



Leader gene of *Corynebacterium pseudotuberculosis* may be useful in vaccines against caseous lymphadenitis of goats: a bioinformatics approach

Eliane Macedo SOBRINHO SANTOS^{1,2)*}, Anna Christina ALMEIDA³⁾,
Hércules Otacílio SANTOS⁴⁾, Alex Sander Rodrigues CANGUSSU⁵⁾,
Deborah Aires ALMEIDA⁵⁾ and Kattyanne Souza COSTA⁶⁾

¹⁾Department of Dentistry, Universidade Estadual de Montes Claros, Minas Gerais, 39400-000, Brazil

²⁾Instituto Federal do Norte de Minas Gerais, Campus Araçuaí, Minas Gerais, 39600-000, Brazil

³⁾Instituto de Ciências Agrárias da UFMG, Campus Montes Claros, 39400-000, Brazil

⁴⁾Instituto Federal do Amazonas, Campus São Gabriel da Cachoeira, Amazonas, 69750-000, Brazil

⁵⁾Univeridade Federal do Tocantins, Campus Gurupi, Tocantins, 77402-000, Brazil

⁶⁾Research and Development Laboratory of Vallée S.A., Montes Claros, Minas Gerais, 39400-000, Brazil

ABSTRACT. We conducted an *in silico* analysis to search for important genes in the pathogenesis of Caseous Lymphadenitis (CL), with prospects for use in formulating effective vaccines against this disease. For this, we performed a survey of proteins expressed by *Corynebacterium pseudotuberculosis*, using protein sequences collected from the NCBI GenPept database and the keywords “caseous lymphadenitis” and “*Corynebacterium pseudotuberculosis*” and “goats”. A network was developed using the STRING 10 database, with a confidence score of 0.900. For every gene interaction identified, we summed the interaction score of each gene, generating a combined association score to obtain a single score named weighted number of links (WNL). Genes with the highest WNL were named “leader genes”. Ontological analysis was extracted from the STRING database through Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A search in the GenPept database revealed 2,124 proteins. By using and plotting with STRING 10, we then developed an *in silico* network model comprised of 1,243 genes/proteins interconnecting through 3,330 interactions. The highest WNL values were identified in the *rplB* gene, which was named the leader gene. Our ontological analysis shows that this protein acts effectively mainly on Metabolic pathways and Biosynthesis of secondary metabolites. In conclusion, the *in silico* analyses showed that *rplB* has good potential for vaccine development. However, functional assays are needed to make sure that this protein can potentially induce both humoral and cellular immune responses against *C. pseudotuberculosis* in goats.

KEY WORDS: *in silico*, network, ontological analysis, *rplB*

J. Vet. Med. Sci.

80(8): 1317–1324, 2018

doi: 10.1292/jvms.16-0581

Received: 16 November 2016

Accepted: 28 May 2018

Published online in J-STAGE:

25 June 2018

Bioinformatics analyses can add value to molecular biology studies. Therefore, on the basis of interest in experiments to analyze risk factors or potential therapeutic targets, gene interaction maps and the position of genes are very relevant [4] in identifying genes involved in a group and determining the most important gene, called the leader gene [17, 30, 40, 41]. However, no studies have yet identified the leader genes involved in Caseous Lymphadenitis (CL) disease.

CL is a chronic bacterial infectious disease that affects sheep and goats and is responsible for economic losses in many countries [12, 14], including Brazil [1]. Since the disease is spread worldwide, and it has considerable economic importance [32, 46], researchers have increasingly focused on investigating its pathogenesis. *Corynebacterium pseudotuberculosis* is a significant animal pathogen and it is the etiological agent of this disease [46]. However, the genetic determinants of *C. pseudotuberculosis* virulence are still poorly characterized [14] and proteins that are bottlenecks for the action of these microorganisms in the pathogenesis of CL are still unknown or little studied.

The majority of these proteins can be virulence factors or they can positively modulate virulence genes or encode virulence factors, allowing *C. pseudotuberculosis* to exert pathogenic characteristics. Advances in the genomics field have already allowed

*Correspondence to: Sobrinho Santos, E. M.: elianemsobrinho@hotmail.com

©2018 The Japanese Society of Veterinary Science



This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License. (CC-BY-NC-ND 4.0: <https://creativecommons.org/licenses/by-nc-nd/4.0/>)

the characterization of many *C. pseudotuberculosis* genes, mainly those related to virulence, such as phospholipase D (*pld*), *aroB*, *aroQ*, *fagA*, *fagB*, *fagC*, and *fagD*, Heat shock proteins, *recA*, and *rpoB* [9]. More recent genes identified includes *dak2*, *fagA*, *fagB*, the NlpC/P60 protein family, and the LPxTG putative protein family [16]. For this reason, it is of great interest from the scientific community to find proteins and genes that are bottlenecks in the action of *C. pseudotuberculosis*: genes working as leader genes in the virulence of these bacteria. Leader genes can become interesting candidates for developing new vaccines, more efficient therapies, and for diagnosis and illness control; CL is still currently managed by rudimentary prophylaxis. In the case of CL, the leader genes can be seen as the main virulence factors of the bacterium because they are genes that encode possible antigenic proteins, or at least may directly or indirectly influence other genes that are responsible for the virulence characteristics.

CL disease is expressed as external and visceral forms, either separately or together [2, 47]. Experimental infections have shown that *C. pseudotuberculosis* strains with mutations in the *pld* gene are unable to cause typical CL abscesses and disseminate within the host, revealing the toxin's key role in establishing disease [20]. Studies indicate that *pld* expression is reduced when *C. pseudotuberculosis* is exposed to heat shock and increased when the bacterium infects macrophages [26, 27]. Two genes were found to be activated in *C. pseudotuberculosis* only during macrophage infection: one encoding a non-ribosomal peptide synthetase and the other encoding a subunit of the propionyl-CoA-carboxylase enzyme [25]. Several genes encoding exported proteins have been identified, including the ones involved in the uptake of iron and the formation of adhesins and fimbriae, important structures for the initial stages of infection [13].

