Identification of membrane-associated proteins with pathogenic potential expressed by *Corynebacterium pseudotuberculosis* grown in animal serum

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**Abstract**

**Objective:** Previous works defining antigens that might be used as vaccine targets against *Corynebacterium pseudotuberculosis*, which is the causative agent of sheep and goat caseous lymphadenitis, have focused on secreted proteins produced in a chemically defined culture media. Considering that such antigens might not reflect the repertoire of proteins expressed during infection conditions, this experiment aimed to investigate the membrane-associated proteins with pathogenic potential expressed by *C. pseudotuberculosis* grown directly in animal serum.

**Results:** Its membrane-associated proteins have been extracted using an organic solvent enrichment methodology, followed by LC–MS/MS and bioinformatics analysis for protein identification and classification. The results revealed 22 membrane-associated proteins characterized as potentially pathogenic. An interaction network analysis indicated that the four potentially pathogenic proteins ciuA, fagA, OppA4 and OppCD were biologically connected within two distinct network pathways, which were both associated with the ABC Transporters KEGG pathway. These results suggest that *C. pseudotuberculosis* pathogenesis might be associated with the transport and uptake of nutrients; other seven identified potentially pathogenic membrane proteins also suggest that pathogenesis might involve events of bacterial resistance and adhesion. The proteins herein reported potentially reflect part of the protein repertoire expressed during real infection conditions and might be tested as vaccine antigens.

**Keywords:** *Corynebacterium pseudotuberculosis*, Caseous lymphadenitis, Sheep, Goat, Antigens, Virulence factors, Pathogenesis, Bovine fetal serum

**Introduction**

*Corynebacterium pseudotuberculosis* is the causative agent of caseous lymphadenitis in sheep and goats, which is an infectious disease responsible for a high level of economic losses in the livestock sector [1]. During the years between 1972 and 2011, at least 39 vaccine models against *C. pseudotuberculosis* were proposed by researchers worldwide; however, no product presented satisfactory effectiveness, offering complete protection to the animals in a herd [2].

Recently, efforts have been made to characterize the bacterial exoproteome and discover novel secreted antigens for use as vaccine candidates against *C. pseudotuberculosis* infection. In particular, the use of high-throughput proteomic approaches allowed the identification of more than 100 extracellular proteins [3–8]. Albeit such advances, those results might not reflect the repertoire of proteins expressed during infection
conditions, since all works used synthetic and chemically
defined culture media, and did not investigate the mem-
brane proteins, which might involve several virulence
factors important for bacterial infection. Thus, this study
aimed to investigate the membrane-associated proteins
with pathogenic potential expressed by \textit{C. pseudotuber-
culosis} grown directly in animal serum.

\textbf{Main text}

\textbf{Bacterial strain and growth conditions}

All the chemicals and other reagents were purchased
from Sigma-Aldrich (St. Louise, MO, USA) unless oth-
erwise stated. Strain VD57 of \textit{C. pseudotuberculosis} was
used in this study, which has been previously used as the
virulent reference strain [6, 7]. The bacteria were rou-
tinely maintained in Brain Heart Infusion (BHI) broth at
37 °C. For this proteomic study, commercial bovine fetal
serum (BFS) was used as integral culture medium, heated
at 56 °C for 30 min prior to use to inactivate complement
system proteins [8]. An overnight BHI culture (24 h) of
\textit{C. pseudotuberculosis} was inoculated in triplicate (1:10)
separately into 150 mL of pre-warmed BFS and incu-
bated at 37 °C, with agitation at 150 rpm. Growth curves
were monitored by optical density (OD) at 595 nm, and
100 mL of bacterial culture was retrieved at the mid-
exponential growth phase (OD595 nm = 0.7). The bacte-
rial pellets were recovered by centrifugation at 6000 rpm
for 20 min at 4 °C, then washed three times with sterile
phosphate-buffered saline (PBS), and the final pellets
were stored at −70 °C until used for membrane protein
extraction.

