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Short Communication

The Robustness to Cell Disruption of the Heavy Metal-Resistant *Pseudomonas fluorescens* BA3SM1 Isolated from Tidal Flat Sediments

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Abstract

A bacterial strain (BA3SM1) was isolated from the tidal flat sediments collected in west of Cherbourg seaport (France). The biochemical characteristics and the partial 16S rDNA gene sequencing of isolate, BA3SM1, have allowed to identify it as *Pseudomonas fluorescens*. When cultivated, this strain exhibits a very high tolerance to metal contamination [minimal inhibitory concentration = 10 mM (650 mg/L) for zinc (Zn), 9 mM (560 mg/L) for copper (Cu), and 3 mM (350 mg/L) for cadmium (Cd)]. To attempt to explain this high tolerance, future studies will be undertaken to analyze stress proteins produced by BA3SM1 in the presence of heavy metal excess. For this aim, several protein extraction procedures were tested, that is, biochemical, sonication, and high-pressure treatments. Our results show that BA3SM1 is very resistant to cell disruption, because an efficient protein release was only obtained with a drastic physical protocol: a treatment by a hypoosmotic shock followed by three passages through a French press at 159 MPa. This preliminary work is essential because it recommends a convenient protein extraction procedure to perform quality 2D-electrophoresis, from a tidal flat strain particularly resistant to heavy metals and to cell disruption. In further studies, proteomic analysis will allow to understand the resistance mechanisms developed by this strain to counteract heavy metal stresses. A better knowledge of these mechanisms will give us more information about the abilities of BA3SM1 to be used for bioremediation applications.

Key words: tidal flat bacterium; heavy metal resistance; cell disruption; protein extraction; high pressures; hypoosmotic shock; 2D-electrophoresis

Introduction

IN THE MARINE ENVIRONMENT, the tidal flat area is naturally submitted to wide variations of many parameters such as salinity, temperature, water turbulence, or light. Moreover, for the last decades, it has been severely affected by heavy metals resulting from the increase of human activities (Zhou *et al.*, 2007a, 2007b). Although various physicochemical processes have been developed for treating these pollutants, these approaches are often prohibitively expensive, nonspecific, disruptive to the sites, and nonadapted to vast ecosystems

such as the tidal flat area (Srivastava and Majumder, 2008). In this specific environment, bioremediation with microorganisms seems to be an attractive alternative to conventional techniques. The application of micro-organisms for the remediation of heavy metals is a recent field of research in environmental engineering, and several studies have demonstrated the efficiency of metal removal by bacteria (Brar *et al.*, 2006; Chen *et al.*, 2007; Park *et al.*, 2008; Srivastava and Majumder, 2008; Wasi *et al.*, 2008). The microbioremediation focuses on the transformation of the metal, and its subsequent biosorption and biomineralization, which is the formation of insoluble metal precipitates due to interactions with microbial metabolic products (Brar *et al.*, 2006; Park *et al.*, 2008; Srivastava and Majumder, 2008; Wasi *et al.*, 2008; Kazy *et al.*, 2009). Among the bacterial species able to develop important adaptation strategies to survive in polluted habitats, there is *Pseudomonas fluorescens*. This species is present in various

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environments, and is known for its stress resistance (Stockwell and Loper, 2005). It is frequently found in contaminated area, and its survival strategies were described especially against pollutants like hydrocarbons, pesticides, or heavy metals (Sharma *et al.*, 2006; Vasudevan *et al.*, 2007; Wasi *et al.*, 2008); nevertheless, no special focussing has been investigated on strains living in the tidal flat area. In this drastic environment, *P. fluorescens* strains have to adapt to numerous stresses to survive (Azam and Malfatti, 2007), and probably have to develop high-performance resistance mechanisms, making them interesting candidates for bioremediation studies.

