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# Comparative Proteome Analysis of *Bacillus anthracis* with pXO1 Plasmid Content

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***Bacillus anthracis*** **the causative agent of anthrax, is an important pathogen among the** ***Bacillus cereus*** **group of species because of its physiological characteristics and its importance as a biological warfare agent. Tripartite anthrax toxin proteins and a poly-D-glutamic acid capsule are produced by** ***B. anthracis*** **vegetative cells during mammalian hosts infection and when cultured in conditions that are thought to mimic the host environment. To identify the factors regulating virulence in** ***B. anthracis*** **the whole cell proteins were extracted from two** ***B. anthracis*** **strains and separated by narrow range immobilized pH gradient (IPG) strips (pH 4-7), followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins that were differentially expressed were identified by the peptide fingerprinting using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS). A total of 23 proteins were identified as being either upregulated or downregulated in the presence or absence of the virulence plasmid pXO1. Two plasmid encoded proteins and 12 cellular proteins were identified and documented as potential virulence factors.**

***Keywords***: *B. anthracis*, 2-DE, MALDI-TOF MS, virulence factor, pXO1

Anthrax is a zoonotic disease caused by the Gram-positive, non-motile, endospore-forming, rod-shaped, facultative anaerobe *Bacillus anthracis* (Mock and Fouet, 2001). The infection and its severity depend on the route of entry of the *B. anthracis* spore. The most fatal form of anthrax is caused by spore inhalation (Dixon *et al*., 1999). During the course of infection, the spores germinate inside host macrophages to form fast-dividing vegetative cells that secrete three classical toxins encoded by the plasmid pXO1. The protective antigen (PA) facilitates the formation of two distinct bipartite virulence factors, edema factor (EF) and lethal factor (LF) as common components. PA binds to specific host receptors and translocates EF and LF in to cells. In this way, these virulence factors affect their detrimental activities within cells.

The virulence of *B. anthracis* is governed by complex regulatory circuits whose underlying mechanisms are not fully understood (Bourgogne *et al*., 2003; Fouet and Mock, 2006). Some observations have suggested that the expression of both plasmid-encoded and chromosomally-encoded genes is essential for virulence. AtxA, a pXO1-encoded protein, plays a central role in virulence regulation. Furthermore, several chromosomal and plasmid genes are regulated, either positively or negatively, by this master transcription regulator (Dai *et al*., 1995; Hoffmaster and Koehler, 1999; Bourgogne *et al*., 2003; Fouet and Mock, 2006). Two pXO2 encoded regulatory proteins, AcpA and AcpB, regulate capsule synthesis and are thought to act downstream of AtxA (Fouet and Mock, 2006). Impinging on virulence factor expression through cellular regulators such as AbrB, a chromosomally-encoded transition state regulatory protein and sigma factor $\sigma^H$ have also been shown to modulate AtxA synthesis (Strauch *et al*., 2005).

Environmental factors such as $CO_2$ and temperature have also been shown to play important roles in virulence factor regulation (Sirard *et al*., 1994; Dai and Koehler, 1997; Fouet and Mock, 2006). It has not yet been determined whether the induction of toxin and capsule gene expression by $CO_2$-bicarbonate is exerted through the AtxA protein or through some independent mechanism. Differences in the susceptibilities of warm-blooded and cold-blooded animals to *B. anthracis* have been described previously. However, temperature is not a critical factor for germination; instead, temperature appears to be an important signal for the regulation of toxin production in an AtxA dependent manner (Lee and Schneewind, 2001).

Genome sequencing of *B. anthracis* strains has been initiated to better understand its biochemistry and metabolism, which may provide insights for novel drug targets. The accumulation of genomic information combined with advances in protein separation and identification techniques has advanced the technology of proteomics (Cash, 2000). High-throughput, two-dimensional electrophoresis (2-DE) displays and furthers the identification of the protein complement of the genome that is expressed in an organism, which may lead to the identification of disease-specific markers, factors involved in virulence and virulent strains of pathogenic bacteria (Poxton and Brown, 1979). Extensive genomic and bioinformatic analyses have suggested the participation of proteins other than the three classical proteins in anthrax pathogenesis (Ariel *et al*., 2002, 2003).