\textbf{Membrane protein extraction}

Firstly, a delipidation step was performed to remove most
of the lipoarabinomannans (LAMs) and phosphatidylinos-
itol mannosides (PIMs), as previously described [9, 10].
The final pellets were subjected to a low-hydrophobicity
membrane protein extraction protocol [11], using 30
volumes of 9% 1-butanol for 3 h with shaking (150 rpm)
at room temperature, followed by 6000 rpm for 20 min
at 4 °C. The procedure was applied twice more, and the
supernatants of each extraction were dried by rota-evap-
at 4 °C. The procedure was applied twice more, and the
final pellets were subjected to a low-hydrophobicity
membrane protein extraction protocol [11], using 30
volumes of 9% 1-butanol for 3 h with shaking (150 rpm)
at room temperature, followed by 6000 rpm for 20 min
at 4 °C. The procedure was applied twice more, and the
supernatants of each extraction were dried by rota-evap-
oration (50 °C). The resulting protein extracts from each
replicate were resuspended in sterile water, and their
protein concentrations were determined by the Lowry
method (DC-reagent Kit, Bio-Rad Laboratories) using
bovine serum albumin (BSA) as a standard.

\textbf{Liquid chromatography and tandem mass spectrometry}
(LC–MS/MS)

The three protein extracts were pooled together for
homogenization, then shared into six equal replicates
for mass spectrometry analysis, as previously described
[12]. Prior to mass spectrometry analysis, aliquots of
150 µg protein of each replicate were subjected to over-
night acetone precipitation at −20 °C to remove any
contaminants. Protein precipitates were centrifuged at
13,000 rpm for 20 min at 4 °C, and after the acetone
was removed, the resulting pellets were resuspended in
100 µL of NH4HCO3 (50 mM, pH 9.7). The sample prepa-
ration and the mass spectrometry analysis were per-
formed according to a previously described protocol [13].
Briefly, the peptide separation was performed by liquid
chromatography (nanoACQUITY UPLC™, Waters Cor-
poration, Milford, MA, USA) and the mass detection of
peptides was performed on a hybrid quadrupole-time-
of-flight mass spectrometer (Q-TOF micro, Micromass,
Alliedscienpro, Quebec, Canada) equipped with a nano-
electrospray Z spray source. Data from were processed
using MassLynx (v 4.1, Waters Corporation), and the
raw files from the Q-TOF micro (MS/MS data) were
converted into the PKL (peak list) format; the files of
the six replicates were merged into one file for the database
search, which is provided as Additional file 1.

Searches employing MS/MS data were performed
using the open-source software X!Tandem (www.
thegpom.org/tandem/) and the specific database for \textit{C. pseudotuberculosis} [14]; the search parameters included
the enzyme entry set for trypsin, peptide tolerance of
100 ppm, fragment mass tolerance of 1.2 Da and a maxi-
mum of one missed cleavage. Cysteine carbamido meth-
ylation and methionine oxidation were set as fixed and
variable modifications, respectively. Search results were
validated through the Log of the E score, and peptide
spectrum matches were considered significant when the
Log expectation value reported by X!Tandem was smaller
than −3, meaning that the probability of a peptide match
being a false positive is less than 0.005.

\textbf{Bioinformatics analysis}

The bioinformatics analysis used sequences and acces-
sion numbers from the non-redundant reference strains
C231, Cp267 or E19. The identified proteins underwent
prediction of subcellular localization in the CELLO2GO
web server (http://cello.life.nctu.edu.tw/cello2go/), with
the E-value set at 0.001 [15]. All the proteins were also
subjected to prediction of protein topology in the TOP-
CONS web server (http://topcons.cbr.su.se/), which iden-
tifies transmembrane and signal peptide regions [16].
The proteins that were in silico classified as exclusively
intracellular or that presented no transmembrane-helix
or signal peptides were excluded from further predic-
tions since they were not considered to be membrane
associated. The pathogenic potential of the selected
proteins was determined using the MP3 web server (http://metagenomics.iiserb.ac.in/mp3/), with a threshold value set at 0.2 [17]. The sequences were subjected to protein–protein interaction analysis using the STRING web server (http://string-db.org/) [18]. STRING was used in multiple sequences mode, and only interactions with the highest confidence levels (> 0.900) were kept, as recommended by the authors [19]. The interaction network was constructed with the whole set of target proteins, with the maximum number of interactors to show set at 110 proteins (five interactors per target protein), and the final metabolic pathway maps involving the pathogenic target proteins were searched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database as previously described [20].

