Full Length Research Paper

Proteomic analysis of chromate response in *Staphylococcus saprophyticus* isolated from a fly ash dumping site

Kouadjo Zaka Ghislaine Claude and Zézé Adolphe*

Groupe de Recherches sur les Biotechnologies Végétale et Microbienne, Ecole Supérieure d'Agronomie, Département d'Agriculture et Ressources Animales, Institut National Polytechnique Houphouet-Boigny, BP 1093 Yamoussoukro, Côte d'Ivoire

*Staphylococci s*pecies resistant to chromate were previously isolated from a fly ash dumping site. To further understand the mechanisms developed by these bacteria to tolerate chromate, a proteomic analysis was performed to identify proteins involved in chromate stress response of *Staphylococcus saprophyticus* isolated from a fly ash dumping site. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two dimensional (2D) electrophoresis gels revealed several proteins visualized as up- and down- regulated in presence of Cr (VI) (500 µg/ml). Using matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS), a putative ribosome-associated protein Y [*S. saprophyticus* subsp. *saprophyticus* ATCC 15305], and a pyruvate dehydrogenase E1 component beta subunit [*S. saprophyticus* subsp. *Saprophyticus* ATCC 15305] were identified as up-regulated during chromate stress. The putative ribosome-associated protein Y is homologous to the ribosomal protein (gi24349644) expressed in *Shewanella oneidensis* in response to an acute chromium stress. The chromate stress upregulated pyruvate dehydrogenase from *S. saprophyticus* was highly similar to a pyruvate dehydrogenase (AF23026) upregulated in *Staphylococcus aureus* during osmotic stress. These results show that these proteins may be used as genetic systems by *S. saprophyticus* to resist chromate pollution and other stresses within the fly ash (FA) dumping site.

Key words: Fly ash, chromate resistant bacteria, *Staphylococci*, SDS-PAGE, 2D electrophoresis.

INTRODUCTION

In South Africa, coal is the most important natural source of energy. It is used in several industries such as electricity production. Of many environmental issues concerned with coal industry, the one due to flyash (FA), a by-product resulting from coal combustion process in electricity production is widespread. FA management is difficult because of its texture as fine particles and its lightness and can easily contaminate the air. Its management is often done by dumping, and this process requires large area of land. In 2010 for example, the electricity company (Eskom) in South Africa produced 36.01 million of tons of ash, but only sold 2 million of tons and recycled 5.6%, disposing of 33.89 million of tons in dams and ash dumps (Eskom, 2010). Moreover, fly ash dumping poses huge environmental problems since it was shown that it contains toxic elements or pollutants (Fernandez et al., 1994). A recent thorough investigation of a FA dumping site in South Africa revealed that this site was highly polluted with chromate (Kouadjo and Zeze, 2011). In its oxidized form (Cr (VI)), chromate is highly water soluble while the reduced Cr (III) form is not soluble (Cervantes et al., 2001). In the oxidized form, chromate is highly toxic

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*Ccorresponding author. E-mail: youhe.deba@gmail.com.

Abbreviations: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; **MALDI-TOF MS,** matrix assisted laser desorption ionisation time-of-flight mass spectrometry.

and human exposure can result in ulceration of the skin (Ye et al., 1999; Levina et al., 2001, O'Brien et al., 2005). However, the reduced form, (Cr (III)), in small concentrations, appears to be an essential nutrient for plant, animal and human life (Nieboer and Jusys, 1988) and has been shown to be involved in glucose and lipid metabolism in mammals (Anderson, 1989). Altogether, the reduced form is less toxic. Therefore, the transformation of the Cr (VI) form of chromate to Cr (III) and the maintenance of this status in environments should constitute a priority in developing strategies for the removal of chromium toxicity. Of many technologies, the microbe based technologies such as bioremediation provide an alternative to conventional methods for chromate removal (Suzuki et al., 1992; Park et al., 2000; Ackerley et al., 2004a; Vatsouria et al., 2005; Barak et al., 2006; Viamajala et al., 2007; Opperman et al., 2008). Recent studies of the microbiology of a fly ash dumping site revealed the existence of *Staphylococci* species able to resist and reduce chromate in its less toxic form (Kouadjo and Zeze, 2011). It was then important to understand the mechanisms through which these *Staphylococci* resist and reduce chromate. Further investigation using genomic approaches allowed the identification of some genetic systems used by these bacteria to resist chromate toxicity (Kouadjo and Zeze, 2013).

