



Identification of 11 new exoproteins in *Corynebacterium pseudotuberculosis* by comparative analysis of the exoproteome



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ABSTRACT

This study involves the comparison between the exoproteomes of two different strains of *Corynebacterium pseudotuberculosis*, the etiologic agent of caseous lymphadenitis in small ruminants. In a previous study, based on a gel-free system (TPP-LC/MS^E), 70 exoproteins for the strain 1002 and 67 for the strain C231, totaling 93 different extracellular proteins for *C. pseudotuberculosis*, were identified. In the present work, we have used 2D gel electrophoresis to resolve the extracellular proteins of both strains, which were then digested with trypsin, analyzed by MALDI-TOF/TOF and identified with the software MASCOT[®]. A total of 45 extracellular proteins of *C. pseudotuberculosis* were identified by this approach. The comparative analysis between the strains 1002 and C231 identified 13 and 3 strain-specific proteins, respectively, 11 of which are novel. These newly identified proteins may play an important role in the physiology and virulence of *C. pseudotuberculosis*.

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1. Introduction

Corynebacterium pseudotuberculosis is a Gram-positive, facultative, intracellular pathogen, responsible for caseous lymphadenitis (CLA) in small ruminants [1] and causes great economic losses in the goat and sheep industries worldwide [2]. Currently, there are no efficient tests, vaccines, or drugs available for early diagnosis, effective prevention, or treatment.

Abbreviations: CLA, caseous lymphadenitis; LC-MS, liquid chromatography – mass spectrometry; PLD, phospholipase D; PSE, potentially surface exposed; TPP, three-Phase partitioning; 2-DE, two-dimensional electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization – time-of-flight; MS, mass spectrometer; HSP, heat shock protein; RT, room temperature.

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The phospholipase D (PLD), a potent exotoxin with sphingomyelinase activity, is the main virulence factor of *C. pseudotuberculosis* [3]. Other virulence factors include ABC type iron transporters (important for disease progression) [4] and a serine protease [5]. Extracellular and secreted proteins are important virulence factors, associated with cell adhesion, cell invasion, survival and proliferation in the host cell, and escape from the immune system; and have high potential for the development of drugs or vaccines to target bacterial pathogens [6]. Therefore, the exoproteome and secretome of *C. pseudotuberculosis* are of interest for developing effective drugs or vaccines to combat CLA.

Here, using two-dimensional electrophoresis (2-DE) along with mass spectrometry MALDI-TOF/TOF, we have generated exoproteome maps for the *C. pseudotuberculosis* 1002 (Cp 1002) and *C. pseudotuberculosis* C231 (Cp C231) strains. Based on this methodology it was possible to identify 11 extracellular proteins of *C. pseudotuberculosis* that had not been detected in the previous

study, based on gel-free approach TPP-LC/MS^E [7]. After combining the results (93 extracellular proteins) obtained from TPP-LC/MS^E, and 2-DE-MALDI-TOF/TOF methodologies, a total of 104 extracellular proteins were successfully characterized in *C. pseudotuberculosis*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The wild-type *C. pseudotuberculosis* strains Cp 1002 and Cp C231 were routinely maintained in Brain Heart Infusion (BHI) broth or bacteriological agar plates at 37 °C. To extract extracellular proteins, the strains were cultured in chemically defined medium (CDM) [(Na₂HPO₄·7H₂O (12.93 g/L), KH₂PO₄ (2.55 g/L), NH₄Cl (1 g/L), MgSO₄·7H₂O (0.20 g/L), CaCl₂ (0.02 g/L), and 0.05% (v/v) Tween 80]; 4% (v/v) MEM Vitamins Solution 100× (Invitrogen); 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) at 37 °C [8] until the set point of exponential growth (DO_{600nm} = 1.3) was reached.