The proteomic components (both intracellular and secreted)

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of *B. anthracis* have been subjected to recent analysis to identify functionally active genes, subcellular proteins, disease-specific proteins, and immunoreactive proteins (Francis *et al*., 2005; Lamonica *et al*., 2005; Wang *et al*., 2005; Chitlaru *et al*., 2006). It is clear that regulatory factors that are not secreted into the intercellular space can modulate virulence expression (Okstad *et al*., 1999; Gohar *et al*., 2002). Thus, changes in expression of these regulatory factors might alter the virulence activity of the bacterium in host cells. In our study we used 2-DE and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) techniques to investigate the differences in the cellular proteins of two *B. anthracis* strains in the presence or absence of the virulence regulatory plasmid pXO1. Several proteins were found to be upregulated or downregulated in both strains under induced toxin expression conditions. Expressed proteins that had clear differences in intensity and were uniquely present in only one strain were subjected to mass spectrometry and further identification.

## Materials and Methods

### Bacterial culture and sample preparation for 2-DE

*B. anthracis* Sterne (pXO1$^+$, pXO2$^-$) and plasmids deleted Δ Sterne (pXO1$^-$, pXO2$^-$) strains were used in this study. Cells were grown aerobically in 100 ml Nutrient broth yeast extract (NBY) medium [containing 0.8% (w/v) nutrient broth (Difco Laboratories, USA), 0.3% yeast extract (Becton, Dickinson and Company, USA), and 0.5% glucose] supplemented with 0.9% $NaHCO_3$ and 5% $CO_2$ at a temperature mimicking host conditions (37°C). The cells were collected at the late exponential phase by centrifugation for 10 min. The cells were treated with lysis buffer (9.5 M urea, 2.5% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS), 40 mM dithiothreitol (DTT), 0.12% carrier ampholytes, 0.0012% bromophenol blue) and solubilized by sonication on ice as described by Wang *et al.* (2005).

### 2-DE separation of proteins and spot quantitation

Isoelectric focusing (IEF) was performed using immobilized pH gradient (IPG) dry stripes (pH 4.0-7.0 NL, 7 cm, Bio-Rad Laboratories, USA). Samples of 60 μg were applied to IPG dry stripes using an in-gel sample rehydration technique according to the manufacturer's protocol (Bio-Rad Laboratories) and essentially as described by Jung *et al.* (2008). Gel electrophoresis was carried out on 12% polyacrylamide gels. Protein spot detection and MALDI-TOF MS analyses were performed next, followed by the gel staining with Coomassie blue (Poxton and Brown, 1979). Following automatic spot detection on 2-DE gels using PDQuest software (version 7.0, Bio-Rad Laboratories) the gels were manually warped and their common spots were matched to generate average gels. Protein spots of two different samples were compared and analyzed. Differentially expressed protein spots on two different gels were selected for further characterization.

### In-gel digestion, MALDI-TOF MS analysis and database search

Selected protein spots were digested enzymatically in-gel in a manner similar to that described by Seo *et al*. (2004, 2008) using modified porcine trypsin (Promega, USA). Gel pieces were washed with 50% acetonitrile to remove sodium dodecyl sulfate (SDS) and salt and dried to remove the solvent. They were then rehydrated with trypsin (8-10 ng/μl) and incubated for 16 h at 37°C. To terminate the of proteolytic reaction 5 μl of 0.5% trifluoroacetic acid were added. Peptides were extracted with 50% aqueous acetonitrile.

