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Identification of genes and proteins involved in the pleiotropic response to arsenic stress in *Caenibacter arsenoxydans*, a metalloresistant beta-proteobacterium with an unsequenced genome

Christine Carapito [a,1], Daniel Muller [b,1], Evelyne Turlin [c], Sandrine Koechler [b], Antoine Danchin [c], Alain Van Dorsselaer [a], Emmanuelle Leize-Wagner [a], Philippe N. Bertin [b], Marie-Claire Lett [b,*]

[a] *LSMBO, Laboratoire de Spectrométrie de Masse Bio-Organique ECPM, Bâtiment R5, 67087 Strasbourg, France CNRS-UMR 7509/Université Louis-Pasteur, Strasbourg, France*
[b] *Génétique Moléculaire, Génomique, Microbiologie, UMR 7156, CNRS et Université Louis-Pasteur, 28, rue Goethe, 67000 Strasbourg, France*
[c] *Unité de Génétique des Génomes Bactériens URA 2171 CNRS Département Structure et Dynamique des Génomes, 28, rue du Docteur Roux, Institut Pasteur, Paris, France*

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Abstract

The effect of high concentrations of arsenic has been investigated in *Caenibacter arsenoxydans*, a β-proteobacterium isolated from an arsenic contaminated environment and able to oxidize arsenite to arsenate. As the genome of this bacterium has not yet been sequenced, the use of a specific proteomic approach based on nano-high performance liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) studies and de novo sequencing to perform cross-species protein identifications was necessary. In addition, a random mutational analysis was performed. Twenty-two proteins and 16 genes were shown to be differentially accumulated and expressed, respectively, in cells grown in the presence of arsenite. Two genes involved in arsenite oxidation and one in arsenite efflux as well as two proteins responsible for arsenate reduction were identified. Moreover, numerous genes and proteins belonging to various functional classes including information and regulation pathways, intermediary metabolism, cell envelope and cellular processes were also up- or down-regulated, which demonstrates that bacterial response to arsenic is pleiotropic.

Keywords: Arsenic stress; Two-dimensional gel electrophoresis; Tandem mass spectrometry; De novo sequencing; Cross-species identification; Transposon mutational analysis

1. Introduction

Arsenic is widely distributed in many environments, released from both natural and anthropogenic sources. Arsenic can be trapped in combination with sulfur (e.g. realgar As_4S_4, orpiment As_2S_3 and arsenopyrite FeAsS). These insoluble forms can be mobilized from the solid to the aqueous phase by both chemical or biological transformations, including microbial transformations, supporting the contamination of the aquifers by the two inorganic forms (As[III] and As[V]), both of which are toxic for living organisms. The presence of arsenic in drinking water aquifers is critical to the health of millions of people worldwide [1]. Several studies have demonstrated that, due to their impact on speciation and mobilization of arsenic in the environment, bacteria play a major role in the biogeochemical cycle of this element [2,3]. Most investigations dealing with the interactions between arsenic and bacteria have focused exclusively on the mechanisms of resistance that include the transformation of this metalloid by methylation, reduction or oxidation and the active efflux of As[III] (for review see [3,4]). In contrast, the other cellular functions involved in the adaptation of these microorganisms to toxic concentrations of arsenic remain to date largely

Abbreviations: CDM, chemically defined medium; IEF, isoelectric focusing; nanoLC-MS/MS, nano-high performance liquid chromatography tandem mass spectrometry.

* Corresponding author. Tel.: +33 3 90 24 19 97; fax: +33 3 90 24 20 28.
E-mail address: lett@gem.u-strasbg.fr (M.-C. Lett).

[1] These authors contributed equally to this work.

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unknown. The knowledge of regulatory cellular networks controlled by arsenic will provide a better understanding of the mechanisms implicated in the colonization of toxic environments supported by arsenic cycling.

With this aim in view, we used a proteomic strategy to investigate the regulatory cellular network involved in the adaptation to a high concentration of arsenic in *Caenibacter arsenoxydans* strain ULPAs1 [5], an arsenic resistant β-proteobacterium. Proteome expression profiling constitutes an attractive tool to understand biological processes in a comprehensive manner. To gain insight into the effect of arsenic on bacterial physiology we compared the protein profiles of ULPAs1 cells grown with and without this metalloid. Indeed, nanoLC-MS/MS analysis and de novo sequencing interpretation of the MS/MS spectra followed by sequence similarity database searches lead in most cases to successful cross-species protein identifications [6–9]. As a complement, this approach was combined with a mutational analysis in which a collection of mutants obtained by random gene transposition was screened to identify differential gene expression in the presence of arsenic. A previous study has already led by such a procedure to the identification and sequencing of *aoxA* and *aoxB*, the genes encoding the two subunits of the arsenite oxidase responsible for the transformation of As[III] to As[V] [10].

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strain ULPAs1 has been previously isolated from industrial activated sludge contaminated with arsenic and described in Weeger et al., 1999 [11]. It was cultivated in a chemically defined medium (CDM) as described previously [11]. Cultures were grown in liquid medium in flasks placed on a rotary shaker at 25 °C. Induced cells were obtained by adding a solution of sodium arsenite ($NaAsO_2$) to obtain a final concentration of 2.66 mM As [III]. Mutants induced by arsenic were obtained by random insertion of a mini-Tn*5* as described by Muller et al., 2003 [10]. Minimal inhibitory concentrations (MIC) were determined according to the procedure of Lim and Cooksey [12]. Briefly, bacterial suspensions were transferred in triplicate from liquid culture to solid CDM plates supplemented with increasing concentrations of arsenite. The MIC was defined as the As[III] or As[V] concentration that inhibited confluent growth on plates after 3 days at 25 °C.

2.2. DNA manipulation

DNA manipulation was carried out according to standard protocols as described by Sambrook et al. [13]. Total DNA of strain ULPAs1 was isolated using the Wizard© Genomic DNA purification kit (Promega). Templates for I-PCR (Inverse-PCR) were prepared as previously described [10]. DNA flanking the mini-Tn*5* insertion was amplified by PCR with Taq DNA polymerase (Gibco BRL) with the minitransposon-specific primer 5′-AGATCTGATCAAGAGACAG-3′ (I-end) and 5′-ACTTGT GTATAAGAGTCAG-3′ (O-end) [14].