2.2. Three-phase partitioning

An optimized TPP protocol was used to extract extracellular proteins [9]. The cultures were centrifuged for 20 min at 2700 × g. Supernatants were filtered using 0.22 μm filters, ammonium sulfate was added to the samples at 30% (w/v) and the pH of the mixtures was adjusted to 4.0. Next, an equal volume of N-butanol was added to each sample. The samples were vigorously vortexed and left to rest for 1 h at room temperature, then centrifuged for 10 min at 1350 × g at 4 °C. The interfacial precipitate was collected in 1.5 mL microcentrifuge tubes and re-suspended in 1 mL Tris 20 mM + 10 μL protease inhibitor. The protein concentration was determined using a standard curve [10].

2.3. Two-dimensional electrophoresis (2-DE)

Approximately 300 μg of protein was dissolved in 450 μL of rehydration buffer (Urea 7 M, thiourea 2 M, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate 2%, Tris 40 mM, bromophenol blue 0.002%, DTT 75 mM, IPG Buffer 1%). Samples were applied to 18 cm pH 3–10 NL strips (GE Healthcare). Isoelectric focusing (IEF) was performed using the apparatus IPGphor 2 (GE Healthcare) under the following voltages: 100 V 1 h, 500 V 2 h, 1000 V 2 h, 10000 V 3 h, 10000 V 6 h, 500 V 4 h. The strips were kept at 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) and an additional 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). The isolated proteins were separated in 12% acrylamide/bis-acrylamide gels with an Ettan DaltSix II system (GE Healthcare). To visualize the separated proteins, gels were stained with Coomassie blue G-250 staining solution. Three biological replicates of 2-DE gels were scanned using an Image Scanner (GE Healthcare), and the Image Master 2D Platinum 7 (GE Healthcare) software was used to analyze the generated images. Reproducible images detected by the software were manually analyzed to eliminate possible artifacts.

2.4. In-gel tryptic digestion of proteins

Protein spots were excised from the gels using an *Ettan Spot Picker* (GE Healthcare), and fragments containing the excised spots were washed with ultra-sterile water for 5 min and dehydrated with acetonitrile (ACN) for 20 min. Subsequently, the fragments were

dried in a speed vac. Protein digestion was performed by adding 10 μL of a stock solution of trypsin (Promega, Sequencing Grade Modified Trypsin) (33 ng/mL—ca. 1.5 μM) to each tube for 60 min at 4 °C. After removal of excess trypsin, samples were incubated at 58 °C for 30 min. Digestion was interrupted by adding 1 mL of 5% formic acid (v/v). The extraction of peptides was performed using 30 mL of formic acid solution (5%–50% ACN), and the sample was subjected to ultrasound. The peptides were concentrated to a volume of 10 mL by a speed vac and desalinated and concentrated using ZIP-TIP C18 tips (Eppendorf) [11]. Finally, the samples were stored at –20 °C for subsequent analysis by mass spectrometry.

2.5. Mass spectra and database search

Analyses by MS and MS/MS modes were performed using a MALDI-TOF/TOF mass spectrometer Autoflex IIITM (Brucker Daltonics, Billerica USA). The equipment was controlled in a positive/reflector using the FlexControlTM software. Calibration was performed using samples of standard peptides (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) (Brucker Daltonics, Billerica, USA). The peptides were added to the alpha-cyano-4-hydroxycinnamic acid matrix, applied on an AnchorChipTM 600 plate (Brucker Daltonics, Billerica, USA) and analyzed by Autoflex III. The search parameters included 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 for identifying proteins with the program MASCOT[®] (<http://www.matrixscience.com>) and compared with the NCBI databases.

2.6. In silico predictions of protein sub-cellular localization

To predict the sub-cellular localization of proteins, we used the following programs: SurfG+ v1.0 [12], SecretomeP v2.0 [13] and TatP v1.0 [14].