However, recently, the use of other methodologies such as transcriptomic and proteomic analyses have allowed a better mapping of genes involved in chromate tolerance (Brown et al., 2006; Chourey et al., 2006; Thompson et al., 2007; Henne et al., 2009). In this paper, we used a proteomic approach in order to identify proteins that were differentially expressed in the genome of *Staphylococcus saprophyticus* isolated from a fly ash dumping site when exposed to acute chromate stress.

MATERIALS AND METHODS

Bacterial culture conditions

Staphylococcal strains (*Staphylococcus aureus*, *Staphylococcus arlettae*, *Staphylococcus saprophyticus*, *Staphylococcus pasteuri* and *Staphylococcus epidermidis*) previously isolated from a FA dumping site and characterized as chromate resistant and reducing bacteria (Kouadjo and Zeze, 2011) were used. These strains were cultivated at 37°C in 500 ml of culture. The nutrient medium consisted (g/l) of: KH_2PO_4, 0.9; $Na_2HPO_4.2 H_2O$, 1.2; NH_4Cl, 0.5; yeast extract, 0.9; Na pyruvate, 2.0; $NaH-CO_3$, 0.2; $MgSO_4.7H_2O$ and 0.5 (pH 7) (Vatsouria et al., 2005) was amended with 0 or 500 µg/ml of potassium dichromate, used as source of Cr (VI). After five days, cells of the species with high resistance and tolerance aptitude were harvested from three cultures per growth condition by centrifugation and washed twice in phosphate buffered saline (PBS). Cell pellets were stored at -80°C until processed. Bacterial chromate reduction aptitude was determined by the method described by Kouadjo and Zeze (2011).

Protein extraction

Cell pellets collected in 2 ml microtube were immediately mixed with PBS. Proteins were extracted by grinding cells with a sonicator (Bandelin Sonopuls). The supernatant were collected by centrifugation (5,000 x g) and the proteins were precipitated with 20% trichloro acetic acid (TCA) for 1 h at 4°C. The mixture was centrifuged and then the collected pellet was air-dried and resuspended in 200 µl of urea buffer [9 M urea, 2 M thiourea, 4% (w/v) 3-(3-cholamidopropyl dimethylammonio)-1-propanesulfonate CHAPS)], after washing with ice-cold acetone. The resulting solution was homogenized by vortex (180 xg) for 1 h and unbroken cells and debris were removed by low-speed centrifugation. The final supernatant was collected as the whole protein extract in 2 ml sterile microtubes and kept at 4°C. The amount of protein was estimated using Bradford assay (Bradford, 1976).

One-dimension electrophoresis gel

The protein patterns were analyzed in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-Protean III electrophoresis cell (Bio-Rad Hercules, CA, USA). Approximately 100 µg/µl of proteins mixed with ßmercapto-ethanol was loaded and electrophoresed at 80 V in the stacking gel and at 120 V in the resolving gel, in running buffer composed with: 0.025 M Tris-HCl, pH 8.3, 0.192 M glycine, 0.1% (w/v) SDS. Page ruler™ with a known weight was used as marker.

Proteins were visualized by PharosFXTM molecular imager and scanner (Bio-Rad) after gel overnight staining in 50 ml of coomassie brilliant blue R-250 solution and destaining with 250 ml methanol, 50 ml glacial acetic acid and 200 ml of distilled water.

