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

Leader gene in caseous lymphadenitis of goats

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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.

Keywords: in silico, network, ontological analysis, rplB
INTRODUCTION

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 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/)
“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 = \( TETO(\log(\text{CONT.NUM}(N);2);1) \); 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].

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*’s 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 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 which it is inserted, as well as the protein primary function in STRING, indicating its participation in the *C. pseudotuberculosis*'s 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]. 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 \textit{rplB} leader gene with the phospholipase \textit{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, \textit{aroQ}, and \textit{aroB}.

Two dehydroquinate enzymes called \textit{aroQ} (3-dehydroquinate dehydratase) and \textit{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 means 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 \textit{C. pseudotuberculosis} where the \textit{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 \textit{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.

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support.


26. McKean SC, Davies JK, Moore RJ: 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* 2007, 153(Pt 7):2203-2211.16


**Fig. 1.** Gene interaction map and up- and down-regulated genes involved in *C. pseudotuberculosis*’s action in CL in goats.

**Legend:** 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.
Fig. 2. Characterization of the leader gene
Legend: (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.

Fig. 3. Network model for leader gene *rplB*
Legend: (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.
Table 1 Network statistics for *C. pseudotuberculosis*’ action in CL in goats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
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</thead>
<tbody>
<tr>
<td>Number of nodes</td>
<td>1,243</td>
</tr>
<tr>
<td>Number of edges</td>
<td>3,330</td>
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<tr>
<td>Average node degree</td>
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<td>Clustering coefficient</td>
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<td>Expected number of edges</td>
<td>3,143</td>
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<td>PPI enrichment p-value</td>
<td>0.0005</td>
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Table 2 Description of the leader gene in *C. pseudotuberculosis*’ action in the CL in goats.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Official name</th>
<th>Protein primary function in STRING</th>
<th>Cluster</th>
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<tbody>
<tr>
<td>rplB</td>
<td>50S ribosomal protein L2</td>
<td>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)</td>
<td>A (Leader class)</td>
</tr>
</tbody>
</table>

Source: STRING, V.10
Table 3 Ontological analysis of the results in *C. pseudotuberculosis*'s action in CL in goats showing 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).

<table>
<thead>
<tr>
<th>Pathway ID</th>
<th>Pathway description</th>
<th>Count in gene set</th>
<th>False discovery rate</th>
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<tr>
<td>1100</td>
<td>Metabolic pathways</td>
<td>344</td>
<td>1.94e-43</td>
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<tr>
<td>1110</td>
<td>Biosynthesis of secondary metabolites</td>
<td>182</td>
<td>1.36e-24</td>
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<td>3010</td>
<td>Ribosome</td>
<td>53</td>
<td>1.88e-11</td>
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<tr>
<td>1230</td>
<td>Biosynthesis of amino acids</td>
<td>88</td>
<td>8.33e-10</td>
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<tr>
<td>1200</td>
<td>Carbon metabolism</td>
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<td>1120</td>
<td>Microbial metabolism in diverse environments</td>
<td>87</td>
<td>3.03e-09</td>
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<tr>
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<td>Phenylalanine, tyrosine, and tryptophan biosynthesis</td>
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<td>400</td>
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<td>330</td>
<td>Arginine and proline metabolism</td>
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<td>520</td>
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<td>Pentose phosphate pathway</td>
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<td>2-Oxocarboxylic acid metabolism</td>
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<td>Two-component system</td>
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<tr>
<td>250</td>
<td>Alanine, aspartate and glutamate metabolism</td>
<td>18</td>
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<td>910</td>
<td>Nitrogen metabolism</td>
<td>10</td>
<td>0.0198</td>
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<td>51</td>
<td>Fructose and mannose metabolism</td>
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<td>0.0379</td>
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<td>670</td>
<td>One carbon pool by folate</td>
<td>12</td>
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