3. Results

3.1. The proteome reference maps of two *C. pseudotuberculosis* strains and protein identifications by MALDI-TOF/TOF

After 2-DE, 85 spots for Cp 1002 and 80 spots for Cp C231 were detected in the extracellular proteome. The electrophoretic patterns of the proteins were similar between the strains, with the majority of spots concentrated in the acidic range of the gels (p.I 3–5). After acquiring the results from images of the gels, spots were excised from the gels, submitted to tryptic digestion and analyzed by MALDI-TOF MS/MS. MASCOT software identified 55 of the 85 protein spots (65%) in Cp 1002 (Fig. 1) and 45 (56%) in Cp C231 (Fig. 2). Because of low amounts or post-translational modifications of the proteins, some spots could not be identified. Altogether, we identified 45 proteins, 29 of which are common to both strains (Supplementary file 1), 13 of which are unique to Cp 1002 (Supplementary file 2) and 3 of which are exclusive to Cp C231 (Supplementary file 3). Some protein spots were identified as the same protein, possibly revealing the presence of isoforms because of post-translational modifications.

3.2. Comparative analysis between the strains exoproteomes

The exclusive proteins to Cp 1002 are involved in several molecular functions (Supplementary file 2), acting on distinct

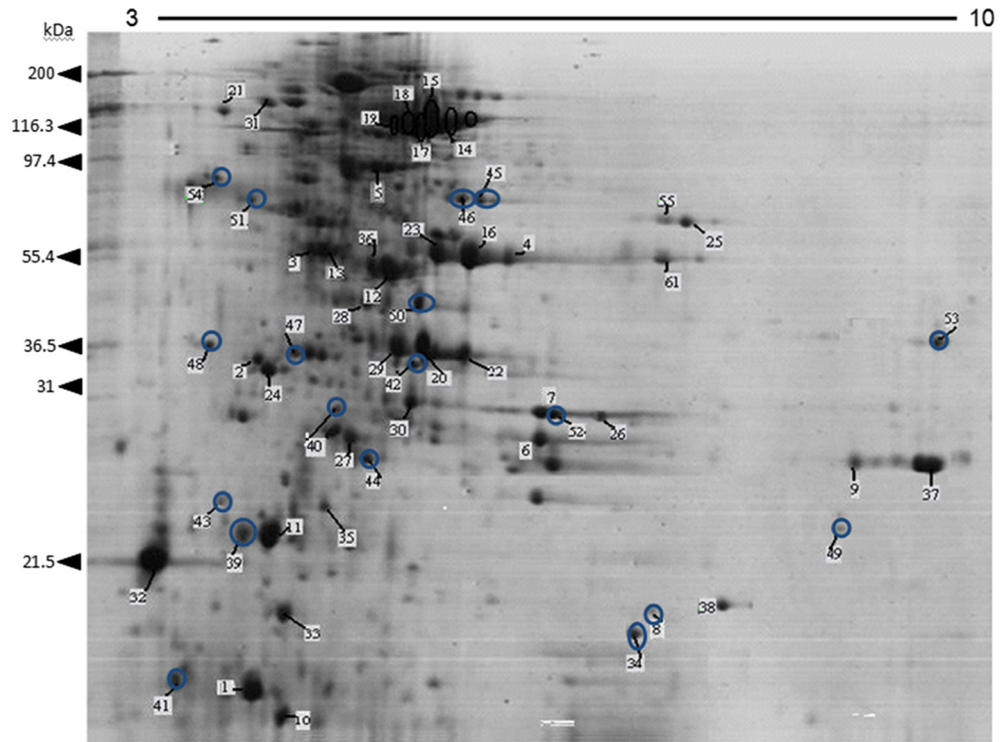


Fig. 1. 2-DE of extracellular proteins of strain 1002 stained with colloidal Coomassie. Electrophoretic profile using 3–10 NL strips. Spots with numbers were identified by MS. Spots with blue circles indicate protein spots taken exclusively from strain 1002. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