Protein preparation for 2D-gel

Protein samples (250 µg) were mixed separately with 0.8% (w/v) dithiothreitol (DTT), 0.2% (v/v) ampholytes, pH range 3 to 10 (Bio-Rad) with 0.002% (w/v) bromophenol blue to a final volume of 125 µl in urea buffer. Samples were then used passive overnight rehydration linear 7 cm immobilized pH gradient (IPG) strips (pH range 4 to 7 (Bio-Rad)) at room temperature. The strips were used for the first dimension by isoelectric focusing (IEF) using the PROTEAN IEF cell.

Two-dimensional gel electrophoresis

IEF strips containing the proteins, were equilibrated twice for 15 min each with gentle shaking in an equilibration buffer (6 M urea, 2% (w/v) SDS, 0.05 M Tris-HCl, pH 8.8, 20% (v/v) glycerol, 0.002% bromophenol blue), firstly containing 1% (w/v) DTT and secondly 2.5% (w/v) iodoacetamide. The strips after equilibration were loaded onto a 12% SDS-PAGE gels.

Proteins were electrophoresed in running buffer at 100 V. Proteins were visualized by coomassie staining R-250 and imaged with a PharosFXTM plus molecular imager scanner (Bio-Rad).

2D gel image analysis

In order to detect significant differences in protein abundance between growth conditions, a comparative analysis of 2D-PAGE from the no chromate gels, and the chromate gels was carried out using the PDQuest 8.0.1 software (Bio- Rad). Each growth condition was represented by three experimental replicates (derived from three independent batch cultures). After automated detection and matching of spots, manual editing was performed for every identified spots. Only spots displaying reproducible change patterns in at least two of the three replicate gels were considered as differentially expressed.
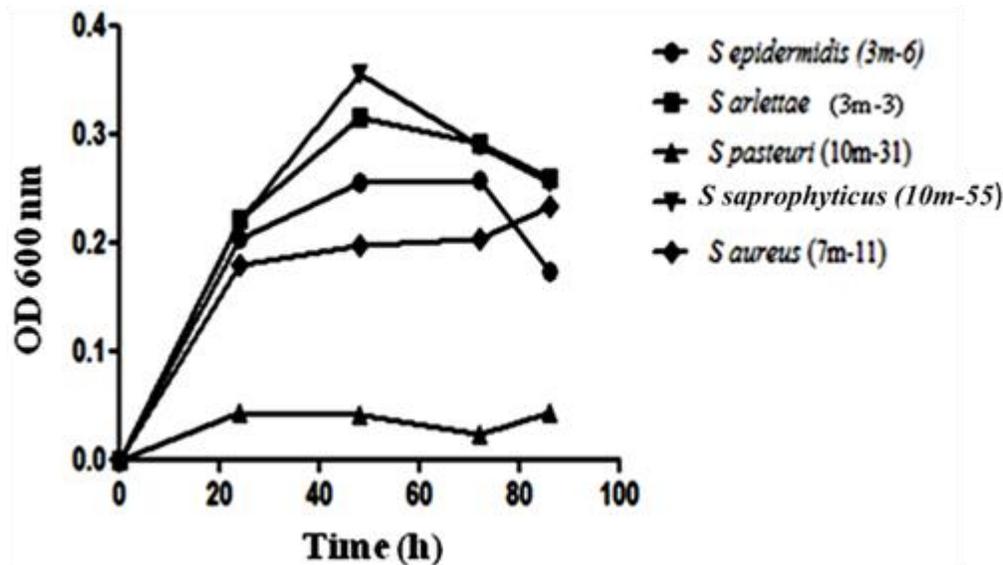
Figure 1. Growth curves of *S. epidermidis*, *S. arlettae*, *S. pasteuri*, *S. saprophyticus* and *S. aureus* in the presence of chromate (500 µg/ml).

