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Review article

***Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence**

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Abstract – *Corynebacterium pseudotuberculosis* is the etiological agent of caseous lymphadenitis (CLA), a common disease in small ruminant populations throughout the world. Once established, this disease is difficult to eradicate because drug therapy is not effective and because the clinical detection of infected animals is of limited efficiency. We reviewed the microbiological, biochemical and taxonomic features of *C. pseudotuberculosis*, general aspects of infection, the main virulence determinants and currently available commercial vaccines. We also examined the current molecular strategies for the study of virulence in *C. pseudotuberculosis*, including the latest research on the identification of novel virulence factors and genes, which will help us to better understand the biology of this microorganism. This knowledge may also contribute to the development of improved CLA vaccines, including subunit and DNA-based types, as well as to improve the diagnosis, treatment and control of this disease.

***Corynebacterium pseudotuberculosis* / caseous lymphadenitis / pathogenesis / virulence / vaccine**

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1. INTRODUCTION

The genus *Corynebacterium* belongs to a suprageneric group of actinomycetes that also includes the genera *Mycobacterium*, *Nocardia* and *Rhodococcus* [46, 87, 100, 102]. These gram-positive bacteria (*Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* species), termed the CMN group, constitute a very heterogeneous group; however, most of the species share particular characteristics, such as: (i) a specific cell wall organization, mainly characterized by the presence of a huge polymer complex composed of peptidoglycan, arabinogalactan and mycolic acids [5, 26–28, 39, 45, 48] and (ii) high G+C content (47–74%) [39, 40, 43, 80]. The genomes of several species of this group have already been completely sequenced; this fact reflects the considerable medical, veterinary and biotechnological importance of these organisms (Tab. I).

Corynebacterium pseudotuberculosis is an important animal pathogen. It is the etiological agent of a disease that is commonly called caseous lymphadenitis (CLA) or cheesy gland [114]. This disease is found in all the world's major sheep and goat production areas, causing significant economic losses [85, 114].

In this review, we present the main microbiological characteristics of *C. pseudotuberculosis*. Bacterial virulence determinants, including previously reported vir-

ulence factors and recently identified molecules, are discussed, with emphasis on the molecular strategies that have been used to identify and study such determinants. The aspects regarding CLA are also covered, focusing on the currently-available commercial and experimental vaccines.

2. MICROBIOLOGICAL, BIOCHEMICAL AND TAXONOMIC FEATURES OF *C. PSEUDOTUBERCULOSIS*

2.1. Microbiological aspects

C. pseudotuberculosis was isolated from bovine farcy in 1888 by Nocard. Preisz, in 1894, was the first to completely describe this microorganism and to observe its resemblance to the diphtheria bacillus. Synonyms for *C. pseudotuberculosis* were *Bacillus pseudotuberculosis ovis*, *Bacillus pseudotuberculosis*, *Corynebacterium ovis* and Preisz-Nocard bacillus [59, 72].

This microorganism is a facultative intracellular pathogen that exhibits pleomorphic forms, such as coccoids and filamentous rods, ranging in size from 0.5 μm to 0.6 μm by 1.0 μm to 3.0 μm [17, 28, 72, 97]. It is a non-sporulating, non-capsulated and non-motile bacterium; however, it has fimbriae [17, 46, 72]. This bacterium is a facultative anaerobe and grows best at

37 °C, at a pH of 7.0 to 7.2 [17, 72, 97]. It grows sparse initially on the agar surface and then becomes organized in clumps or in palisades, taking on a cream to orange coloration; colonies are dry, opaque and concentrically ringed. Growth in fluid medium develops as a granular deposit with a surface pellicle [17, 72, 77]. Haemolysis on blood agar is variable, but large zones develop in the presence of *Rhodococcus equi* [17]. *C. pseudotuberculosis* toxin inhibits the action of staphylococcal β -lysin [59].

C. pseudotuberculosis stains Gram-positive and when stained by Albert's or Neisser's method, volutin granules can be visualized. These metachromatic granules are clearly observed in the bacillary form, but are absent from coccoid cells; they contain high-energy phosphate reserves [46, 72].

