

# Differential Exoproteome Analysis of Two *Corynebacterium pseudotuberculosis* Biovar *Ovis* Strains Isolated from Goat (1002) and Sheep (C231)

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Received: 19 February 2013 / Accepted: 17 April 2013 / Published online: 23 May 2013  
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**Abstract** *Corynebacterium pseudotuberculosis* is the etiologic agent of caseous lymphadenitis a chronic infectious disease affecting small ruminants. The 2D-DIGE technique was used to compare the exoproteomes of two *C. pseudotuberculosis* biovar *ovis* strains isolated from goat (strain 1002) and sheep (strain C231). Seventeen proteins differentially produced were identified here. Nine proteins appeared over-produced in the exoproteome of 1002 goat strain and 8 in that of C231 sheep strain. These proteins were related to various biological functions, such as the cell envelope, respiratory metabolism and proteolysis. This proteomic analysis revealed strain-specific exoproteins although each of the corresponding genes was found in both strain genomes. Such differential expression pattern may reflect inter-strain differences in adaptation to a specific host, in pathogenicity and or in antigenicity of this pathogenic bacterium.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00284-013-0388-4) contains supplementary material, which is available to authorized users.

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## Introduction

*Corynebacterium pseudotuberculosis* is a Gram-positive, facultative intracellular pathogen, and the etiologic agent of caseous lymphadenitis (CLA), a chronic infectious disease affecting small ruminants [9]. Despite a huge impact of CLA in small ruminant herds, *C. pseudotuberculosis* is still poorly documented and our understanding of its pathogenesis is partial. A few virulence factors of *C. pseudotuberculosis* were identified in previous works, including phospholipase D [15], toxic cell wall lipids [12], iron transporters belonging to the ABC proteins family [5] and a serine protease [32].

Comparative proteomics has been used to identify virulence factors and to gain further information about the physiology of various pathogens such as *Listeria monocytogenes* [29], *Staphylococcus aureus* [17] or *Clostridium perfringens* [27]. These studies took account of secreted/extracellular proteins, a protein fraction which indeed contains factors involved in adhesion and invasion of the

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host cell, and in survival, persistence and intracellular proliferation [29].

We recently reported the use of a gel-free proteomic approach to compare the exoproteomes of two *C. pseudotuberculosis* strains 1002 and C231 that both belong to the biovar *ovis*, but were isolated from different hosts: goat and sheep, respectively. This study enabled detection of qualitative and quantitative changes between the exoproteome of the two strains. Proteins identified were associated with bacterial physiology and included putative virulence factors [22]. However, such approach only gives a partial view of the protein sample contents. More sensitive techniques, such as the two-dimensional differential in-gel electrophoresis (2D-DIGE), have been described and allow to achieve a more complete analysis of proteomes. This multiplex technique allows analyzing differences between protein samples resolved on the same gel, in the presence of an internal standard, enabling more accurate results. 2D-DIGE demonstrated advantages over the conventional 2-DE in quantitative studies [1]. Here, 2D-DIGE was used to identify quantitative changes in the exoproteomes of *C. pseudotuberculosis* 1002 and C231.

## Materials and Methods

### Bacterial Strains and Culture Conditions

*Corynebacterium pseudotuberculosis* biovar *ovis* strain 1002, isolated from a caprine host in Brazil, and *C. pseudotuberculosis* biovar *ovis* strain C231, isolated from an ovine host in Australia, were maintained in brain–heart–infusion broth (BHI—HiMedia Laboratories Pvt. Ltd., India) at 37 °C. For proteomic analysis, both strains were cultivated in chemically defined medium (CDM) [(Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O (12.93 g/L), KH<sub>2</sub>PO<sub>4</sub> (2.55 g/L), NH<sub>4</sub>Cl (1 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.20 g/L), CaCl<sub>2</sub> (0.02 g/L) and 0.05 % (v/v) Tween 80]; 4 % (v/v) MEM Vitamins Solution 100× (Invitrogen, Gaithersburg, MD, USA); 1 % (v/v) MEM Amino Acids Solution 50× (Invitrogen); 1 % (v/v) MEM Non-Essential Amino Acids Solution 100× (Invitrogen); and 1.2 % (w/v) filter-sterilized glucose) [19].