Peptide analysis was performed using an Ultraflex MALDI-TOF MS (Bruker Daltonics, Germany). In brief, peptides were evaporated with an $N_2$ laser at 337 nm using a delayed extraction approach and were accelerated with a 20-kV injection pulse for time of flight analysis. The search program Profound, developed by Rockefeller University (http://prowl.rockefeller.edu/prowl-cgi/profound.exe), was used against the online NCBInr database with free access on the internet (based on a Z score of greater than 1.65) and MASCOT developed by Matrix Science Ltd. (http://www.matrixscience.com), was used for protein identification by peptide mass fingerprinting. The search parameters were a maximum of one missed cleavage by trypsin, fixed modification of oxidation, charged state of +1, and a mass tolerance of ±1 Da.

### Protein location prediction

The cellular locations of the identified *B. anthracis* proteins were predicted using PSORTb v.3.0 (http://www.psort.org/psortb/, Gardy *et al*., 2005). In addition to PSORTb, additional sequence analyses were performed in combination with Prosite (http://www.expasy.ch/prosite/) and Pfam (http://pfam.sanger.ac.uk/). The presence of both cleavable and uncleavable N-terminal signal sequences, transmembrane domains, lipoprotein signals, were predicted by PSORTb, and the presence of specific sequence features such as the cell wall anchoring LPXTG motif and the S-layer homology (SLH) domain, were predicted by PROSITE and Pfam. Proteins that lacked such sequence features were categorized as cytosolic.

## Results

The total cellular proteins of *B. anthracis* Sterne (pXO1$^+$ pXO2$^-$) and Δ Sterne (pXO1$^-$ pXO2$^-$) were extracted after growing in NBY-$CO_2$ medium, which is known to induce the expression of toxin and capsule proteins (Chitlaru *et al*., 2006). A combination of two pre-mass spectrometric methods was used to identify the proteins from *B. anthracis* vegetative cells protein separation by p*I* using IEF on IPG strips and protein separation by molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). According to the reported theoretical distribution of *B. anthracis* cellular proteins in a 2-DE reference gel approximately 60% of the proteins had theoretical p*I* values between 4 and 7 (Wang *et al*., 2005). Considering the theoretical distribution of proteins and the difficulty of handling the alkaline proteins we considered a pH range of 4-7 as a standard analytical window for our experiments. From the Coomassie blue-stained gel in the p*I* range of 4-7, a total of 147 protein spots were identified 26 of them were either up- or down-regulated between the two protein samples. A total of 3 protein spots were observed in *B. anthracis* Sterne but were not present on the 2-DE gel of *B. anthracis* Δ Sterne. Equivalent amounts of proteins (60 μg) were used for the analysis and the differentially expressed 29 protein spots on the gel were excised and destained, followed by in-gel digestion using trypsin for further analysis. The masses of the resulting peptide fragments were measured by MALDI-TOF MS, resulting in protein identification. A total of 23 proteins were identified from the 29 processed protein spots. The information regarding these proteins and their cellular functions are listed in Table 1 and 2, and the spots