One way to control CL is to identify and remove the infected herd animals and vaccinate healthy animals [3, 12, 47]. However, the suppressive nature of CL vaccination is checked after prolonged immunization with bacterins, toxoids, combined, and live vaccines with persistent disease [3, 47]. Vaccines, often unable to protect animals, need to be better studied using genomic, transcriptomic, and proteomic approaches to elucidate virulence mechanisms and identify vaccine candidates and diagnostic tests for CL. Animals infected with *C. pseudotuberculosis* can contaminate meat and milk, creating a risk of zoonotic infection in humans [3, 47]. There are currently no commercial vaccines capable of protecting animals susceptible to this disease, and since *C. pseudotuberculosis* can infect both animals and humans, the development of new vaccines is needed for effective control [3, 44].

In this study, we conducted an *in silico* analysis to search for important genes in the pathogenesis of CL, with prospects of use in formulating effective vaccines against this disease, as well as their application in helping to control CL. Therefore, the leader genes identified in this study could be used as promising molecular tools to formulate effective CL vaccines and for use as targets in diagnosing the disease.

MATERIALS AND METHODS

Screening proteins expressed in *C. pseudotuberculosis*

In this study, we performed a survey of proteins expressed by *C. pseudotuberculosis*, using protein sequences collected from the NCBI GenPept database, until January 2016, focusing on analyzing peptides that are related with the keywords “caseous lymphadenitis”, “*Corynebacterium pseudotuberculosis*”, and “goats”.

Development of network representing protein-protein associations

A network was developed using the STRING 10 database (<http://string-db.org/>) [42, 43] with “Experiments”, “Databases”, “Textmining”, “Co-expression”, “Neighborhood”, “Gene fusion”, and “Co-occurrence” as input options and a confidence score of 0.900, with maximum number of interactors to show no more than 0 interactors for the 1st and 2nd shells. STRING is a searchable database used for determining interacting genes/proteins extracted from diverse curated and public databases with information on direct and indirect functional interactions/associations. These interactions are derived from different sources, such as manually-curated databases, primary databases, Medline abstracts, and a large collection of full-text articles, algorithms, and co-expression analyses using genomic information, and interactions observed in one organism that are systematically transferred to others via pre-computed orthology relationships [43]. As an input, we selected genes from the list of proteins that would be part of the survey in NCBI GenPept database.

Identification of leader genes in *C. pseudotuberculosis*' role in CL

For every gene interaction identified, we summed the interaction score of each gene, generating a combined association score. This score was adjusted and multiplied by 1,000 [8, 17, 31] to obtain a single score named the weighted number of links (WNL). This variable represents gene-gene interaction strength. Genes with the highest WNL values were defined as “leader genes” [8, 17, 31], since these are the candidate genes that may play an important role in *C. pseudotuberculosis* action.

Cluster numbers were calculated using the following equation: Cluster number = $TETO (LOG (CONTNUM (N); 2); 1)$; in this equation the mathematical symbolism of Microsoft Excel is represented in the Portuguese version, where N stands for WNL values of genes involved in the *C. pseudotuberculosis*'s action in CL. The interaction network was not expanded since the number of nodes and edges increased.

Leader genes showed the highest rank; other genes with lower WNL scores were listed in alphabetical descending order. Genes that presented no interaction were called orphan genes [31]. Interacting genes were classified as upregulated or downregulated, as previously described [31].