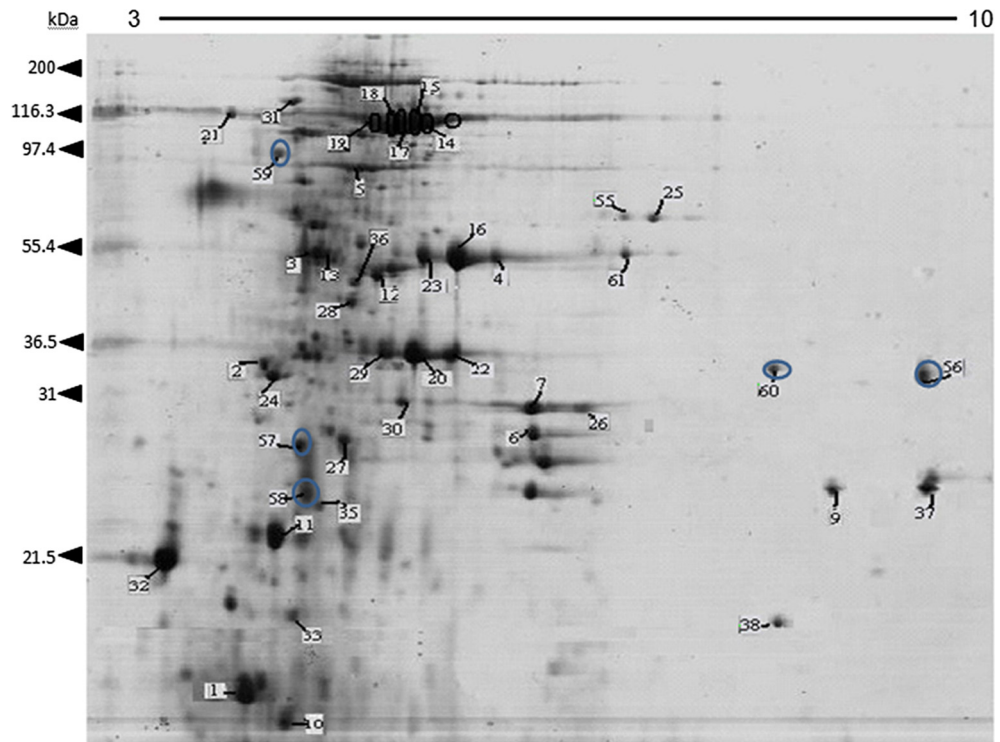


Fig. 2. 2-DE of extracellular proteins of strain C231 stained with colloidal Coomassie. Electrophoretic profile using 3–10 NL strips. Spots with numbers were identified by MS. Spots with blue circles indicate protein spots taken exclusively from strain C231. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

biological processes. The predominant functional group among the proteins detected is predicted to be involved in stress oxidative and cellular metabolism. Others process such as: transcription, translation, transport and biosynthesis were also identified. The set exclusive proteins to *Cp C231* (Supplementary file 3) are likely related to virulence, biosynthesis and cell wall biogenesis. Proteins with unknown functions were also identified for both strains. According to the NCBI database, one specific hypothetical protein (ADL09626) showed no similarity to any other protein in the *Corynebacterium* genus or other bacterial genera, suggesting that it is unique to the *C. pseudotuberculosis* species. This observation motivates further studies to explore the role of ADL09626 in *C. pseudotuberculosis* physiology and pathogenesis.

Bioinformatics analyses revealed that among the set of exclusive proteins *Cp 1002*, only the Open Reading Frames (ORF) which encodes for a putative DsbG protein (ADL21555) is not present in the genome of *Cp C231*, this protein belongs to the superfamily of thioredoxin and acts as a chaperone in the formation of disulfide bonds in proteins secreted by *Escherichia coli* [15]. However, DNA sequences encoding for all proteins present in exclusive proteome *Cp C231*, are present in the genome of *Cp 1002*.

Identical proteins with unknown functions were also identified for both strains. According to the NCBI database, one specific hypothetical protein (ADL09626) showed no similarity to any other protein in the *Corynebacterium* genus or other bacterial genera, suggesting that it is unique to the *C. pseudotuberculosis* species. This observation motivates further studies to explore the role of ADL09626 in *C. pseudotuberculosis* physiology and pathogenesis.