### Three-Phase Partitioning

The three-phase partitioning protocol (TPP) was used to extract extracellular proteins [24]. Briefly, each strain was cultivated in triplicate on CDM and after reaching late exponential growth (DO<sub>600nm</sub> = 1.3). Bacterial cells were pelleted by centrifugation for 20 min at 2,700×g, and culture supernatants were filtered using 0.22 μm filters. Ammonium sulfate 6 g/L was added to supernatant samples, and the pH was set to 4.0. *N*-butanol 20 mL/L was

then added to each 20 mL of sample, left 1 h at room temperature and centrifuged for 10 min at 1,350×g at 4 °C. The interfacial precipitate was re-suspended in 1 mL Tris–HCl (20 mM, pH 7.2) + 10 μL Protease Inhibitor Mix (GE Healthcare, Piscataway, NJ, USA).

### 2D-DIGE

For 2D-DIGE experiment, the protein samples (50 μg) of each strain were labeled separately with 400 pmol of either Cy3 or Cy5 CyDye DIGE Fluor minimal dyes (GE Healthcare, Piscataway, NJ, USA), and an internal standard was prepared using 25 μg of each bacterial protein extract (*Cp1002* + *CpC231*) and labeled with 400 pmol of Cy2 (GE Healthcare). The labeled samples were then mixed and combined with a sample buffer containing 7 M urea, 2 M thiourea, 4 % CHAPS, 0.2 % DTT and 0.002 % bromophenol blue and applied to 18 cm pH 3–10 NL strips (GE Healthcare). Isoelectric focusing was performed using the IPGphor 2 (GE Healthcare) for a total of 60,000 Vh. The strips were then brought to equilibrium for 15 min in 10 mL of equilibration buffer I (Tris–HCl 50 mM pH 8.8, urea 6 M, glycerol 30 %, SDS 2 %, bromophenol blue 0.002 %, 100 mg dithiothreitol) followed by 15 min in 10 mL of equilibration buffer II (Tris–HCl 50 mM pH 8.8, urea 6 M, glycerol 30 %, SDS 2 %, bromophenol blue 0.002 %, iodoacetamide 250 mg). After equilibration, proteins were separated in 12 % acrylamide/bis-acrylamide gels with an Ettan DaltSix II system (GE Healthcare), and the gels were then scanned between low fluorescence glass plates at a 50 nm resolution. The three fluorophores were imaged at excitation wavelengths of Cy3/580 nm, Cy5/670 nm and Cy2/520 nm, and gel images were cropped and analyzed using the Image Master 2D Platinum 7.0 DIGE Software (GE Healthcare). Spots with at least a 2.0-fold volume ratio change and ANOVA *t* test *P* value less than 0.01 were selected for identification.

### In-Gel Trypsin Digestion, Mass Spectrometry and Protein Identification

Protein spots were excised from the gels using an Ettan Spot Picker (GE Healthcare) and in-gel digestion was carried out using trypsin enzyme (Promega, Sequencing Grade Modified Trypsin, Madison, WI, USA). The peptides were then concentrated to a volume of 10 μL using a speed vacuum, desalinated and concentrated using ZIP-TIP C18 tips (Eppendorf, Hamburg, Germany). The samples were subsequently analyzed by MS and MS/MS modes, using a MALDI-TOF/TOF mass spectrometer Autoflex III<sup>TM</sup> (Bruker Daltonics, Billerica USA). The equipment was controlled in a positive/reflector way using the Flex-Control<sup>TM</sup> software (Bruker Daltonics), and calibration

was performed using standard peptide samples (angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39, somatostatin 28, bradykinin Fragment 1–7, renin Substrate tetradecapeptide porcine) (Bruker Daltonics). The peptides were added to the alpha-cyano-4-hydroxycinnamic acid matrix, applied on an Anchor-Chip™ 600 plate (Bruker Daltonics) and analyzed by Autoflex III. The search parameters were as follows: peptide mass fingerprint; enzyme; trypsin; fixed modification, carbamido methylation (Cys); variable modifications, oxidation (Met); mass values, monoisotopic; maximum missed cleavages, 1; and peptide mass tolerance of 0.05 % Da (50 ppm). The results obtained by MS/MS were used to identify proteins utilizing the MASCOT® (<http://www.matrixscience.com>) program and compared to NCBI databases.