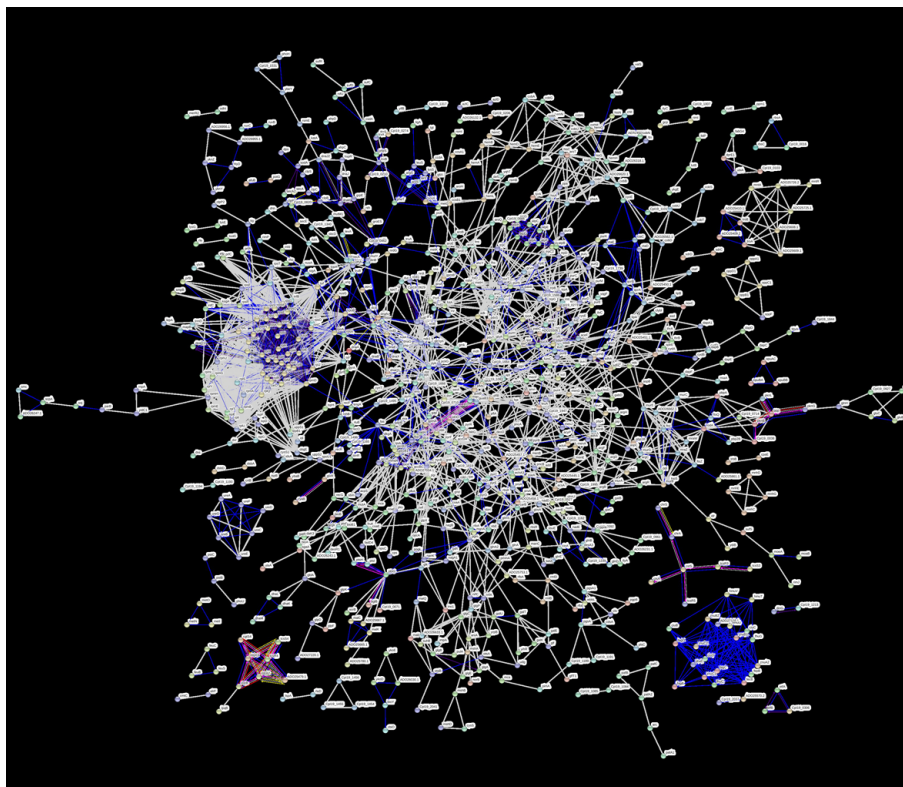


Fig. 1. Gene interaction map and up- and down-regulated genes involved in *C. pseudotuberculosis*'s action in CL in goats. Nodes represent proteins (splice isoforms or post-translational modifications are collapsed, i.e. each node represents all the proteins produced by a single, protein-coding gene locus); Node Size: small nodes: protein of unknown 3D structure, large nodes: some 3D structure is known or predicted; Node Color: colored nodes: query proteins and first shell of interactors, white nodes: second shell of interactors; Edges represent protein-protein associations (associations are meant to be specific and meaningful, i.e. proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other); Action Types: activation; inhibition; binding; catalysis; phenotype; posttranslational modification; reaction; transcriptional regulation; Action effects: positive; negative; unspecified.

Ontological characterization of network model for C. pseudotuberculosis action in CL

Ontological analysis was performed for additional information from the STRING database. Significant pathways were identified from some subgroups, such as cellular components, molecular functions, biological processes, and the pathological phenomenon was shown in the network. This information was obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical analyses

The genes were ranked according to this parameter in clusters, by the clustering method K-means. To evaluate the differences between various classes based on WNL, a Kruskal Wallis test was performed. Statistical significance was set at a *P*-value <0.001. Other statistical data were extracted from the STRING platform.

RESULTS

Proteins of C. pseudotuberculosis action in CL were identified in the GenPept database

A search of the GenPept database for keywords “caseous lymphadenitis”, “*Corynebacterium pseudotuberculosis*”, and “goats” revealed 2,124 proteins. Of these, 577 proteins were excluded from further analysis because they were hypothetical.

Network shows interaction between proteins of C. pseudotuberculosis' action in CL

Using STRING 10, it was possible to obtain a model showing gene/protein interaction maps including genes with increased or decreased expression during *C. pseudotuberculosis*'s action in CL (Fig. 1). This map of protein interaction shows the action of each protein in the context of the phenomenon under study; this means that some genes were downregulated while others were upregulated in order to allow *C. pseudotuberculosis* to promote CL.

By using and plotting with STRING 10, we then developed the in silico network model comprised of 1,243 genes/proteins

Table 1. Network statistics for *C. pseudotuberculosis*'s action in CL in goats

Parameters	Results
Number of nodes	1,243
Number of edges	3,330
Average node degree	5.36
Clustering coefficient	0.736
Expected number of edges	3,143
PPI enrichment <i>P</i> -value	0.0005

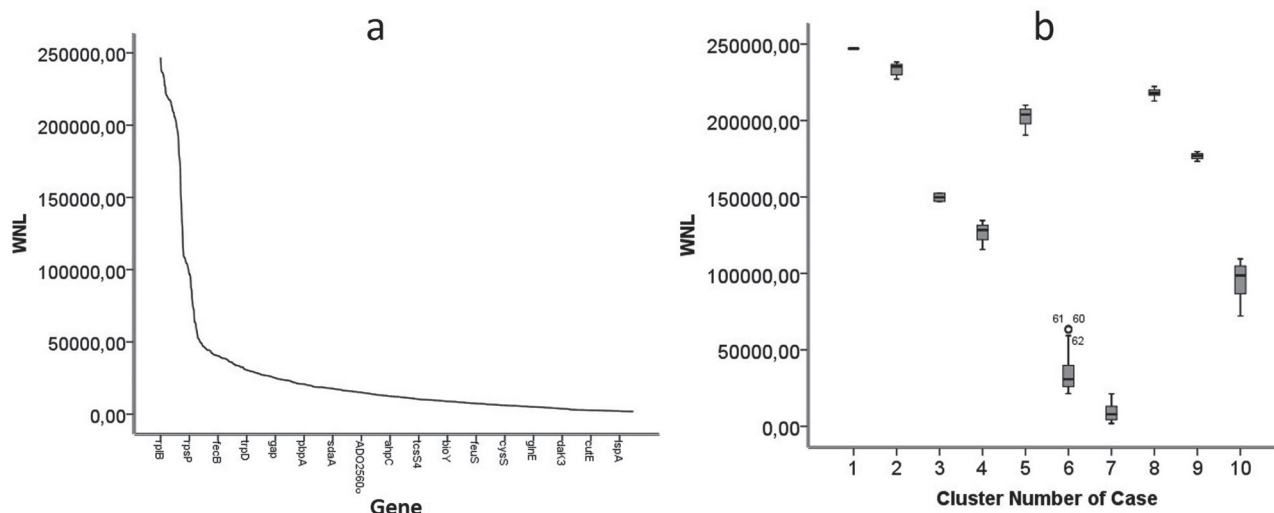


Fig. 2. Characterization of the leader gene. (a) WNL value for each gene in the data sets. The highest WNL values were identified for the *rplB* gene. Clustering analysis of WNL identified that only the *rplB* gene belonged to the largest cluster, which is the 'leader' class. (b) Data analysis related to clustering and distribution of genes by cluster. Graph represents the cluster number of each case against the WNL.