3.3. Analysis of *C. pseudotuberculosis* extracellular proteins using different approaches

Pacheco et al. [7] detected 70 and 67 (for a total of 93) proteins, respectively, from the exoproteomes of strains *Cp 1002* and *Cp C231* using the TPP/LC-MS^E method. This combined method of 2-DE and MALDI-TOF/TOF techniques allowed us to identify 42 proteins in *Cp 1002* and 32 proteins in *Cp C231*, resulting in a total of 45 different extracellular proteins identified for *C. pseudotuberculosis*. Despite our lower number of identifications, it is possible to demonstrate the presence of 11 proteins that were not previously detected by Pacheco et al. [7] (Table 1). Combining the results obtained by both studies, 81 and 73 extracellular proteins were characterized for strains *Cp 1002* and *Cp C231*, respectively, totaling 104 exported proteins for *C. pseudotuberculosis* (Supplementary file 4). Fig. 3 shows the distribution of proteins identified by the different approaches.

Table 1
List of the 11 proteins not identified by TPP/LCMS^E.

Protein	ID (NCBI) ^a of strains		Gel identification	
	1002	C231	1002	C231
Chaperone GroEL	ADL21673	ADL11255	Yes	Yes
Chaperone protein DnaK	ADL21757	ADL11343	Yes	Yes
Hypothetical protein	ADL21702	ADL11289	Yes	Yes
Hypothetical protein	ADL21703	ADL11290	Yes	Yes
Hypothetical protein	ADL21704	ADL11291	Yes	Yes
Elongation factor P	ADL21021	ADL10611	Yes	Yes
Enolase	ADL20605	ADL10195	Yes	No
Glyceraldehyde-3-phosphate dehydrogenase	ADL20991	ADL10581	Yes	No
ABC-type transporter	ADL20391	ADL09988	Yes	No
Carbonic anhydrase	ADL20477	ADL20477	Yes	No
Manganese superoxide dismutase	ADL21849	ADL11437	Yes	No

^a Access number of proteins (NCBI genome project at 40687 and 40875).

4. Discussion

In this study, a comparative analysis between the extracellular proteins of *C231* and *1002 C. pseudotuberculosis* strains was performed. Exoproteome differences observed between these strains may result from differences in hosts and geographic locations (*Cp 1002* was isolated from goats in Brazil and *Cp C231* from sheep in Australia). We could not detect spots referent to PLD and CP40 proteins in *Cp 1002* gels, similar to the findings by Pacheco et al. [7]. PLD is the most important virulence factor in *C. pseudotuberculosis*, the non-production of this exotoxin in *Cp 1002* can promote the inability this strain to spread in host, directly influencing in pathogenesis process of this pathogen [16].

The cytoplasmic proteins identified in the extracellular extracts may be exported by non-classical secretion. According to previous work, the detected proteins, elongation factor Tu, GroEL, Enolase, Glyceraldehyde-3-phosphate dehydrogenase and superoxide dismutase (SodA), were found to be dependent on a non-classical secretion pathway via SecA [13,17–19]. After sequencing the genomes of strains *Cp 1002* and *Cp C231*, it was observed that both carry two *SecA* genes (*SecA1* and *SecA2*), possibly involved in the *C. pseudotuberculosis* secretion systems [20]. *SecA2* has been described in *Bacillus subtilis* [17] and pathogenic bacteria, such as *Listeria monocytogenes* [18] and *Mycobacterium tuberculosis* [19].

A classic proteomics technique, based on 2-DE, led us to identify 11 proteins not detected using the TPP-LC/MS (gel-independent) approach in a previous study [7]. The combination of both techniques was found to be complementary, as it increased the previous number of identified extracellular proteins from 93 to 104. The differences observed in both studies may be associated with the separation methods, which involve distinct buffers for sample solubilization, and the physical–chemical properties of each protein. Another question that deserves attention for proteomics involving prokaryotes is the growth phase in which the studies are held. This growth phase issue is a possible explanation for the non-identification by Pacheco et al. [7] of 11 proteins described in the present work, as the extractions of proteins in our study occurred at the late-exponential growth phase, whereas Pacheco et al. [7] performed their extractions in the early exponential phase.