Protein identification by mass spectrophotometry (MS) and phylogenetic analyses

Protein spots were excised manually from stained gels and transferred into sterile microcentrifuge tubes. Proteins were digested in the gel with approximately 120 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) dissolved in 25 mM ammonium bicarbonate for 6 h at 37°C. Digestion was stopped by adding 50 µl of 1% (v/v) trifluoroacetic acid (TFA) and incubating for 2 h at room temperature before storage at 4°C until further analysis. The resulting peptides were extracted, and samples were subjected to analysis by mass spectrometry with matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager DE Pro Biospectrometry workstation (Applied Biosystems, Forster City, CA, USA) to generate peptide mass fingerprinting (PMF). All searches were performed as described by Thomas et al. (2010). Identified proteins by MALDI-TOF MS were classified individually by their putative function as previously defined by Bevan et al. (1998) and Ndimba et al. (2003). Sequence homology search was performed using basic local alignment search tool (BLAST) (Altschul et al., 1990) and sequences aligned using CLUSTALW algorithms (Thompson et al., 1994). Phylogenetic analyses were conducted with MEGA 4 software (Tamura et al., 2007) and phylogenetic trees constructed using the neighbor-joining method (Saitou and Nei, 1987).

RESULTS

Proteomic expression assays in the chromate resistant *Staphylococcus saprophyticus* using SDS-PAGE

S. saprophyticus showed the highest chromate resistance (Figure 1) and a strong potential to reduce chromate (Figure 2). Consequently, this species was used for further analysis. After four days of growth, total proteins extracted from *S. saprophyticus* cultivated in media with and without Cr (VI) (500 µg/mL) were separated by