Table 2. Description of the leader gene in *C. pseudotuberculosis*' action in the CL in goats

Gene name	Official name	Protein primary function in STRING	Cluster
rplB	50S ribosomal protein L2	One of the primary rRNA binding proteins. Required for association of the 30S and 50S subunits to form the 70S ribosome, for tRNA binding and peptide bond formation. It has been suggested to have peptidyltransferase activity; this is somewhat controversial. Makes several contacts with the 16S rRNA in the 70S ribosome (By similarity) (280 aa)	A (Leader class)

Source: STRING, V.10.

interconnecting through 3,330 interactions (Table 1) with a clustering coefficient equal to 0.736. Clustering coefficient measures the degree to which the nodes of a graph tend to cluster. Evidence suggests that the nodes of most interaction networks, and especially protein interaction networks, tend to create cohesive groups characterized by a high density of loops. The probability of this occurring tends to be greater than the average probability of a bond being established, randomly, between two nodes. The clustering coefficient greater than zero, as shown in Table 1, shows this network interconnectivity.

Network suggests *rplB* as putative host-derived leader gene in *C. pseudotuberculosis* action in CL

The WNL values for each gene in the dataset are displayed in Fig. 2a. The highest WNL values were identified in the *rplB* gene. Clustering analysis of WNL identified only the *rplB* gene belonging to the largest cluster, which is the 'leader' class. Data analysis related to clustering and distribution of genes by cluster are represented in Fig. 2b. A preliminary k-means analysis revealed a cluster number of 10.

Results were validated using the Kruskal Wallis test, which revealed a statistically significant difference in WNL. In particular, statistical analysis showed that the leader gene, *rplB*, had a significantly greater WNL than other gene classes ($P < 0.001$). Table 2 shows more information on the leader gene obtained in this study. This table shows the official name of *rplB* and the cluster in

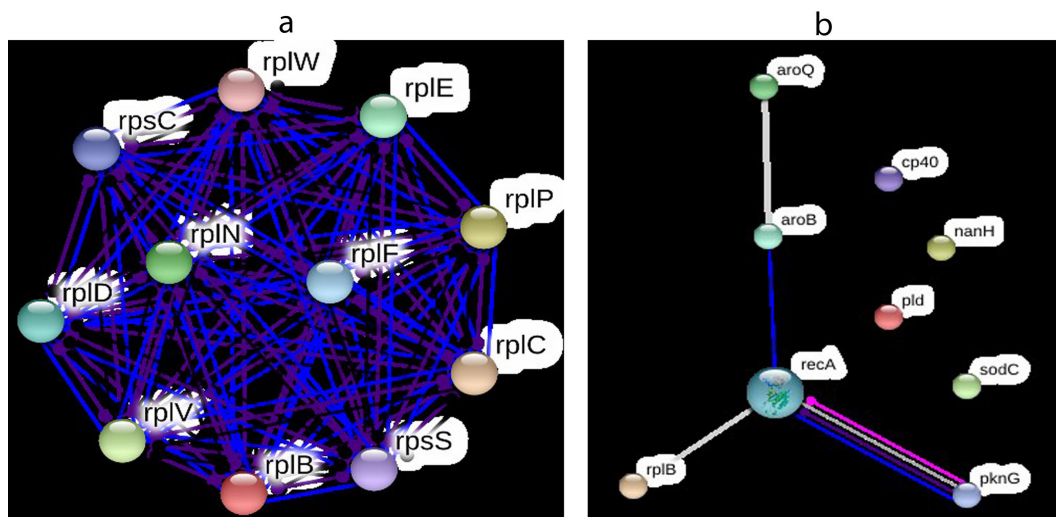


Fig. 3. Network model for leader gene *rplB*. (a) An action view of a leader gene for *C. pseudotuberculosis*' action in the CL in goats using STRING 10. (b) A diagram of the relationship between *rplB* and other proteins that have been used in formulating vaccines against CL.

which it is inserted, as well as the protein primary function in STRING, indicating its participation in the *C. pseudotuberculosis*' action in CL in goats.

Figure 3a shows the binding partners of the leader gene for *C. pseudotuberculosis*' action in the CL in goats, using STRING. Similarly, Fig. 3b shows a diagram of the relationship between *rplB* and other proteins that have been used in formulating vaccines against CL. We can predict the direct or indirect interaction of the leader gene with *recA*, *AroQ*, and *AroB* proteins, which suggests its participation in the expression of enzymes involved in the biosynthesis of nutritive compounds for the bacterium, such as phenylalanine, tyrosine, and tryptophan.

Functional enrichments in the network

The network presented significant pathways belonging to various subgroups, such as cellular components, molecular functions, biological processes, and the pathological phenomenon obtained from the KEGG database. Table 3 shows the gene ontology (GO) analysis of biological processes related to the identified molecular complexes, such as Metabolic pathways, Biosynthesis of secondary metabolites, Ribosome, Biosynthesis of amino acids, Carbon metabolism, and Microbial metabolism in diverse environments.

DISCUSSION

In this study, our main aim was to identify genes/proteins that may be true bottlenecks in the action of *C. pseudotuberculosis* in CL. The genes/proteins acting as leader genes may be promising candidates for use in efficient vaccines for goats to protect them against CL. This model should be able to integrate a maximum number of genes/proteins from biological processes that are typically altered in the action of *C. pseudotuberculosis* in CL. Elucidating the protein behavior of *C. pseudotuberculosis* in CL using a network plays a significant role in the design of new vaccines.

Gene expression depends on its interaction with other genes and regulation, therefore functional relationships among biological molecules can be linked to protein-protein interactions [18]. In our study, we highlight one gene that can regulate the activities of *C. pseudotuberculosis* in CL by interfering with the expression of other genes/proteins. The *rplB* gene was classified as a bottleneck, thus acting as a leader gene in the network. Being a leader gene, *rplB* can regulate many biological processes. The network of the leader gene *rplB* (Fig. 3a) shows that it acts directly or indirectly on other genes such as: *rpsC* (30S ribosomal protein S3), *rpsS* (30S ribosomal protein S19), *rplD* (50S ribosomal protein L4), *rplW* (50S ribosomal protein L23), *rplC* (50S ribosomal protein L3), *rplN* (50S ribosomal protein L14), *rplE* (50S ribosomal protein L5), *rplF* (50S ribosomal protein L6), *rplP* (50S ribosomal protein L16), and *rplV* (50S ribosomal protein L22).