Results

_Corynebacterium pseudotuberculosis_ strain VD57 has been directly cultivated in bovine fetal serum, and its membrane-associated proteins have been extracted using an organic solvent enrichment methodology, followed by LC–MS/MS analysis for protein identification. One hundred and sixteen proteins were identified by this methodology and in silico characterized according to their subcellular localization, topology and pathogenic potential (Additional file 2). Bioinformatics analysis revealed that 35 sequences were classified as membrane, cell wall or extracellular proteins and presented at least one transmembrane-helix or a signal peptide. The in silico prediction of potentially pathogenic proteins revealed that 22 of the identified membrane-associated proteins might be associated with _C. pseudotuberculosis_ pathogenesis, and 11 are as yet uncharacterized proteins. The final list of identified membrane-associated proteins with predicted pathogenic potential is presented in Table 1.

The existence of cooperative work among the 22 target pathogenic proteins was investigated using the STRING server to construct interaction networks. The results indicated that the proteins CiuA and FagA are biologically connected in the same network, which was constructed with 12 interactor proteins and 39 interaction edges with the highest confidence levels (> 0.900), and was associated with the ABC Transporters KEGG pathway (Fig. 1a). The proteins oppCD2 and oppA4 were biologically connected in another interaction network constructed with 22 interactor proteins and 68 interaction edges with highest confidence levels (> 0.900); three interactor proteins were also associated with the ABC Transporters KEGG pathway (Fig. 1b).

Discussion

The presented results indicate that the combination of organic solvent enrichment methodology with LC–MS/MS and bioinformatics analysis was effective in identifying 22 potentially pathogenic membrane associated proteins. The high number (50%) of potentially pathogenic proteins that are still uncharacterized clearly demonstrates that a greater effort is needed to investigate the virulence determinants of _C. pseudotuberculosis_. Among the 11 identified well characterized antigens, only the proteins ciuA, fagA, OppA4 and OppCD2, which were associated with the ABC Transporters KEGG pathway (Fig. 1a, b, respectively), have consistent scientific evidence of participation in _C. pseudotuberculosis_ pathogenesis.

The CiuA protein is expressed by the operon ciuAB-CDE (ABC-type transporter and siderophore biosynthesis-related proteins) [21–23], and an experimental work using an iron-acquisition-deficient mutant strain of _C. pseudotuberculosis_, which had the ciuA gene disrupted, revealed that such strain had reduced intracellular viability during infection in murine model. These results suggested that siderophores in such intracellular bacteria improve cell viability by helping the pathogen to strip

| # | Protein names | Gene names |
|---|---------------|------------|
| 1 | Iron ABC transporter substrate-binding protein | ciuA CpC231_0982 |
| 2 | FagA protein | fagA CpC231_0028 |
| 3 | Oligopeptide-binding protein oppA | oppA4 CpC231_1167 |
| 4 | Oligopeptide transport ATP-binding protein OppD | oppCD2 CpC231_0260 |
| 5 | Mycosubtilin synthase subunit B | mycB CpC231_1794 |
| 6 | ESX-3 secretion system protein eccC3 | eccC3 CpC231_0404 |
| 7 | ABC transporter inner membrane protein | CpC231_1467 |
| 8 | Transmembrane transport protein MmpL | mmpL CpC231_1939 |
| 9 | LpqU family protein | LpqU CpC231_0712 |
| 10 | Surface antigen | CpC231_0173 |
| 11 | Copper resistance D domain-containing protein/Cytochrome c oxidase caa3 assembly factor (Caa3_CtaG) | copD CpC231_1627 |
| 12 | Uncharacterized protein | CpC231_0196 |
| 13 | Uncharacterized protein | CpC231_0252 |
| 14 | Uncharacterized protein | CpC231_1761 |
| 15 | Uncharacterized protein | CpC231_1862 |
| 16 | Uncharacterized protein | CpC231_1904 |
| 17 | Uncharacterized protein | CpC231_2052 |
| 18 | Uncharacterized protein | CpC231_0195 |
| 19 | Uncharacterized protein | CpE19_0622 |
| 20 | Uncharacterized protein | CpC231_0905 |
| 21 | Uncharacterized protein | CpE19_1910 |
| 22 | UPF0182 protein | CpC231_0555 |

Table 1 Membrane-associated proteins with predicted pathogenic potential expressed by Corynebacterium pseudotuberculosis grown in bovine fetal serum
iron from chelators such as transferrin, lactoferrin and hemoglobin-haptoglobin complexes [24]. The identification of FagA in this study, as well as its connection with CiuA in the same interaction network, might indicate a possible complementation of functions between the two operons ciuABCDE and FagABCD for the uptake of iron by *C. pseudotuberculosis*. This finding is in accordance with the experimental evidence reported in a previous study using a recombinant *C. pseudotuberculosis* strain carrying a fagA-lacZ fusion, which indicated that the putative fagABC operon was highly expressed iron-limited media and that this operon was regulated by iron concentration in vitro. Another fagB(C) mutant showed reduced virulence compared to wild-type in a goat model of caseous lymphadenitis, indicating that the expression of the fag genes in the host also appears to contribute to virulence [25].