one-dimensional SDS-PAGE. Figure 3 shows the protein patterns for this strain in each treatment. It was shown that several proteins were up- and down- regulated in presence of Cr (VI). For example, when *S. saprophyticus* was cultivated without chromium, it was shown that most of the proteins expressed have molecular weights higher than 40 KDa (Figure 3). However, when this species was under acute chromium stress, most of these proteins were not detectable, showing that they were down regulated except five proteins at 60, 55, 45 and 40 KDa which were equally expressed in both conditions. The most up-regulated proteins had lower molecular weights of 10, 12 and 20 KDa).

2-D gel electrophoresis (2-DGE) shows differentially expressed proteins under chromate stress

In order to realize a thorough analysis of differentially expressed proteins, total extracted proteins were separated in a 2-DGE electrophoresis, focused and resolved using pH range 4 to 7 IPG strips. A proteome map of proteins expressed in *S. saprophyticus* with and without chromium was obtained (Figure 4). The figure confirmed presence of down and up regulated proteins in chromium stress condition as observed in the 1D SDS-PAGE patterns. Using PDQuest, an average of 193 abundant spots was visualized in the treatment without chromium and 183 spots in the treatment with chromium, after comassie staining. Detail characteristics of some spots are given in Table 1. Out of 25 proteins analyzed, 13 proteins with molecular weight ranging from 10 to 38 KDa were up-regulated except one with 60 KDa. This confirms the result
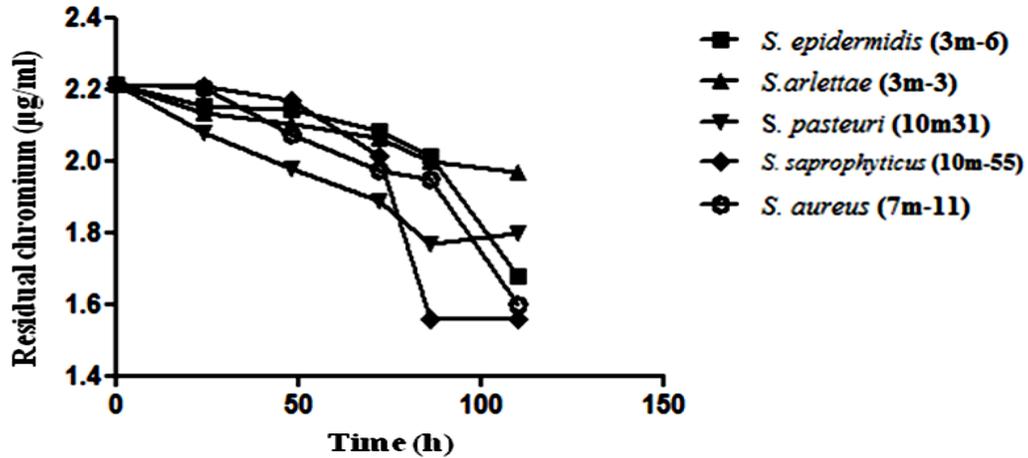
Figure 2. Chromate reduction potential of *S. arlettae*, *S. pasteuri*, *S. saprophyticus* and *S. aureus* cultivated in the presence of Cr (VI) (500 µg/ml).
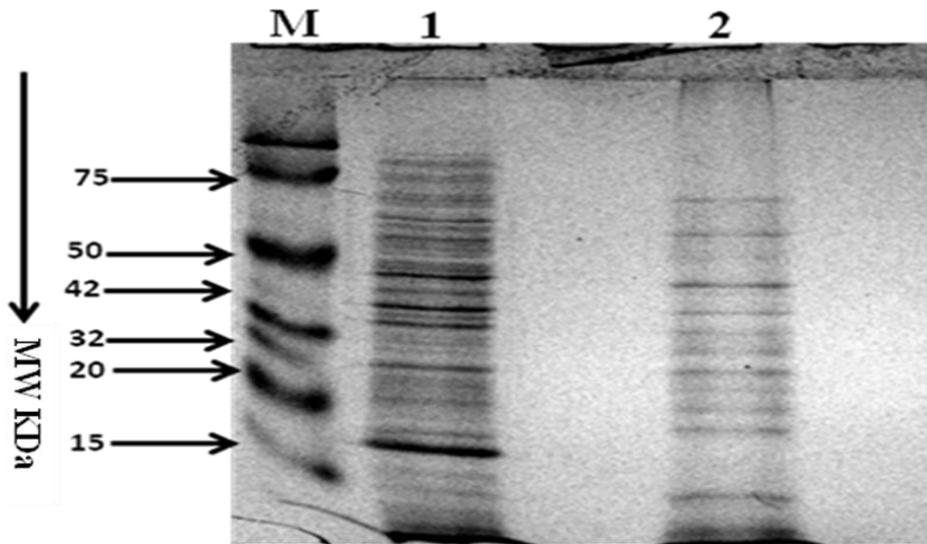