### Bioinformatics Analysis

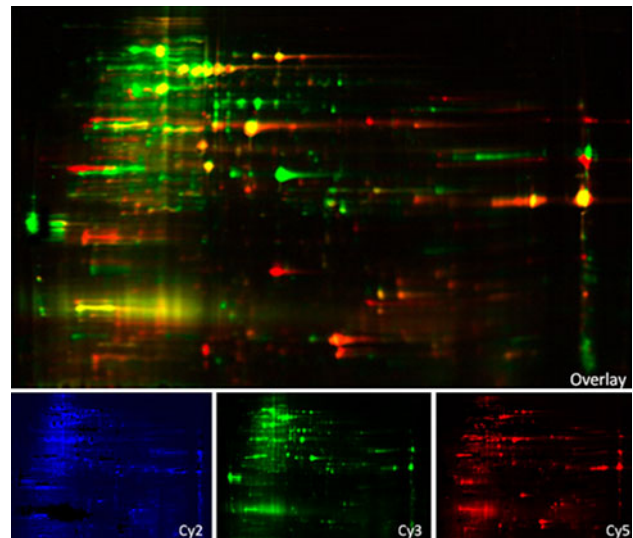
The Blast2GO program was used to classify the protein's functionality [8]. To predict the sub-cellular localization of proteins, we used the following programs: SurfG+v1.0 [2], SecretomeP v2.0 [3] and TatP v1.0 [4].

## Results and Discussion

### 2D-DIGE and Mass Spectrometry

In this study, the 2D-DIGE was used for a quantitative analysis and a comparison of the exoproteomes of *C. pseudotuberculosis* 1002 and C231, isolated from goat and sheep, respectively (Fig. 1). Using this technique, 18 spots were found differentially produced, selected and subjected to mass spectrometry analysis. Nine out of these 18 proteins were specifically found over-produced in strain 1002 supernatants (Supplementary file 1 and Fig. 2) and 8 were over-produced in strain C231 supernatants (Supplementary file 2 and Fig. 2). Of note, the genes corresponding to each of these proteins are found in both 1002 and C231 genomes suggesting that the differences observed at the proteome level are due to strain-specific abilities in expressing these particular genes, rather than differences in gene content.

Interestingly, various selected spots actually contained the same unique protein. For example, trehalose corynomycolyl transferase C was found at the expected molecular mass in C231 (spot 53; Supplementary file 2) and at a lower observed mass in 1002 (spot 87; Supplementary file 1), suggesting a proteolytic cleavage occurred in strain 1002. Cytochrome c oxidase sub-unit 2 was found over-produced in C231 in two different spots, at the expected size but significantly lower pI (spot 147; Supplementary

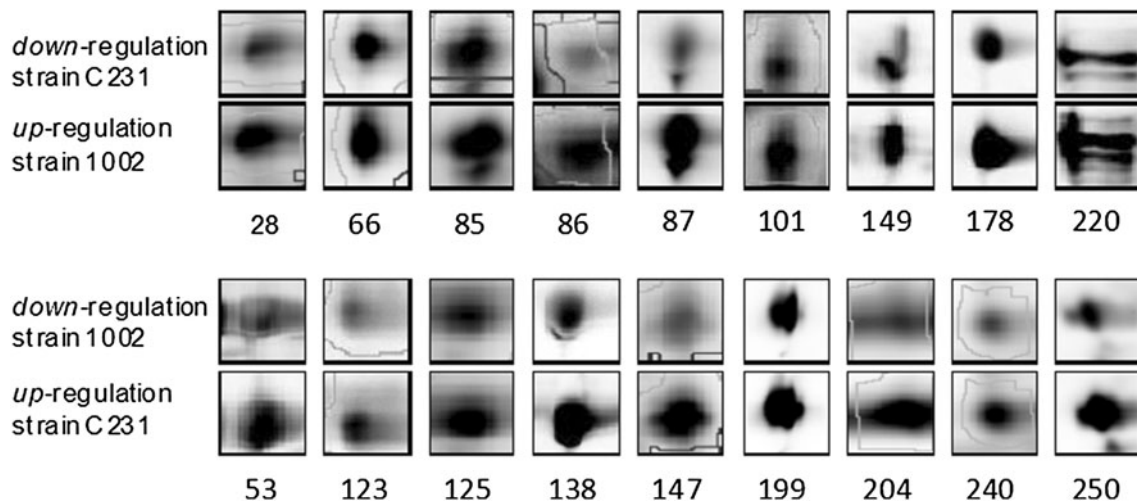


**Fig. 1** Comparative display of differential in-gel electrophoresis (DIGE) of the extracellular fractions of strains 1002 and C231 of *C. pseudotuberculosis*. Overlay of the three color images Cy3 (colored green), Cy5 (colored red) and Cy2 (colored blue) derived from a single gel