Studies involving the action of *rplB* in *C. pseudotuberculosis* are scarce in the literature. One of the primary rRNA-binding proteins in *rplB* seems to have peptidyltransferase activity (STRING, V.10), interfering the cell growth. Our ontological analysis has shown that the protein acts effectively mainly on Metabolic pathways and Biosynthesis of secondary metabolites, as shown in Table 3.

In some microorganisms such as *E. coli*, cell growth is aligned to a specific period of the cell cycle DNA replication. However, the mechanism of this coordination is not well studied. Ribosome biogenesis also correlates with cell growth [33]. It is believed that the cellular concentration of ribosomes determines the rate of protein synthesis, which controls the rate of bacterial growth. This suggests that a factor that is required to assemble ribosomes may couple the initiation of DNA replication with cell growth [6].

Table 3. Ontological analysis of the results in *C. pseudotuberculosis*'s action in CL in goats

Pathway ID	Pathway description	Count in gene set	False discovery rate
1100	Metabolic pathways	344	1.94e-43
1110	Biosynthesis of secondary metabolites	182	1.36e-24
3010	Ribosome	53	1.88e-11
1230	Biosynthesis of amino acids	88	8.33e-10
1200	Carbon metabolism	59	1.31e-09
1120	Microbial metabolism in diverse environments	87	3.03e-09
400	Phenylalanine, tyrosine, and tryptophan biosynthesis	22	0.000127
230	Purine metabolism	49	0.000801
190	Oxidative phosphorylation	22	0.00089
860	Porphyrin and chlorophyll metabolism	29	0.00089
500	Starch and sucrose metabolism	17	0.00113
260	Glycine, serine and threonine metabolism	24	0.00158
20	Citrate cycle (TCA cycle)	16	0.00162
240	Pyrimidine metabolism	34	0.00254
620	Pyruvate metabolism	19	0.00254
630	Glyoxylate and dicarboxylate metabolism	14	0.00378
330	Arginine and proline metabolism	17	0.00583
520	Amino sugar and nucleotide sugar metabolism	23	0.00629
10	Glycolysis/Gluconeogenesis	22	0.00768
30	Pentose phosphate pathway	16	0.00768
970	Aminoacyl-tRNA biosynthesis	22	0.00768
1210	2-Oxocarboxylic acid metabolism	16	0.00768
2020	Two-component system	19	0.00768
250	Alanine, aspartate and glutamate metabolism	18	0.0114
910	Nitrogen metabolism	10	0.0198
51	Fructose and mannose metabolism	12	0.0379
670	One carbon pool by folate	12	0.0379

The significant pathways represented as: cellular components, molecular functions, biological processes, and the pathologic phenomenon according to the Kyoto Encyclopaedia of Genes and Genomes resulting from the leader genes cluster, carried out with the advanced function of STRING ($P < 0.01$ with Bonferroni correction).

A study identified the inhibitory activity of *lplB* (ribosomal protein L2) by N-terminal sequence analysis and immunoblotting with an antibody specific for L2. Since L2 is required for ribosome biogenesis, which is coupled to the cell growth, it is possible that it may inhibit the function of DnaA to affect the initiation process [6]. Therefore, this essential protein, which is one of the most evolutionarily ancient among ribosomal proteins, may act to coordinate the initiation of DNA replication with cell growth [29].

To our knowledge, this is the first study to hypothesize that the *rplB* protein is an important candidate for developing CL vaccines in goats. Other proteins have been the target of previous studies, but some disadvantages found in each study necessitated a search for novel proteins that can make more efficient vaccines.

Phospholipase D (PLD) has been used in vaccine development against CL because of its immunogenic characteristics [28]. The vaccines that are currently produced for CL control generally use formalin-inactivated PLD-rich *C. pseudotuberculosis* culture supernatants because PLD is considered the major protective antigen [19, 22, 34], or they use DNA as a vaccine [11]. However, conventional attenuated vaccines induce greater and more durable cytotoxic T lymphocyte and humoral responses [5, 10] with only a single dose in mice [5]. A strain of *C. pseudotuberculosis* lacking the *pld* gene deleted from the chromosome, called Toxminus was used in a vaccine; after a single vaccination with this attenuated strain, sheep were immune to challenge with a wild strain [20, 21].

Notably, from the results obtained, it was not possible to find a relationship of the *rplB* leader gene with the phospholipase *pld*, which is one of the proteins most studied for controlling CL. The hypothesis raised in this study is that the leader gene promotes its action in CL through interactions with RecA, *aroQ*, and *aroB*.

Two dehydroquinate enzymes called *aroQ* (3-dehydroquinate dehydratase) and *aroB* (3-dehydroquinate synthase) were also studied and used in formulating vaccines against CL. One study attenuated these genes using allelic exchange, and it was found that such strains did not cause disease in murine models, being a potential vector for vaccines against CL [38, 39]. This mean that these two genes are potential candidates for vaccine development against CL.

The protein RecA (Recombinase A) is present in eubacteria in general and is a highly conserved protein among bacterial organisms [7, 24, 36]. It participates in homologous recombination, DNA repair, and the SOS response. Specifically, RecA binds stretches of single-stranded DNA and unwinds duplex DNA [23]. A study was conducted to corroborate and deepen the understanding of results of the literature and generated isogenic mutants of *C. pseudotuberculosis* where the *recA* gene was mutated. Recombination 10 to 12 times yielded a mutant compared to the parental strain, suggesting a vector vaccine [35].