The PCR products were purified and sequenced. The sequencing chemistry used AmpliTaq FS DNA polymerase and BIGDYE TM terminators (version 1). Sequence reactions were analyzed with an Applied Biosystems 373XL sequencer. Database searches and sequence analyses were performed using the BLAST program [15].

DNA probes, corresponding to part of the coding region in which the Tn*5* has inserted, were generated by PCR amplification with oligonucleotides given in Table 3 and by using the PCR DIG Probe synthesis kit (ROCHE) according to the manufacturer's instructions.

2.3. Analytical two-dimensional gel electrophoresis

Late exponential phase cells (100 ml) were harvested by centrifugation. The cell pellets were washed with the CDM [10] and resuspended in 1 ml of distilled water. After DNase and RNase treatment, cells were disrupted with a "FP120 FastPrep Cell disruptor" (Bio101) (two times 30 s at maximum speed with 1-min intervals on ice). Cell debris were removed by ultracentrifugation for 60 min at 90 000 × *g*.

Isoelectric focusing (IEF) was conducted using the horizontal Multiphor II system (Pharmacia) at a temperature of 20 °C. For analytical gels, 60 μg of protein were solubilized in 400 μl of rehydration solution (0.5% (v/v) Pharmalyte 3–10, 8 M urea, 65 mM DTT, 2% (v/v) Nonidet P40), and loaded onto a 18 cm pH 4–7 immobilized pH gradient strip (IPG) using the in-rehydration technique [16]. Such a procedure leads to a good protein resolution almost without background. For preparative gels, 120 μg of protein was solubilized as mentioned above. For both analytical and preparative gels, focusing was performed for 3 h at 300 V, 1 h at 750 V, 30 min at 1500 V, 16 h at 2500 V and 2 h at 3500 V (total = 50 kVh). The IPGs were equilibrated as previously described [17,18]. The second dimension was performed with 11.5% (w/v) SDS-polyacrylamide gels using the Protean II xi 2D Multicell system (BioRad). Proteins were stained with silver nitrate and gels were digitalized using a JX-330 scanner (Sharp). Digitized 2-D gel patterns were edited and matched using the PDQUEST software package (PDI, Humington Station).

To account for unspecific variations, eight gels obtained by using two independent protein preparations extracted from four independent cultures, were performed for each condition (in the presence or in the absence of arsenite). Protein levels were expressed as percentage volume, which corresponds to the percentage ratio between the volume of a single spot and the total volume of all spots present in a gel. The mean values of spot intensity were calculated by comparing the eight gels together. Spots showing more than 15% variation within the same condition were not considered.

2.4. Sample preparation and mass spectrometry analysis

In situ digestion of the gel spots was performed with an automated protein digestion system, MassPREP Station (Waters, USA). The gel plugs were washed three times with a

mixture of 50%/50% NH_4HCO_3 (25 mM)/ACN. The cysteine residues were reduced with dithiothreitol at 57 °C for 30 min and alkylated with iodoacetamide at room temperature for 20 min. After dehydration with acetonitrile, the proteins were digested in gel with 20 µl of 12.5 ng/µl of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM NH_4HCO_3 overnight and at room temperature. Then a double extraction was performed, first with 60% acetonitrile in 5% formic acid and a second extraction with 100% acetonitrile. The resulting tryptic peptides were analyzed by nanoLC-MS/MS. These analyses were performed using a CapLC capillary LC system (Waters, Milford, Massachusetts, USA) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer Q-TOF II (Waters, USA). Chromatographic separations were conducted on a reverse-phase (RP) capillary column (Pepmap C18, 75 µm i.d., 15 cm length, LC Packings) with a flow rate of 200 nl/min accomplished by a pre-column split. An external calibration was performed using a 2 pmol/µl GFP ([Glu^1]-Fibrinopeptide B from Sigma) solution.

Mass data acquisitions were piloted by MassLynx software (Waters, USA) using automatic switching between MS and MS/MS modes.

2.5. Data interpretation and protein identification

In a first step, the complete .pkl files were submitted to protein database searches via a local Mascot™ (MatrixScience, London, UK) server. Searches were done with a mass tolerance of 50 ppm in MS mode and 0.25 Da in MS/MS mode. One missed cleavage per peptide was allowed and variable modifications were taken into account such as carbamidomethylation of cysteine and oxidation of methionine. Searches were performed without constraining protein molecular mass or isoelectric point and without any taxonomic restriction.

In the case of unsuccessful protein identification in the protein databases with Mascot™, the generated MS/MS spectra were individually interpreted in order to deduce a partial or complete amino acid sequences. These interpretations were performed using specialized de novo sequencing softwares: the PepSeq program (Waters, USA) and the PEAKS Studio program (Bioinformatics Solutions, Canada).

The deduced amino acid sequence fragments were submitted to the MS-BLAST program [6] provided at the EMBL to perform cross-species protein identifications. In the contrary to common BLAST programs, optimized for long and accurate protein sequences, this program has been manipulated to be adapted to data produced by mass spectrometry analysis. Indeed, the direct submission of the numerous redundant putative sequence candidates deduced from nanoLC-MS/MS analysis is possible with this MS-BLAST program. We used the MS-BLAST specifically modified PAM30MS scoring matrix, no filter was set and the nrdb95 database was used for the searches. The statistical evaluation of the results and the validation of the matches was performed according to Shevchenko et al. [6].

2.6. β-Galactosidase assays

The activity of the β-galactosidase reporter protein was determined by a standard assay using ortho-nitrophenol β-D-galactopyranoside (ONPG) essentially as described by Miller [19]. Briefly, this enzyme cleaves uncolored ONPG to produce yellow ortho-nitrophenol. The quantity of this compound, which is directly proportional of the activity of the enzyme, is determined by spectrophotometry at 540 nm.

After 48 h of ULPAs1 mutants culture in CDM, the medium was supplemented with 0.66 mM As[III] to induce the genes controlled by arsenite and cultures were further grown for 4 h. Whereas the cultures containing the metalloid solution ceased growth, the OD of the control cultures without metals increased, but remained in the recommended range of 0.3–0.9 [19].