file 2) and at an observed mass slightly higher than the theoretical mass (spot 125; Supplementary file 2). These observations suggest the occurrence of post-translational modifications, which are commonly detected in studies based in gel-dependent system [25]. Such post-translational modifications were shown to regulate several cellular functions, for example, in *S. aureus*, post-translational modifications (phosphorylation) play a key role in pathogenesis, through modulation of adhesion to and invasion of host cells [21]. The exact nature of the putative post-translational modifications observed here is still to be determined. Whether and how such modifications can affect pathogenesis and or host specificity in *C. pseudotuberculosis* remains unknown.

### Predicting the Sub-Cellular Localization of Identified Exoproteins

In silico predictions of sub-cellular localization of *C. pseudotuberculosis*, extracellular proteins were performed using the software SurfG+ [2]. The predictions point out the presence of 13 secreted proteins, 2 proteins possibly associated with the cell wall and 1 cytoplasmic protein. The protein predicted as cytoplasmic was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 101, Supplementary file 1); this protein is associated with the lipid biosynthesis, carbohydrate metabolism and oxidation process. However, despite being predicted as a cytoplasmic protein, several studies have detected GAPDH in different sub-cellular locations (membrane, cell surface and extracellular) and developing different functions; due to these features, this



**Fig. 2** Differential expression pattern of the proteins spots. Expanded views of the proteins spots differentially produced, between the strains 1002 and C231, identified by MS/MS

protein is classified as moonlighting proteins; these proteins show distinct functional behavior depending on the cell type, cellular localization and multiple binding sites [14]. Several of the moonlighting functions of GAPDH have been studied in different Gram-positive and Gram-negative pathogens, showing that this protein plays important role during the adhesion and invasion process to host cell [11, 18, 23]. The functional prediction of GAPDH by Blast2GO in *C. pseudotuberculosis* shows the presence of two distinct functions: (1) oxireduction activity and (2) binding nucleotide showed the moonlight behavior this protein in this pathogen (Supplementary file 1). However, further studies are needed to uncover the true moonlighting role of GAPDH in *C. pseudotuberculosis*.

#### Functional Classification of Differentially Produced Proteins

The differentially produced proteins identified here were related to various physiological functions. Two proteins (spots 204 and 250) found in strain C231, and one protein (spot 149) found in 1002 were of unknown function when submitted to a Blast2GO search [8]. Their putative role in the physiology and/or virulence of *C. pseudotuberculosis* thus remains to be determined. Four proteins detected in 1002 strain (spot 28, 66, 85 and 86, Supplementary file 1) and one protein detected in C231 strain (spot 199, Supplementary file 2) are associated with cell envelope and have a hydrolase activity. Such hydrolases partially digest the cell wall during the bacterial growth and thus participate in the turnover of peptidoglycan during the cell growth, in cell division or in cell autolysis [31]. Peptidoglycan is essential for cell viability and shape. It also

controls the internal osmotic pressure [13]. Among, the detected proteins with hydrolases activity, spot 28 (Supplementary file 1) was identified as a neuraminidase in 1002 supernatants. This protein acts in the hydrolysis process of glycoproteins by cleaving sialic acid residues. Moreover, it was shown to be involved in the virulence of pathogens like *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*, by promoting cell adhesion and cell invasion [7, 30].

Other proteins are found differentially produced and are also associated with cell envelope: Mycolyltransferases (spot 87, 178, Supplementary file 1; spot 53, 123, Supplementary file 2) were previously shown to be associated with the cell envelope in *Mycobacterium tuberculosis*. This class of proteins is involved in the biosynthesis of corynemycolyl components that are associated with the envelope structure. These components are formed from high molecular weight chain fatty acids and are the largest cell wall constituents of *Mycobacterium*, *Nocardia* and *Corynebacterium* [28]. Changes in the quantity and structure of these fatty acids can affect the permeability, fluidity and other physical characteristics of the bilayer membrane of these bacteria and consequently may influence bacterial growth [33]. Furthermore, these constituents of the cell envelope also play important roles in the mechanisms of pathogenicity, due to their ability to form an impermeable asymmetric lipid bilayer that contributes to the resistance and survival of the bacteria in the hostile environment of macrophages [10].