Figure 3. SDS-PAGE. The profiles of proteins extracted from; lane 1, *S. saprophyticus* cultured in a medium without chromium; 2, *S. saprophyticus* cultures in a medium supplemented with chromium (500 µg/ml). M, Molecular weight marker. Some bands represented up and down regulated proteins expressed in 2 media are indicated by arrows. SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

found with the SDS-PAGE that most of up-regulated proteins were those with low molecular weights. Out of the 11 down regulated proteins, seven had molecular weights ranging from 50 to 80 KDa.

Spot sequencing and identification of two up-regulated proteins

Protein identification was done using the peptide masses of each sample obtained by MALDI-TOF MS. The result was submitted to MASCOT and comparative research using MSDB and NCBI databases Swiss-Prot was performed. This process resulted in the identification of protein spots. Results with a MOWSE score greater than 81 was considered statistically significant at p <0.05. A putative ribosome-associated protein (gi73663273) and a pyruvate dehydrogenase E1 component beta subunit (gi|73663004) belonging both to *S. saprophyticus* were identified (Table 2). The putative ribosomal protein belongs to the super family cl00250, RaiA ("ribosome-associated inhibitor A", also known as Protein Y (PY), YfiA, and SpotY. This is a stress-response protein that binds the ribosomal subunit interface and arrests translation by interfering with amino-
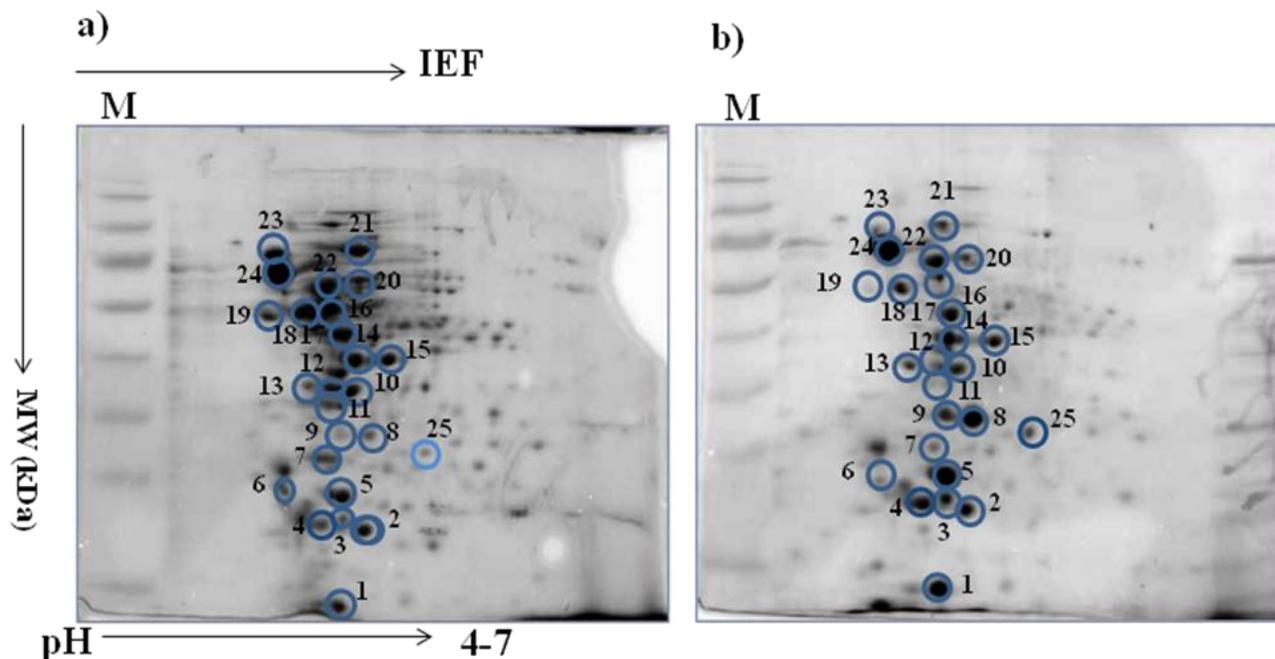
Figure 4. Coomassie-stained two-dimensional polyacrylamide gels of total cellular protein samples collected from (a) *S. saprophyticus* cultured without chromium and (b) *S. saprophyticus* challenged with Cr(VI) (500 µg/ml). Average spot densities from triplicate gels were compared for the unchallenged and Cr(VI) challenged *S. saprophyticus*. Example of distinct protein spots whose expression was up- or down-regulated threefold or greater are indicated by circles. Spots 3 and 4 from gel b) were excised and identified by MALDITOF-MS fingerprinting. M, Molecular weight on each gel. MALDI-TOF MS, matrix assisted laser desorption ionisation time-of-flight mass spectrometry.

acyl-tRNA binding to the ribosomal A site. When this putative ribosome-associated protein from *S. saprophyticus* was phylo-genetically compared to ribosomal proteins expressed in other microorganisms under chromate stress (Figure 5), it showed homologies to a ribosomal protein (gi24349644) from *Shewanella oneidensis* (Chourey et al., 2006). Moreover, a sequence comparison of the chromate stress upregulated pyruvate dehydrogenase from *S. saprophyticus* with the pyruvate dehydrogenase up regulated in *S. aureus* during osmotic stress showed high similarity between the two proteins (Figure 6).

DISCUSSION

Microorganisms implement several strategies to survive in extreme environments. These strategies are often linked to specific changes in proteins whose biological functions are related to tolerance to toxic compounds. Comparative analysis of proteomes are then made to study the differential expression of proteins in organisms surviving in extreme environments to study the mechanisms involved in the survival process (Van Wijk, 2001). Chromate resistant Staphylococci species were isolated from a fly ash dumping site (Kouadjo and Zeze, 2011). Proteomic approaches were used in this study in order to identify proteins expressed in these *Staphylococci* species

