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Preliminary serological secretome analysis of *Corynebacterium pseudotuberculosis*

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Abstract

Caseous lymphadenitis (CLA) is a chronic disease affecting small ruminants that is caused by *Corynebacterium pseudotuberculosis* and is responsible for significant economic losses. Various *C. pseudotuberculosis* secreted proteins are known to react with sera from infected goats. Mapping of the secretome would help us understand the pathogenesis of CLA. We identified six immunoreactive secreted proteins of *C. pseudotuberculosis* by 2D-Western blotting, using sera from goats with CLA, and characterized them by mass spectrometry. This preliminary information will give support to future studies aimed at the development of efficient vaccines and diagnostic kits.

Keywords: *C. pseudotuberculosis*, Caseous lymphadenitis, Secretome.

1. Introduction

Caseous lymphadenitis (CLA) is a chronic disease affecting small ruminants; it is caused by infection with *Corynebacterium pseudotuberculosis* and is responsible for significant worldwide economic losses due to decreases in both the productivity and the reproductive performance of infected animals [1]. The lack of efficient immunoprophylaxis against CLA results in ineffective management of this disease in animals, facilitating its dissemination [2]. Efficient vaccines against CLA and diagnostic kits for this disease are still not available, in part due to a lack of sufficient information concerning newly-characterized *C. pseudotuberculosis* virulence determinants [3].

Only a few genes and their products have been identified as factors that contribute to the virulence of *C. pseudotuberculosis*, including phospholipase D (PLD) [4,5], the *fagABC* operon involved in iron acquisition by the cell [6] and the protease CP40 [7]. Chaplin et al. [8] developed a DNA vaccine encoding PLD to immunize sheep, but they achieved only partial protection against challenge with *C. pseudotuberculosis.* Similar results were obtained when sheep were immunized with a formalin-inactivated subunit vaccine [9]. CP40 protease has been reported as a possible candidate for the development of vaccines, based on Western blot analysis with serum samples from sheep experimentally infected with *C. pseudotuberculosis* [10].

To date, the search for immunogenic proteins has been carried out in a non-exhaustive manner, using various extraction and separation techniques [11,12]. A comprehensive analysis of the entire set of proteins expressed by *C. pseudotuberculosis* strains is needed in order to identify the best

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candidate proteins for immunoprophylactic or diagnostic applications. Bacterial secreted proteins have a various biological functions, ranging from toxicity to more subtle alterations of the host cell for the benefit of the invader; they are an important part of the pathogenic process [13].

There has not been much conclusive research concerning pathogenesis or even the immune response against *C. pseudotuberculosis* infection, compared to research on other important veterinary pathogens, such as *Mycobacterium tuberculosis* [14]. Mapping of the *C. pseudotuberculosis* secretome, followed by characterization of expressed proteins and assessment of their immunogenic potential would be ideal for shedding light on the pathogenesis of specific strains and the host immune response that they provoke, paving the way for the development of more efficient vaccines and diagnostic kits. We examined antigenic proteins of the secretome of *C. pseudotuberculosis* strain 1002 cultivated in chemically defined medium (CDM) using serological proteome analysis (SERPA) [15].

2. Material and Methods

2.1 *Bacterial strain and growth conditions*

Corynebacterium pseudotuberculosis strain 1002, originally isolated from an infected goat in Brazil [16], was routinely maintained in Brain Heart Infusion broth (BHI) and characterized by biochemical and molecular methods, as previously described [17]. For SERPA, bacteria were grown at 37ºC under agitation (100 rpm), for 24 h in 1 L of chemically defined medium (CDM), until reaching the exponential growth phase (OD600nm = 1.3). The CDM contained 0.067M of phosphate buffer, 0.05% (v/v) Tween 80 (Sigma), 4% (v/v) 100X minimal Essential Medium (MEM) Vitamin Solution (Invitrogen), 1% (v/v) of 50X MEM Amino Acids Solution (Invitrogen), 1% (v/v) 100X MEM Non-Essential Amino Acids Solution (Invitrogen) and 1.2% (w/v) filter-sterilized glucose, as previously described [18].

2.2 *Extraction of secreted proteins*

Corynebacterium pseudotuberculosis exoproteins were obtained according to a previously described three-phase partitioning (TPP) protocol [19]. Briefly, bacterial cells were separated from the supernatant by centrifugation at 4,000 rpm for 20 min at 4ºC. The supernatant was filtered through a 0.22 µm membrane (filter) and 30% (w/v) ammonium sulphate was added. The pH was adjusted to 4.0 and *n*-butanol was added at a ratio of 1:1, and the sample was vortexed. After 1h of incubation at room temperature, the precipitate at the interface was collected and re-suspended in 1 mL of 20 mM Tris-HCl buffer pH 7.4 with 10 µL of protease inhibitor (GE).