**Table 1.** Differentially expressed *B. anthracis* cellular protein between two strains as identified by MALDI-TOF

| Spot number | Accession no. | *B. anthracis* Ames (Accession no.) | Proteins | MW (kDa) | p*I* | No. of peptides | % coverage |
|---|---|---|---|---|---|---|---|
| 1 | NP_842945 | BA0401 | Tellurium resistance protein | 21.18 | 4.5 | 45 | 88% |
| 2 | NP_842892 | BA0345 | Alkyl hydroperoxide reductase, subunit C | 20.87 | 4.8 | 19 | 76% |
| 3 | NP_845193 | BA2849 | NLP/P60 family protein | 37.05 | 4.6 | 38 | 88% |
| 4 | NP_847538 | BA5364 | Eno phosphopyruvate hydratase | 46.44 | 4.7 | 66 | 100% |
| 5 | NP_844655 | BA2267 | Alcohol dehydrogenase | 37.51 | 5.2 | 89 | 100% |
| 6 | NP_847539 | BA5365 | phosphoglyceromutase | 56.61 | 4.9 | 25 | 100% |
| 7 | NP_846517 | BA4283 | peptidyl-prolyl cis-trans isomerase, cyclophilin-type | 15.64 | 5.4 | 27 | 100% |
| 8 | NP_843535 | BA1035 | MarR family transcriptional regulator | 18.24 | 5.6 | 81 | 92% |
| 9 | NP_847074 | BA4873 | Alanine dehydrogenase | 40.15 | 5.2 | 89 | 100% |
| 10 | NP_846156 | BA3909 | 2-oxoglutarate ferredoxin oxidoreductase subunit beta | 31.58 | 5.7 | 14 | 100% |
| 11 | NP_846421 | BA4184 | Pyruvate dehydrogenase complex E1 component, alpha subunit | 41.42 | 5.5 | 44 | 94% |
| 12 | NP_842858 | BA0309 | 1-pyrroline-5-carboxylate dehydrogenase | 56.44 | 5.4 | 32 | 91% |
| 13 | NP_843388 | BA0876 | Acyl-CoA synthetase | 58.57 | 5.4 | 71 | 85% |
| 14 | NP_843613 | BA1127 | S-layer protein, putative | 24.81 | 7.0 | 57 | 100% |
| 15 | NP_843714 | BA1242 | Hypothetical protein | 28.02 | 5.1 | 27 | 82% |
| 16 | NP_842582 | BA0011 | Glutamine amidotransferase subunit PdxT | 21.30 | 5.1 | 37 | 86% |
| 17 | NP_845609 | BA3321 | Phosphoserine aminotransferase | 41.37 | 5.4 | 53 | 93% |
| 18 | NP_ 842705 | BA0137 | DNA-directed RNA polymerase, Alpha | 38.75 | 4.83 | 28 | 85% |
| 19 | NP_844606 | BA2214 | Hypothetical protein | 36.15 | 4.9 | 14 | 82% |
| 20 | NP_847490 | BA5314 | Tyrosyl-tRNA synthetase | 47.3 | 5.3 | 23 | 82% |
| 21 | NP_844387 | BA1981 | Siderophore biosynthesis protein, putative | 69.9 | 5.1 | 35 | 79% |
| 22 | NP_052750 | pXO1-54 | S-layer protein | 44.91 | 6.7 | 25 | 81% |
| 23 | NP_052809 | pXO1-113 | Spore germination protein XA | 55.1 | 6.6 | 28 | 78% |

were labeled on the Coomassie blue-stained protein gel (Fig. 1).

Under the induced toxin expression conditions used in this study total of 15 proteins were identified in *B. anthracis* Sterne. Of these 15 proteins, 13 were upregulated (3- to 15-fold), and 2 were expressed only in *B. anthracis* Sterne. Eight proteins were identified as upregulated (3- to 9-fold) in *B. anthracis* Δ Sterne (Table 3). The identities of the protein spots were established by their positions in the protein map and were confirmed by the MALDI-TOF MS analysis.

## Discussion

Although the 2-DE method is considered as a standard method for separating proteins, technical limitations are common as many gene products have similar physicochemical properties and are difficult to separate each other in 2-DE experiments. In *B. anthracis* it has been demonstrated that most protein spots are displayed close together in the region of pH 4-7 (Wang *et al*., 2005). Protein identification of a given spot might be hampered by contamination by nearby spots. In our study we selected protein spots that were clearly separated and had clear differences in intensity.

The expression levels of extracellular proteins also differ between strains in the presence or absence of the plasmid pXO1 (Lamonica *et al*., 2005). This difference might result from the presence of plasmid-encoded regulators such as AtxA. Likewise, in *B. anthracis* toxin expression is regulated by the transition state regulator AbrB in a growth phase-dependent manner (Saile and Koehler, 2002). In our study, we have also observed changes in cellular protein expression levels in the presence or absence of pXO1.