2.2. Biochemical properties

Cell wall peptidoglycan is based on *meso*-diaminopimelic acid (*meso*-DAP). Arabinose and galactose are major cell wall sugars. Short-chain mycolic acids (corynomycolic acids, 22–36 carbon atoms) are present [59, 94, 97]. Biochemical reactions of *C. pseudotuberculosis* isolates vary considerably, mainly in their fermenting ability [72, 100, 105]. All strains produce acid, but not gas, from many carbon sources, including glucose, fructose, maltose, mannose, and sucrose [17, 53, 59, 72]. This bacterium is phospholipase D and catalase positive, oxidase negative, and it is beta-hemolytic [59, 77, 100]. Strains isolated from small ruminants generally do not reduce nitrate [17, 72, 100, 114].

A well-established biochemical test for coryneform bacteria identification is the API Coryne system (API-bioMérieux, Inc., La Balme les Grottes, France). This method consists of 21 biochemical tests; it can be performed in 24–48 h. The test contains 20 tubes containing substrates that allow for 11 enzyme tests (pyrazinamidase,

pyrrolidonyl arylamidase, β -galactosidase, alkaline phosphatase, α -glucosidase, *N*-acetylglucosaminidase, β -glucuronidase, and nitrate reduction and gelatin, urea and esculin hydrolysis) and eight carbohydrate fermentation tests (glucose, ribose, D-xylose, mannitol, maltose, lactose, sucrose and glycogen). This system is more reliable and rapid when it is compared with standard identification methods (API-bioMérieux, Inc.). A summary of general biochemical properties of *C. pseudotuberculosis* is presented in Table II.

2.3. Antimicrobial susceptibility

The susceptibility pattern of *C. pseudotuberculosis* to antimicrobial agents varies among isolates obtained from various sources [28, 37, 66]. Muckle and Gyles [77], in a study of 26 strains isolated from lesions of caseous lymphadenitis in goats, reported that all strains were susceptible to the antibiotics ampicillin, chloramphenicol, lincomycin, gentamicin, tetracycline, penicillin G and sulfamethoxazole-trimethoprim. Only three isolates were susceptible to neomycin, and all strains were resistant to streptomycin. Garg et al. [40] reported strains of *C. pseudotuberculosis* that were strongly resistant to penicillin but susceptible to neomycin. A strain highly resistant to streptomycin (500 μ g/mL) was observed in a study of 22 isolates of *C. pseudotuberculosis* from sheep and goat abscesses [90]. Minimal inhibitory concentration (MIC) values for all isolates were similar for the various antimicrobial agents. Later studies also indicated a similarity of MIC values among strains [1, 29, 60]. However, Fernández et al. [35] found higher MIC values for several antimicrobial agents, in an analysis of corynebacteria isolated from ewe mastitis.

Olson et al. [82] grew *C. pseudotuberculosis* as a biofilm, in an attempt to reproduce the environment of a natural infection. They observed that this bacterium was highly resistant to all the drugs that they tested under such growth conditions.

Table 1. The main representatives of the CMN group.