2.7. RNA manipulation

RNA was prepared from strain ULPAs1 cultures grown in 20 ml CDM medium to exponential phase. Cultures were induced by addition of 0.66 mM (50 ppm) As[III] 15 and 30 min before extraction. Bacteria were pelleted by centrifugation and total RNA extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were then treated with DNase I (Gibco-BRL) and quantified spectrophotometrically at 260 nm.

2.8. Quantitative analysis of mRNA

RNA (500 ng or 2 µg) was denatured in 300 µl of RNA dilution buffer (water, 20 × SSC, and formaldehyde in the ratio 5:3:2) at 65 °C for 15 min. The 20 × SSC solution (3 M NaCl–0.3 M trisodium citrate adjusted to pH 7) was previously treated with diethylpyrocarbonate. RNAs were then applied on Hybond N1 nylon filters (Amersham) with a Biodot SF slot blot applicator (BioRad) and fixed by UV irradiation. DIG-labeled probe (20 µl) was hybridized to the immobilized RNA at 50 °C for 24 h with DIG Easy Hyb buffer (Roche). The membrane was washed two times with 20 × SSC–0.1% sodium dodecyl sulfate at room temperature and then two times with 0.2 × SSC–0.1% sodium dodecyl sulfate at 68 °C. The labeled probes were visualized with the CSPD chemiluminescence detection system (Roche) and Hyperfilm-MP X-ray film (Amersham). The image acquisition was performed with the Geldoc 2000 apparatus (BioRad).

3. Results and discussion

3.1. Identification of As-induced proteins

Before evaluating in a global way the effect of arsenic stress on bacterial physiology, we determined the resistance level of strain ULPAs1 to this toxic compound. Minimal inhibitory concentrations (MIC) for As[III] and for As[V] were found to be 6.6 and 200 mM (the limit of solubility of the product in CDM medium), respectively. Proteome modifications generated by a

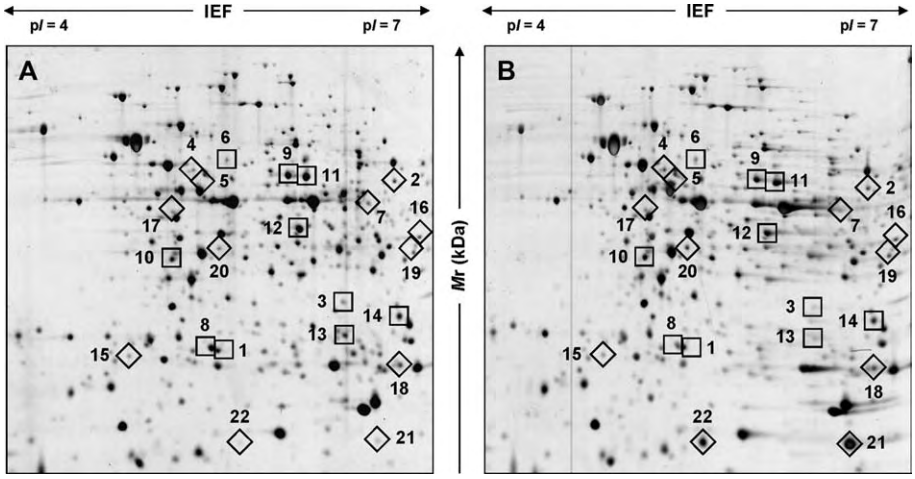

Fig. 1. Comparison of the protein synthesis patterns of *C. arsenoxydans* in the absence (A) or in the presence (B) of arsenite. Cells were grown in MS medium with or without 2.66 mM of As[III] at 28 °C for 30 h. Proteins were separated in IPG pH 4.7 gels in the first dimension and in 11.5% (w/v) polyacrylamide gels in the second dimension. After silver staining, proteins induced (◊) or repressed (□) by arsenic stress were submitted to mass spectrometry. Two-dimensional gels were repeated at least three times for each condition.

concentration of this metalloid corresponding roughly to one-half the MIC were then explored using 2D-electrophoresis. Bacteria were grown in CDM medium [10] to stationary phase and after cell disruption, proteins were separated on 2D SDS-PAGE gels, silver stained, and analyzed by mass spectrometry (see Section 2). Representative patterns of silver-stained proteins are shown in Fig. 1. The overall profile of total soluble proteins in strain ULPAs1 was found to be identical in both growth conditions (Fig. 1a, b). Most proteins were found in the p*I* and mass region ranging from 5 to 7 and 25 to 175 kDa, respectively.

The accumulation level of several polypeptides was affected by at least a factor of two when As[III] was present in the growth medium. To identify these proteins isolated from an organism whose genome has not yet been sequenced, a cross species protein identification strategy was used, as described by Shevchenko et al. [6,8]. This strategy allows the identification of proteins that are not present in the databases using error-tolerant algorithms as described in Fig. 2. When comparing control and stressed samples, we found that among 652 spots, 630 (96.5%) were similar, while 22 (3.5%) showed changes (increase or decrease) by a factor of at least 2. The latter were identified on the basis of the sequences specified in Table 1. Among those, approximately two thirds were homologous to proteins of *Ralstonia solanacearum*, a phylogenetically related bacterium. A relatively good correlation was observed between both theoretical and experimental isoelectric points and molecular masses of the identified proteins. An example of de novo sequencing interpretation of MS/MS spectra and the resulting MS-BLAST results is presented in Fig. 3. Most proteins which displayed a decreased accumulation level in the presence of arsenic (7 out of 22) play a role in intermediary metabolism. In particular, several proteins involved in the metabolism of methionine were accumulated at a lower level in the presence of As[III], e.g. proteins similar to the two isoforms of the adenosylhomocysteinase (spots 9 and 11) and a methionine aminopeptidase (spot 3) in *R. solanacearum*, and a 5,10-methylenetetrahydrofolate reductase (spot 14) in *Chromobacterium*