Of the proteins that were identified and differentially expressed on the 2-DE gel, most were functionally related to cellular metabolism and might be required to sustain life. The proteins that were identified and upregulated in *B. anthracis* Δ Sterne were involved in cellular metabolic processes (BA0876), protein folding (BA4283), energy production (BA5364, BA4184), amino acid biosynthetic processes (BA0309), and DNA binding activities (BA3909, BA0137). One identified protein had an unknown function (BA1242).

By contrast, in *B. anthracis* Sterne several ion-binding proteins (BA2267, BA5365, BA4873, and BA2214) were upregulated. Two were involved in zinc ion binding activities. One of the classical anthrax toxin subunits, LF, is a metalloprotease that has zinc as a cofactor (Klimpel *et al*., 1994). In a previous study, it was shown that proteins involved in Zn ions transport

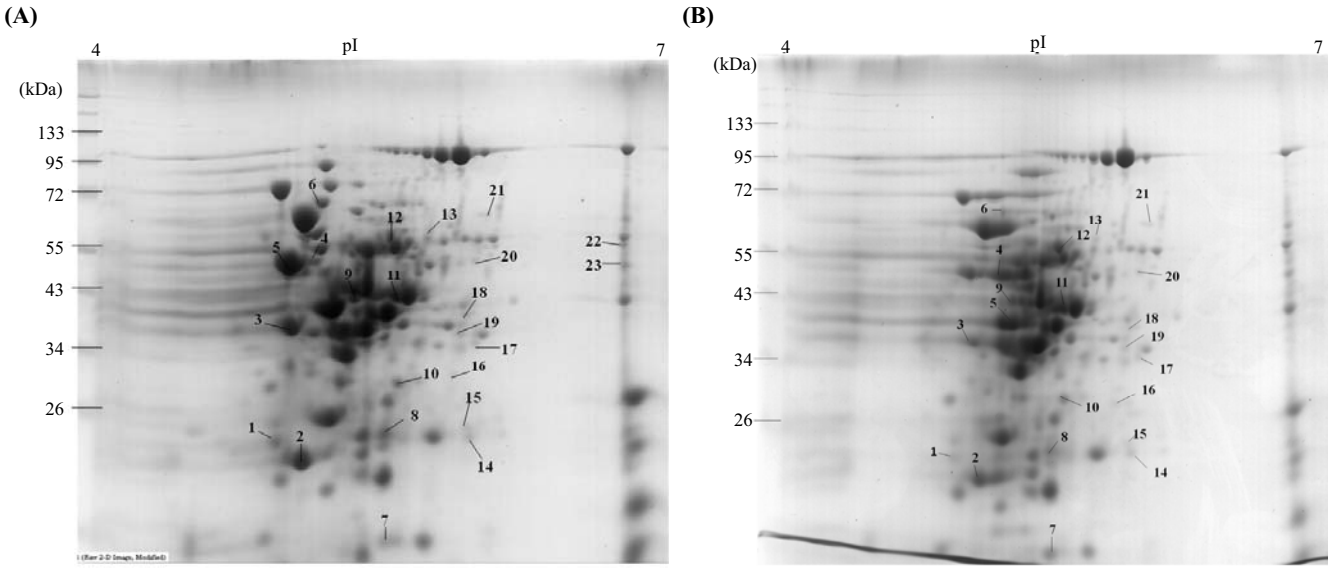


**Fig. 1.** 2-DE gel presenting whole cell protein spots within the range of pH 4-7 (A) Gel representing the cellular proteins of *B. anthracis* Sterne. Differentially expressed proteins that were subjected to further identification are marked on the gel. (B) Gel representing the proteins of *B. anthracis* Δ Sterne.