Consequently, the objectives of this preliminary work were (1) to isolate a *P. fluorescens* strain from tidal flat sediments collected in a moderately metal-contaminated site to the west of Cherbourg seaport, (2) to study the ability of this strain to survive to heavy metal stresses, (3) and to compare the efficiency of different protein extraction protocols on this strain, to achieve quality 2D-electrophoresis. In further studies, proteomic analysis will be used to understand the mechanisms involved in the heavy metal resistance of this strain. A better understanding of these mechanisms will allow to determine if BA3SM1 is a promising agent for the bioremediation of polluted marine sediments.

Materials and Methods

Bacterial strain isolation and growth conditions

BA3SM1 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 the Cherbourg seaport: 49°39'N–001°40'W, France). Long-term storage was performed 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, BA3SM1 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) (pH = 7.4). Cultures were inoculated with 1 mL of bacterial suspension from a 24-h-old preculture. All samples were harvested at the end of the exponential growth phase (15 h), so that the bacterial concentration was always 2.10^8 cells/mL, which corresponds to 0.330 absorbance units at $\lambda = 600$ nm.

Identification and characterization of bacteria

Identification of the BA3SM1 strain was carried out by performing Gram staining, mobility, shape, oxidase, catalase, and other complementary biochemical tests using API 20NE system (bioMérieux, France). To confirm the identification of the bacterium, a partial 16S rDNA gene sequencing was performed (Genoscreen, France).

Minimal inhibitory concentration (MIC) determination

MICs of the strain BA3SM1 were determined in the media described above, supplemented with increasing concentrations of a given metal ($ZnCl_2$, $CuCl_2$, $CdCl_2$). The cultures were incubated for up to 30 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.

Preparation of protein extracts with different extraction procedures

Biochemical treatments. A cell culture (100 mL) was centrifugated at $10,000\times g$ for 10 min. The pellet was resuspended in a lysis buffer (0.1 M Tris-HCl, pH 7.5) containing either 0.1, 0.5, or 1.0% lysozyme; or 0.5% lysozyme and 1.5% Triton X-100; or 0.5% lysozyme and 1.5% sodium dodecyl sulphate (SDS). The mixture was incubated for 30 min or 1 h at +4°C to inhibit the protease activity, with occasional stirring. At the end of the incubation, cells were harvested by centrifugation for 10 min at $10,000\times g$ to remove enzymatic and chemical agents. The pellet was then resuspended in 10 mL of ultrapure water to achieve a hypoosmotic shock to induce the intracellular protein release.

Sonication. A cell culture (100 mL) was centrifugated at $10,000\times g$ for 10 min. The pellet was washed twice and resuspended in 10 mL of ultrapure water (hypoosmotic shock). Cells were disrupted by sonication (sonicator ultrasonic processor XL, Misonix, Farmingdale, NY) using a microtip (maximal acoustic power: 168 W) in an ice-water bath. Sonication was undertaken at a frequency of 20 kHz. Several acoustic powers (30, 50, 100, and 125 W) and sonication times (8, 15, and 30 min) were tested with 2-s on/2-s off cycles. An experiment was also carried out with 10 mL of Tris EDTA/phenylmethanesulfonyl fluoride (TE/PMSF) buffer (10 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM PMSF, pH 7.5) instead of ultrapure water (Sauer and Camper, 2001; Sonawane *et al.*, 2003).

Freezing–thawing cycles/sonication. A cell culture (100 mL) was centrifugated at $10,000\times g$ for 10 min. The pellet was washed twice and resuspended in 10 mL of TE/PMSF buffer. The sample was submitted to one or five freeze–thaw cycles. Then, cells were disrupted by sonication (sonicator ultrasonic processor XL, Misonix) using a microtip in an ice-water bath at 20 kHz, 30 W during 10 min, with 2-s on/2-s off cycles.

High pressures. The culture (100 mL) was centrifugated at $10,000\times g$ for 10 min. The pellet was washed twice and resuspended in 10 mL of ultrapure water (hypoosmotic shock). Cells were disrupted through a precooled French press cell (French pressure cell 40 K, Thermo IEC, Needham Heights, MA). The influence of the number of passages (one to five passages) and the applied pressure (120 MPa or 159 MPa) were tested in relation to protein release.