Among the 11 proteins newly identified in this study (Table 1), 3 have unknown functions (hypothetical proteins) and 8 have been related to various physiological functions and virulence factors. GroEL (HSP60) and DnaK (HSP70) are 2 of the major and best-studied chaperone proteins that play a key role in bacterial physiology and infection [21]. Elongation factor P (EFP) is a highly conserved protein in prokaryotes that stimulates the peptidyl-transferase activity of 70S ribosomes and enhances the synthesis of certain dipeptides initiated by *N*-formylmethionine [22]. The *efp* gene is essential for *E. coli* growth and protein synthesis [22]. In *Salmonella enterica*, it is involved in virulence and stress response [23]. Enolase is an enzyme of the glycolytic pathway that catalyzes the reversible conversion of 2-phosphoglycerate (2-PGE) into phosphoenolpyruvate (PEP). Bacterial enolases exhibit fibronectin-binding activity and influence colonization, invasion and persistence in host tissues [24]. In *Bacillus anthracis*, this protein acts as an immunodominant antigen [25] and may contribute to raising the invasive potential of the pathogen [26]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is present in cellular surfaces and secretomes of various pathogens; it is also associated with the *Streptococcus agalactiae* virulence mechanism because of its ability to modulate the host immune system during infection [27]. ABC-type transporters are essential for nutrient uptake and the secretion of various molecules, and they are involved in translation of mRNA and DNA repair [28]. ABC transporters can also play important roles in cell viability, virulence and bacterial

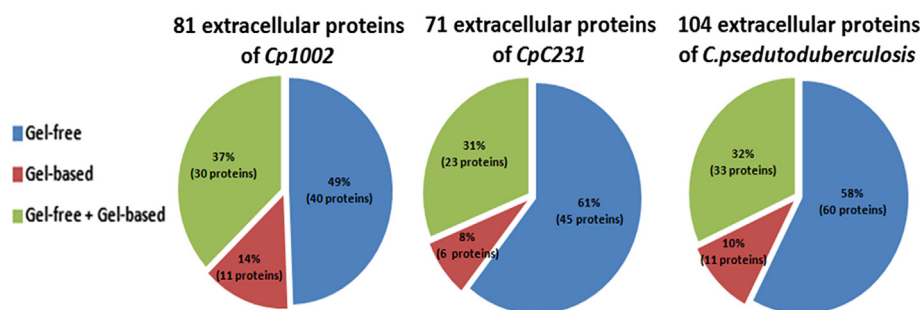


Fig. 3. Distribution of the identification of extracellular proteins of *C. pseudotuberculosis* by different approaches. Proteins detected by both techniques (2DE/MALDI-TOF/TOF + TPP-LC-MS^E) are in blue; proteins identified by the 2DE/MALDI-TOF/TOF technique are in red; and proteins identified by Pacheco et al. (2011) (TPP-LC-MS^E) are in green.

pathogenicity, such as the iron ABC uptake system [29]. Carbonic anhydrase maintains pH homeostasis and is involved in respiration, ion transportation, and bacterial growth [30,31]. Manganese superoxide dismutase (MnSOD) is an isoform of the superoxide dismutase family and an important component of the antioxidant defense mechanism that acts to eliminate reactive oxygen species (ROS) [32,33]. In *Enterococcus faecalis*, the MnSOD contributes to survival of the bacterium in macrophages, which is potentially important during the pathogenesis process [34].

In conclusion, the observed difference between the exoproteome of the strains *Cp 1002* and *Cp C231*, can influence in pathogenesis, antigenicity and adaptation specific-hosp of each isolated. Therefore, our data demonstrated which use of complementary proteomic techniques is an efficient strategy for the characterization of bacterial exoproteomes. These new identified proteins increase the extracellular protein catalog of *C. pseudotuberculosis* and validate former *in silico* predictions. In addition, these proteins may represent new potential targets for use in prophylaxis against CLA.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.micpath.2013.05.004>.

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