1. Bacterial strain isolation and growth conditions

P. fluorescens BA3SM1 is isolated and stored as described in a previous study (Poirier et al., 2009). The selective culture medium used to isolate this strain is *Pseudomonas* CFC agar, containing cetrimide, fucidin and cephalotin as selective agents (Merck KgaA, Germany). For all experiments, *P. fluorescens* BA3SM1 was cultivated in nutrient broth (Biokar Diagnostics, Beauvais, France) at 22 °C under shaking. Cultures (100 mL) were inoculated with a bacterial suspension from a 24-h-old preculture to obtain an initial cellular concentration of approximately 10^5 cells mL^{-1}.

To determine the effect of metals on bacterial growth and proteome, the nutrient broth was supplemented with different Cd, Zn or Cu concentrations (0–3 mM, 0–10 mM and 0–9 mM for Cd, Zn and Cu, respectively) using 1 g metal ions L^{-1} standard solutions (Cd, Zn or Cu atomic absorption spectrometry (AAS) standard in 0.1 M HCl, VWR International SAS, France). In the AAS standards, Cl^- is the anionic component. To have the same nutrient concentration in metal supplemented and control cultures, the nutrient broth powder was directly rehydrated with the appropriate volume of the metal standard solution. The pH was then adjusted to 7.3 (nutrient broth pH) with NaOH, and distilled water was added to reach a final culture volume of 100 mL. For 2D-PAGE analysis, cultures were harvested during the exponential growth phase, when the bacterial concentration was close to 3×10^8 cells mL^{-1}, which corresponds to an absorbance of 0.5 at 600 nm (Spectronic 20 Genesis, Rochester, NY, USA).

2.2. Identification and characterization of bacteria

The identification of the BA3SM1 strain was carried out in a previous study (Poirier et al., 2009).

2.3. Growth kinetics

For each metal, growth kinetics of metal supplemented and control cultures were followed simultaneously by absorbance measurements at 600 nm using an Apollo-1 microplate reader (Berthold Technologies GmbG & Co. KG, Bad Wildbad, Germany). Data acquisition was performed with the MikroWin 2000 4.0 version software (Mikrotek Laborsysteme GmbH, Overath, Germany). Absorbance values were converted into cell concentrations (cells mL^{-1}) using a calibration curve relating absorbance at 600 nm to cell concentration determined by cell counting with a Thoma chamber. This calibration curves for *P. fluorescens* BA3SM1 cultivated in nutrient broth and controls were constructed and it was made sure that the same curves could be also used for the metal supplemented cultures. The natural logarithm (ln) of the cell concentration was used for the graphic representation of growth kinetics. This allows a more precise determination of the growth rate. All experiments were performed in triplicate.

2.4. 2D-PAGE analysis of soluble proteins

2.4.1. Preparation of protein extracts

The metal concentrations chosen for the proteomic analysis were 0.4 mM, 1.5 mM and 1.5 mM for Cd, Zn and Cu, respectively. For each metal, this concentration corresponds to approximately 15% of the minimal inhibitory concentration determined in a previous study (Poirier et al., 2009), thus the resistance mechanisms developed by *P. fluorescens* BA3SM1 can be compared between the three metals. These concentrations are high enough to induce metabolic changes in *Pseudomonas* species (Miller et al., 2009; Poirier et al., 2008; Sharma et al., 2006). Furthermore, at these concentrations, the BA3SM1 growth remained satisfactory; consequently, the cells were able to develop effective resistance mechanisms. For protein extraction, metal supplemented and control cultures were treated as described by Poirier et al. (2008). In short, after cell lysis with a French Press, the resulting homogenates were ultracentrifuged for 1 h at 218 000 × *g* at 4 °C (Optima L-100 XP Beckman Coulter, SW 40 Ti swing rotor) to pellet down cell debris. The supernatants were purified, concentrated and stored as described by Poirier et al. (2008). The protein concentration of the extracts was determined according to Bradford (1976).

2.4.2. 2D-electrophoresis

For isoelectric focusing (IEF), 500 μg of proteins was solubilized in a rehydration buffer containing 8 M urea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 20 mM dithiothreitol (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 4–7, Bio-Rad) were submitted to IEF (Protean IEF Cell, Bio-Rad) with the following voltage/time process: 150 V ramping for 6.5 h, 750 V ramping for 6.5 h, 1500 V ramping for 6.5 h, 5000 V ramping for 6.5 h and 10 000 V ramping for 6 h, resulting in a total voltage of 76 800 Vh. After IEF separation, strip treatment and second dimension run were carried out as described by Poirier et al. (2008). Proteins were visualized by gel staining with Coomassie Blue according to Candiano et al. (2004). Gels were scanned using the GS-800 Imaging densitometer (Bio-Rad) and analyzed using the PDQuest software 7.3.0 version (Bio-Rad), which automatically detects and quantifies protein spots, after resizing and alignment of images. For each experiment (control and metal supplemented cultures), four replicates were analyzed. The cropped images were normalized by a filtration procedure (filtered image) and then the 3D Gaussian spots were created (Gaussian image). After spot detection, the software created an average gel for each experimental condition (MatchSet Master). The average gels obtained for the three metals were compared to the average gel obtained for the control. After the automatic spot detection, each gel scan was controlled visually in order to eliminate artefacts from the spot list. Only spots that were detectable on all gels of a sample set were considered for evaluation, while other spots were excluded. The statistical significance of the spot intensity difference between the average gels was conducted with Bio-Rad PDQuest software. *p* values were determined with Student *t*-test analysis. For quantitative analysis, all spots were quantified on Gaussian images. The following formula was used to calculate the quantity of Gaussian spots: spot height × σ_x × σ_y. Spot height is the peak of the Gaussian representation of the spot in optical densitometry (OD) units, σ_x is the standard deviation of the Gaussian distribution of the spot in the direction of the *x* axis, and σ_y is the standard deviation in the direction of the *y* axis in image units (IUs). This formula results in units of OD × IU^2. The spot quantity was normalized for each gel individually and expressed as a percentage of the total spot quantity on the gel (relative spot quantity). For comparison, spot quantity means and their standard deviations were calculated. Spots, whose values did not overlap when the standard deviations were taken into account, were considered to be differentially expressed. The fold changes were calculated. A 1.5-fold difference in abundance with a Student *t*-test *p*-value of less than 0.05 was considered significant.

The protein spots of interest were excised and stored at −20 °C prior to mass spectrometry analysis.