An experiment was also carried out with 10 mL of TE/PMSF instead of ultrapure water.

Protein concentration measurement. After each treatment, samples were centrifugated for 10 min at $10,000\times g$ at +4°C to pellet cell debris. Protein concentration in the supernatant was determined according to Bradford (1976). All the measurements were carried out in duplicate. The detection limit was 0.005 mg/mL (Bradford, 1976).

Purification and concentration of the protein extracts. All protein extracts were purified and concentrated five times with a centrifugal concentrator (10 kDa cutoff size, Amicon Ultra-4 Millipore, Bedford, MA); and stored at –80°C until analysis.

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SDS-PAGE analysis

Samples with a sufficient protein concentration (higher than 3 mg/mL) were separated by 12.5% SDS-PAGE (Laemmli, 1970). Electrophoresis was performed during 90 min at 100 V using the Mini Protean 3 apparatus (Bio-Rad, Hercules, CA). Proteins were then visualized by staining with Bio-Safe Coomassie Blue (Bio-Rad).

2D-electrophoresis tests

For isoelectric focusing (IEF), 250 or 500 µg proteins were solubilized in a rehydration buffer containing 8 M urea, 4% (w/v) 3-[3[cholamidopropyl]diamethylammomio]-1]propan-sulfonate (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 or pH 4–7, Bio-Rad) were submitted to IEF (Protean IEF Cell, Bio-Rad) with the following voltage/time process: 150 V for 1 h, 350 V for 15 min, 750 V for 45 min, 1,500 V for 1 h, and 3,500 V for 18 h (adapted from Vilain *et al.*, 2004). All steps were performed with rapid ramping. 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 Bio-Safe

Coomassie Blue (Bio-Rad). The gels were then analyzed using the PD-Quest software 7.3.0 version (Bio-Rad).

Results

Identification and characterization of bacteria

The API 20NE system has identified BA3SM1 to be *Pseudomonas fluorescens*. This identification was confirmed by the partial 16S rDNA gene sequencing.

MIC determination

The MIC values for the three heavy metals tested are 10 mM (650 mg/L) for zinc (Zn), 9 mM (560 mg/L) for copper (Cu), and 3 mM (350 mg/L) for cadmium (Cd). As a result, BA3SM1 is metal resistant.

Influence of the extraction procedure on the protein concentration of extracts and SDS-PAGE analysis

The concentrations of the protein extracts are indicated for each protocol in Table 1.

◀T1

Biochemical treatments. With the use of lysozyme only, during 30 min at +4°C, the protein concentration is lower than 1 mg/mL for the three enzyme concentrations tested (0.1, 0.5, and 1.0%). At a fixed concentration (0.5%) and for a longer action time (1 h), protein concentration increased at least threefold, to finally reach 3.15 mg/mL. When a detergent like Triton X-100 or SDS was used in combination with the lysozyme, protein extraction proved to be more efficient, a maximal protein concentration of 8.50 mg/mL being reached after

