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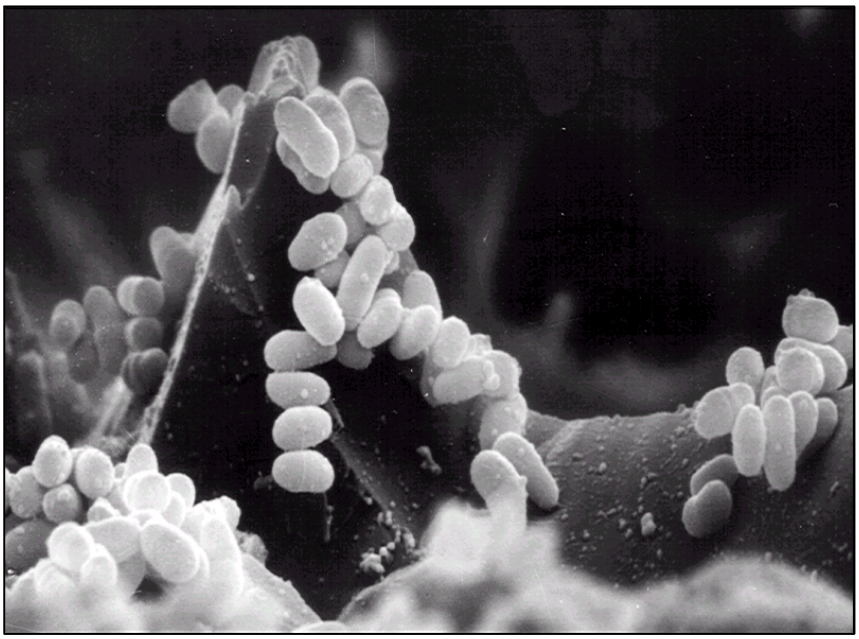
IDENTIFICAÇÃO DE SEQÜÊNCIAS DE DNA CODIFICADORAS DE PROTEÍNAS EXPORTADAS DA *Corynebacterium pseudotuberculosis* ATRAVÉS DA UTILIZAÇÃO DO SISTEMA DE TRANSPOSIÇÃO *in vivo* BASEADO NO TnFuZ



Belo Horizonte

Departamento de Biologia Geral

Instituto de Ciências Biológicas

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Dissertação apresentada ao Curso de Mestrado do Departamento de Biologia Geral do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Mestre em Genética.

Orientador: Vasco Azevedo, D.V.M, M.Sc, Ph.D, **Livre docente.**

Co-orientador: Anderson Myioshi, B. Sc, M. Sc., Ph.D.

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SUMÁRIO

RESUMO

A linfadenite caseosa é uma doença que acomete caprinos e ovinos e tem como agente etiológico a bactéria *Corynebacterium pseudotuberculosis*. Apesar da ampla distribuição mundial desta doença e de sua grande importância econômica, a imunoprofilaxia contra a infecção causada pela *C. pseudotuberculosis* ainda não é eficaz na redução da incidência da doença. Além disso, os mecanismos moleculares da virulência e patogenicidade desta bactéria ainda não estão bem definidos, principalmente no que diz respeito à identificação de produtos secretados e ancorados. Tendo em vista a importância desta bactéria, esta dissertação de mestrado propôs-se identificar seqüências gênicas de *C. pseudotuberculosis* codificadoras de proteínas exportadas através da utilização de um sistema de transposição *in vivo* baseado no TnFuZ. Para tanto, fez-se necessária a otimização do protocolo para a eletrotransformação desta bactéria a fim de dar-se continuidade a este trabalho. O protocolo resultante proporcionou uma eficiência de transformação 10 vezes maior que aquelas obtidas através dos protocolos já existentes. Com este protocolo otimizado, pôde-se partir para o próximo passo, ou seja, a mutagênese de *C. pseudotuberculosis* através do sistema TnFuZ. Foi possível identificar e isolar 34 clones de *C. pseudotuberculosis* que exibiam o fenótipo fosfatase alcalina positivo. Através do seqüenciamento do DNA genômico dos clones selecionados, foram identificados 21 loci que codificam subunidades fimbriais, proteínas de transporte e também proteínas de função hipotética e/ou desconhecida, as quais podem, ou não, estar relacionadas à virulência e à patogenicidade deste microrganismo. Enfim, estes loci representam promissores candidatos para o desenvolvimento de linhagens vacinais atenuadas ou mesmo alvos para ação de drogas.

ABSTRACT

Corynebacterium pseudotuberculosis is the etiological agent of caseous lymphadenitis (CLA), a worldwide chronic disease that affects sheep and goats. Despite all economic losses caused by CLA, there is not much information about molecular mechanisms of the pathogenesis of this bacterium. The immune prophylaxis against the infection has not enough efficacy to reduce the illness incidence. The molecular mechanisms of pathogenesis and virulence of this bacterium are not well defined yet, especially in reference to the identification of its secreted and anchored products. Taking into account the importance of this bacterium, this master degree dissertation is centered on: review the principal characteristics of *C. pseudotuberculosis* including relevant issues in CLA, a general vision on molecular techniques employed until now in the study of virulence factors of this bacterium and with this information identify genetic sequences from *C. pseudotuberculosis* coding for exported proteins. The system is based on an *in vivo* transposition employing TnFuZ. First of all, the electrotransformation protocol was optimized for this bacterium. The resulting protocol propitiated an efficiency transformation rate 10-fold higher compared with the previous protocols. With this optimized protocol the next step was to perform the mutagenesis of *C. pseudotuberculosis* using the TnFuZ system. It was possible to identify 34 *C. pseudotuberculosis* clones that possess a detectable PhoZ activity. By using this system, we were able to identify 21 different loci coding for fimbrial and transport subunits, and also for hypothetical and unknown function proteins from *C. pseudotuberculosis*, which represents promising target genes in the development of attenuated vaccine strains or targets for drugs action.

APRESENTAÇÃO

1. Introdução geral

Corynebacterium é um gênero bastante heterogêneo, composto de espécies bacterianas encontradas em uma grande variedade de nichos ecológicos. A maioria das espécies não é patogênica, encontrada no solo e nas superfícies das mucosas. As poucas espécies patogênicas estão envolvidas em infecções agudas e crônicas nos homens e animais (Merchant & Packer, 1967; Deb & Nath, 1999). As espécies pertencentes a este grupo exibem algumas características comuns: (1) são Gram-positivas; (2) apresentam células pleomórficas; (3) são imóveis; (4) não formam cápsula; e (5) não esporulam (Merchant & Packer, 1967; Buxton & Fraser, 1977). Além disto, todas as espécies deste grupo contêm, em sua parede, ácido micólico (Takahashi *et al.*, 1997). Suas células podem apresentar-se individualmente, aos pares ou em paliçada. Exibem grande diversidade na necessidade de oxigênio, podendo ser aeróbias, microaerófilas ou anaeróbias facultativas (Merchant & Packer, 1967; Buxton & Fraser, 1977; Jones & Collins, 1986; Coyle & Lipsky, 1990).

O conteúdo de G+C nos genomas das espécies do gênero *Corynebacterium* é bastante alto, variando de 50% a 70% (Deb & Nath, 1999; Riegel & Funke, 2000). Supõe-se que essas espécies possuam um cromossomo circular e estima-se que o tamanho total do genoma das espécies desse gênero varie de 3 a 6,5 Mb (Redenbach *et al.*, 2000). Métodos moleculares como a hibridização DNA-DNA e a análise do rDNA da subunidade 16S vêm sendo amplamente utilizados na identificação de espécies deste gênero e determinação do grau de similaridade entre linhagens relacionadas (Coyle & Lipsky, 1990; Vaneechoutte *et al.*, 1995; Riegel *et al.*, 1995; Takahashi *et al.*, 1997; Hou *et al.*, 1997).

Fazem parte deste grupo: *Corynebacterium diphtheriae, C. pseudotuberculosis, C. xerosis, C. pseudodiphtheriticum, C. kutscheri, C. efficiens, C. minutissimum, C. striatum, C. renale, C. cystitidis, C. pilosum, C. mycetoides, C. matruchotii, C. flavescens, C. vitarumen, C. glutamicum, C. callunae, C. bovis, C. equi* e *C. pyogenes* (Jones &. Collins, 1986). Entre as espécies patogênicas, uma possui grande importância na saúde humana, *C. diphtheriae*, que causa difteria, e quatro têm grande importância na saúde animal, *C. pyogenes, C. renale, C. equi* e *C. pseudotuberculosis* (Merchant & Packer, 1967).