2.3 *2D-PAGE-Western blot*

Two-dimensional electrophoretic separation was carried out, as previously described [20], with minor modifications. Secreted proteins (150 µg) were dissolved in 2-DE sample buffer (8 M urea, 2 M thiourea, 4% CHAPS, 1% (v/v) carrier ampholyte pH 3.0-5.6, 80 mM dithiothreitol (DTT), 40 mM Tris-base and bromophenol blue. The mixture was used for overnight rehydration of 11 cm immobilized pH gradient (IPG) strips (Immobiline DryStripTM Gels, pH 3.0-5.6 NL [GE Healthcare]). Isoelectric focusing (IEF) was carried out at room temperature for 24.5 h (maximum voltage of 3,500 V and maximum current of 50 µA). After equilibration for 15 min in a 50 mM Tris HCl (pH 8.8) buffer solution containing 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol and 0.001% (v/v) bromophenol blue and 10 mg/mL DTT, the strips were equilibrated for 15 min in the same solution, except that the DTT was replaced by 25 mg/mL iodoacetamide. The proteins were resolved in 2D electrophoresis in 12% polyacrilamyde gels under denaturing conditions, using a Protean IIxi system (Biorad). Protein spots were visualized by staining with Coomassie blue G-250 (GE Healthcare). For each protein sample, three 2D gels were stained to visualize proteins and six 2D gels were electroblotted onto polyvinylidene difluoride membranes (*Owl* system) for 1 h, with an electric current of 0.4 A. The membranes were blocked overnight at 4º C in 5% non-fat milk in phosphate buffered saline pH 7.5 with 0.05% Tween 20 (PBS-T). The membranes were then incubated at room temperature for 1 h in PBS-T with sera (at a proportion of 1:100 v/v PBS-T:serum) obtained from animals either infected or uninfected with *C. pseudotuberculosis*. The membranes were then washed with PBS-T three times for 5 min and incubated for 1h with an anti-goat IgG peroxidase antibody produced in rabbits (Sigma), diluted 1:1000 in PBS-T solution. Antibody-tagged protein spots were detected with DAB peroxidase substrate solution.

2.4 *Identification of immunoreactive proteins*

Membranes were digitally scanned and immunoreactive proteins matched to 2D gel images of the samples were identified using the Melanie software (GeneBio). All spots reactive in 2D-Western blots were selected from an analogous 2D stained gel and manually excised. The excised gel fragments were incubated overnight with 25mM bicarbonate/50% acetonitrile (ACN) solution until completely destained. After drying, gel fragments were placed in 50 mM ammonium bicarbonate solution with 20ng/µL sequencing-grade modified trypsin (Promega Biosciences, CA, USA). Digestion was run at 37º C overnight. The peptides were extracted using 5% formic acid/50% acetonitrile solution, concentrated in a SpeedVac (Savant, USA) to a volume of about 10 µL, desalted using ZipTip® C18 plates (C18 resin, P10; Millipore Corporation, Bedford, MA, USA) and eluted with 0.1% trifluoroacetic acid solution containing 50% ACN. The sample extract was mixed at a 1:1 ratio with matrix (10 mg/mL recrystallized α-

cyano-4-hydroxycinnamic acid) to a final volume of 1 µL and then spotted onto an MTP AnchorChip™ 600/384 (Bruker Daltonics) for matrix-assisted laser desorption/ ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) (LIFT technology, Autoflex III™; BrukerDaltonics, Billerica, USA) analysis. Ionization was performed in MS/MS (PSD-LIFT technology) by irradiation of a nitrogen laser (337 nm) operating at 50 Hz. Data were acquired at a maximum accelerating potential of 25 kV in the positive and reflector modes. Trypsin and keratin contamination peaks were excluded from the mass spectra and MS/MS results were used to search the *C. pseudotuberculosis* 1002 (Gen Bank: CP001809.1) protein database using MASCOT software (http://www.matrixscience.com/).