TABLE 1. PROTEIN CONCENTRATION OF FINAL EXTRACTS OBTAINED WITH THE DIFFERENT EXTRACTION METHODS

Protein extraction method		Protein concentration after ultrafiltration (mg/mL)
Biochemical treatment	0.1% lysozyme, 30 min, +4°C	0.35 ^a
	0.5% lysozyme, 30 min, +4°C	0.95 ^a
	1% lysozyme, 30 min, +4°C	0.55 ^a
	0.5% lysozyme, 1 h, +4°C	3.15 ^a
	0.5% lysozyme + 1.5% Triton X-100, 1 h, +4°C	4.30 ^a
	0.5% lysozyme + 1.5% SDS, 1 h, +4°C	8.50 ^a
Ultrasonication	30 W, 10 min in ultrapure water	0.02
	30 W, 10 min in TE/PMSF buffer	0.15
	50 W, 10 min in ultrapure water	0.01
	50 W, 15 min in ultrapure water	0.02
	50 W, 30 min in ultrapure water	0.01
	100 W, 10 min in ultrapure water	0.01
	125 W, 10 min in ultrapure water	0.01
Freezing–thawing cycles + ultrasonication	one freeze–thaw cycle + 30 W, 10 min in TE/PMSF buffer	0.20
	five freeze–thaw cycles + 30 W, 10 min in TE/PMSF buffer	1.00
High pressures	one passage at 120 MPa in ultrapure water	2.25
	two passages at 120 MPa in ultrapure water	2.55
	three passages at 120 MPa in ultrapure water	3.55
	four passages at 120 MPa in ultrapure water	2.25
	five passages at 120 MPa in ultrapure water	2.00
	three passages at 159 MPa in ultrapure water	8.00
	three passages at 120 MPa in TE/PMSF buffer	2.50

^aThese protein concentration values are overestimated due to lysozyme, as revealed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

TE/PMSF, Tris EDTA/phenylmethylsulfonyl fluoride.

F1 ▶

SDS treatment. The SDS-PAGE profiles of protein extracts with a concentration higher than 3 mg/mL are shown in Fig. 1 (lanes 1–3). The sample protein bands are hardly visible; only one band clearly appears on the gel, corresponding to the lysozyme (14.4 kDa). This result reveals that the residual presence of lysozyme and detergents in the extracts has disturbed the protein measurement by the Bradford method and has contributed to an important overestimation of the amount of extracted proteins. In these three extracts, the amount of bacterial proteins was so low that no clear bands appeared on the gel. Taking these results into account, *P. fluorescens* BA3SM1 seems to be particularly insensitive toward biochemical treatments.

Sonication. Sonication applied in ultrapure water, or in TE/PMSF buffer with or without freeze–thaw cycles, proved to be inefficient to achieve a substantial release of soluble proteins from the *P. fluorescens* BA3SM1. Consequently, this strain is an example of a special biological material for which cell disruption proved to be particularly difficult.

High pressures. At 120 MPa in ultrapure water, three passages have been required to reach a maximal protein concentration (3.55 mg/mL). For a higher pressure of 159 MPa, this value increased twofold to reach 8.00 mg/mL. When passages in the French press were carried out in TE/PMSF buffer instead of ultrapure water, protein extract concentrations were slightly lower (2.5 mg/mL instead of 3.55 mg/mL). So, for BA3SM1, an osmotic shock combined with three passages at 159 MPa were necessary to obtain a sufficient protein release that confirms its high resistance to disruption. The SDS-PAGE profiles of protein extracts with a

concentration higher than 3 mg/mL are shown in Fig. 1 (lanes 4–5). Numerous well-separated bands are visible. Both profiles are similar whatever the applied pressure (120 MPa for lane 4 and 159 MPa for lane 5). Taking this result into account, we have decided to extract proteins with the French press (three passages at 159 MPa in ultrapure water) to achieve the first 2D-electrophoresis tests.

2D-electrophoresis tests. The results of the protein separation by 2D-electrophoresis are shown in Fig. 2.