Dentre estas, *C. pseudotuberculosis*, um parasita intracelular facultativo, é de especial importância para a pecuária mundial, pois é o agente etiológico da linfadenite caseosa que acomete caprinos e ovinos. A linfadenite caseosa consiste numa doença infecto-contagiosa crônica também conhecida como "mal-do-caroço". Caracteriza-se, geralmente, pela hipertrofia dos gânglios linfáticos localizados pelas diversas regiões do corpo do animal (Ayers, 1977). *C. pseudotuberculosis* raramente infecta humanos. Poucos casos já foram descritos e relatam a ocorrência de linfadenite subaguda e crônica, contraída pelo contato com animais infectados (Euzéby & Guérin-Faublée, 2000). Esta bactéria está presente em todo o mundo, no entanto é mais comumente encontrada em regiões como Austrália, Nova Zelândia, América do Sul e do Norte, e África do Sul (Merchant & Packer, 1967). No Brasil, a região Nordeste concentra aproximadamente 93% do rebanho caprino nacional com oito milhões de cabeças, representando 1,2% do total mundial. É também a região onde se observa a maior incidência de linfadenite caseosa, visto que os rebanhos são criados soltos na caatinga, cuja vegetação caracterizada pela presença de espinhos, favorece a disseminação da doença, que pode ocorrer diretamente entre os animais ou ainda através de contaminação da água, do solo e dos alimentos, expondo-os também indiretamente. Uma vez que a caprino e ovinocultura nesta região são atividades rurais de grande importância para pequenos e médios produtores e também constituem as principais fontes econômicas e de nutrição dessa população, a linfadenite caseosa trata-se de um sério problema social. As perdas econômicas, acarretadas pela linfadenite caseosa, podem ser evidenciadas pela diminuição da produção de leite, da desvalorização da carne e da pele devido às cicatrizes geradas (www.snagricultura.org.br/artigos/artitec-caprinos.htm**).**

Não há evidência clara sobre a rota mais comum de infecção, mas acredita-se que a ingestão de comida e água contaminadas, bem como o contato com animais infectados, possa desencadeá-la. O microrganismo penetra por lesões na pele e, ao ser carreado pelas vias linfáticas, forma abcessos característicos. Abscessos externos supuram, danificando a pele, e os abscessos viscerais levam à perda de peso, resultando em morbidez, deficiência reprodutiva e, em alguns casos, na morte do animal, gerando assim importantes perdas econômicas para os criadores (Buxton & Fraser, 1977; Ayers, 1977; Hodgson *et al.*, 1990).

Atualmente, não existe um tratamento realmente eficiente para a linfadenite caseosa. O uso de antibióticos não é aconselhável visto que o tratamento além de bastante longo

(dura de semanas a meses) não é totalmente eficaz. Inspeções periódicas do rebanho, isolamento dos animais doentes, tratamento e desinfecção de qualquer tipo de ferimento superficial, além da limpeza das instalações, são algumas das medidas profiláticas que podem ajudar a conter essa doença (www.snagricultura.org.br/artigos/artitec-caprinos.htm). Há vários relatos na literatura de testes de vacinas vivas atenuadas contra *C. pseudotuberculosis*, que mostraram proteger parcialmente os animais e reduzir a infecção (Eggleton *et al.*, 1991; Ribeiro *et al.*, 1991; Simmons *et al.*, 1997). Em 2000, a Empresa Baiana de Desenvolvimento Agrícola (EBDA) lançou uma vacina viva atenuada, a cepa 1002 contra a linfadenite caseosa de caprinos e ovinos. Essa vacina foi testada em campo e em laboratório e apresentou uma eficiência de 83% (www.bahia.ba.gov.br/seagri/ebda/index.html).

Apesar de sua importância como um patógeno animal, muito pouco se sabe sobre os mecanismos moleculares e as bases genéticas da virulência e patogenicidade desta bactéria (Coyle & Lipsky, 1990; Hodgson et al., 1990). Um dos fatores mais estudados é o gene que codifica a fosfolipase D, uma poderosa exotoxina que hidrolisa lisofosfatidilcolina e esfingomielina, e que desempenha um papel considerável na patogênese da linfadenite caseosa em caprinos, além de ser responsável pelo efeito letal quando administrada em camundongos (Sutherland et al., 1989; Egen et al., 1989; Hodgson et al., 1990). Outro fator de virulência, uma serina protease corinebacteriana de 40kDa (CP40), é reconhecida como um antígeno protetor contra a linfadenite caseosa em ovinos (Wilson et al., 1995). Sendo assim, informações básicas sobre a síntese, regulação, secreção e modo de ação de outras proteínas são ainda necessárias para elucidar seu papel na doença.

2. Delineamento da dissertação

Este manuscrito apresenta-se subdividido em seções de acordo com os objetivos propostos:

1. Em sua primeira seção, uma revisão da literatura sobre *Corynebacterium pseudotuberculosis*.

Esta dissertação de mestrado inicialmente propôs-se fazer um trabalho de síntese sobre as características mais relevantes de *C. pseudotuberculosis*, tais como: características microbiológicas, bioquímicas, taxonômicas, aspectos gerais da infecção, linfadenite caseosa, fatores de virulência, além de um apanhado geral sobre as estratégias moleculares já utilizadas para o estudo destes fatores em *C. pseudotuberculosis.*

2. Em sua segunda seção encontram-se os resultados, os quais estão organizados em duas partes:

2.1. Otimização de um protocolo de eletrotransformação da *C. pseudotuberculosis*,

Dados da literatura têm demonstrado que os protocolos de eletrotransformação, disponíveis para bactérias do gênero *Corynebacterium*, dependem não só da qualidade e quantidade do DNA empregado mas também da quantidade e viabilidade das células a serem transformadas; atingindo nas melhores condições (1μg de DNA ultrapuro e aproximadamente 10^9 células viáveis) uma eficiência de transformação de cerca de 10^4 unidades formadoras de colônia (UFC) por μg de DNA (Songer *et al.*, 1991; van der Rest *et al.*, 1999; Tauch *et al.*, 2002). Uma vez que os métodos até hoje disponíveis são dispendiosos, seus resultados de difícil reprodução e geralmente limitados a uma linhagem específica, o desenvolvimento de um protocolo mais eficiente e adequado de eletrotransformação de *C. pseudotuberculosis* fez-se necessário para a continuidade deste trabalho de dissertação. O trabalho referente a esta parte visa contribuir para que tais problemas sejam superados. Desta forma partiu-se para a otimização e o estabelecimento de um protocolo de transformação mais eficiente para *C. pseudotuberculosis*.

2.2. Identificação de seqüências de DNA codificadoras de produtos exportados da *C. pseudotuberculosis* utilizando-se o sistema de transposição TnFuZ.

A compreensão dos mecanismos de patogenicidade em bactérias depende da identificação e caracterização de genes e produtos gênicos que estejam envolvidos no processo de infecção. O desenvolvimento de técnicas de biologia molecular associado ao progresso da genética, da bioquímica, da fisiologia e da microbiologia, nas últimas duas décadas, tem levado à identificação e caracterização de vários fatores de virulência, assim como o desenvolvimento de novos agentes antimicrobianos e vacinas. As estratégias utilizadas baseiam-se na expressão e transferência gênica, mutações aleatórias ou dirigidas, comparação genômica e sequenciamento.

Dentre estas estratégias, a técnica de mutagênese aleatória vem sendo considerada uma poderosa ferramenta na identificação de genes relacionados à virulência e patogenicidade bacteriana. A estratégia de mutagênese aleatória pode ser empregada de diversas maneiras, uma delas é na produção de fusões transcricionais ou traducionais entre um gene (conhecido ou não) e um gene repórter incorporado a um transposon. Considerando que, após a fusão, o produto do gene repórter permaneça ativo, a proteína de fusão resultante poderá ser detectada por métodos estabelecidos. Esta estratégia tem sido útil na identificação e caracterização de novos produtos gênicos, principalmente no que diz respeito à identificação de genes que codifiquem produtos secretados, associados à superfície e/ou ao espaço periplasmático. Este subgrupo de proteínas exportadas compreende muito dos fatores de virulência, candidatos a vacinas de subunidade e alvos para terapia de drogas. Sendo assim, a identificação e caracterização destes produtos gênicos são cruciais para a melhor compreensão dos mecanismos utilizados pelos microrganismos patogênicos para interagir, sobreviver e causar danos ao hospedeiro.