Representative	Status	Importance	Sequenced strain	Genome size (Mbp)	GC contents (%)	Reference
<i>Corynebacterium diphtheriae</i>	Complete	Causal agent of the disease diphtheria in humans	NCTC 13129	2.488	53	[20]
<i>Corynebacterium efficiens</i>	Complete	Production of glutamate and other amino acids and compounds	YS-314	3.147	63	[81]
<i>Corynebacterium glutamicum</i>	Complete	Production of glutamate, other amino acids (L-lysine) and compounds	ATCC 13032	3.309	53	[55]
<i>Mycobacterium avium</i>	In progress	Causes tuberculosis in birds and disseminated infections in immunocompromized humans (the elderly, children, and especially patients with AIDS)	104	5.480	68	http://www.tigr.org/db/mdb/mdbinprogress.html
<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>	Complete	Causative agent of Johne's disease, or paratuberculosis, a chronic severe intestinal infection. The disease affects domestic and free-ranging ruminants, but has also been reported in primates, rabbits, stoats and foxes	k10	4.829	69	[88]
<i>Mycobacterium bovis</i>	Complete	Causative agent of classic bovine tuberculosis, but it can also cause the disease in humans, especially if contaminated milk is consumed without prior pasteurization. This is a fully virulent strain	AF2122/97	4.345	65	http://www.sanger.ac.uk/Projects/M_bovis/ [41]
<i>Mycobacterium bovis</i>	In progress	Causative agent of classic bovine tuberculosis, but it can also cause the disease in humans. This is the strain that is used to produce BCG (Bacille de Calmette et Guérin) vaccine, a well-known tuberculosis vaccine	BCG	4.400	57	http://www.pasteur.fr/recherche/umites/L-gmb/mycogenomics.htm
<i>Mycobacterium leprae</i>	Complete	Causative agent of human leprosy	TN	3.268	57	[38]
<i>Mycobacterium smegmatis</i>	In progress	Generally non-pathogenic mycobacterium capable of causing soft tissue lesions. This bacterium was initially isolated from human smegma. It is associated with soft tissue lesions following trauma or surgery	MC2 155	7.040	57	http://www.tigr.org/db/mdb/mdbinprogress.html

Table I. Continued.

Representative	Status	Importance	Sequenced strain	Genome size (Mbp)	GC contents (%)	Reference
<i>Mycobacterium tuberculosis</i>	Complete	Causative agent of tuberculosis. It is highly contagious, infecting approximately 80% of the patient's social contacts	CDC1551	4.403	65	[36]
<i>Mycobacterium tuberculosis</i>	Complete	Causative agent of tuberculosis. Unlike some clinical isolates, it retains full virulence in animal models of tuberculosis and is susceptible to drugs and receptive to genetic manipulation	H37Rv	4.411	65	[25]
<i>Mycobacterium tuberculosis</i>	In progress	Causative agent of tuberculosis. It was subsequently found that this strain is one of the most wide-spread and virulent <i>Mycobacterium tuberculosis</i> strains	210	4.400	57	http://www.tigr.org/db/mdb/mdbinprogress.html
<i>Nocardia farcinica</i>	Complete	The causative agent of nocardiosis, affecting the lung, central nervous system, and cutaneous tissues of humans and animals. This species exhibits a greater degree of virulence than the more common <i>Nocardia asteroides</i>	IFM 10152	6.021 (Chromosome) 0.184 (Plasmid pNF1) 0.087 (Plasmid pNF2)	70 67 68	[56]
<i>Rhodococcus</i> sp.	In progress	Microbe capable of degrading a wide variety of polychlorinated biphenyls	RHA1	9.700		http://www.rhodococcus.ca

Table II. Biochemical characteristics of *C. pseudotuberculosis*.

Biochemical characteristics			
Acid production		Hydrolysis	
Glucose	+	Esculin	–
Arabinose	d	Hippurate	–
Xylose	–	Urea	+
Rhamnose	–	Tyrosine	–
Fructose	+	Casein	–
Galactose	+		
Mannose	+	Phosphatase	+
Lactose	–	Pyrazinamidase	–
Maltose	+	Methyl red	+
Sucrose	d	Nitrate reduction	d
Trehalose	–	Catalase	+
Raffinose	–	Oxidase	–
Salicin	–	Lipophilism	–
Dextrin	d		
Starch	–		

+: more than 90% are positive; d: 21–89% are positive; –: more than 90% are negative or resistant.

2.4. Taxonomy

Classification of *C. pseudotuberculosis* was originally based on morphological and biochemical characteristics [59, 77]. Nitrate reductase production was used by Biberstein et al. [8] to distinguish the *equi* biovar (isolated from horses and cattle; nitrate reduction positive) from the *ovis* biovar (isolated from sheep and goats; nitrate reduction negative). Later, Songer et al. [100] reached the same conclusion using restriction endonuclease (*EcoRV* and *PstI*) analyses of chromosomal DNA, and based on nitrate reduction data. More recently, the same result was also observed with restriction fragment length polymorphisms of 16S-rDNA [29, 105, 111]. Connor et al. [28] used pulsed-field gel electrophoresis, associated with biochemical analysis, for the characterization of *C. pseudotuberculosis* isolates.