**Table 2.** Identified proteins and their cellular function

| Accession no. | Proteins | Cellular function[a] |
|---|---|---|
| BA5364 | Eno phosphopyruvate hydratase | Magnesium ion binding; Phosphopyruvate hydratase activity |
| BA4283 | Peptidyl-prolyl cis-trans isomerase, cyclophilin-type | Peptidyl-prolyl cis-trans isomerase activity |
| BA3909 | 2-oxoglutarate ferredoxin oxidoreductase subunit beta | Catalytic activity; Thiamin pyrophosphate binding |
| BA4184 | Pyruvate dehydrogenase complex E1 component, alpha subunit | Glycolysis; Oxidation reduction |
| BA0309 | 1-pyrroline-5-carboxylate dehydrogenase | Glutamate and proline biosynthetic process; Oxidation reduction |
| BA0876 | Acyl-CoA synthetase | Metabolic process |
| BA1242 | Hypothetical protein | - |
| BA0401 | Tellurium resistance protein | Stress response; Antibacterial resistance |
| BA0345 | Alkyl hydroperoxide reductase, subunit C | Cell redox homeostasis; Oxidation reduction |
| BA2849 | NLP/P60 family protein | - |
| BA2267 | Alcohol dehydrogenase | Zinc ion binding; Oxidation reduction; Alcohol dehydrogenase (NAD) activity |
| BA5365 | Phosphoglyceromutase | Glycolysis; Manganese ion binding |
| BA1035 | MarR family transcriptional regulator | Transcription factor activity |
| BA4873 | Alanine dehydrogenase | Oxidation reduction; Binding |
| BA1127 | S-layer protein, putative | - |
| BA0011 | Glutamine amidotransferase subunit PdxT | Glutamine catabolic process |
| BA3321 | Phosphoserine aminotransferase | L-serine biosynthetic process |
| BA2214 | Hypothetical protein | Proteolysis; zinc ion binding |
| BA0137 | DNA-directed RNA polymerase, Alpha | DNA binding; Transcription |
| BA1981 | Siderophore biosynthesis protein, putative | Siderophore-iron transmembrane transporter activity |
| BA5314 | Tyrosyl-tRNA synthetase | Tyrosyl-tRNA aminoacylation |
| pXO1-54 | S-layer protein | - |
| pXO1-113 | Spore germination protein XA | Pathogenesis; Spore germination |

[a] Cellular function were searched through UniPort – UniPortKB (http://www.uniprot.org/uniprot/).

**Table 3.** Identified differentially regulated protein of *B. anthracis* strain at 37°C

| Spot number | Gene ID | Proteins | *M*W (kDa) | p*I* | Loc[a] |
|---|---|---|---|---|---|
| | | Protein up-regulated in *B. anthracis* Δ Sterne | | | |
| 4 | BA5364 | Eno phosphopyruvate hydratase | 46.44 | 4.7 | M |
| 7 | BA4283 | Peptidyl-prolyl cis-trans isomerase, cyclophilin-type | 15.64 | 5.4 | C |
| 10 | BA3909 | 2-oxoglutarate ferredoxin oxidoreductase subunit beta | 31.58 | 5.7 | C |
| 11 | BA4184 | Pyruvate dehydrogenase complex E1 component, alpha subunit | 41.42 | 5.5 | M |
| 12 | BA0309 | 1-pyrroline-5-carboxylate dehydrogenase | 56.44 | 5.4 | C |
| 13 | BA0876 | Acyl-CoA synthetase | 58.57 | 5.4 | C |
| 15 | BA1242 | Hypothetical protein | 28.02 | 5.1 | C |
| 18 | BA0137 | DNA-directed RNA polymerase, alpha | 38.75 | 4.83 | C |
| | | Protein up-regulated in *B. anthracis* Sterne | | | |
| 1 | BA0401 | Tellurium resistance protein | 21.18 | 4.5 | C |
| 2 | BA0345 | Alkyl hydroperoxide reductase, subunit C | 20.87 | 4.8 | C |
| 3 | BA2849 | NLP/P60 family protein | 37.05 | 4.6 | E |
| 5 | BA2267 | Alcohol dehydrogenase | 37.51 | 5.2 | C |
| 6 | BA5365 | Phosphoglyceromutase | 56.61 | 4.9 | C |
| 8 | BA1035 | MarR family transcriptional regulator | 18.24 | 5.6 | C |
| 9 | BA4873 | Alanine dehydrogenase | 40.15 | 5.2 | C |
| 14 | BA1127 | S-layer protein, putative | 24.81 | 7.0 | M |
| 16 | BA0011 | Glutamine amidotransferase subunit PdxT | 21.30 | 5.1 | C |
| 17 | BA3321 | Phosphoserine aminotransferase | 41.37 | 5.4 | C |
| 19 | BA2214 | Hypothetical protein | 36.15 | 4.9 | E |
| 20 | BA5314 | Tyrosyl-tRNA synthetase | 47.3 | 5.3 | C |
| 21 | BA1981 | Siderophore biosynthesis protein, putative | 69.9 | 5.1 | C |
| 22 | pXO1-54 | S-layer protein | 44.91 | 6.7 | CW |
| 23 | pXO1-113 | Spore germination protein XA | 55.1 | 6.6 | M |