Neste sentido, devido à escassez de informações a respeito dos mecanismos moleculares de virulência e patogenicidade de *C. pseudotuberculosis*, a utilização da técnica de mutagênese aleatória inédita nesta bactéria, foi utilizada visando a identificação de seqüências de DNA codificadoras de produtos exportados por esta bactéria. Para tanto, utilizou-se o sistema de transposição *in vivo* baseado no TnFuZ.

O TnFuZ é uma ferramenta genética de descoberta de proteínas exportadas por bactérias Gram-positivas. Baseia-se em um elemento transponível (Tn*4001*) combinado ao

gene da fosfatase alcalina (*phoZ*) de *Enterococcus faecalis*, cujas regiões codificadoras do promotor e do peptídeo sinal foram removidas. Uma vez que a fosfatase alcalina está ativa somente quando secretada, a inserção deste transposon em loci gênicos que codificam proteínas exportadas levará a fusões que resultarão em células secretando fosfatase e que poderão ser facilmente detectadas pela visualização *in vitro* do produto da degradação de um substrato revelador por esta proteína. Os resultados obtidos aqui poderão contribuir para a identificação de novos genes cujos produtos, exportados ou ancorados à membrana, estejam relacionados à invasão e sobrevivência deste patógeno no hospedeiro.

3. Em sua terceira seção encontram-se as conclusões e perspectivas do trabalho.

4. Finalmente, em sua quarta seção encontram-se as referências bibliográficas desta dissertação.

REVISÃO DA LITERATURA

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

Veterinary Research, 37: 201-218 (2006)

Anexo 1 (.pdf)

RESULTADOS

An improved protocol for electrotransformation of *C. pseudotuberculosis*

Veterinary Microbiology, 114:298-303. (2006)

Anexo 2 (.pdf)

In vivo insertional mutagenesis in *Corynebacterium pseudotuberculosis:* an efficient means to identify DNA sequences encoding exported proteins

Applied and Environmental Microbiology, 72:7368-7372 (2006)

Anexo 3 (.pdf)

CONCLUSÕES

E

PERSPECTIVAS

CONCLUSÕES

Nas condições do presente estudo, é possível concluir que:

1. A otimização do protocolo para a eletrotransformação de *C. pseudotuberculosis* nos permitiu alcançar uma eficiência de transformação 10 vezes mais alta que aquela dos protocolos até hoje disponíveis.

   1.1. Este protocolo foi de grande utilidade para a continuidade dos estudos genéticos de *C. pseudotuberculosis* aqui realizados, os quais poderão ser empregados para auxiliar num melhor entendimento dos mecanismos moleculares de virulência e patogenicidade desta bactéria.

2. Foi comprovada a eficácia da utilização do sistema de transposição *in vivo* baseado no TnFuZ em *C. pseudotuberculosis* com o intuito de identificar seqüências gênicas codificadoras de produtos exportados.

   2.1. O emprego deste sistema possibilitou a obtenção de cerca de 1500 clones recombinantes, dos quais 34 clones exibiram o fenótipo fosfatase alcalina positivo.

   2.2. A partir do sequenciamento das regiões que flanqueiam as inserções do transposon no genoma dos clones selecionados identificamos 21 loci que apresentaram similaridade com seqüências depositadas em banco de dados.

   2.3. As seqüências obtidas, similares a genes previamente identificados, correspondem a prováveis proteínas de membrana, tais como subunidades fimbriais e sistemas de transporte. Foram encontradas também seqüências codificando proteínas de função hipotética e desconhecida.

3. Estes resultados são promissores, uma vez que as seqüências gênicas identificadas podem estar relacionadas à virulência e patogenicidade da *C. pseudotuberculosis*

PERSPECTIVAS

Este trabalho abre perspectivas para:

1. Avaliar a possível utilização das linhagens mutantes como vacinas ou mesmo como vetores vivos vacinais. Para tanto serão necessários:

   1.1. Ensaios para testar a capacidade de persistência das linhagens mutantes da *C. pseudotuberculosis* no modelo de infecção murino, comparada à persistência da linhagem selvagem.

   1.2. Testar a possibilidade das linhagens mutantes em conferir imunidade protetora contra o desafio com a linhagem selvagem de *C. pseudotuberculosis* em ensaios de imunização de camundongos susceptíveis à infecção.

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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 virulence 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 μg/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 I. The main representatives of the CMN group.

Representative	Status	Importance	Sequenced strain	Genome size (Mbp)	GC contents (%)	Reference
Corynebacterium diphtheriae	Complete	Causal agent of the disease diphtheria in humans	NCTC 13129	2.488	53	[20]
Corynebacterium efficiens	Complete	Production of glutamate and other amino acids and compounds	YS-314	3.147	63	[81]
Corynebacterium glutamicum	Complete	Production of glutamate, other amino acids (L-lysine) and compounds	ATCC 13032	3.309	53	[55]
Mycobacterium avium	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/tdb/mdb/mdbinprogress.html
Mycobacterium avium subsp. *paratuberculosis*	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]
Mycobacterium bovis	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]
Mycobacterium bovis	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 Guèrin) vaccine, a well-known tuberculosis vaccine	BCG	4.400	57	http://www.pasteur.fr/recherche/unites/Lgmb/mycogenomics.htm
Mycobacterium leprae	Complete	Causative agent of human leprosy	TN	3.268	57	[38]
Mycobacterium smegmatis	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/tdb/mdb/mdbinprogress.html

Table I. Continued.

Representative	Status	Importance	Sequenced strain	Genome size (Mbp)	GC contents (%)	Reference
Mycobacterium tuberculosis	Complete	Causative agent of tuberculosis. It is highly contagious, infecting approximately 80% of the patient's social contacts	CDC1551	4.403	65	[36]
Mycobacterium tuberculosis	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]
Mycobacterium tuberculosis	In progress	Causative agent of tuberculosis. It was subsequently found that this strain is one of the most wide-spread and virulent Mycobacterium tuberculosis strains	210	4.400	57	http://www.tigr.org/tdb/mdb/mdbinprogress.html
Nocardia farcinica	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 *Nocardia asteroides*	IFM 10152	6.021 (Chromosome) 0.184 (Plasmid pNF1) 0.087 (Plasmid pNF2)	70 67 68	[56]
Rhodococcus 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 (*Eco*RV and *Pst*I) 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

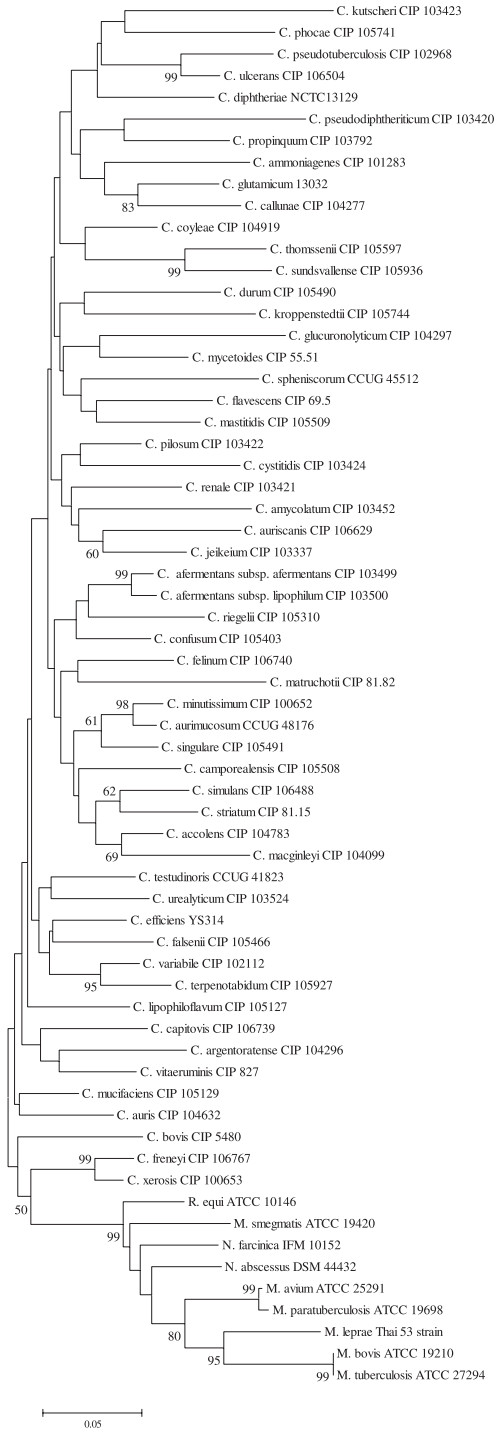

Figure 1. Dendrogram representing the phylogenetic relationships of the CMN group (*Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* species) obtained by the neighbor-joining method [96]. The tree was derived from the alignments of *rpoB* gene sequences. The phylogenetic distances were 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 administrated 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-ΔPLD) 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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An improved protocol for electrotransformation of *Corynebacterium pseudotuberculosis*

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Abstract

We developed an improved protocol for the electrotransformation of *Corynebacterium pseudotuberculosis*, testing variations of parameters in the procedures that are routinely used for the preparation of electrocompetent cells of this species, including (i) culture conditions, (ii) cell growth phase, (iii) electroporation solutions and (iv) quantity of plasmid DNA. We obtained the greatest efficiency of transformation when the cells were grown until the stationary phase and then washed with 10% glycerol electroporation solution. The transformation efficiency was inversely proportional to the quantity of plasmid DNA. The transformation efficiency reached 10^5 colony-forming units (cfu)/μg plasmid DNA. This protocol would be useful for genetic studies of *C. pseudotuberculosis*.