The oppA4 and oppCD2 proteins are expressed by the operon oppBCDA (oligopeptide permease) and are located in the plasma membrane, presenting functions generally related to cell nutrition and the uptake of peptides from the extracellular environment. An experiment using an OppD-deficient *C. pseudotuberculosis* strain demonstrated that this mutant presented a reduced ability to adhere to and infect macrophages compared to the wild-type bacteria [26]. Transcriptomics experiments have also demonstrated that the in vitro replication of *C. pseudotuberculosis* is decreased when it is grown under stressful conditions (acid, osmotic and thermal shock stresses), possibly due to the repression of the opp operon genes [27].

Concerning the other potentially pathogenic proteins identified in this study (Table 1), they did not have the function experimentally studied in *C. pseudotuberculosis*.
but functional information from the respective heterologous proteins in other bacterial species revealed that some of them might also contribute to host–pathogen interactions and microbial virulence, as briefly discussed below.

Mycosubtilin synthase subunit B (mycB) integrates the gene cluster responsible for the biosynthesis of mycosubtilin, which has been demonstrated to present in vitro hemolytic activity [28]; the ESX-3 secretion system protein (eccC3) is a type VII secretion system that is essential for bacterial viability and is involved in iron uptake [29]. The transmembrane transport protein MmpL (mmpL) is associated with survival in macrophages and drug resistance [30], as well as with bacterial viability and virulence [31]; the lpqU family protein (lpqU) might play a role in bacterial viability and resistance [32, 33]; the copper resistance D domain-containing protein (copD) integrates a cluster of copper tolerance genes that have the main function of protecting bacteria from copper intoxication [34]. According to Uniprot annotation, the surface antigen (CpC231_0173) presents an adhesion domain, which is found in adhesin proteins, and the ABC transporter inner membrane protein (CpC231_1467) belongs to the ABC-4 integral membrane protein family and presents a permease protein domain.

In conclusion, as growing *C. pseudotuberculosis* directly in animal serum exposes the bacteria to real host constituents, the membrane proteins reported herein potentially reflect part of the protein repertoire expressed during sheep and goat caseous lymphadenitis, and might contribute for the future development of a satisfactory vaccine model. Based on the identification of potentially pathogenic antigens and protein–protein interaction network, it is suggested that *C. pseudotuberculosis* pathogenesis might be associated with the transport and uptake of nutrients, bacterial resistance, cell viability and adhesion.

**Limitations**

Although this work provides a list of promising and relevant membrane-associated antigens of *C. pseudotuberculosis* grown in animal serum, that can be used in future vaccine tests, without any further functional characterization the overall nature of the present work will be only a preliminary and descriptive one. Thus, experimental validation of the in silico results are required to confirm that the identified antigens are really needed for bacterial pathogenesis and reflect part of the protein repertoire expressed under real infection conditions.

**Additional files**

Additional file 1. Mass spectrometry data of LC–MS/MS analysis in PKI format.

Additional file 2. List of identified proteins in LC–MS/MS analysis and respective bioinformatics analysis.

**Abbreviations**

BHI: Brain Heart Infusion; BFS: bovine fetal serum; OD: optical density; PBS: phosphate-buffered saline; LAMs: lipoarabinomannans; PIMs: phosphatidylinositol mannosides; BSA: bovine serum albumin; LC–MS/MS: liquid chromatography and tandem mass spectrometry; KEGG: Kyoto Encyclopedia of Genes and Genomes.

**Authors’ contributions**

JTR, BLB, PCBVB, and MCS carried out the bacterial cultivation, antigen preparation and the mass spectrometry experiments and analyzed the spectrum data; MCAS and TJS carried out the bioinformatic analysis of the sequenced data; JTR and BLB analyzed the final data and drafted the manuscript; RWP, LFMC, VA and RM conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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