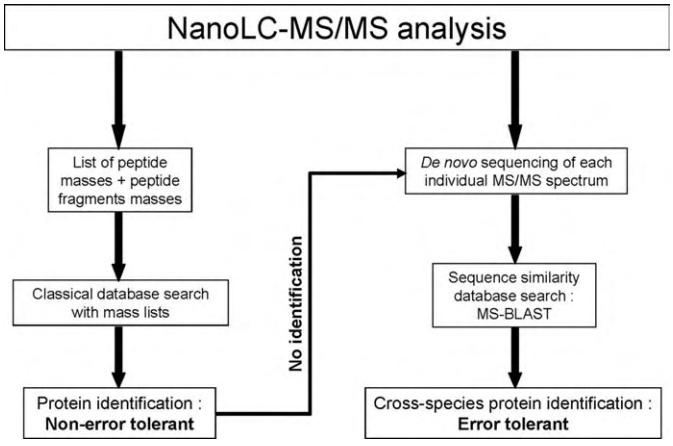

Fig. 2. The strategy of cross-species protein identification starting from a nanoLC-MS/MS analysis. When the classical non-error-tolerant mass-similarity-based search way is not successful, the de novo sequencing strategy is used. This procedure consists in the de novo sequencing interpretation of the MS/MS spectra to deduce amino acid sequence tags followed by the submission of these sequence tags to MS-BLAST algorithms for sequence similarity searches. This kind of searches enable the identification of proteins even if they are not available in the databases by homology with related and sequenced organisms. This is possible as this procedure is tolerant to punctual amino acid errors in the contrary to mass-similarity-based algorithms.

violaceum (Table 1). Proteins that were significantly accumulated in bacterial cells subjected to arsenic stress belong to different functional classes. Some of them are homologous to proteins involved in translation, namely the elongation factor Tu (spot 5) and the tRNA synthetases of various amino acids (spots 4 and 7). Two proteins are similar to proteins involved in the metabolism of purines and pyrimidines in *R. solanacearum*, phosphoribosylformylglycinamidine cyclo-ligase (spot 10) and dihydroorotase (spot 16), respectively, and one protein showed homology with a peptidase (spot 2) of the same organism that may play a role of modulator of gyrase activity. Two proteins involved in the synthesis of membrane components were also induced in strain ULPAs1 in the presence of arsenic: a hypothe-

tical protein in *Leptospira interrogans* belonging to the nucleoside-diphosphate-sugar epimerase family (spot 19) and a D-alanine-D-alanine ligase (spot 20) in *Bordetella pertussis*. These growth conditions also resulted in the increased accumulation of two proteins showing homology with arsenate reductases (spots 21 and 22) in *Shigella flexneri* (Table 1).

3.2. Analysis of arsenic-responsive gene fusions

In a previous work, we constructed a collection of approximately 4000 mutants generated by a *lacZ*-containing reporter gene transposon [10]. Among 16 clones showing arsenic-induced expression we isolated two mutants characterized by an arsenite-oxidase minus phenotype (Table 2, group D). Their analysis permitted the identification of the genes *aoxA* and *aoxB*, coding respectively for the small Rieske-type subunit and the large catalytic subunit of the arsenite oxidase [10]. For the other mutants, the sequencing of the regions flanking the Tn*5*-derivative led to the identification of 14 putative proteins, which have been classified in functional groups as defined by Moszer [20] (Table 2). In mutants M11, M13 and M16 (Group A), the predicted proteins showed similarities with proteins found either in *Burkholderia cepacia* (exonuclease R) or in *R. solanacearum* (two transcription regulators) involved in information and regulation pathway. The coding DNA sequences (CDSs) flanking the transposon in M3, M9, M17, M33, M39, (group B) display high similarities with enzymes involved in intermediary metabolism found in *Bordetella pertussis* (carboxynorspermidine decarboxylase), in *R. solanacearum* (fumarase and probable FDHD protein), in *Rubrivivax gelatinosus* (NAD(P)H-nitrite reductase) and in *Ralstonia metallidurans* (UDP-3-*O*-[3-hydroxymyristoyl] glucosamine *N*-acyltransferase) while in M20, M28, M31, M36, (Group C), the putative proteins display similarities with proteins found in the cell membrane and involved in bacterial motility in *R. solanacearum* (flagellar FLIL transmembrane protein), in membrane structure in *R. metallidurans* (hypothetical lipoprotein) or in energy metabolism or cell division in *Methylobacillus flagellatus* (predicted GTPases and ATPase involved in chromosome partitioning). Group D (M1, M2, M31) contains three proteins involved in arsenic resistance, including the two proteins implicated in transformation of the most mobile form of arsenic (As[III]) to the less mobile (As[V]) [10], as well as a putative efflux pump. Group E (M18) contains one probable protein showing no significant similarity with any protein in databases.

As demonstrated by the quantitative β-galactosidase assay, all the genes coding for these putative proteins were significantly induced in presence of 0.66 mM As[III]. These results were confirmed by the quantitative analysis of mRNA synthesized in induced and non-induced bacteria. In each functional group one mutant was chosen and total RNA was extracted from cells grown in the absence (abs) of arsenic or exposed to As[III] for 15 or 30 min. Hybridization was performed with a probe corresponding to the part of the coding region in which the transposon is inserted. A control was done for each strain with a 16S-rDNA

Table 1a
Identification of *C. arsenoxydans* arsenic regulated proteins

Spot number	Spot intensity No arsenic (–) [a]	Spot intensity Arsenic (+) [a]	Intensity Ratio +/–	MW (kDa)/p*I* (theoretical)	MW (kDa)/p*I* (experimental)
A. Information and regulation pathways					
1	2845	6352	2.5	23772/4.92	23550/5.39
2	577	1225	2	49360/5.69	47110/6.19
3	717	167	0.2	29815/5.91	31300/5.84
4	545	1177	2	47708/4.98	50130/5.33
5	abs	8946	> 1000	43167/5.43	47260/5.35
6	608	1207	2	52342/5.35	52340/5.40
7	abs	1550	> 1000	43496/5.97	43660/5.97
8	4108	2030	0.5	26995/5.38	25280/5.37
B. Intermediary metabolism					
9	8867	3664	0.5	51949/5.78	48710/5.52
10	2187	5699	2.5	37151/5.22	37960/5.26
11	10501	5107	0.5	51949/5.78	49200/5.59
12	9084	4355	0.5	38218/5.42	39140/5.55
13	1665	abs	< 0.001	23787/5.83	26610/5.86
14	4819	2229	0.5	30913/6.25	28560/6.48
15	664	1329	2	27214/5.01	24100/5.00
16	539	1401	2.5	37816/6.43	38100/6.55
17	abs	1180	> 1000	40939/5.23	42400/5.26
C. Cell envelope and cellular processes					
18	1036	2688	2.5	25894/5.91	23360/6.31
19	270	1344	5	37973/7.70	37980/6.50
20	617	1499	2.5	33558/5.16	38060/5.39
D. Other functions					
21	abs	18757	> 1000	18826/5.95	17980/5.99
22	abs	8640	> 1000	18826/5.95	17870/5.43

[a] Intensity value for each spot was measured using the PDQUEST software package. The mean values of spot intensity were calculated by comparing the eight gels obtained for each condition (+ or – As). Spots showing more than 15% variation were not considered.