A close relationship between *C. pseudotuberculosis* and *C. ulcerans* was suggested by the fact that these organisms are unique among the corynebacteria in producing phospholipase D [15, 44]. Moreover, some strains of *C. ulcerans* and *C. pseudotuberculosis* can produce diphtheria toxin (DT). Furthermore, some non-toxigenic strains are converted to toxigeny (DT production) by β -phages from toxinogenic *C. diphtheriae* [15, 23, 24, 44].

Molecular methods, including nucleic acid hybridization and 16S rRNA gene sequence analysis, have been used to determine the degree of relatedness of many different corynebacterial species and strains [54, 62, 95, 107]. Riegel et al. [95] found that some strains of *C. pseudotuberculosis* and *C. ulcerans* belong to a monophyletic group, based on phylogenetic analysis of small-subunit rDNA sequences that are only found in the CMN group. They also

concluded that the *equi* and *ovis* biovars of *C. pseudotuberculosis* should not be classified as subspecies, due to their high genomic similarity. In two other independent studies [54, 107], *C. pseudotuberculosis* was found to be closely related to *C. ulcerans*.

More recently, analysis of partial gene sequences from the β -subunit of RNA polymerase (*rpoB*) has been shown to be more accurate for the identification of *Corynebacterium* species than analyses based on 16S rDNA [61, 62]. This method has also been successfully used to identify mycobacterial species [63]. Although the *rpoB* gene is a powerful identification tool, many authors propose that it may be used to complement the 16S rRNA gene analysis in the phylogenetic studies of *Corynebacterium* and *Mycobacterium* species [61–63, 74]. We have constructed a phylogenetic tree based on *rpoB* gene sequences of reference strains from the CMN group (Fig. 1). Based on this phylogenetic tree, we can observe a clear relationship between *C. pseudotuberculosis* and *C. ulcerans*. Moreover, analysis using the *rpoB* gene allowed the identification of the group that these two species belong to, as previously observed [61, 62].

3. GENERAL ASPECTS OF *C. PSEUDOTUBERCULOSIS* INFECTION

Though *C. pseudotuberculosis* was originally identified as the causative microorganism of CLA in sheep and goats, this bacterium has also been isolated from other species, including horses, in which it causes ulcerative lymphangitis and pigeon fever in cattle, camels, swine, buffaloes, and humans [89, 97, 114, 117].

3.1. Transmission

The potential of *C. pseudotuberculosis* to survive for several weeks in the environment likely contributes to its ability to spread within a herd or flock [4, 117].

Transmission among sheep or goats occurs mainly through contamination of superficial wounds, which can appear during common procedures, such as shearing, castration and ear tagging, or through injuries of the animal's bodies generated by other traumatic events. Not infrequently, contaminated sheep cough bacteria onto skin cuts of other sheep, constituting another means of transmission [84, 114]. In cattle, as well as in buffaloes, there is evidence of mechanical transmission of this bacterium by houseflies and by other Diptera, though the natural mechanisms of infection with *C. pseudotuberculosis* are not well documented [97, 116, 117].

3.2. Human cases

Human infection caused by *C. pseudotuberculosis* is a rare event, and most of the reported cases have been related to occupational exposure; one case, diagnosed in 1988, involved the ingestion of raw goat meat and cow milk [89]. About 25 cases of infection of humans with this microorganism have been reported in the literature [67, 73, 89].

Peel et al. [89] reviewed 22 cases, in which infected humans were generally presented with lymphadenitis, abscesses, and constitutional symptoms. Mills et al. [73] described suppurative granulomatous lymphadenitis in a boy, due to contact with contaminated farm animals. Liu et al. [67] reported a *C. pseudotuberculosis* infection in a patient's eye, due to an ocular implant.

In most cases, the patients received antibiotic therapy and the affected lymph nodes were surgically removed [67, 73, 89].