during chromate stress. In order to select the most resistant *Staphylococcal* species for proteomic analyses, a growth assay was realized in presence of chromate (500 µg/mL); an acute chromium concentration. Of the five *Staphylococci* species previously identified as resistant to chromate, *S. saprophyticus* was selected as a model species for further analyses. Indeed, the growth assay showed that this species had a better growth curve compared to the four other species. Moreover, it exhibited the strongest potential to reduce chromate. It was interesting to understand the specific changes in the proteome of this species under chromium stress. When SDS-PAGE analyses were performed, differentially expressed proteins could be identified. SDS-PAGE profiles allowed the detection of bands representing proteins that were up and down regulated in *S. saprophyticus* response to chromate stress. This technique revealed that most of down-regulated proteins were high molecular proteins while low molecular weight proteins were up-regulated. Such approach was previously used for the charac-terization of proteins induced by chromate stress (Thacker and Madamwar, 2005; Abskaaron et al., 2009). In order to gain a better insight in the proteomic changes that occured in *S. saprophyticus* under chromate stress, a 2D analysis was performed. Differences in the proteome of bacteria grown under conditions of stress and normal conditions were analyzed by comparing the expression

Table 1. Analysis of 25 spots identified using 2D electrophoresis.

Spot	Regulation	Molecular weight (MW, kDA)	Isoelectric point
1	+	10	5.35
2	+	18	5.45
3	+	18.2	5.35
4	+	18	5.25
5	+	19	5.35
6	-	19	5.16
7	-	21	5.25
8	+	23	5.48
9	+	23	5.30
10	+	31	5.40
11	-	25	5.45
12	-	31	5.30
13	+	31	5.15
14	+	32	5.45
15	+	38	5.65
16	-	50	5.35
17	-	43	5.15
18	-	50	5.15
19	-	50	4.80
20	-	60	5.45
21	-	80	5.45
22	+	60	5.15
23	-	80	5
24	=	65	5
25	+	22	5.75

+, Up regulation; -, down regulation; =, no signal variation.

Table 2. Identification by MALDI-TOF MS of two protein spots from *Staphylococcus saprophyticus* cultivated in media with chromium (500 µg/ml).

Spot accession number	Protein name and species	Predicted MW/p*I*	MOWSE score	Regulation
4 (gi|73663273)	Putative ribosome-associated protein Y [*S. saprophyticus*] ATCC 15305]	22.156/5.34	102	+
3 (gi|73663004)	Pyruvate dehydrogenase E1 component beta subunit [*S. saprophyticus* ATCC 15305]	35.216/4.62	142	+

+, Up regulations during chromium stress; MALDI-TOF MS, matrix assisted laser desorption ionisation time-of-flight mass spectrometry.

profile of proteins identified by 2-DE analysis. The application of these techniques showed that the *S. saprophyticus* isolated from the fly ash dumping site was able to induce various changes of its proteome in order to survive acute chromium stress as was already shown for different microorganisms (Llagostera et al., 1986; Chourey et al., 2006; Brown et al., 2006, Miranda et al., 2005; Henne et al., 2009). The identification of genetic systems involved in chromate toxicity challenging systems in native *Staphylococci* species isolated from a fly ash dumping site is a very important step if we were to use these organisms for bioremediation purposes. Recently, the use of genomic analyses allowed the detection and identification of an ATP-binding cassette (ABC) transporter, a DNA helicase and a nirD protein that were used by these bacteria as chromate challenging systems

Figure 5. Phylogenetic analysis of ribosomal protein from *Staphylococcus saprophyticus* (gi|73663273). Ribosomal proteins up regulated in *Shewanella oneidensis* MR-1 in presence of acute chromate stress (gi|24349644, gi|24345648, gi|24345647, gi|24345655, gi|24345643, gi|24345654, gi|24345645, gi|24345646, gi|24345633, gi|24345644, gi|24345651, gi|24345657, gi|24345642, gi|24345658, gi|24345634, gi|24345649 and gi|24345659 were used for comparison). The tree was constructed by the neighbor-joining method using Mega version 4.