Table 1b
Identification of *C. arsenoxydans* arsenic regulated proteins

Spot number	Sequences [a]	Putative function [b]	Accession number[c]	Induction
A. Information and regulation pathways				
1	FLMIEQQIR HVTTLEIEPEL LQELALRK	Probable protein-L-isoaspartate *O*-methyltransferase [*Ralstonia solanacearum*]	Q8Y1J3	2.5
2	MGQGVNYVTGDYSR IQYPVEEITIAGN TVEAAYNIAR GASGFWVE NSEGASVYA KSTFLLDVTGK LAAPETIG	Putative PMBA protein [*Ralstonia solanacearum*]	Q8Y0U8	2
3	AGVTTGELDR LGSEVLDYITPFVK AELTYECM MFILGEEPALAK TAEDIEGMR	Probable methionine aminopeptidase protein [*Ralstonia solanacearum*]	Q8XZJ2	0.2
4	SGAAYAVIIGED LELNSLGNAEER GIGAVTDIVEK NPAMQEMVGGA TPIVEPTALFAR LWDDLGL	Histidyl-tRNA synthetase [*Ralstonia solanacearum*]	Q8Y029	2
5	LLDQGQAGDNVGVLLR FLLPVEDVFSISGR TQVTTCTGVEMFR ALDSYIPTPER FPGDDLPIIK LTAAIATVLS TTLTAAIA IIVFLNK EHILLAR	Elongation factor Tu [*Ralstonia solanacearum*]	Q8XGZ0	> 1000
6	NYFYPDLPK TPLLELVTEPDMR CDGNMQEGSFR EIDAAPVSAAQLAVLLHR NYFYPDLPK FMEDAINYEVR SAAEAVAYAK QIELIEDGGR IIDDVLAANTK	Aspartyl/glutamyl-tRNA (Asn/Gln) amidotransferase subunit B [*Ralstonia solanacearum*]	Q8Y3C6	2
7	LSGAPIGIGQLLK VLLAQEIVAR VEAGTVVVQVGK IDGAVISDK GGTDQK NLLVGR	Probable tyrosyl-tRNA synthetase protein [*Ralstonia solanacearum*]	Q8Y240	> 1000
8	LLTFMMEDPR MLHDALDAFAR KYDMDLETIR	Hypothetical protein PhoU [*Nitrosomonas europaea*]	Q82TX5	0.5
B. Intermediary metabolism				
9	GVTEETTTGVHR SKFDNLYGCR ESLVDGIKR ATDVMIAGK	Adenosylhomocysteinase [*Ralstonia solanacearum*]	Q8Y387	0.5
10	EVDAGDALVEAIRKPFAK NCGIGMTVIV LNADFHG PVLVSGTDRGTK	Phosphoribosylformylglycinamidine cyclo-ligase [*Ralstonia solanacearum*]	Q8XW52	2.5

(continued)

Table 1b *(continued)*

Spot number	Sequences [a]	Putative function [b]	Accession number[c]	Induction
11	ESLVDGIK ATDVMIAGK ESLVDGIKR FDNLYGCGR IAETEMPGLMAIR VAVLAGYGDVGK TEETTTGVHR	Adenosylhomocysteinase [*Ralstonia solanacearum*]	Q8Y387	0.5
12	ELTGDLYFGQPR TPAAITGGLELGVGR GYWIDAASTLR DLPLAEIESIIR VGLVGWR	3-Isopropylmalate dehydrogenase [*Ralstonia solanacearum*]	Q8XXX5	0.5
13	MDEGGLVSDDLIIGLV LILLGAPGAGK AGTPLGLAAK VTGEELLQR	Adenylate kinase [*Bordetella pertussis*]	P39068	< 0.001
14	FSDMCGAELPR FYNADAYFR AFGLDVVTD SAGHDAAPHLSC SLEFFPPK SYNDDTDSIR DDLASYVR	5,10-Methylenetetrahydrofolate reductase [*Chromobacterium violaceum*]	A-E016913	0.5
15	AVLAASLFHYGQHTVQEAK GAGEILLTSMDR IIPCLDVTAGR FMSEQGIAMR IVVAIDAK GVNFLELR TGLDAI	Imidazole glycerol phosphate synthase [*Burkholderia multivorans*]	Q845U7	2
16	FLGTDSAPHPK YYCLPVLK ALVAAATSG VVFEHITTK	Dihydroorotase [*Ralstonia solanacearum*]	Q8Y249	2.5
17	LLIEEGADIK DPAEIEASK FNFDSNALYR	Succinyl-CoA synthetase, beta chain [*Bordetella pertussis*]	Q7WKM5	> 1000
C. Cell envelope and Cellular processes				
18	GCGGGILSESMAR LEWINSLAPLAAK	Probable 3-demethylubiquinone-9 3-methyltransferase protein [*Ralstonia solanacearum*]	A-L646061	2.5
19	YGNVMASR LYESLVSR VVVLSTDK FYIGDLR	Hypothetical nucleoside diphosphate sugar epimerase [*Leptospira interrogans*]	Q9S4H0	5
20	SLVPMAAK VGVLFGGR YEAVVLVEQFV HGVPTP SLAELAA ALPLVEI	D-Alanine-D-alanine ligase [*Bordetella pertussis*]	Q7WFS4	2.5
D. Other functions				
21	YNVLFLCTGNSAR WGFEDPAAATGTDEE SIMAEAMINTMGK	Hypothetical arsenate reductase [*Shigella flexneri*]	Q7UC02	> 1000
22	SIMSEALIATMG NVLFLCTGNSAR	Hypothetical arsenate reductase [*Shigella flexneri*]	Q7UC02	> 1000

[a] Peptide sequences obtained by de novo sequencing and matching with protein sequences present in databases.