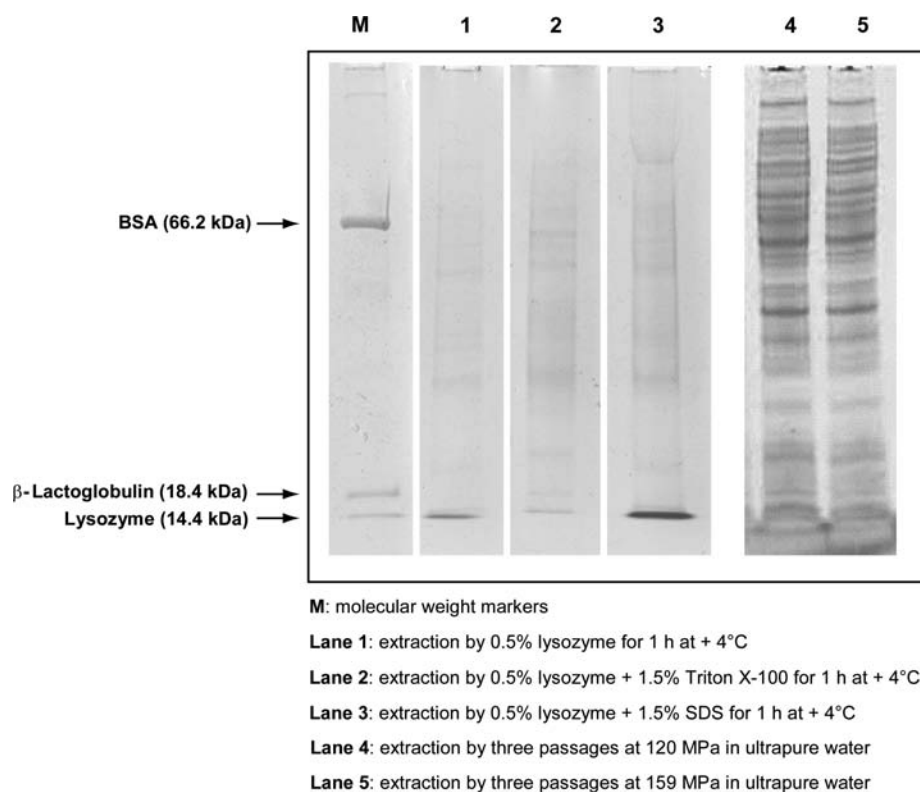
When the 2D-electrophoresis was started with 250 µg proteins and with pH 3–10 gradient strips (Fig. 2A), poorly defined spots were observed, mainly located at low pH_i values. The PD-Quest software detected only 340 ± 31 spots ($n=4$); moreover, many horizontal streaks are present. Consequently, this first result is unexploitable. When the 2D-electrophoresis was performed with 500 µg proteins and using strips with a pH 4–7 gradient (Fig. 2B), spots were well defined and distributed on the totality of the gel surface. In these new conditions, the PD-Quest software detected 1238 ± 31 spots ($n=4$).

◀ F2

Discussion

P. fluorescens BA3SM1 has shown a high level of resistance to all the metals tested, because 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 an MIC of 127 mg/L for *Pseudomonas aeruginosa*, and Chen *et al.* (2006) 190 mg/L for *Pseudomonas putida*, whereas Bender and

FIG. 1. SDS-PAGE profiles of *Pseudomonas fluorescens* proteins extracted by various protocols. Proteins (50 µg) were loaded in each well.