A hypothetical protein (spot 225, Supplementary file 1) showed conserved domains the calcium ion binding. Calcium and calcium-binding proteins are involved in numerous bacterial processes, such as chemotaxis, sporulation, virulence, molecules transport, phosphorylation,

separation and stability of the cell envelope [20]. Several studies showed that calcium also influences the formation of biofilms. Thickness of *P. aeruginosa* biofilm increase in the presence of CaCl<sub>2</sub> compared to growth in medium without CaCl<sub>2</sub> supplementation [26]. The formation of biofilm by *V. cholerae* is dependent upon calcium and biofilms dissolve when the medium is depleted of calcium [16].

Bacteria that show characteristics of aerobic or anaerobic facultative growth, such as *C. pseudotuberculosis*, need oxygen as exogenous electron acceptor for respiration. The Cytochrome *c* oxidase sub-unit 2 (spot 125, 147, Supplementary file 2) is involved in this process [6], and was detected in this work.

#### Exploring *C. pseudotuberculosis* Exoproteome by Different Proteomics Approaches

The results obtained here were compared to those obtained by Pacheco et al. [22] in a previous study. Most of the differentially produced proteins identified in this work were shown over-produced as well in Pacheco et al. [22]. However, this study revealed some discrepancies with this previous study. Proteins ADL20134, ADL20574 and ADL21814 were indeed found over-produced in 1002 here (Table 1), whereas they were previously reported over-produced in C231 [22] and vice versa for proteins ADL11400 and ADL10895. We also identified some additional proteins, for example, lysozyme M1 (spot 86,

**Table 1** Analysis comparative between the results obtained by TPP/LC-MS<sup>E</sup>

| Protein   | In this work                        | Pacheco et al. [22]                     |
|---|-------------------------------------|---|
| Secreted hydrolase<br>Resuscitation-promoting factor RpfB | Over-produced in 1002 strain        | Over-produced in C231 strain            |
| Trehalose corynomocolyl transferase B                     |                                     |   |
| Trehalose corynomocolyl transferase C                     | Over-produced in C231 strain        | Over-produced in 1002 strain            |
| Cytochrome <i>c</i> oxidase sub-unit 2                    |                                     |   |
| Neuraminidase   | Significant expression <sup>b</sup> | Not significant expression <sup>a</sup> |
| Hypothetical protein                                      |                                     |   |
| Metalloendopeptidase                                      |                                     |   |
| Lysozyme  | Detected                            | Not detected <sup>c</sup>               |
| M1 GPDH   |                                     |   |
| Hypothetical protein                                      |                                     |   |

<sup>a</sup> Proteins not differentially expressed according to the *t* test and *P* value

<sup>b</sup> Proteins differentially expressed according to the *t* test and *P* value

<sup>c</sup> Proteins not detected in the exoproteome analysis by TPP-LC/MS<sup>E</sup> (Pacheco et al. [22])

Supplementary file 1), GPDH (spot 101, Supplementary file 1) and hypothetical protein (spot 250, Supplementary file 2) that were not previously detected. Such differences may be linked to the experimental procedure used: these differentially produced or additional proteins may indeed be revealed in samples prepared, in this work, from cultures in late exponential growth phase whereas they did not appear in Pacheco et al. [22] where proteins were prepared from cultures in early exponential phase. On the other hand, sensitivity of the 2D-DIGE technique (as compared to the gel-free approach adopted in Pacheco et al. [22].) might also explain the additional proteins that were identified here. These results demonstrated that comparative analysis, combining different proteomic approaches, is a powerful strategy to characterize a proteome.

#### Conclusions

Exoproteome analysis of *C. pseudotuberculosis* strains 1002 and C231 revealed differential production patterns, which may be related to differences in host adaptation, pathogenicity or antigenicity of this pathogen. The genes corresponding to the differentially produced proteins are present in both strains, suggesting differences related to the ability of each strain to express these genes in these growth conditions. The results obtained here complement previous comparative genomic and proteomic studies, adding data regarding the biology and virulence of *C. pseudotuberculosis*. Investigating the proteomes of *C. pseudotuberculosis* strains isolated from various hosts will help understand how bacteria are able to adapt to specific hosts, and provide excellent candidates for targeted studies of the molecular basis of *C. pseudotuberculosis* pathogenesis in small ruminant.

**Acknowledgments** We are thankful to the Center for Study of Structure and Function of Biomolecules of the Institute of Biological Sciences of the Federal University of Minas Gerais and to the Genomic and Proteomic network of the State of Pará. The current work was supported by CNPq, FAPEMIG and FAPESPA.

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