[b] Putative function, between brackets is indicated the organism in which the protein with greatest homology is found according to amino acid conservation and sequence coverage.

[c] Accession number corresponding to the homolog protein.

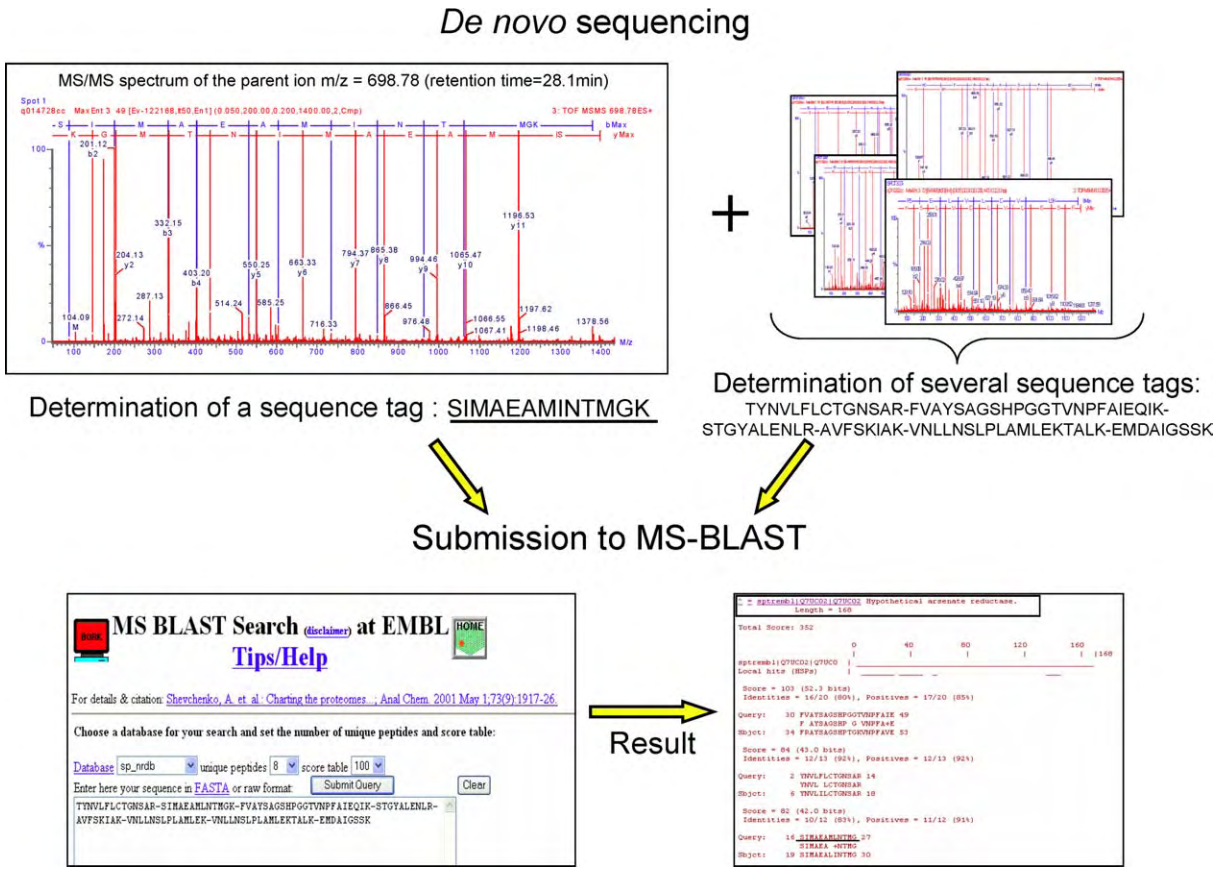

Fig. 3. Example of the treatment of nanoLC-MS/MS results by de novo sequencing. The individual MS/MS spectra are treated and interpreted by de novo sequencing to deduce a partial or complete amino acid sequence. Here the spectra are treated with the PepSeq program (Waters, Milford, Massachusetts, USA). Then all deduced sequence tags are submitted to the MS-BLAST program (http://dove.embl-heidelberg.de/Blast2/) to perform sequence similarity searches in the available databases and thus identify the proteins by sequence homologies in available phylogenetically related organisms.

probe. The synthesis of reporter gene mRNA was significantly up-regulated in each mutant when exposed to arsenic, while the control remained stable (only one control shown) (Fig. 4).

3.3. Biological functions of proteins and genes induced by arsenic

The combination of a mutational analysis and a proteomic approach led to the identification of 16 genes and 22 proteins that are regulated by arsenic. They belong to different functional categories, including information and regulation pathways, intermediary metabolism, cell envelope (see above). This demonstrates that the response of this bacterium to the toxic metalloid is not limited to changes in arsenic speciation but is rather pleiotropic, since it results in a great variety of biological effects.

Genes and proteins, classified in the intermediary metabolism category (B) are involved in various metabolic pathways, such as amino-acids and protein synthesis, TCA cycle, carbohydrates and metabolism suggesting that arsenic affects the general physiology of the cell, including energy metabolism. The most dramatic change was found with the disappearance of spot 13 corresponding to the adenylate kinase, involved in ADP synthesis (Table 2). This is in agreement with the observation that arsenic has a negative effect on ADP and ATP production in *E. coli* [21,22]. However, adenylate kinase is essential for the cell: it makes ADP from AMP and ATP. Since the growth of strain ULPAs1 was not inhibited in the presence of the arsenic concentration used in the 2D-protein electrophoresis experiment, it is likely that either adenylate kinase was modified post-translationally moving to another position in the gel, or like in *E. coli* [23] the cell harbors an isoform of this enzyme.