3.3. Caseous lymphadenitis

Caseous lymphadenitis causes significant economic losses to sheep and goat producers worldwide, mainly due to the reduction of wool, meat and milk yields, decreased reproductive efficiencies of affected animals and condemnation of carcasses and skins in

abattoirs [3, 83]. The manifestations of CLA in small ruminants are characterized mainly by bacteria-induced caseation necrosis of the lymph glands. The most frequent form of the disease, external CLA, is characterized by abscess formation in superficial lymph nodes and in subcutaneous tissues. These abscesses can also develop internally in organs, such as the lungs, kidneys, liver and spleen, characterizing visceral CLA [72, 91]. In some cases, the infection produces few obvious clinical signs in the animal, remaining unrecognized until a post-mortem examination has been carried out, making it difficult to obtain definitive data about the prevalence of this disease [3, 17, 83].

3.4. Epidemiology of CLA

Recent epidemiological surveys have examined the prevalence of CLA in different countries [2, 3, 6, 11, 28, 85]. Among flocks surveyed in Australia, the average prevalence of CLA in adult sheep was 26% [85]. Forty-five percent of the farmers interviewed in a study in the United Kingdom had seen abscesses in their sheep; however, this could be an overestimation of CLA prevalence since few farmers had investigated the causes of the abscesses [11]. Twenty-one percent of 485 culled sheep examined in Canadian slaughterhouses had CLA [3]. This disease remains an important subject of veterinary concern throughout the world.

3.5. Diagnosis and control of CLA

Controlling CLA with antibiotics is not an easy task, since viable bacteria stay protected inside abscesses due to the thick capsule that surrounds them [91, 103, 114]. It is generally agreed that the best strategy to control the disease is vaccination of healthy animals, along with the identification/removal of infected animals [13, 71, 84, 114]. However, the difficulties associated with the early clinical identification of infected animals can be a hindrance to such a strategy.

Several serodiagnostic tests have been developed to overcome the problem of clinical identification of CLA, but most have been reported to lack either sensitivity or specificity [14, 16, 70, 71, 104, 114, 118]. Nevertheless, some enzyme-linked immunosorbent assay (ELISA)-based diagnostic tests have been reported to be effective in control and eradication programs [32, 33, 110]. Recently, ELISA tests to detect gamma interferon (IFN- γ), as a marker of cell-mediated immunity against *C. pseudotuberculosis*, have been developed [71, 86, 93]. The IFN- γ ELISA test appears to be more sensitive than the normal antibody ELISA in detecting prior infection in goats, and it does not seem to be affected by vaccination in sheep [71]. Another novel strategy that holds promise for the diagnosis of CLA is the use of polymerase chain reaction (PCR) tests specific for *C. pseudotuberculosis* to identify bacteria isolated from abscesses [21].

4. FROM PROTEINS TO DNA: COMMERCIAL AND EXPERIMENTAL VACCINES

4.1. Commercial vaccines

Most of the currently-available commercial vaccines for caseous lymphadenitis are combined with vaccines against other pathogens. These include *Clostridium tetani*, *Cl. perfringens*, *Cl. septicum*, *Cl. novyi* and *Cl. chauvoei* [85, 91, 103, 114]. These vaccines are based on inactivated phospholipase D (PLD) and are called toxoid vaccines.

Paton et al. [84], in an analysis of the effectiveness of a combined toxoid vaccine against CLA, reported a reduction in the number and size of CLA lung abscesses and a decrease in the spread of this disease within the flock. However, in another study [85], it was reported that although 43% of the farmers applied commercial CLA vaccines, only 12% used them correctly. It was concluded that adjustments in vaccination