Characterization of the corynebacterial protease 40 (CP40) protein revealed it to be a serine protease. This protein is hydrophobic [36, 38] and has a leader sequence containing an Ala-x-Ala cleavage motif at the C-terminus, similar to that of PLD. CP40 is likely secreted because it was found in the *C. pseudotuberculosis* culture supernatant [45]. Owing to its similarity to PLD, it is supposed to present a good immunogenic activity that can be used in the composition of vaccines against CL in goats. One study indicated that the recombinant protein CP40 induced a specific immune response against *C. pseudotuberculosis* in mice that was able to afford protection after challenge, regardless of the adjuvant used in the formulation [15].

Another study indicated that *C. pseudotuberculosis* SpaC, NanH, SodC, and PknG proteins may play significant roles in virulence and pathogenicity. In this study, the characterization and evaluation of the vaccine potential of these proteins were conducted *in silico* [37] and showed promising results according to the authors.

The network shown in Fig. 3b highlights that the leader gene can interfere directly or indirectly in the action of some of the proteins already used in the formulation of vaccines against CL, such as: *aroB*, *aroQ*, *recA*, and *pknG*. From the current study, we suggest that *rplB* can be used for developing a multi-peptide vaccine. These epitopes must be evaluated experimentally to determine their actual potential for protection. Currently, there are great advances in molecular biology, mainly in the optimization of vaccine formulation, which means that efforts are needed to make the results less expensive and more accurate. Through these advances in molecular biology and genomics, we aim at discovering target genes, new diagnostics, and more efficient vaccines for CL control.

The *in silico* analyses performed show that *rplB* presents good potential as a candidate for vaccine development. This protein appeared as a bottleneck in *C. pseudotuberculosis*' action in CL in goats; thus, working as a leader gene, it can interfere with the action of several other proteins. Several studies are needed to make sure that this protein can potentially induce both cellular and humoral immune responses against *C. pseudotuberculosis* in goats. Future studies on the three-dimensional structure, molecular mechanism, pathogenicity, virulence, and vaccine development potential depend on the production of this protein in large quantities; these studies would contribute to preventing CL disease in goats worldwide.

Although bioinformatic analyses have only theoretical results, these studies offer a great opportunity to raise hypotheses that can be later tested in the laboratory.

ACKNOWLEDGMENTS. This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Ministério da Agricultura, Pecuária e Abastecimento (MAPA), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil. We thank Dr. Guimarães and Dra. Farias for technical support.

REFERENCES

1. Alves, J. T., Veras, A. A., Cavalcante, A. L., de Sa, P. H., Dias, L. M., Guimaraes, L. C., Morais, E., Silva, A. G., Azevedo, V., Ramos, R. T., Silva, A. and Carneiro, A. R. 2016. Complete Genome Sequence of *Corynebacterium pseudotuberculosis* Strain PA01, Isolated from Sheep in Para, Brazil. *Genome Announc.* **4**.
2. Baird, G. J. and Fontaine, M. C. 2007. *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. *J. Comp. Pathol.* **137**: 179–210. [Medline] [CrossRef]
3. Bastos, B. L., Dias Portela, R. W., Dorella, F. A., Ribeiro, D., Seyffert, N., Castro, T. L. P., Miyoshi, A., Oliveira, S. C., Meyer, R. and Azevedo, V. 2012. *Corynebacterium pseudotuberculosis*: immunological responses in animal models and zoonotic potential. *J. Clin. Cell Immunol.* **1**(S4): 005.
4. Bragazzi, N. L., Giacomelli, L., Sivozhelezov, V. and Nicolini, C. 2011. Leader Gene: A Fast Data-mining Tool for Molecular Genomics. *J. Proteomics Bioinform.* **4**: 4.
5. Chaplin, P. J., De Rose, R., Boyle, J. S., McWaters, P., Kelly, J., Tennent, J. M., Lew, A. M. and Scheerlinck, J. P. 1999. Targeting improves the efficacy of a DNA vaccine against *Corynebacterium pseudotuberculosis* in sheep. *Infect. Immun.* **67**: 6434–6438. [Medline]
6. Chodavarapu, S., Felczak, M. M. and Kaguni, J. M. 2011. Two forms of ribosomal protein L2 of *Escherichia coli* that inhibit DnaA in DNA replication. *Nucleic Acids Res.* **39**: 4180–4191. [Medline] [CrossRef]
7. Clark, A. J. and Sandler, S. J. 1994. Homologous genetic recombination: the pieces begin to fall into place. *Crit. Rev. Microbiol.* **20**: 125–142. [Medline] [CrossRef]
8. Covani, U., Marconcini, S., Giacomelli, L., Sivozhelevov, V., Barone, A. and Nicolini, C. 2008. Bioinformatic prediction of leader genes in human periodontitis. *J. Periodontol.* **79**: 1974–1983. [Medline] [CrossRef]
9. D'Afonseca, V., Moraes, P. M., Dorella, F. A., Pacheco, L. G., Meyer, R., Portela, R. W., Miyoshi, A. and Azevedo, V. 2008. A description of genes of *Corynebacterium pseudotuberculosis* useful in diagnostics and vaccine applications. *Genet. Mol. Res.* **7**: 252–260. [Medline] [CrossRef]
10. Davis, H. L., Mancini, M., Michel, M. L. and Whalen, R. G. 1996. DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. *Vaccine* **14**: 910–915. [Medline] [CrossRef]
11. De Rose, R., Tennent, J., McWaters, P., Chaplin, P. J., Wood, P. R., Kimpton, W., Cahill, R. and Scheerlinck, J. P. 2002. Efficacy of DNA vaccination by different routes of immunisation in sheep. *Vet. Immunol. Immunopathol.* **90**: 55–63. [Medline] [CrossRef]
12. de Sá Guimarães, A., do Carmo, F. B., Pauletti, R. B., Seyffert, N., Ribeiro, D., Lage, A. P., Heinemann, M. B., Miyoshi, A., Azevedo, V. and Guimarães Gouveia, A. M. 2011. Caseous lymphadenitis: epidemiology, diagnosis, and control. *IIOAB J.* **2**: 11.
13. Dorella, F. A., Estevam, E. M., Pacheco, L. G., Guimarães, C. T., Lana, U. G., Gomes, E. A., Barsante, M. M., Oliveira, S. C., Meyer, R., Miyoshi, A. and Azevedo, V. 2006. In vivo insertional mutagenesis in *Corynebacterium pseudotuberculosis*: an efficient means to identify DNA sequences encoding exported proteins. *Appl. Environ. Microbiol.* **72**: 7368–7372. [Medline] [CrossRef]
14. Dorella, F. A., Pacheco, L. G., Oliveira, S. C., Miyoshi, A. and Azevedo, V. 2006. *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. *Vet. Res.* **37**: 201–218. [Medline] [CrossRef]
15. Droppa-Almeida, D., Vivas, W. L., Silva, K. K., Rezende, A. F., Simionatto, S., Meyer, R., Lima-Verde, I. B., Delagostin, O., Borsuk, S. and Padilha, F. F. 2016. Recombinant CP40 from *Corynebacterium pseudotuberculosis* confers protection in mice after challenge with a virulent strain. *Vaccine* **34**: 1091–1096. [Medline] [CrossRef]