Keywords: *Corynebacterium pseudotuberculosis*; Plasmid transformation; Electroporation

1. Introduction

Corynebacterium pseudotuberculosis is a Gram-positive intracellular pathogen that causes a chronic disease condition in sheep and goats, known as caseous lymphadenitis (CLA). This bacterium is distributed worldwide, but it has a higher prevalence in certain regions, namely Australia, New Zealand, South Africa, the United States of America and Brazil (Merchant and Packer, 1967; Buxton and Fraser, 1977). CLA is characterized by the formation of necrotic lesions, typically located in superficial lymph nodes and in the lungs. Economic losses due to CLA are caused by reduced wool and meat production and increased culling and condemnation of carcasses and skins in slaughterhouses (Ayers, 1977).

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There is not much information concerning the molecular mechanisms of virulence and pathogenicity of this bacterium (Coyle and Lipsky, 1990); efficient methods for the genetic manipulation of *C. pseudotuberculosis*, such as transformation protocols, would be useful. To date, there has been only one report of electrotransformation of *C. pseudotuberculosis*; there was a maximum transformation efficiency of 10^4 colony-forming units (cfu)/μg of plasmid DNA (Songer et al., 1991). Laborious methods, including a two-step transformation procedure, medium supplementation, protoplast transformation and high-intensity electric fields, have been used for the transformation of *C. glutamicum*, *C. diphtheriae* and other species of Corynebacteria. However, these complex and time-consuming methods are difficult to reproduce, and the protocols are usually limited to one particular bacterial strain (Katsumata et al., 1984; Serwold-Davis et al., 1987; Liebl et al., 1989; Bonamy et al., 1990; Songer et al., 1991; van der Rest et al., 1999; Jang and Britz, 2000; Tauch et al., 2002). In order to overcome these difficulties, we developed an improved protocol for the electrotransformation of five different strains of *C. pseudotuberculosis*, which was achieved by systematic analysis of previously established parameters used for the transformation of Corynebacteria.

2. Material and methods

2.1. Bacterial strains, growth conditions and plasmid

Escherichia coli DH5α strain containing the pEC-K18*mob2* plasmid (Table 1) was aerobically grown in Luria-Bertani medium (LB, Difco Laboratories, Detroit, USA) at 37 °C. The *C. pseudotuberculosis* strains were aerobically grown in Brain Heart Infusion (BHI, Acumedia Manufacturers, Inc., Baltimore, MD, USA) medium at 37 °C, supplemented or not with glycine (1.5%, v/v). Plasmid-containing transformants were selected by the addition of kanamycin (50 μg/ml for *E. coli* and 25 μg/ml for *C. pseudotuberculosis*).

2.2. DNA manipulation and bacterial transformation

pEC-K18*mob2* was extracted from *E. coli* by the alkaline lysis procedure (Birnboim and Doly, 1979). Plasmid DNA quantification was performed by spectrophotometry (UV mini 1240 UV–vis Spectrophotometer, Shimadzu), as previously described (Sambrook et al., 1989).

Electrocompetent cells were prepared as follows (the following improved protocol corresponds to three different assays with similar results, Table 2): a single colony of *C. pseudotuberculosis* was grown overnight, diluted in fresh medium (1:50) and then incubated for 72 h to an optical density at 600 nm ($OD_{600\ nm}$) = 1.0–1.5. In some trials, 1.5% glycine (v/v) was added to the culture at $OD_{600\ nm}$ = 0.5, with growth till $OD_{600\ nm}$ = 1.0–1.5. Cells were then harvested by centrifugation at 5000 rpm at 4 °C for 20 min. The cell pellets were washed four times with 40 ml of ice-cold 10% glycerol in water (v/v). Following the last centrifugation, the cells were resuspended in 1 ml 10% glycerol. Aliquots (100 μl) were frozen in dry ice and methanol and were stored at −70 °C.

Electroporation assays were performed as follows: one aliquot of electrocompetent cells was thawed on ice for 5 min, and plasmid DNA was added. The mixture was then transferred to an electroporation

Table 1
Bacterial strains and plasmid used for electrotransformation of *Corynebacterium pseudotuberculosis*

Bacterium/plasmid	Strain	Description	Source/reference
C. pseudotuberculosis	T1	Wild strain, isolated from goats	Dr. Roberto Meyer Universidade Federal da Bahia
C. pseudotuberculosis	T2	Wild strain, isolated from goats	Dr. Roberto Meyer
C. pseudotuberculosis	1002	Attenuated strain, isolated from goats	Dr. Roberto Meyer
C. pseudotuberculosis subsp. *ovis*	CIP 102968T	Wild strain, isolated from sheep	Dr. Mario Vaneechoutte University Hospital, Ghent Belgium
C. pseudotuberculosis	HJ ULCC06.334.4	Wild strain, isolated from goats	Dr. Mario Vaneechoutte
pEC-K18*mob2*		Corynebacteria replicative-vector	Tauch et al. (2002)

Table 2
Outline of the electroporation parameters that gave the best results for *Corynebacterium pseudotuberculosis* transformation

Parameter	Optimal condition
Culture density	Stationary phase (1.0–1.5 OD at 600 nm)
Medium supplementation	Strain-dependent
Electroporation solution	10% ice-cold glycerol
DNA concentration	Less than 100 ng
Heat shock	Strain-dependent

cuvette (interelectrode distance: 0.2 cm); the samples were electroporated by using the Gene Pulser System™ (Bio-Rad, CA, USA), with the parameters: (i) 2.5 kV (equivalent to 12.5 kV/cm); (ii) 200 Ω and (iii) 25 μF (van der Rest et al., 1999). Immediately after the electric pulse, 1.5 ml of Brain Heart Infusion (BHI) medium was added to the cell culture, which was then incubated for 2 h at 37 °C, without agitation. In some trials, the cells were exposed to heat shock at 46 °C in a water bath during 6 min, after electroporation. Finally, aliquots of cell suspensions were plated on selective BHI agar plates and were incubated at 37 °C for 48 h to recover transformants.

3. Results and discussion

3.1. Optimization of growth conditions

We analyzed several parameters to determine the optimal growth conditions to prepare electrocompetent *C. pseudotuberculosis* cells, including media supplementation (with or without glycine) and growth phase [($OD_{600\ nm}$) = 0.5 (exponential-phase) or = 1.5 (stationary-phase)], before the washing steps.

Efficient transformation of *C. glutamicum*, *C. diphtheriae* and other Gram-positive bacteria depends on glycine supplementation (Dunny et al., 1991; Serwold-Davis et al., 1987; Kalscheuer et al., 1999; van der Rest et al., 1999; Jang and Britz, 2000; Lee et al., 2002; Tauch et al., 2002). Glycine is thought to inhibit the cross-linking of the peptidoglycan layer (Hammes et al., 1973; Akhtar et al., 2000). However, Serror et al. (2002) found that glycine did not improve *Lactobacillus delbrueckii* electrotransformation.

We examined whether glycine can improve the transformation efficiency of *C. pseudotuberculosis* strains (Table 1). The cells were grown in BHI medium until $OD_{600\ nm}$ = 0.5 was reached, and glycine was added to a final concentration of 1.5% (v/v). The bacterial culture was then incubated for a further 36 h or until $OD_{600\ nm}$ = 1.0–1.5. Finally, electrocompetent cells were prepared as described in Section 2.