Arsenic treatment revealed a decreased synthesis of several enzymes involved in sulfur metabolism, such as an adenosylhomocyteinase. Recently, Fauchon et al. [24] have shown that yeast cells treated by the metal Cd^{2+} respond by converting most of the sulfur assimilated by the cells into glutathione, thus reducing the rate of sulfur used in protein synthesis. More importantly, Haugen et al. [25] have demonstrated the existence of a similar response in yeast in the presence of arsenic. The results presented here further supports a modification of the sulfur assimilation profile in cells subjected to arsenic stress, in agreement with the fact that arsenic shows a high affinity with sulfhydryl groups [26,27].

Several proteins and genes involved in the synthesis of cell envelope components (category C) were up-regulated, suggesting that both the organization (lipoproteins, peptidoglycan and LPS) as well as the functioning (transport and mobility) of the bacteria may be changed in cells grown in the presence of arsenite. In particular, we observed the up-regulation of a pu-

Table 2
Identification of *Caenibacter arsenoxydans* arsenic induced protein coding genes

Name	Gene name	Putative function [a]	Accession number [b]	%Identity (identical amino acids/total amino acids)	Induction [c]
A. Information transfer and regulation pathways					
M11	*rnr*	Exoribonuclease R [*Burkholderia cepacia* R18194]	AY728030	63% (245/385)	2
M13		Probable transcription regulator protein [*Ralstonia solanacearum*]	AY728029	40% (93/229)	3
M16		Probable transcription regulator protein [*Ralstonia solanacearum*]	AY728031	51% (139/269)	3
B. Intermediary metabolism					
M3		Carboxynorspermidine decarboxylase [*Bordetella pertussis*]	AY728032	68% (251/365)	3
M9	*fumC*	Probable fumarate hydratase class II (Fumarase) protein [*Ralstonia solanacearum*]	AY728025	58% (153/261)	2
M33	*fdhD*	Probable FDHD protein homolog [*Ralstonia solanacearum*]	AY728035	64% (175/271)	2
M39	*nirB*	NAD(P)H-nitrite reductase [*Rubrivivax gelatinosus* PM1]	AY728037	76% (333/434)	2,5
M17		UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase [*Ralstonia metallidurans* CH34]	AY728027	62% (52/83)	3,5
C. Cell envelope and cellular processes					
M36	*fliL*	Probable flagellar FLIL transmembrane protein [*Ralstonia solanacearum*]	AY728028	50% (33/65)	3,5
M31		ATPases involved in chromosome partitioning [*Methylobacillus flagellatus* KT]	AY728036	48% (109/225)	2,5
M20		Hypothetical lipoprotein [*Ralstonia metallidurans* CH34]		33% (50/151)	3,5
M28		Predicted GTPases [*Methylobacillus flagellatus* KT]	AY728033	66% (184/275)	2,5
D. Other functions					
M30	*acr3*	Putative arsenite efflux pump ACR3 and related permeases [*Erwinia carotovora*]	AY728034	58% (176/301)	4
M1	*aoxA*	Arsenite oxidase small subunit [*Caenibacter arsenoxydans*]	AF509588	Published	
M2	*aoxB*	Arsenite oxidase catalytic subunit [*Caenibacter arsenoxydans*]	AF509588	Published	
E. Similar to unknown proteins					
M18		Conserved hypothetical protein [*Pseudomonas syringae*]	AY728026	22% (163/711)	3

[a] Functional classification of protein coding genes as referred by I. Moszer (1998) [20], between brackets is indicated the organism in which the protein with greatest homology is found according to sequence conservation.
[b] Accession number corresponding to sequence determined in *Caenibacter arsenoxydans*.
[c] Quantitative enzyme assay-fold induction in the presence of 4 mM AsIII.

Table 3
List of primers which were used in transcription analysis

Genetic locus (inserted orf)		Primer set	Amplified size (nt)	Primer name and sequence (5'—3')	
M 9	*fumC*	M9a/M9b	107	M9a	AACACCACAATCCCGAGT
				M9b	CAGCATCACGCAGAAACT
M 16		M16a / M16b	345	M16a	GGTTGTGCATGGAGACAA
				M16b	TACGGAAGCAGTGCTACG
M 18		M17a/M17b	128	M18a	TCAGCATCAATGGCATCT
				M18b	ATGGATTTCGCTGGTTTC
M 20		M20a/M20b	266	M20a	CAGGCGGAATACTGGTTT
				M20b	CCATGTGCATGATGCTTG
M 1	*aox*	AOXA1/AOXA2	1400	AOXA1	AGCACTCGATCTTTTGCAG
				AOXA2	CGCTGCAAATGGGATACG
16S RNA	*rrn*	rD1/fD1	1451	rD1	AAGCTTAAGGAGGTGATCCAGCC
				fD1	AGAGTTTGATCCTGGCTCAG

tative gene coding an arsenite efflux pump (*acr3*). This is consistent with the modifications involved in the adaptation of cells to an arsenic-rich toxic environment. In addition, different proteins and genes involved in arsenic transformations were also detected (category D). Mutagenesis approach led to the identification of *aoxA* and *aoxB* genes, involved in synthesis of the small Rieske subunit and of the large catalytic subunit, respectively, of the arsenite oxidase [10]. The identification of arsenic-regulated proteins indicated that ULPAs1 strain is harboring two *arsC* genes, encoding arsenate reductase, the expression of which was strongly induced by the presence of arsenite in the medium. These two functional *arsC* genes are probably the indicators of the presence of at least one *ars* operon in the genome of this strain. The fact that *aox* knock-out mutants still display resistance to arsenic, but at rate lower than that observed in the wild type [10], can also be connected to the occurrence in ULPAs1 of one or two *ars* operon(s) that also include an efficient arsenite efflux pump. The *acr3* gene, found among the Tn*5*-insertion mutants, may belong to such an operon. These results demonstrate that arsenite oxidase may have a second role. Lebrun et al. [28] have shown that arsenite oxidase is an ancient bioenergetic enzyme, which might have given a phenotypical benefit to microbes living in arsenic rich environment. Indeed, arsenite oxidase activity is considered as a resistance mechanism converting highly toxic arsenite to relatively less toxic arsenate as well as a potential energetic mechanism. Nevertheless, and although this energetic activity has easily been demonstrated for chemolithotrophic bacteria, these microorganisms have not been shown to derive major energy from arsenite oxidation in growth experiments [3,29,30], which is also the case for *C. arsenoxydans* [10].