16. Galvão, C. E., Fragoso, S. P., de Oliveira, C. E., Forner, O., Pereira, R. R. B., Soares, C. O. and Rosinha, G. M. S. 2017. Identification of new *Corynebacterium pseudotuberculosis* antigens by immunoscreening of gene expression library. *BMC Microbiol.* **17**: 202. [Medline] [CrossRef]
17. Giacomelli, L. and Nicolini, C. 2006. Gene expression of human T lymphocytes cell cycle: experimental and bioinformatic analysis. *J. Cell. Biochem.* **99**: 1326–1333. [Medline] [CrossRef]
18. Gursoy, A., Keskin, O. and Nussinov, R. 2008. Topological properties of protein interaction networks from a structural perspective. *Biochem. Soc. Trans.* **36**: 1398–1403. [Medline] [CrossRef]
19. Hodgson, A. L., Carter, K., Tachedjian, M., Krywult, J., Corner, L. A., McColl, M. and Cameron, A. 1999. Efficacy of an ovine caseous lymphadenitis vaccine formulated using a genetically inactive form of the *Corynebacterium pseudotuberculosis* phospholipase D. *Vaccine* **17**: 802–808. [Medline] [CrossRef]
20. Hodgson, A. L., Krywult, J., Corner, L. A., Rothel, J. S. and Radford, A. J. 1992. Rational attenuation of *Corynebacterium pseudotuberculosis*: potential cheesy gland vaccine and live delivery vehicle. *Infect. Immun.* **60**: 2900–2905. [Medline]
21. Hodgson, A. L., Tachedjian, M., Corner, L. A. and Radford, A. J. 1994. Protection of sheep against caseous lymphadenitis by use of a single oral dose of live recombinant *Corynebacterium pseudotuberculosis*. *Infect. Immun.* **62**: 5275–5280. [Medline]
22. Johnson, V. G. and Nicholls, P. J. 1994. Identification of a single amino acid substitution in the diphtheria toxin A chain of CRM 228 responsible for the loss of enzymatic activity. *J. Bacteriol.* **176**: 4766–4769. [Medline] [CrossRef]
23. Karlin, S., Weinstock, G. M. and Brendel, V. 1995. Bacterial classifications derived from recA protein sequence comparisons. *J. Bacteriol.* **177**: 6881–6893. [Medline] [CrossRef]
24. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. and Rehauer, W. M. 1994. Biochemistry of homologous recombination in *Escherichia coli*. *Microbiol. Rev.* **58**: 401–465. [Medline]
25. McKean, S., Davies, J. and Moore, R. 2005. Identification of macrophage induced genes of *Corynebacterium pseudotuberculosis* by differential fluorescence induction. *Microbes Infect.* **7**: 1352–1363. [Medline] [CrossRef]
26. McKean, S. C., Davies, J. K. and Moore, R. J. 2007. Expression of phospholipase D, the major virulence factor of *Corynebacterium pseudotuberculosis*, is regulated by multiple environmental factors and plays a role in macrophage death. *Microbiology* **153**: 2203–2211. [Medline] [CrossRef]
27. McKean, S. C., Davies, J. K. and Moore, R. J. 2007. Probing the heat shock response of *Corynebacterium pseudotuberculosis*: the major virulence factor, phospholipase D, is downregulated at 43 degrees C. *Res. Microbiol.* **158**: 279–286. [Medline] [CrossRef]
28. Moussa, I. M., Ali, M. S., Hessain, A. M., Kabli, S. A., Hemeg, H. A. and Selim, S. A. 2016. Vaccination against *Corynebacterium pseudotuberculosis* infections controlling caseous lymphadenitis (CLA) and oedematous skin disease. *Saudi J. Biol. Sci.* **23**: 718–723. [Medline] [CrossRef]
29. Nakagawa, A., Nakashima, T., Taniguchi, M., Hosaka, H., Kimura, M. and Tanaka, I. 1999. The three-dimensional structure of the RNA-binding domain of ribosomal protein L2; a protein at the peptidyl transferase center of the ribosome. *EMBO J.* **18**: 1459–1467. [Medline] [CrossRef]
30. Nicolini, C. 2006. Nanogenomics for medicine. *Nanomedicine (Lond.)* **1**: 147–152. [Medline] [CrossRef]
31. Orlando, B., Bragazzi, N. and Nicolini, C. 2013. Bioinformatics and systems biology analysis of genes network involved in OLP (Oral Lichen Planus) pathogenesis. *Arch. Oral Biol.* **58**: 664–673. [Medline] [CrossRef]