Some researchers have found that an efficient electrotransformation protocol also depends on the quantity and viability of the cells to be transformed (Bonamy et al., 1990; Songer et al., 1991; van der Rest et al., 1999; Tauch et al., 2002). We tested glycine medium supplementation in different culture growth phases (lag-, exponential- and stationary-phase, data not shown); and, surprisingly, cells grown in BHI until $OD_{600\ nm}$ = 1.0–1.5 gave the highest number of viable cells, about 10^{10} cells/ml per strain. Consequently, these growing conditions were adopted during all experiments.

We noted only a modest transformation efficiency improvement attributable to glycine in HJ ULCC06.334.4 strain, reaching 1.07×10^5 transformants/μg of plasmid DNA (Table 7). In the CIP 102968T strain, the glycine supplementation together with heat shock proved to be the best combination for electrotransformation, reaching 5.6×10^4 transformants/μg of plasmid DNA (Table 6). In the other strains, glycine had only a minor influence on transformation efficiency, even when large quantities of DNA were added (Tables 3–5).

3.2. Electroporation solution

Various electroporation solutions have been used to wash electrocompetent cells of Gram-positive bacteria, such as: (i) 275 mM sucrose, 2.4 mM K_2HPO_4 and 0.6 mM KH_2PO_4 at pH 7.4 (Songer et al., 1991), (ii) 7 mmol/l HEPES and 1 mmol/l $MgCl_2$ at pH 6.0 (O'Sullivan and Fitzgerald, 1999), (iii) 0.4 M sucrose, 1 mM $MgCl_2$ and 5 mM KH_2PO_4 at pH 6.0 (Serror et al., 2002) and (iv) 1 mM Tris–HCl at pH 7.5 and 10% glycerol (v/v) (Tauch et al., 2002). Less complex solutions have also been used, including (i) ice-cold glycerol 10% (v/v) (van der Rest et al., 1999; Tauch et al., 2002) and (ii) warm glycerol plus sucrose (Lee et al., 2002), with similar results to ours.

When the cells were washed with ice-cold glycerol 10% (v/v), the transformation efficiency was 10^5 cfu/μg of plasmid DNA, which is similar to or higher than

Table 3
Effects of DNA concentration, glycine supplementation and heat shock on the transformation efficiency of the T1 strain of *Corynebacterium pseudotuberculosis*

DNA (ng)		Transformants/μg DNA		Gly + HS
		Gly	HS	
0	0	0	0	0
1	1.5×10^5	3.0×10^4	1.8×10^4	7.8×10^4
10	2.4×10^4	5.8×10^4	3.6×10^3	6.5×10^4
100	1.5×10^4	2.6×10^4	1.3×10^3	2.4×10^4
500	8.2×10^3	4.9×10^3	3.2×10^2	7.05×10^3
1000	1.2×10^3	9.3×10^3	2.3×10^2	4.9×10^3

Gly, 1.5% glycine; HS, heat shock. All electroporation assays were carried out under the following basic conditions: cells cultivated in Brain Heart Infusion to an $OD_{600\ nm} = 1.0 – 1.5$; when necessary, 1.5% glycine was added at $OD_{600\ nm} = 0.5$; ice-cold 10% (v/v) glycerol was used as an electroporation solution. The electroporation parameters were 25 μF, 200 Ω and 2.5 kV (12.5 kV/cm). A heat shock of 46 °C in a water bath for 6 min was applied after electroporation.

that obtained with more complex solutions (Songer et al., 1991; O'Sullivan and Fitzgerald, 1999; Tauch et al., 2002; Serror et al., 2002). Consequently, we adopted ice-cold glycerol 10% (v/v) as the electroporation solution for our electrotransformation procedures. The pellet was washed four times with this solution in order to avoid arching during the electroporation assay.

3.3. Quantity of plasmid DNA

Large quantities of plasmid DNA are normally used in protocols for the transformation of Corynebacteria and other Gram-positive bacteria (Bonamy et al., 1990; Kurusu et al., 1990; Kalscheuer et al., 1999; van der Rest et al., 1999; Lee et al., 2002; Tauch et al., 2002). We used various quantities of plasmid DNA, ranging from 1 to 1000 ng, to determine the optimal concentration for *C. pseudotuberculosis* transformation in electroporation assays. Based on several studies (Bonamy et al., 1990; Kurusu et al., 1990; Kalscheuer et al., 1999; van der Rest et al., 1999; Lee et al., 2002; Tauch et al., 2002), we expected a linear relationship between plasmid DNA quantity and the number of transformants. However, different from these reports, we found that 100 ng of plasmid DNA was sufficient for high transformation efficiency.

The highest transformation efficiencies were obtained with 1 ng of plasmid DNA, for all the strains and under all conditions. Transformation efficiency was inversely proportional to the quantity of plasmid DNA in all strains (Tables 3–7), reaching 10^4–10^5 transformants/μg of plasmid DNA, as postulated by Songer et al. (1991). Glycine supplementation alone, as well as in combination with heat shock treatment, resulted in a modest increase in transformation efficiencies, mainly in the T2 (Table 4), 1002 (Table 5) and CIP 102968T (Table 6) strains. These results were only obtained with large quantities of DNA.

Table 4
Effects of DNA concentration, glycine supplementation and heat shock on transformation efficiency in the T2 strain of *Corynebacterium pseudotuberculosis*

DNA (ng)		Transformants/μg DNA		Gly + HS
		Gly	HS	
0	0	0	0	0
1	4.0×10^4	4.0×10^3	4.0×10^3	2.8×10^4
10	1.8×10^3	8.0×10^2	8.0×10^2	4.0×10^3
100	3.53×10^2	5.6×10^2	2.13×10^2	1.46×10^2
500	3.45×10^2	1.06×10^2	80	5.84×10^2
1000	16	34	5	3.08×10^2

See Table 3 for abbreviations and general electroporation assay conditions.

Table 5
Effects of DNA concentration, glycine supplementation and heat shock on transformation efficiency in strain 1002 of *Corynebacterium pseudotuberculosis*

DNA (ng)		Transformants/μg DNA		Gly + HS
		Gly	HS	
0	0	0	0	0
1	2.54×10^5	2.13×10^4	1.64×10^5	3.25×10^4
10	8.09×10^4	4.58×10^4	2.48×10^4	4.73×10^3
100	4.8×10^3	9.94×10^3	1.2×10^2	4.45×10^4
500	6.56×10^2	6.36×10^3	8	3.65×10^3
1000	5.64×10^2	5.6×10^3	12	1.88×10^3

See Table 3 for abbreviations and general electroporation assay conditions.

3.4. Host restriction-modification system

Transformation efficiency can be severely affected by heterologous plasmid DNA from other species of Corynebacteria, mainly *C. glutamicum*, due to host restriction-modification systems, which can almost completely degrade such DNA (Liebl et al., 1989; Bonamy et al., 1990; Schafer et al., 1994a,b; Ankri et al., 1996a,b; van der Rest et al., 1999; Jang and

Table 6
Effects of DNA concentration, glycine supplementation and heat shock on transformation efficiency in the CIP 102968T strain of *Corynebacterium pseudotuberculosis*

DNA (ng)		Transformants/μg DNA		Gly + HS
		Gly	HS	
0	0	0	0	0
1	2.6×10^4	4.0×10^3	8.0×10^3	5.6×10^4
10	2.0×10^3	4.23×10^3	8.0×10^2	6.0×10^3
100	6.1×10^2	1.85×10^2	1.2×10^2	6.8×10^2
500	52	1.28×10^2	16	62
1000	8	51	4	47

See Table 3 for abbreviations and general electroporation assay conditions.

Britz, 2000; Tauch et al., 2002). It is also known that cells exposed to stress conditions, such as heat, have reduced activity in such systems (Schafer et al., 1994a,b). Consequently, various protocols adopt a heat shock treatment, following electroporation, in order to reduce the activity of these restriction-modification systems, increasing the transformation efficiency of these bacteria (Schafer et al., 1994a,b; van der Rest et al., 1999; Tauch et al., 2002).

We tested whether heat shock would improve the transformation efficiency of *C. pseudotuberculosis* strains. Immediately after electroporation, we resuspended the cells in 1.5 ml of BHI medium and incubated them for 6 min in a water bath at 46 °C. The cell suspension was then incubated at 37 °C for 2 h without agitation. Finally, the cells were plated on selective BHI agar plates and incubated at 37 °C for 48 h to recover transformants.