3. Results and Discussion

Bacterial growth within a host resulting in infection is a consequence of colonization, adherence, invasion, evasion of the immune response and toxigenesis caused by the bacterial cell. This feat can be accomplished by a bacterial strain through temporal expression of a panoply of virulence genes (the virulon), in response to appropriate environmental stimuli. Characterization of when, which and what amounts of virulence factors are expressed in response to certain stimuli is necessary for understanding the pathogenesis of bacterial species [13]. The dynamics of the immune response to infection can only be fully understood if we characterize the bacterial proteins responsible for eliciting the immune response. The advent of genomics has made this approach feasible, since information concerning an immunogenic protein can be traced back to the genome, and thence to the regulon that is involved. Several species of Actinobacteria have been the subject of proteomic analysis, including *Mycobacterium avium*, *Mycobacterium tuberculosis*, *Rhodococcus equi* and *Corynebacterium diphtheriae*, yielding insight into the relationships between the host and the bacterial parasite [21,22,23,24,25]. The secretome of *C. pseudotuberculosis* was first studied by Braithwaite et al. [12], who extracted proteins from a culture supernatant with ammonium sulphate; they found seven proteins with molecular weights between 14 and 64 kDa, five of which reacted with sera obtained from goats infected with *C. pseudotuberculosis*. A later study described the reaction of a pool of sera obtained from goats suffering from CLA against 11 *C. pseudotuberculosis* secreted proteins with molecular weights ranging from 24 to 125 kDa [19]; these proteins induced an increase in the serum concentration of IFN-γ in goats infected with this bacterium [26]. We made a follow up of that study. The excreted-secreted antigens of *C. pseudotuberculosis* were obtained by culturing the 1002 strain in CDM [18], with subsequent extraction of secreted proteins by TPP [19], and a 2D-PAGE-Western blot. Twenty -three immunoreactive spots were detected using sera obtained from animals with CLA; due to time and budget constraints, only six of these proteins were identified by MALDI-TOF-MS/MS (Figure 1 and Table 1). Immunoproteomic methods, such as SERPA [15], have been used to identify biomarkers and target antigens for developing diagnostic kits based on antibody/antigen detection, as well as to develop vaccines and treatments for various infectious diseases. Due to the unfeasibility of targeting many proteins simultaneously, our objective was to

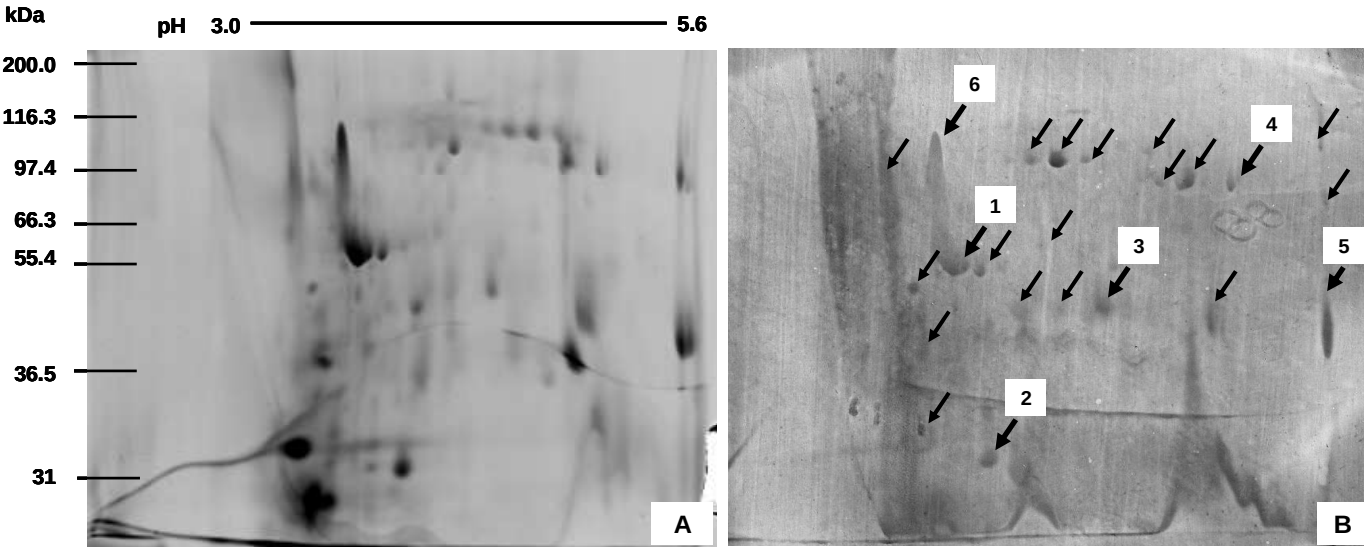

Figure 1. Serological proteome analysis of secreted proteins of *Corynebacterium pseudotuberculosis* using serum from infected goats. A) 2D-PAGE with 150µg of sample for analysis of *C. pseudotuberculosis* secreted proteins. Spots were detected in the gels stained with Coomassie G-250. B) 2D-PAGE-Western blot analysis of *C. pseudotuberculosis* secreted proteins with 150µg sample. The black arrows indicate 23 immunoreactive spots detected by anti-goat IgG peroxidase antibody produced in rabbits. Numbers correspond to the proteins identified in Table 1.