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gi|73663004    MAQMTMVQAINNALKTELQNDENVLLFGEDVGVNGGVFRVTEGLQKEFGEDRVFDTPLAE 60
AF235026       MAQMTMVQAINDALKTELKNDQDVLIFGEDVGVNGGVFRVTEGLQKEFGEDRVFDTPLAE 60
               ***********:******:**::**:*********************************

gi|73663004    SGIGGLALGLTTQGYRPVMEIQFLGFVFEVFDSVAGQLARTRFRSGNSKQAPVTIRAPFG 120
AF235026       SGIGGLAMGLAVEGFRPVMEVQFLGFVFEVFDAIAGQIARTRFRSGGTKTAPVTIRSPFG 120
               *******:**::.:*:*****:**********::***:********.:* ******:***

gi|73663004    GGVHTPELHADNLEGILAQSPGLSVVIPSNPYDAKGLLISAIRSNDPVVYLEHMKLYRSF 180
AF235026       GGVHTPELHADNLEGILAQSPGLKVVIPSGPYDAKGLLISSIRSNDPVVYLEHMKLYRSF 180
               ***********************.*****.*********:*******************

gi|73663004    RDEVPEEEYTIEIGKANVKQEGNDITLIAYGAMVQESLKAAEELEKEGYSVEVIDLRTVQ 240
AF235026       REEVPEEEYTIDIGKANVKKEGNDISIITYGAMVQESMKAAEELEKDGYSVEVIDLRTVQ 240
               *:*********:*******:*****::*:********:*******:*************

gi|73663004    PIDIETLVASVEKTGRAVVVQEAQRQAGVGAAVASELAERAILSLDAPIARVAAADTVYP 300
AF235026       PIDVDTIVASVEKTGRAVVVQEAQRQAGVGAAVVAELSERAILSLEAPIGRVAAADTIYP 300
               ***::*:**********************.:**:*******:***.*******:**

gi|73663004    FTQAENVWLPNKNDIVEKAKATLEF 325
AF235026       FTQAENVWLPNKNDIIEKAKETLEF 325
               **************:**** ****
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Figure 6. Alignment of the pyruvate dehydrogenase (gi|73663004) up regulated in *Staphylococcus saprophyticus* in chromate stress and the pyruvate dehydrogenase AF235026 up regulated in *S. aureus* during osmotic stress.*identical amino acid; :conserved substitutions.

(Kouadjo and Zeze 2013). Proteomic analyses also offered the possibilities to identify genes and proteins involved in chromate stress (Brown et al., 2006; Henne et al., 2009). The sequencing of two spots after 2-D electro-phoresis that represented two up-regulated proteins in *S. saprophyticus*, allowed the identification of a putative ribosome-associated protein (gi73663273) and a pyruvate dehydrogenase E1 component beta subunit (gi|73663004). The putative ribosome-associated protein (gi73663273) belongs to a stress-response protein superfamily. Moreover, ribosomal associated proteins have been reported to be up-regulated during chromate stress in *S. oneidensis* (Chourey et al., 2006) and in *Pseudomonas putida* (Thompson et al., 2010). The putative ribosome-associated protein (gi73663273) is certainly a genetic system used by *S. saprophyticus* to survive acute chromium stress condition. Pyruvate dehydrogenases have been found to be regulated in *Arthrobacter* sp. (Henne et al., 2009), and in *Escherichia coli* (Ackerley et al., 2006). The pyruvate dehydrogenase E1 component beta subunit (gi|73663004) which is up-regulated in *S. saprophyticus* might be used by this species to combat chromate stress. Moreover, this protein may also be involved in challenging osmotic stress since it is homolo-gous to a pyruvate dehydrogenase up-regulated in *S. aureus* (AF235026) during osmotic stress (Vilhelmsson and Miller, 2002) (Figure 6). This is the first report of the use of proteomic approaches for the identification of proteins involved in chromate stress response in micro-organisms isolated from a fly ash dumping site. A global proteomic mapping will be needed for a thorough investi-gation of all the *Staphylococci* proteome involved in acute chromate challenge.

ACKNOWLEDGEMENTS

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