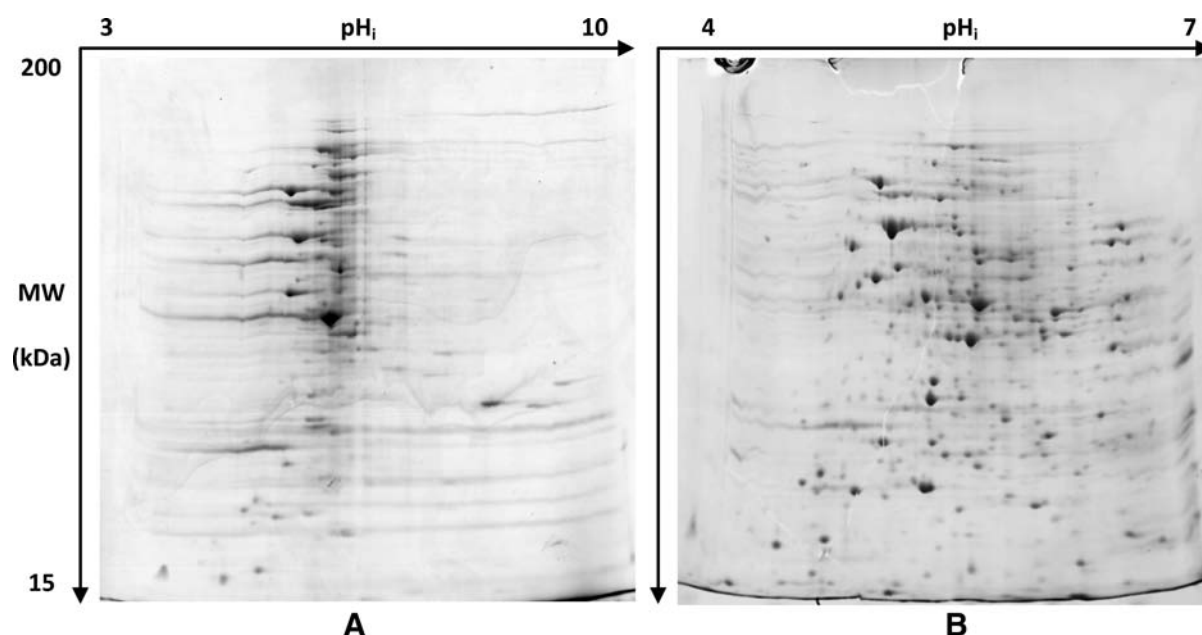


FIG. 2. 2D-electrophoresis gels of *P. fluorescens* hydrosoluble proteins. The protein extraction was performed by combination of a hypoosmotic shock and high pressures (three passages in the French Press at 159 MPa). (A) Proteins (250 μ g), strips with a pH 3–10 gradient, and Coomassie blue staining were used. (B) Proteins (500 μ g), strips with a pH 4–7 gradient, and Coomassie blue staining were used.

Cooksey (1986) obtained a value of 6.35 mg/L for *Pseudomonas syringae*, and Tom-Petersen *et al.* (2001) 3 mg/L for *Pseudomonas fluorescens*. For Zn, MICs range between 50 mg/L for *Pseudomonas* sp. (Malik and Jaiswal, 2000) and 3,139 mg/L for *Pseudomonas aeruginosa* (De Vincente *et al.*, 1990). For Cd, some *Pseudomonas* strains were incapable of growth at Cd concentrations above 1.1 mg/L (Zawadzka *et al.*, 2007), whereas other strains could tolerate concentrations as high as 1,000 mg/L (*Pseudomonas* sp.: Malik and Jaiswal, 2000; *Pseudomonas putida*: Hu and Zhao, 2007). By comparing our results with those quoted previously, it appears that our *Pseudomonas* strain presents a high metal resistance.

Literature often reports sonication and biochemical lysis as means to disrupt Gram-negative cells and to release intracellular hydrosoluble biomolecules (Willcox *et al.*, 2001; Hanouille *et al.*, 2004; Sharma *et al.*, 2006; Kobayashi *et al.*, 2007; Bar *et al.*, 2007), but these treatments were inefficient to obtain a substantial release of soluble proteins from BA3SM1. In our study, the lysozyme activity is surely low at +4°C compared to the one at its optimal temperature; nevertheless, after an action time of 1 h, the enzyme should have been efficient, as it was demonstrated by other authors (Hanouille *et al.*, 2004; Kobayashi *et al.*, 2007). Moreover, the presence of lysozyme in extracts, as revealed by SDS-PAGE, contributes to an important overestimation of the protein concentration, making impossible the achievement of an electrophoresis in good conditions. Concerning the sonication, all the tested treatments were totally inefficient for our strain. However, sonication conditions similar to ours were applied successfully in several *Pseudomonas* proteomic studies (*P. aeruginosa*: Vilain *et al.*, 2004; *P. putida*: Sauer and Camper, 2001). Consequently, BA3SM1 is an example of a special biological material for which cell disruption proved to be particularly difficult. The strong resistance of this strain could be due to the special robustness of its envelope resulting from adaptations to