Table 1. Antigenic proteins of *Corynebacterium pseudotuberculosis* identified by MALDI-TOF MS/MS. [a] Accession numbers in Entrez Protein (NCBI Genome CP001809.1). [b] Theoretical molecular weights (Mr). [c] Theoretical isoelectric points (pI)

Spot/Protein description	[a]Protein ID/NCBI	[b]Mr(kDa)/ [c]pI	MASCOT score (%)	Coverage (%)	Peptide Sequence	Ion score
1 Resuscitation-promoting factor RpfB	gi|302330380	40.31/5.06	298	13	K.AGVTVGDKDIVYPGLTEK.I	70
					K.TVFTQIAAATVKDVLAER.G	114
					K.VQASQGWGAWPACTSK.L	122
2 Putative secreted protein	gi|302330462	24.39/5.34	174	27	K.AKDFADTLPEPLR.N	34
					K.DFADTLPEPLR.N	26
					K.LGPNEHQAMNVHWFNLSTLQGGSTR.L	86
					R.VIALIEGTIATEASPCTFLPTAALFEVK.L	28
3 NlpC/ P60 protein	gi|302331099	36.63/5.62	115	6	R.GAVIDPLTNAVSAENPQNAIDR.A	115
4 Putative efflux system protein	gi|302331553	59.55/5.60	232	14	R.VLVEGTVEPIR.T	24
					R.DQLISAALDAAR.T	38
					K.TKPLYPVEIELTGNR.D	54
					K.NREPIKLPSEAVYQENNAK.K	26
					R.TVTVGNTTDIIAEITGGELKPGDK.V	90
5 Surface layer protein A (Spl A)	gi|302329946	38.67/5.90	530	32	R.VVEAWAHSPSMNR.N	39
					K.ASSPDRPTVYLLNGGDGGEGR.A	26
					R.GHATPEQMWGPMGSDYNR.Y	72
					R.YNDAVVMAEDLR.G	80
					R.GTEVYVSNASGVAGGHDILANPR.F	132
					R.LQSLNIPADFNLR.N	60
					R.NTGTHSWSYWQDDLR.A	120
6 Metalloendopeptidase-like protein	gi|302205783	24.83/7.23	64	7	K.IVVHTPAMGTLTSPYGMR.W	27

identify polypeptide chains that display immunoreactivity, thus narrowing the number of targets for further experiments. Six of the spots that displayed immunoreactivity by SERPA (Figure 1) and were identified by MALDI-TOF-MS/MS (Table 1) were consistent with five proteins identified by data-independent MS acquisition (LC-MSE) and six proteins predicted *in silico* by SurfG *plus* studies performed by our team to characterize the total *C. pseudotuberculosis* exoproteome, independent of the 2D-PAGE Western blot analysis [27]. Four immunoreactive secreted proteins that we identified, namely, resuscitation-promoting factor B (RpfB), Nlp/P60 protein, putative efflux system protein and surface layer protein A (SlpA), have previously been reported from other bacterial species. Rpf homologues are widespread throughout the Actinobacteria [28] and have the ability to stimulate the growth of dormant mycobacteria, especially *Mycobacterium tuberculosis* [29]. NlpC/P60 belongs to the peptidase family and plays a role in turnover of the bacterial cell wall [30]. Previously characterized NlpC/P60 proteins include *Listeria monocytogenes* secreted autolysin P60 [31], *Bacillus subtilis* autolysins [32] and *Escherichia coli* membrane-associated lipoprotein [33]. The bacterial efflux system is composed of proteins that act as a continuous channel for the extrusion of substrates within the cell envelope into the external environment. This mechanism may be involved in various transport functions, including efflux of toxins, metabolites and drugs [34,35]. Surface layer protein A (Spl A) has been described from several bacterial species, in which it has various roles, including nutrient uptake, colonization, antiphagocytosis and exclusion of noxious substances [36]. Putative secreted protein (spot 2) was found to be similar to protein sequences of other Actinobacteria, based on protein BLAST/NCBI (National Center for Biotechnology Information), but it has not been described. Another secreted protein, metalloendopeptidase-like protein belongs to the family of metalloproteases [37], which are common in pathogenic bacteria, including *Pseudomonas aeruginosa* (degradation of host connective tissues) [38], *Clostridium spp*. (neurotoxin activity) [39], *Bacillus anthracis* (lethal toxin) [40] and *Listeria monocytogenes* (enzyme maturation) [41]. Secreted proteins of similar molecular weight have been found in other studies [12,19], through extraction of proteins by other methods and resolution by unidimensional electrophoresis [12,14], but they were not identified as being the CP40 protease [7]. Our preliminary results indicate that SERPA coupled with mass spectrometry analysis is a useful strategy for the identification of these six antigens. Studies are underway to develop a protocol for the detection of the other spots that remained unidentified. These findings may help identify proteins that can induce protective immunity or elicit immune responses with diagnostic value for CLA.

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