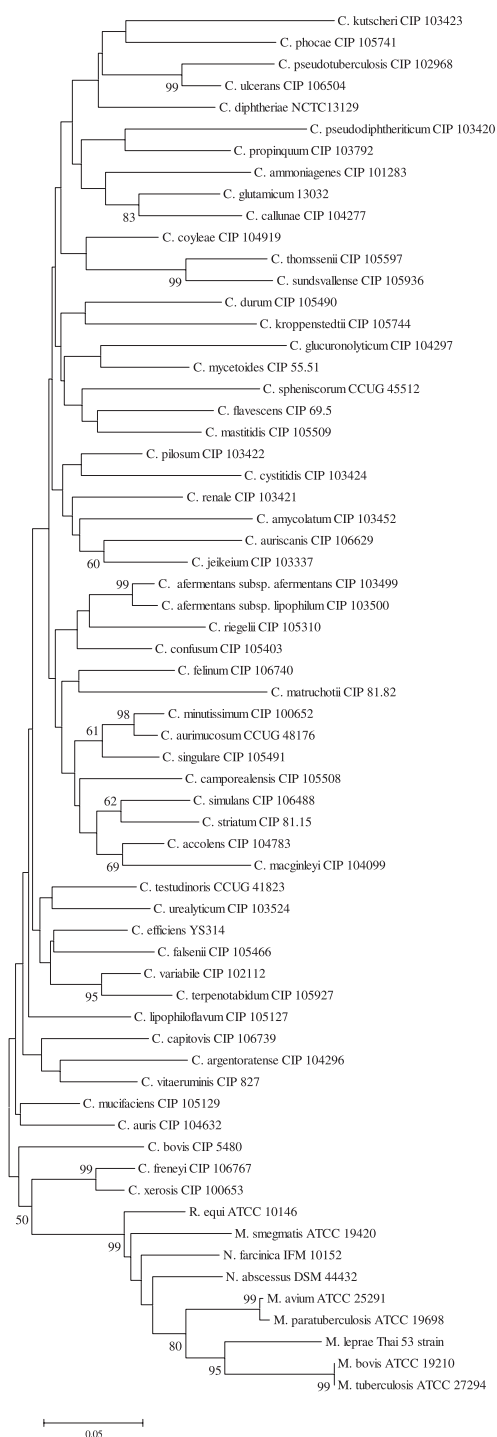


Figure 1. Dendrogram representing the phylogenetic relationships of the CMN group (*Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* species) obtained by the neighbor-joining method [96]. The tree was derived from the alignments of *rpoB* gene sequences. The phylogenetic distances were calculated by the software MEGA 3 [64]. The support of each branch, as determined from 1 000 bootstrap samples, is indicated by the value at each node (in percent).

programs would dramatically diminish the prevalence of CLA.

Not all the vaccines licensed for use in sheep can be used to vaccinate goats. Moreover, while the recommended vaccination program for sheep consists of two priming doses in lambs and yearly boosters in adult sheep, revaccination is recommended at six-month intervals in goats [85, 114].

A live attenuated vaccine strain of *C. pseudotuberculosis*, strain 1002, has been licensed for use in Brazil since 2000. It is already being produced industrially and is available in a liquid form that must be administered yearly to the animals, subcutaneously; a lyophilized version is also being developed by the Empresa Baiana de Desenvolvimento Agrícola (<http://www.ebda.ba.gov.br>). This live vaccine was reported to confer around 83% protection against CLA in goats in experimental assays and in field trials.

4.2. Experimental vaccines

C. pseudotuberculosis Toxminus (pld mutant) has been used as a live bacterial vector to deliver heterologous antigenic proteins [75]. Five heterologous genes (the gene coding for *Mycobacterium leprae* 18-kDa antigen, *Taenia ovis* 45W gene, *Babesia bovis* 11C5 antigen, the *Dichelobacter nodosus* gene encoding mature basic protease (*bprV*) and *Anaplasma marginale* ApH antigen), plus a genetically inactivated analogue of PLD, were used to construct plasmids expressing foreign genes in the Toxminus strain. Three proteins elicited specific antibody responses in experimentally vaccinated sheep. The expression by Toxminus of mature basic protease (*bprV*) of *D. nodosus* fused to the carboxy-terminus of *Mycobacterium leprae* 18-kDa antigen against ovine footrot [76] was also tested. Though the animals were not protected from footrot, this live recombinant vaccine was capable of eliciting a humoral immune response, and it may be capable of successfully delivering a foreign antigen.