Arsenic has been shown to induce DNA damage in eukaryotic cells. Studies based on the analyses of cell lines in culture, animal models or on clinical studies have shown that this toxic metalloid interferes with DNA synthesis and repair, and as a consequence is an efficient agent for carcinogenesis, teratogenesis as well as mutagenesis [31–33]. A recent study, based on the incubation of *E. coli* cells with subinhibitory concentration of sodium arsenite [34], described the effect of arsenic on several apparently unrelated cellular functions, including general recombination, conjugation, transposition,

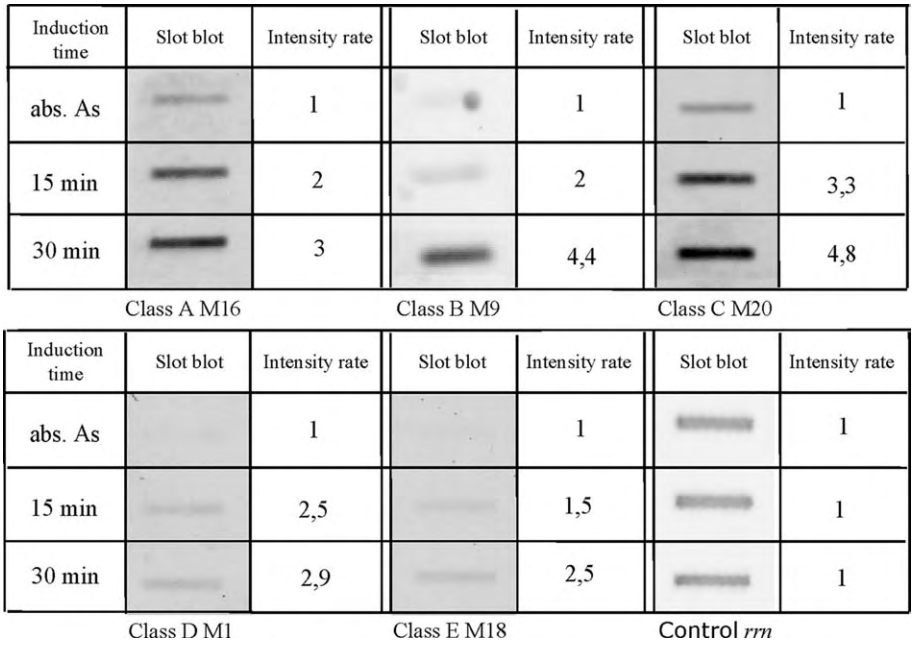

Fig. 4. Slot blot analysis of mRNAs synthesized in induced and non-induced ULPAs1 wild strain. Total RNA was extracted from cells grown in absence of arsenic (abs As) or exposed to arsenite for 15 or 30 min (0.66 mM). Hybridization was performed with the probe corresponding to part to the coding region in which the transposon is inserted. Probes correspond to genes detected in M16, M9, M20, M1 and M18, respectively.

plasmid stability [34]. Moreover, two studies, using a proteomic approach, and investigating the interaction of *E. coli* K12 with cadmium (Cd) and that of *Pseudomonas aeruginosa* with arsenic showed that the exposure of cells to these toxic compounds stimulated the synthesis of cadmium or arsenic regulated-proteins. Some of them were identified as taking part in stress regulons (heat-shock, oxygen stress, general stress and/or SOS response) or in the formation of *S*-adenosylmethionine involved in the synthesis of amino acids [35,36]. On the other hand, Rossbach et al. [37], by using a mutagenesis approach, identified in *P. fluorescens*, a sensor/regulator protein, the expression of which is regulated by Cd. In the case of strain ULPAs1, while the results obtained by the mutagenesis approach suggest an impact of arsenic on RNA degradation and on the regulation of gene expression, the data generated by the proteomic approach clearly show that arsenic interferes with amino acids and proteins synthesis (category A). The latter results can be connected with the observation made by Wilkins et al. [38], showing that different isoforms of the elongation factor Tu can be differentially expressed in response to a pH stress. The fact that these two approaches did not show an extensive overlap may be related to the different methods used. While the mutagenesis approach visualized early and transiently expressed genes (15 and 30 min after arsenic exposure), the two-dimensional protein gels monitored specifically the cytoplasmic late arsenic-induced proteins (after 48 h exposure). The former method is targeting initiation of transcription while the latter is targeting the stability of proteins. Our results show that the response to arsenic stress starts rapidly after exposure to arsenic and lasts during the whole exponential growth of bacteria until stationary phase. By covering different steps and levels of the induction, these two approaches are complementary.

4. Concluding remarks

In this study, we analyzed the molecular response of *Caenibacter arsenoxydans* cells to arsenic stress, by combining both proteomic analysis and transposon mutagenesis. The first approach identified 22 proteins that were differentially accumulated during stationary growth phase and the second approach pointed out 16 genes that were expressed during the early stage of arsenic stress. In addition to the genes involved in arsenite-oxidation (*aoxA* and *aoxB*), previously described [10], the present study led to the identification of one new gene and two new proteins directly involved in arsenic resistance mechanisms. These results provide evidence that the response to arsenic stress also involves other cellular functions including both intermediate and energy metabolism as well as the structure and the functioning of the cell envelope. Such functions may either reflect the toxic effect of arsenic on cells or contribute to the detoxification of this element.

Interestingly, this work provided a convincing demonstration of the efficiency of the cross-species protein identification strategy, as it led to the successful identification of proteins in spite of the lack of knowledge of the genome sequence of the organism while none of its proteins sequence was available in databases. With the constantly increasing number of available and sequenced genomes, the de novo sequencing interpretation of the MS/MS spectra followed by sequence similarity searches enable the extension of the proteomic studies to a

very large set of unsequenced organisms using phylogenetically related sequenced organisms.

Finally, the combination of proteomics and mutagenesis proved to be a powerful tool to decipher the arsenic response of *Caenibacter arsenoxydans*. Since these two approaches focus on different biological objects, proteins or RNAs, they are complementary to investigate the molecular changes involved in bacterial stress response.

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