32. Paton, M. W., Walker, S. B., Rose, I. R. and Watt, G. F. 2003. Prevalence of caseous lymphadenitis and usage of caseous lymphadenitis vaccines in sheep flocks. *Aust. Vet. J.* **81**: 91–95. [Medline] [CrossRef]
33. Paul, B. J., Ross, W., Gaal, T. and Gourse, R. L. 2004. rRNA transcription in *Escherichia coli*. *Annu. Rev. Genet.* **38**: 749–770. [Medline] [CrossRef]
34. Pizzo, M., Covacci, A., Bartoloni, A., Perugini, M., Nencioni, L., De Magistris, M. T., Villa, L., Nucci, D., Manetti, R., Bugnoli, M., *et al.* 1989. Mutants of pertussis toxin suitable for vaccine development. *Science* **246**: 497–500. [Medline] [CrossRef]
35. Pogson, C. A., Simmons, C. P., Strugnell, R. A. and Hodgson, A. L. 1996. Cloning and manipulation of the *Corynebacterium pseudotuberculosis* recA gene for live vaccine vector development. *FEMS Microbiol. Lett.* **142**: 139–145. [Medline] [CrossRef]
36. Roca, A. I. and Cox, M. M. 1990. The RecA protein: structure and function. *Crit. Rev. Biochem. Mol. Biol.* **25**: 415–456. [Medline] [CrossRef]
37. Santana-Jorge, K. T., Santos, T. M., Tartaglia, N. R., Aguiar, E. L., Souza, R. F., Mariutti, R. B., Eberle, R. J., Arni, R. K., Portela, R. W., Meyer, R. and Azevedo, V. 2016. Putative virulence factors of *Corynebacterium pseudotuberculosis* FRC41: vaccine potential and protein expression. *Microb. Cell Fact.* **15**: 83. [Medline] [CrossRef]
38. Simmons, C. P., Dunstan, S. J., Tachedjian, M., Krywult, J., Hodgson, A. L. and Strugnell, R. A. 1998. Vaccine potential of attenuated mutants of *Corynebacterium pseudotuberculosis* in sheep. *Infect. Immun.* **66**: 474–479. [Medline]
39. Simmons, C. P., Hodgson, A. L. and Strugnell, R. A. 1997. Attenuation and vaccine potential of aroQ mutants of *Corynebacterium pseudotuberculosis*. *Infect. Immun.* **65**: 3048–3056. [Medline]
40. Sivozhelezov, V., Braud, C., Giacomelli, L., Pechkova, E., Giral, M., Soullillou, J. P., Brouard, S. and Nicolini, C. 2008. Immunosuppressive drug-free operational immune tolerance in human kidney transplants recipients. Part II. Non-statistical gene microarray analysis. *J. Cell. Biochem.* **103**: 1693–1706. [Medline] [CrossRef]
41. Sivozhelezov, V., Giacomelli, L., Tripathi, S. and Nicolini, C. 2006. Gene expression in the cell cycle of human T lymphocytes: I. Predicted gene and protein networks. *J. Cell. Biochem.* **97**: 1137–1150. [Medline] [CrossRef]
42. Szklarczyk, D., Franceschini, A., Kuhn, M., Simonovic, M., Roth, A., Minguez, P., Doerks, T., Stark, M., Muller, J., Bork, P., Jensen, L. J. and von Mering, C. 2011. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res.* **39**: D561–D568. [Medline] [CrossRef]
43. Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K. P., Kuhn, M., Bork, P., Jensen, L. J. and von Mering, C. 2015. STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res.* **43**: D447–D452. [Medline] [CrossRef]
44. Trost, E., Ott, L., Schneider, J., Schröder, J., Jaenicke, S., Goesmann, A., Husemann, P., Stoye, J., Dorella, F. A., Rocha, F. S., Soares, S. C., D’Afonseca, V., Miyoshi, A., Ruiz, J., Silva, A., Azevedo, V., Burkovski, A., Guiso, N., Join-Lambert, O. F., Kayal, S. and Tauch, A. 2010. The complete genome sequence of *Corynebacterium pseudotuberculosis* FRC41 isolated from a 12-year-old girl with necrotizing lymphadenitis reveals insights into gene-regulatory networks contributing to virulence. *BMC Genomics* **11**: 728. [Medline] [CrossRef]
45. Walker, J., Jackson, H. J., Eggleton, D. G., Meeusen, E. N., Wilson, M. J. and Brandon, M. R. 1994. Identification of a novel antigen from *Corynebacterium pseudotuberculosis* that protects sheep against caseous lymphadenitis. *Infect. Immun.* **62**: 2562–2567. [Medline]
46. Williamson, L. H. 2001. Caseous lymphadenitis in small ruminants. *Vet. Clin. North Am. Food Anim. Pract.* **17**: 359–371, vii. [Medline] [CrossRef]
47. Windsor, P. A. 2011. Control of caseous lymphadenitis. *Vet. Clin. North Am. Food Anim. Pract.* **27**: 193–202. [Medline] [CrossRef]