We observed that heat shock treatment, by itself, did not drastically affect the transformation efficiency of *C. pseudotuberculosis* strains. The exception was the 1002 strain, which when submitted to heat shock treatment after electrotransformation, reached the same transformation efficiency as without heat shock (1.64×10^5 transformants/μg of plasmid DNA, Table 5).

Table 7
Effects of DNA concentration, glycine supplementation and heat shock on transformation efficiency in the HJ ULCC06.334.4 strain of *Corynebacterium pseudotuberculosis*

DNA (ng)		Transformants/μg DNA		Gly + HS
		Gly	HS	
0	0	0	0	0
1	9×10^4	1.07×10^5	1.4×10^5	2.8×10^5
10	4.8×10^3	9.2×10^3	7.2×10^3	3.6×10^3
100	3.98×10^3	3.8×10^4	4.24×10^3	4.1×10^3
500	2.5×10^3	1.31×10^3	2.1×10^3	1.8×10^3
1000	1.5×10^3	6.4×10^3	1.04×10^2	1.7×10^3

See Table 3 for abbreviations and general electroporation assay conditions.

In contrast, the combination of heat shock with glycine supplementation improved the transformation efficiencies of T2 (Table 4) and CIP 102968T (Table 6). This combination (heat shock plus glycine) also gave the best electroporation conditions for the HJ ULCC06.334.4 strain, reaching 2.8×10^5 transformants/μg of plasmid DNA (Table 7).

Moreover, we observed that with large quantities of DNA (500 and 1000 ng), heat shock plus glycine allowed us to reach an approximately 10-fold greater transformation efficiency, compared to the other test conditions, mainly with the T2 strain (Table 4). This will be useful for studies of chromosomal integration with non-replicative plasmids, for which more than 500 ng of DNA is needed.

4. Conclusions

We developed an improved protocol for the electrotransformation of *C. pseudotuberculosis*. Using this new protocol (Table 2), *C. pseudotuberculosis* electrotransformation was improved to 10^5 cfu/μg plasmid DNA, which is 10-fold more efficient than what has been previously reported for electrotransformation of this bacterium (Songer et al., 1991). Although this protocol has worked well for all the strains that we tested, some parameters may need to be adjusted for specific strains. Nevertheless, this improved transformation efficiency was reached with about 100–1000-fold less plasmid DNA than has been used for the transformation of both other Corynebacteria and other Gram-positive bacteria. This protocol will have considerable utility for genetic studies of *C. pseudotuberculosis*.

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In Vivo Insertional Mutagenesis in *Corynebacterium pseudotuberculosis*: an Efficient Means To Identify DNA Sequences Encoding Exported Proteins▽

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The reporter transposon-based system TnFuZ was used to identify exported proteins of the animal pathogen *Corynebacterium pseudotuberculosis*. Thirty-four out of 1,500 mutants had detectable alkaline phosphatase (PhoZ) activity. This activity was from 21 *C. pseudotuberculosis* loci that code for fimbrial and transport subunits and for hypothetical and unknown-function proteins.

Corynebacterium pseudotuberculosis, a gram-positive, facultatively intracellular pathogen, is the main etiological agent of caseous lymphadenitis (CLA), a common disease in sheep and goat populations throughout the world. CLA causes economic damage due to reduced wool and meat production, increased culling rates, and condemnations of carcasses and skins in abattoirs (5). Despite its importance for animal health, *C. pseudotuberculosis* is still poorly characterized, especially regarding genomic information. However, the genomes of several related species, such as *Corynebacterium diphtheriae* (2), *Corynebacterium glutamicum* (10), and *Corynebacterium efficiens* (16), have already been completely sequenced; this information will be helpful for better understanding of the biology of this microorganism.

We used a recently developed reporter transposon-based system, TnFuZ (8), to identify genes encoding exported proteins in *C. pseudotuberculosis*. This system combines a derivative version of the Tn*4001* transposable element with the DNA fragment encoding the mature *Enterococcus faecalis* alkaline phosphatase gene (*phoZ*), whose product is active only when it is located outside the bacterial cytosol (8). Thirty-four out of 1,500 mutants had detectable PhoZ activity. We identified 21 loci coding for fimbrial and transport subunits, and also for hypothetical and unknown-function proteins, in *C. pseudotuberculosis*. These genes are potential targets for the development of new attenuated vaccine strains.

In vivo insertional mutagenesis in *C. pseudotuberculosis* strain T1. The *C. pseudotuberculosis* wild-type strain T1 was isolated from a caseous granuloma found in a CLA-affected goat in Bahia state (Brazil), identified by the API CORYNE battery (Biomerieux, France). Electrocompetent *C. pseudotuberculosis* cells were prepared (4) and transformed with 1 μg of the nonreplicative TnFuZ-containing plasmid (pCMG8). Insertional mutants were isolated by plating on selective brain heart infusion agar plates (Oxoid Ltd., England) containing 25 μg/ml of kanamycin, supplemented with 40 μg/ml of 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma-Aldrich Co.), a substrate that allows recovery of *C. pseudotuberculosis* insertional mutant colonies with positive alkaline phosphatase activity ($PhoZ^+$). We obtained 1,500 kanamycin-resistant *C. pseudotuberculosis* mutants, of which 34 (2.26%) exhibited the $PhoZ^+$ phenotype.

Molecular characterization. After insertional mutagenesis, chromosomal DNA from the 34 selected $PhoZ^+$ mutants was extracted by the 10% lysozyme and phenol-chloroform methods (18) and then directly sequenced using the Big Dye Terminator V3.1 cycle sequencing kit in an ABI 3100 automated DNA sequencer system (Applied Biosystems). The sequencing primer was EnPhoR1 (5′-TGC CTT CGC TTC AGC AAC CTC TGT TTG-3′) (8), and the following PCR protocol was used: 4 min at 95°C and 100 cycles of 30 s at 95°C, 20 s at 50°C, and 4 min at 60°C. Sequences (approximately 200 bp) of interrupted *C. pseudotuberculosis* T1 genes from all 34 mutants were determined.

Nucleotide sequence similarity searches were performed with the BLAST software (http://www.ncbi.nlm.nih.gov/BLAST) service at the National Center for Biotechnology Information (NCBI). The nucleotide sequences were analyzed by searching DNA and protein databases for similarity with sequences of *C. diphtheriae*, *C. efficiens*, and *C. glutamicum* deposited in GenBank. Predicted amino-acid sequences were obtained by using the "Six Frame Translation Tool" service of the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html). Further analyses for the identification of exporting motifs were performed with the following bioinformatics programs: Pfam (http://www.sanger.ac.uk/Software/Pfam/search.shtml), SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/#submission), and PSORT (http://psort.nibb.ac.jp/form.html).

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TABLE 1. *Corynebacterium pseudotuberculosis* DNA-*PhoZ* fusions