Recently, the immune responses of sheep vaccinated with a DNA vaccine expressing the extracellular domain of bovine CTLA-4, fused to HIg and a genetically detoxified phospholipase D (boCTLA-4-HIg- Δ PLD) from *C. pseudotuberculosis* have been investigated [22]. CTLA-4 binds with high affinity to the B7 membrane antigen on antigen-presenting cells (APC), enhancing the humoral immune response to a vaccine antigen. Though the genetically attenuated vaccine was found to be only partially effective against experimental challenge with *C. pseudotuberculosis*, the targeted DNA vaccine provided sheep with a significantly improved antibody response. In order to improve the efficacy of this DNA vaccine, De Rose et al. [31] tested different routes of immunization: (i) intramuscular DNA injection, (ii) subcutaneous DNA injection and (iii) gene gun bombardment. Intramuscular vaccination gave a level of protection similar to that observed with protein vaccination, while subcutaneous and gene gun vaccination did not protect sheep against bacterial challenge.

5. DETERMINANTS OF VIRULENCE

5.1. Phospholipase D

Phospholipase D (PLD) is a potent exotoxin produced by *C. pseudotuberculosis* and it has been considered as the major virulence factor for this bacterium [51, 65].

This exotoxin is a permeability factor that promotes the hydrolysis of ester bonds in sphingomyelin in mammalian cell membranes, possibly contributing to the spread of the bacteria from the initial site of infection to secondary sites within the host [19, 30, 65, 69, 89, 106, 108]. Moreover, it provokes dermonecrotic lesions, and at higher doses it is lethal to a number of different species of laboratory and domestic animals [34, 102]. Damage and destruction of caprine macrophages have been observed during infection with *C. pseudotuberculosis*.

This lethal effect is due to action of PLD [109].

Several of the biological activities of *C. pseudotuberculosis* PLD, as well as its molecular structure, have also been found in sphingomyelinases in the venom of the medically important spider genus *Loxosceles* [7, 10, 30, 102, 108, 112].

The use of an antitoxin has prevented the spread of *C. pseudotuberculosis* within the host; however, it is not able to prevent the development of abscesses [114]. Moreover, vaccination of goats with formalized exotoxin, i.e. with inactive PLD, also prevented the spread of bacteria, following experimental challenge [13].

5.2. Toxic cell-wall lipids

The surface lipids of *C. pseudotuberculosis* have long been described as major factors contributing to its pathogenesis [18, 47, 48, 58]. The toxicity of the extracted lipid material has been demonstrated by the induction of hemorrhagic necrosis following intradermal injection in guinea pigs [58]. Mouse peritoneal macrophages were found to be highly susceptible to the necrotizing action of *C. pseudotuberculosis* surface lipids, but this cytotoxic effect is not observed in rabbit cells [48]. However, infection with *C. pseudotuberculosis* in the guinea pig invariably progresses until death, while guinea pig macrophages are not susceptible to the cytotoxic action of the bacterial lipids [48, 57]. Tashjian et al. [109] observed that *C. pseudotuberculosis* was resistant to killing and digestion by caprine macrophages due to its lipid coat.

A study carried out in mice with 25 isolates of *C. pseudotuberculosis* proposed that there is a direct relationship of the percentage of surface lipids with the induction of chronic abscessation [78].

5.3. New candidates

Recently, it has been proposed that a putative *C. pseudotuberculosis* iron uptake

gene cluster has a role in its virulence [9]. The four genes in this putative operon were identified downstream from the *pld* gene. They were designated as Fe acquisition genes (*fag*) *A*, *B*, *C* and *D*. Since *C. pseudotuberculosis* is an intracellular pathogen, this bacterium must be able to acquire iron from an environment in which this nutrient is scarce. Although there was no alteration in the utilization of iron by a *fagB(C)* mutant in vitro, this mutant had a decreased ability to survive and to cause abscesses in experimentally-infected goats [9].

6. MOLECULAR STRATEGIES FOR THE STUDY OF VIRULENCE IN *C. PSEUDOTUBERCULOSIS*

6.1. Identification of immunodominant peptides

To date, the most widely studied *C. pseudotuberculosis* protein is PLD. It has already been purified, cloned and expressed in *E. coli* [34, 50, 69, 101].