survive in the tidal flat area. Indeed, this zone is submitted to important physicochemical changes and to an accumulation of pollutants, and then it is generally considered as a stressful environment for organisms (Zhou *et al.*, 2007a, 2007b). Consequently, the bacteria living in this specific environment must adapt to numerous stresses (Azam and Malfatti, 2007) by controlling, for example, the biochemical and biophysical properties of their envelope, and/or by producing exopolymers (Beney and Gervais, 2001; Nikolaev, 2004; Mancuso-Nichols *et al.*, 2005; Baysse and O’Gara, 2007; Noghabi *et al.*, 2007; Leuko *et al.*, 2008; Morbach and Krämer, 2008; Saravanan and Jayachandran, 2008; Zhang and Rock, 2008). Such mechanisms, which are commonly mentioned as adaptation strategies by bacteria, could explain the cell wall robustness and the high metal resistance of BA3SM1. Indeed, the tolerance to metals could be a consequence of the biosorption process, a passive phenomenon, which depends totally on physicochemical characteristics of cell wall (Wasi *et al.*, 2008) and consequently, which is affected by environmental factors (Noghabi *et al.*, 2007). In the tidal flat zone, bacteria are exposed to a wide range of stress-inducing fluctuating conditions and probably develop envelope features which facilitate the metal biosorption. Future experiments must be carried out to verify these hypotheses and to explain why BA3SM1 is so hard to disrupt by regular techniques.

Another resistance mechanism allowing survival of tidal flat bacteria in the presence of high concentrations of heavy metals could be the synthesis of specific proteins, as it was demonstrated in other environments (Sharma *et al.*, 2006; Bar *et al.*, 2007; Henne *et al.*, 2009). To verify this hypothesis by proteomic experiments, it is necessary to have concentrated bacterial protein extracts (at least 5 mg/mL). This condition is essential to achieve 2D-dimensional electrophoresis and further protein staining with Coomassie blue, which is a reliable dye for a subsequent mass spectrometry analysis

(Lauder *et al.*, 2001). The first 2D-electrophoresis tests, gave satisfactory results, when 500 μ g proteins and pH 4–7 gradient strips were used. These experimental conditions agree with the ones described by several authors for *Pseudomonas* proteomic studies (Cases *et al.*, 2001; Wehmhöner *et al.*, 2003; Sharma *et al.*, 2006). Qualitatively, spots are well defined, and distributed on the totality of the gel surface. Quantitatively, the PD-Quest software has detected 1238 ± 31 spots ($n = 4$), which is relatively close to the results mentioned in the literature (Sauer and Camper, 2001) for the *Pseudomonas* genus. As a result, this present work appears to be determinant because it allows us to get highly concentrated protein extracts allowing the achievement of high-quality 2D-electrophoresis. In future studies, protein maps will be used to identify specific stress proteins implied in the heavy metal resistance of a *P. fluorescens* strain living in the tidal flat sediments.

Conclusion

It can be concluded based on this study that:

1. *P. fluorescens* BA3SM1 isolated from the tidal flat sediments is heavy metal-resistant. So, it represents a very interesting model of study to characterize the effects of metallic stresses on the bacterial proteome. Moreover, this organism is a very interesting candidate for bioremediation studies.
2. BA3SM1 is particularly resistant toward cell disruption, and this robustness could be due to specific biochemical and biophysical properties of its envelope. It would be interesting to attempt to explain whether this robustness is due to particular envelop properties or/and exopolymers production which could facilitate external metal biosorption.
3. This preliminary work is essential because it suggests a convenient protein extraction procedure to achieve quality 2D-electrophoresis in further studies. Later on, proteomics will allow us to have a better knowledge of the resistance mechanisms developed by BA3SM1 to survive to heavy metal stresses.

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Author Disclosure Statement

The authors declare that no competing financial interests exist.

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