Strain	Function (organism)	Similar protein(s)	% aa[a] identity (% positive)	Transposon insertion site[b]	Phenotype	Identified export signals[c]
Exported proteins						
CZ171049	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	56 (68)	231/490	Early-blue	SP(28–29) and TM (13)
CZ171052	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	56 (67)	166/490	Early-blue	SP (28–29) and TM (18)
CZ171054	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	50 (60)	147/490	Early-blue	SP(28–29) and TM (10)
CZ171057	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	56 (67)	162/490	Early-blue	SP(28–29) and TM (14)
CZ171058	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	70 (86)	193/490	Early-blue	SP(28–29) and TM (5)
CZ171059	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	47 (62)	281/490	Early-blue	SP(28–29) and TM (3)
CZ171060	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	64 (78)	198/490	Early-blue	SP(28–29) and TM (15)
CZ171061	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	60 (74)	318/490	Early-blue	SP(28–29) and TM (17)
CZ171064	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	59 (71)	231/490	Early-blue	SP(28–29) and TM (13)
CZ171071	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	43 (56)	162/490	Early-blue	SP(28–29) and TM (14)
CZ171072	Putative fimbrial subunit (*C. diphtheriae*)	NP 938626	66 (83)	96/490	Early-blue	SP(28–29) and TM (10)
CZ171053	Putative iron transport system binding (secreted) protein (*C. diphtheriae*)	NP 938958	74 (84)	167/298	Early-blue	SP(39–40) and TM (12)
Unknown-function proteins						
CZ171068	Unknown				Early-blue	SP(29–30) and TM (4)
CZ171069	Unknown				Early-blue	SP(31–32) and TM (4)
Membrane proteins						
CZ171047	Putative membrane protein (*C. diphtheriae*)	NP 938972	52 (60)	94/377	Early-blue	SP(32–33) and TM (16)
CZ171050	Putative membrane protein (*C. diphtheriae*)	NP 938972	53 (61)	94/377	Early-blue	SP(32–33) and TM (16)
CZ171062	Putative membrane protein (*C. diphtheriae*)	NP 938972	52 (60)	94/377	Early-blue	SP(32–33) and TM (16)
CZ171074	Putative membrane protein (*C. diphtheriae*)	NP 939774	28 (47)	202/321	Early-blue	TM (13)
Cytoplasmic proteins						
CZ171066	L–Serine dehydratase 1 (*C. diphtheriae*)	NP 938869	78 (84)	457/458	Late-blue	ND
CZ171067	L-Serine dehydratase 1 (*C. diphtheriae*)	NP 938869	39 (53)	457/458	Late-blue	ND
CZ171041	Cystathionine γ-synthase (*C. glutamicum*)	NP 601979	48 (62)	205/382	Early-blue	TM (12)
CZ171042	Hypothetical protein CEO202 (*C. efficiens*)	NP 736812	70 (85)	263/275	Early-blue	TM (13)
CZ171043	Putative Sdr family-related adhesin (*C. diphtheriae*)	NP 940409	44 (63)	949/951	Late-blue	SP(41–42) and TM (13)
CZ171044	Putative sodium:solute symporter (*C. diphtheriae*)	NP 939374	84 (89)	400/552	Early-blue	TM (17)
CZ171045	Putative two-component system sensor kinase (*C. diphtheriae*)	NP 938973	68 (82)	398/403	Early-blue	TM (15)
CZ171046	Hypothetical protein NCgl2271 (*C. glutamicum*)	NP 601554	36 (66)	87/341	Early-blue	SP(29–30) and TM (14)
CZ171063	Hypothetical membrane protein (*C. glutamicum*)	NP 600387	42 (61)	78/157	Early-blue	SP(63–64) and TM (18)
CZ171070	Glycogen operon protein (*C. diphtheriae*)	NP 939914	41 (62)	579/735	Late -blue	TM (5)
CZ171073	Putative proline-betaine transporter (*C. glutamicum*)	NP 602258	30 (44)	388/504	Early-blue	TM (14)
CZ171048	Leucyl-tRNA synthetase (*C. diphtheriae*)	NP 940621	88 (93)	148/960	Late -blue	ND
CZ171051	Phosphoribosyl-ATP pyrophosphatase (*C. efficiens*)	NP 738245	75 (84)	89/91	Late-blue	ND
CZ171055	Aspartate ammonia lyase (*C. efficiens*)	NP 738243	68 (79)	359/538	Late-blue	ND
CZ171056	Putative methylmalonyl coenzyme A mutase small subunit (*C. diphtheriae*)	NP 939625	81 (84)	603/603	Late -blue	ND
CZ171065	Putative uroporphyrin III C-methyltransferase CysG (*C. efficiens*)	NP 737060	33 (48)	648/673	Late-blue	ND

[a] aa, amino acid.
[b] Presented as the last amino acid of the open reading frame product before the transposon/total number of amino acids in the open reading frame product, corresponding to a similar protein in *C. glutamicum*, *C. efficiens*, or *C. diphtheriae*, according to the database.
[c] SP, signal peptide; TM, transmembrane domain; ND, not detected. The number of transmembrane domains found and the cleavage sites of the signal peptide (amino acids) are given in parentheses.

Analyses of regions flanking the transposon insertion sites indicated similarity to 21 different loci, most of them encoding putative membrane proteins, such as fimbrial subunits and transport systems (Table 1). *C. pseudotuberculosis* DNA sequences encoding hypothetical and unknown-function proteins were also identified (Table 1).

In on our analysis, 14 *C. pseudotuberculosis* mutants presented insertions in genes encoding cell envelope-associated

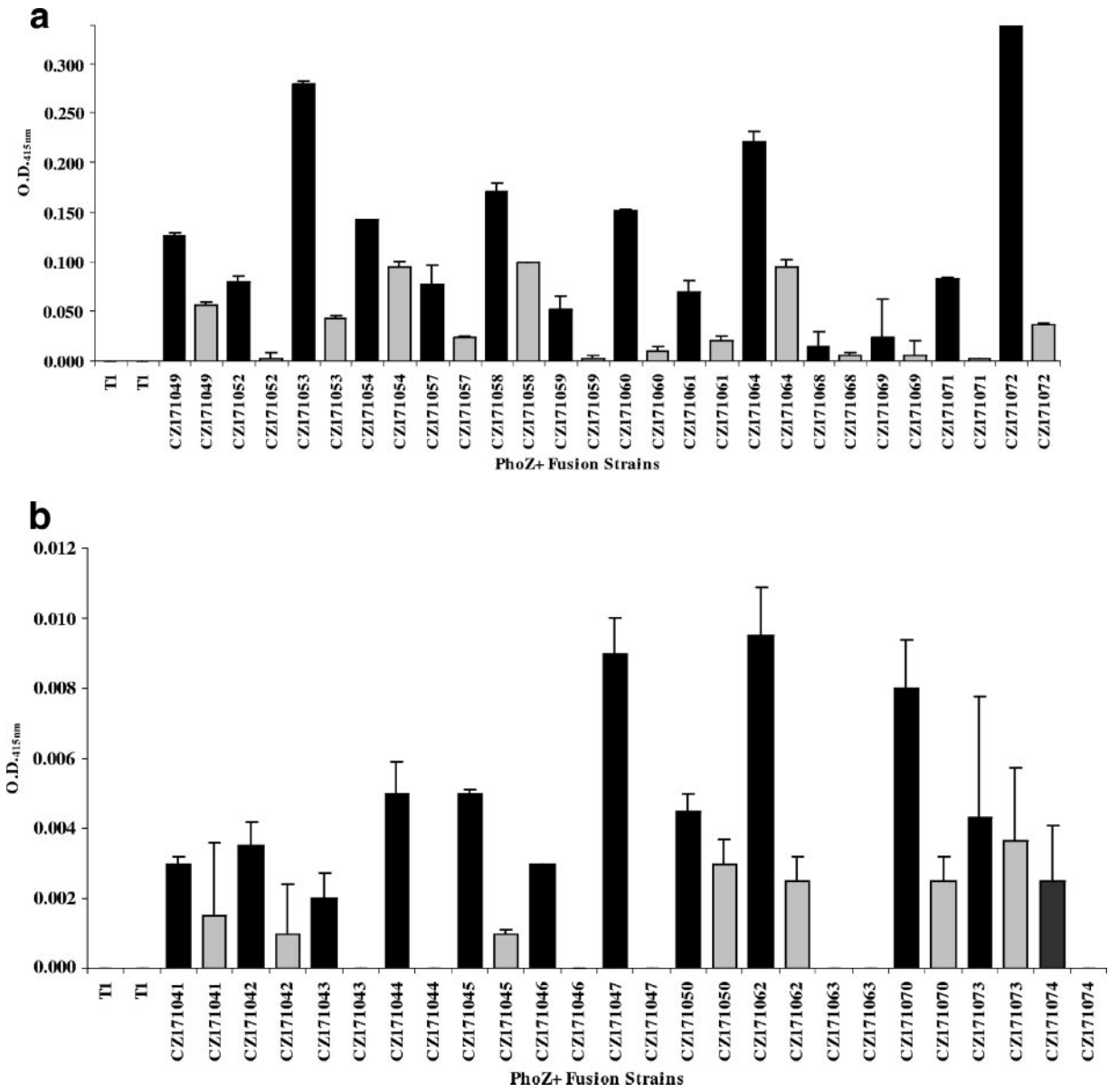

FIG. 1. Quantitative alkaline phosphatase activity of *Corynebacterium pseudotuberculosis* TnFuZ mutants. Alkaline phosphatase activity was measured in filtered and unfiltered culture supernatants, as described in Material and Methods. Shown are results for mutants harboring insertions in gene sequences that encode products homologous to exported proteins (a), membrane proteins (b), or cytoplasmic proteins (c). Values are means of three independent experiments for which the supernatants were collected from the exponential-growth phase. T1, negative-control strain.

proteins, 11 of which had insertions in different positions of the same locus of a putative fimbrial subunit found in *C. diphtheriae* NCTC13129 (2). This putative protein is a surface protein similar to the *Actinomyces viscosus* type 1 fimbrial major subunit precursor FimP. It is involved in bacterial binding to teeth through immobilized salivary statherin and acidic proline-rich protein; consequently, it participates in early plaque development and human mouth colonization (11). Fimbria and pilus proteins are particularly interesting, since fimbria-mediated adhesion is one of the best-studied strategies for host surface colonization by pathogenic microorganisms (6, 7, 13). Normally, these proteins play an important role in early steps of infection, since they are involved with pathogen-host adhesion. Bacterial adherence to host cells or surfaces is often an essential first stage in disease, because it places pathogens at appropriate target tissues. Adhesion to host cells may lead to internalization, either by phagocytosis or by bacterium-induced endocytosis (6, 7).