A protective antigen, corynebacterial secreted protease 40 (CP40) [115], has been identified in *C. pseudotuberculosis* by applying a strategy that involves the local immune response, analyzing the specificity of antibodies produced by B cells [113]. Antibody secreting cells (ASC), obtained from induced infections in sheep, produce antibodies with high specificity. These antibodies are used as probes to screen whole-cell antigens of *C. pseudotuberculosis* by immunoblots. CP40 was one of the earliest antigens recognized in immunoblots of sera. ELISA tests confirmed the results obtained with immunoblots, and field trials with this semipurified antigen showed that CP40 was highly protective against experimentally-induced CLA [113].

Some researchers have analyzed and characterized soluble and insoluble proteins that have immunodominant potential [12, 79]. Though many other immunogenic excreted-secreted components have been

described, using immunoblot techniques [86, 87], these proteins have not been identified. However, they reliably detected CLA infection in goats, and they could be used as vaccine components.

6.2. Generation of mutants

Random chemical mutagenesis, with formic acid, was used by Haynes et al. [49] to produce enzymatically-inactive PLD. This analog protein, though inactive, still had immunological activity [49]. Hodgson et al. [51] and McNamara et al. [68] used site-specific mutagenesis to produce *pld* mutants that had reduced ability to establish infection and were unable to disseminate in sheep and goats.

Site-specific amino acid substitution has also been used to generate genetic inactivation of the *pld* gene in two independent experiments. Tachedjian et al. [106] substituted the His20 in the PLD active site with other amino acids, obtaining mutants that were able to produce a genetically-inactivated version of PLD. After analysis of mutant gene expression, two mutants were selected that retained features useful for toxoid vaccine development. In another study, the inactivated protein, in which His20 was substituted by Ser, gave 44% protection in sheep challenged with the bacterium [52].

A mutant of the *C. pseudotuberculosis* *recA* gene was generated by site-specific inactivation [92]. The mutant had its homologous recombination efficiency decreased 8–10 fold. Nevertheless, in vivo analysis revealed that the mutated *recA* gene did not affect the virulence of this bacterium in mice.

Reduction of virulence of *C. pseudotuberculosis* mutants was obtained by Simmons et al. [98]. Allelic exchange was used to generate *aroQ*-attenuated mutants that were unable to cause CLA in murine models. It was suggested that highly attenuated *aroQ* mutants of *C. pseudotuberculosis* could be used as vaccine vectors [99].

The ability of the *fag* genes to be induced by limited iron was studied by transcriptional fusions with the *lacZ* reporter gene, followed by an assay for β -galactosidase activity [9]. The resultant mutants were grown in both iron-rich and iron-limited media. The mutants expressed very low levels of β -galactosidase activity in iron-rich medium and almost three-fold more in iron-limited medium. Although not well expressed in vitro, this putative operon appears to be induced by limited iron.

Our research group has identified 34 insertional mutants of genes coding for fimbrial and transport subunits, and also for hypothetical and unknown function proteins from *C. pseudotuberculosis*, using random transposon mutagenesis with the TnFuZ transposition system [42], a tool that generates transcriptional and translational fusions with the *phoZ* gene (encoding alkaline phosphatase) of *Enterococcus faecalis*¹. This discovery indicates promising target genes that could contribute to the development of attenuated vaccine strains.

7. FUTURE DIRECTIONS

Despite the various molecular strategies that have been employed, efficient tools for the genetic study of *C. pseudotuberculosis* are still scarce. In fact, the main reason for the lack of molecular investigation of this organism is that the genetics of the genus have been little studied with modern techniques, making it difficult to identify and characterize factors that could be involved in virulence [20]. Nevertheless, other representatives of the CMN group are better characterized, and the genetic tools that have been developed could be directly applicable to *C. pseudotuberculosis* in future studies.

¹ Dorella F.A., Estevam E.M., Pacheco L.G.C., Guimarães C.T., Lana U.G.P., Gomes E.A., Miyoshi A., Azevedo V., unpublished results.

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