[a] Loc, location; C, cytoplasmic/unknown; CW, cell wall; M, membrane; E, extracellular

were upregulated at 37°C temperatures in *B. anthracis* Sterne (Lamonica *et al*., 2005; Chitlaru *et al*., 2006). Thus the upregulation of such proteins might favor toxin synthesis and therefore be beneficial to the bacterium. The other proteins that were identified have different cellular functions, such as amino acid biosynthetic processes (BA0011, BA3321), energy production (BA5365), and stress response (BA0401, BA0345). Several proteins that were identified were noted as probable virulence related factors, listed in Table 4.

The cellular locations of identified proteins were predicted most of them were cytoplasmic (Table 3). Two S-layer proteins were identified in *B. anthracis* Sterne (BA1127, pXO1-54) one was encoded by the pXO1 plasmid. Various functions have been assigned to the S-layer, ranging from shape maintenance to virulence, host recognition evasion, cell adhesion and resistance to phagocytosis. These SLH domain proteins were identified as a virulence factor and a potential vaccine candidate in *B. anthracis* (Ariel *et al*., 2003)

In our study, two pXO1-encoded proteins were identified (pXO1-54, pXO1-113) and noted as a putative virulence factor. The presence of only a few plasmid-encoded proteins might be due to two facts: first pXO1-encoded protein expression is not essential for growth, as was evident by the *B. anthracis* Δ Sterne strain; second the plasmid contains a large number of secreted proteins (17.2% of the predicted plasmid proteome versus 2.6% of the total proteome). Two extracellular proteins were identified in our study (BA2849, BA2214) and were observed to play widespread roles in bacterial cell wall dynamics (Anantharaman and Aravind, 2003). It is unclear if this finding was a result of sample preparation contamination further investigation is needed for the confirmation of this cellular localization. Interestingly, BA2849 contains SH3 (Src homology) domains. However, bacterial proteins possessing SH3 domains were shown to be involved in the virulence of several pathogens, such as the diphtheria toxin repressor DtxR of *Corynebacterium diphtheriae* (Manabe *et al*., 2005; Wylie *et al*., 2005), the invasion protein InlB of *Listeria monocytogenes* (Marino *et al*., 2002), and the iron-dependent repressor IdeR of *Mycobacterium tuberculosis* (Manabe *et al*., 2005).