The DNA flanking the insertion from mutant CZ171053 encodes a putative-iron transport system binding (secreted) protein similar to that from *C. diphtheriae* NCTC13129 (2). This protein is similar to *Escherichia coli* FecB, which belongs to the bacterial solute-binding protein family and is involved in the transport of iron from ferric citrate (20). Proteins related to iron transport are utilized by many bacterial pathogens to perceive iron-limiting conditions of the host and as an envi-

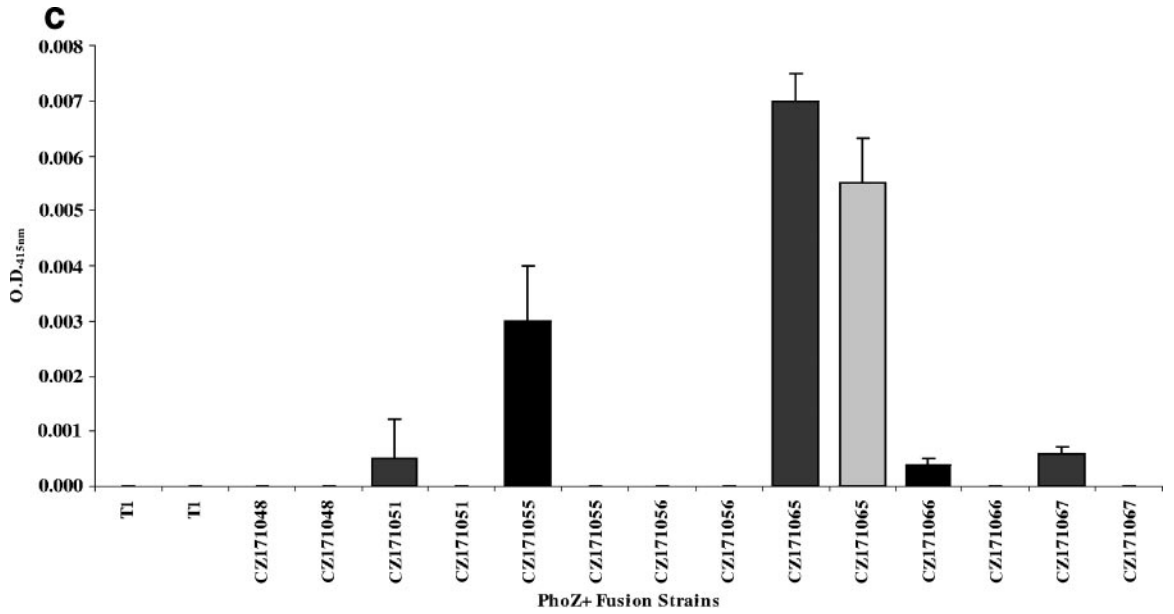

FIG. 1—*Continued.*

ronmental signal to induce expression of virulence factors (12, 17, 19). Moreover, iron modulates the adhesion of *C. diphtheriae* to cells of the human respiratory tract (15). In 2002, Billington et al. identified four genes in *C. pseudotuberculosis* involved in iron acquisition in mammalian hosts and concluded that this mechanism enhanced the capacity of this bacterium to develop a persistent infection in goats (1).

Two unknown genes were identified in the mutants CZ171068 and CZ171069. Although we obtained good-quality DNA sequences, ranging from 150 to 200 nucleotides, analyses of the DNA and amino acid sequences did not reveal any similarity with known sequences in databases. Searches for conserved protein motifs revealed probable cleavage sites in both of the deduced amino acid sequences (between amino acids 29 and 30 for CZ171068 and between amino acids 31 and 32 for CZ171069) of these proteins, indicating a hypothetical signal peptide; these will be investigated further.

The gene identified in mutant CZ171046 is similar to hypothetical protein NCgl2271 from *C. glutamicum* ATCC 13032 (10), which possesses a conserved cutinase domain (Pfam 01083; cutinase). Cutinase is a serine esterase, normally secreted by plant-pathogenic fungi, and it plays an important role in pathogenesis. It hydrolyzes plant cutin, thus facilitating fungus penetration (21, 22). Two cutinase-like proteins have also been found in the genome of *Mycobacterium tuberculosis* (3). Three mutants of this species (CZ171047, CZ171050, and CZ171062) have insertions in the same locus, a gene similar to that of a putative membrane protein of *C. diphtheriae* NCTC13129 (2); they have a conserved PspC (Pfam 04024; COG 1983). Proteins harboring this motif are associated with the cell envelope, functioning as a stress-responsive element (14).

Phenotypic characterization by the alkaline phosphatase activity assay. During the isolation and screening of *C. pseudotuberculosis* T1 insertional mutant colonies for the $PhoZ^+$ phenotype, we observed two types of colonies: (i) "early-blue" colonies (25 out of 34 mutants), detected after 2 days of incubation, and (ii) "late-blue" colonies (9 out of 34 mutants), exhibiting a $PhoZ^+$ phenotype after 3 to 4 days. The phenotypes were confirmed by streaking the colonies onto fresh plates.

We used the alkaline phosphatase assay (9) to monitor the activity level of alkaline phosphatase in filtered (pore size, 0.22 μm; Minisart; Sartorius Ltd., Epsom, United Kingdom) and unfiltered supernatants prepared from mid-log- to stationary-growth-phase bacterial cultures (optical density at 600 nm, 1.0 to 1.5). This approach was used to eliminate a possible background that could be generated by contamination with the remaining portions of the cells; it enabled differentiation between fusion proteins that were actually exported and those that normally remain in the cytoplasm. The alkaline phosphatase activities of *C. pseudotuberculosis* TnFuZ mutants were grouped into three categories, according to the proteins encoded by transposon-interrupted genes. (i) The first category consists of mutants harboring insertions in gene sequences that encode products homologous to exported proteins (Fig. 1a). As expected, this group had the highest levels of phosphatase alkaline activity, even after filtration. Although not all were secreted fusion proteins, we believe that the fimbrial subunit, for example, has weak interactions with the cell membrane and that this membrane is easily breached, making the fimbrial subunit detectable in the supernatant. (ii) The second category consists of mutants harboring insertions in gene sequences that encode products homologous to membrane proteins (Fig. 1b). This group has about 10-fold less alkaline phosphatase activity than the first group. When the supernatant is filtered, there is a significant reduction in alkaline phosphatase activity. Again, we believe that some fusion proteins bind weakly to the cell membrane and would be detectable in the supernatant, even after filtration. (iii) The third category consists of mutants harboring insertions in gene se-

quences that encode products homologous to cytoplasmic proteins (Fig. 1c). These fusion strains have only cell-associated alkaline-phosphatase activity. Only one protein was detectable, at moderate levels: a putative uroporphyrin III C-methyltransferase CysG, similar to that of *C. efficiens*. Since this putative protein is involved in coenzyme metabolism (as with COG 0007.2), being a cytoplasmic protein, it seems that the colony method of screening is not optimal for the identification of fusion proteins that do not remain associated with the cell surface, since it does not eliminate background signals.

This was the first time that the TnFuZ transposition system was used to identify genes coding for exported proteins of *C. pseudotuberculosis*. We identified a great diversity of proteins, including a fimbrial subunit, a protein related to iron uptake, adhesins, and proteins involved in transport, as well as hypothetical proteins and two unknown proteins. These data now constitute the largest collection of exported proteins identified in corynebacteria through genetic screening. Many of the genes that were identified could play an important role in the biology of *C. pseudotuberculosis*, and they are promising targets for the development of attenuated vaccine strains. Further experiments are now in progress in our laboratory in order to determine whether or not these exported proteins are involved in the virulence of this pathogen. We have also been conducting immunization assays to determine if these mutant strains can confer protective immunity against this bacterium.

Nucleotide sequence accession numbers. The ~200-bp nucleotide sequences of interrupted *C. pseudotuberculosis* T1 genes from the 34 selected PhoZ$^+$ mutants have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers CZ171041 to CZ171074.

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