The MarR family transcriptional regulator (BA1035) was indentified in our study as a protein expressed in *B. anthracis* Sterne. Members of the MarR family protein regulate a wide variety of cellular processes, including resistance to multiple antibiotics, organic solvents, household disinfectants, and oxidative stress agents, which are collectively termed the

**Table 4.** Subset of identified proteins that may be related to virulence/pathogenesis

| Gene ID | Proteins | Reference(s) |
|---|---|---|
| BA0309 | 1-pyrroline-5-carboxylate dehydrogenase | Chitlaru *et al*. (2007) |
| BA0345 | Alkyl hydroperoxide reductase | Chitlaru *et al*. (2007) |
| BA0401 | Tellurium resistance protein | Burian *et al*. (1998) |
| BA1035 | MarR family transcriptional regulator | Alekshun *et al*. (1999), Matsumoto *et al*. (2003), Kovacikova *et al*. (2004), Ingavale *et al*. (2005) |
| BA1127 | S-layer protein, putative | Ariel *et al*. (2003) |
| BA1981 | Siderophore biosynthesis protein, putative | Dale *et al*. (2004), Cendrowski *et al*. (2004) |
| BA2849 | NLP/P60 family protein | Manabe *et al*. (2005), Wylie *et al*. (2005), Marino *et al*. (2002) |
| BA4873 | Alanine dehydrogenase | Chitlaru *et al*. (2007) |
| BA5314 | Tyrosyl-tRNA synthetase | Ochsner *et al*. (2007) |
| BA5364 | Eno phosphopyruvate hydratase | Chitlaru *et al*. (2007) |
| pXO1-54 | S-layer protein | Ariel *et al*. (2003) |
| pXO1-113 | Spore germination protein XA | Liu *et al*. (2008) |

BA, *B. anthracis* Ames match; pX01, Plasmid (pXO1) encoded proteins

multiple antibiotic resistance phenotypes (Alekshun and Levy, 1999). They also regulate the synthesis of virulence factors in microbes that infect humans, such as *Yersinia pseudotuberculosis* (Matsumoto *et al*., 2003), *aphA* in *Vibrio cholerae* (Kovacikova *et al*., 2004), and *mgrA* in *Staphylococcus aureus* (Ingavale *et al*., 2005).

Nevertheless, cytoplasmic proteins of pathogenic bacteria have also been shown to be immunogenic during infection (Zysk *et al*., 2000). A total of four proteins were identified in our study (BA0309, BA0345, BA4873, and BA5364) that were demonstrated to be shown as *in vivo* expressed immunogenic proteins by serological proteome analysis (Chitlaru *et al*., 2007). Two were upregulated 3-(BA0309) and 9-(BA5364) fold in *B. anthracis* Δ Sterne, whereas 3 and 10 fold upregulation (BA4873, and BA0345, respectively) was observed in *B. anthracis* Sterne (Table 4).

Several studies have been conducted to identify the virulence regulatory factors of *B. anthracis* using secretome proteins (Chitlaru *et al*., 2004; Lamonica *et al*., 2005; Shahid *et al*., in preparation) and cell wall associated proteins (Ariel *et al*., 2003; Chitlaru *et al*., 2006). In this proteomic study we investigated the participation of intracellular proteins in *B. anthracis* pathogenesis under the influence of a virulence plasmid and growth conditions mimicking host conditions. This is the first approach that compares the cellular proteome under influence of a virulence plasmid and induced toxin expression conditions. Our data represent several immunogenic proteins and putative virulence factors in addition to those identified previously in secretome and cell wall-associated proteome analyses in *B. anthracis*.

Identified proteins that were differentially expressed and the physical and functional roles of these proteins might provide insights into the vegetative *B. anthracis* proteome. The approach used in this study did not address a broad-spectrum comparison of the total vegetative proteins expressed by the two *B. anthracis* strains, but further characterization of these expressed proteins will lead to an improved understanding of the cell biology of *B. anthracis* and other bacilli. Furthermore, this proteomic analysis might be important in future studies aimed at the development of novel antibacterial drugs or human vaccines.

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